



The Role of Unconventional T Cells in the Immune Response to the Gastric Pathogen *Helicobacter pylori*

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the Degree of Doctor of Philosophy**

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*"If we knew what it was we were doing, it
would not be called research, would it?" -
Albert Einstein*

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List of Abbreviations

Abs	antibodies
Ags	antigens
APCs	antigen presenting cells
AT	autoimmune thyroiditis
<i>BabA</i>	blood group antigen-binding adhesion
BrdU	5-bromo-2'-deoxyuridine
C.M. Ags	crude membrane Ags
<i>CagA</i>	cytotoxin associated gene product A
<i>cag</i> -PAI	<i>cag</i> pathogenicity island
CAMPs	cationic antimicrobial peptides
CCUG	culture collection university of göteborg
CD	cluster of differentiation
CLO	<i>Campylobacter</i> -like organism
Cy	cyanine
Cyto Ags	cytoplasmic Ags
DCs	dendritic cells
DETCs	dendritic epidermal T cells
DFMO	diflouromethylornithine
DMSO	dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DTT	dithiothritol
EDTA	ethylenediamine tetraaeric acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GIT	gastrointestinal tract
HBSS	hanks balanced salt solution
HMB-PP	(E)-1-hydroxy-2-methyl-but-2-enyl 4 diphosphate
HP-NAP	<i>Helicobacter pylori</i> -neutrophil activating protein
HPLC	high-performance liquid chromatography

HSCs	hematopoietic stem cells
I	ionomycin
IELs	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
iGb3	isoglobotrihexosylceramide
IL	interleukin
IMPs	inner membrane proteins
IPP	isopentyl pyrophosphate
LDH	lactate dehydrogenase
LL	lower left
LPLs	lamina propria lymphocytes
LPS	lipopolysaccharide
LR	lower right
mAb	monoclonal antibodies
MAIT	mucosal associated invariant T
MALT	mucosal associated lymphoid tissue
mg	milligrams
MHC	major histocompatibility complex
min	minute
ml	milliliter
n	total number
NBI	narrow band imaging
NCTC	national collection of type cultures
NF- κ B	nuclear factor kappa B
NK	natural killer
NKR ⁺ T cell	natural killer receptor ⁺ T cell
NKT cell	natural killer T cell
NLRs	nod-like receptors
nm	nanometer
OMP	outer membrane proteins
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	Phytohaemagglutinin
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PPI	proton pump inhibitor
PRRs	pathogen recognition receptors
RANTES	regulated on activation, normal T expressed and secreted
RNA	Ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription
SabA	sialic acid-binding adhesin
SAT	stool antigen tests
SP-D	surfactant protein D
TBS	tris buffered saline
T _c cells	cytotoxic T cells
TCR	T cell receptor
TFSS	type IV secretion system
TGF- β	tumour growth factor- β
T _h cell	T helper cell
TLRs	toll-like receptors
tPBMLs	total peripheral blood mononuclear leukocytes
T _{reg}	regulatory T cells
UL	upper left
UR	upper right
Ure1	urease 1
VacA	vacuolating toxin A
WCE	whole cell extract
Yr	year
α -GalCer	α -galactosylceramide
MI	microliter

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Abstract

Helicobacter pylori (HP) is a gastric pathogen that is a known causative agent of gastritis, and peptic ulcers. It is a co-factor for the onset of gastric cancer and for these reasons is classified as a type I carcinogen. HP infection elicits an inflammatory T_h1 driven immune response, during which, classical T cells have been considered the primary orchestrators, although this action is rarely effective in clearing infection. Ultimately, the aim of studies in this thesis was to assess the importance of previously overlooked unconventional T cell populations through examination of their immunological responses in the gastric mucosa to HP and to HP derived antigens (Ags).

Firstly, using flow cytometry we phenotypically characterised lymphocyte populations paying particular attention to unconventional T cell subsets [natural killer T cells (NKT cells) and $\gamma\delta^+$ T cells] in the human gastric epithelium (EP) and lamina propria (LP) layers and compared their frequency in HP infection. Results showed that CD4⁺ and CD8⁺ classical T cell numbers were significantly higher and lower respectively in the LP of *H. pylori* infected individuals. Moreover, a significant reduction in the percentage of CD56⁺ NK cells was found in the LP of *H. pylori* positive subjects compared to control samples. The numbers of NKT cells were low in both the EP and LP of control subjects and the frequencies were unchanged in HP infected subjects. Furthermore, when $\gamma\delta^+$ T cells were investigated, numbers were markedly higher in the *H. pylori* infected EP and not in the LP.

In the next experiments, we investigated immunological responses to *H. pylori* antigenic stimulation through proliferation, cytokine and cytotoxicity assays. Here, a variety of HP derived Ags were used to stimulate PBMCs, single cell suspensions derived from the EP, LP or NKT cell clones. The cytokine response of $\gamma\delta^+$ T cells to HP derived Ags was also investigated using $\gamma\delta^+$ T cell clones. The HP derived antigenic stimuli included whole cell extract, cytoplasmic Ags, crude membrane Ags, outer membrane proteins, inner membrane proteins from strains 26695 and J99 and LPS derived from strains NCTC11637 and CCUG17874. A lack of proliferation as measured by the BrdU ELISA was observed in response to many of the HP derived Ags used following antigenic stimulation of NKT cells, PBMCs or cell suspensions from the gastric EP and LP layers. In cytokine studies, using the T_h1/T_h2 11plex FlowCytomix kit, small increases in cytokines (IL-8, IFN- γ and others) were observed following stimulation of NKT cells with some of the HP derived Ags (whole cell extract from HP 26695 and others) while results using PBMCs or gastric lymphocyte populations were variable. In these experiments, when the $\gamma\delta^+$ T cell clones were stimulated with the HP derived Ags, higher levels of IFN- γ , IL-2, IL-4, IL-5, IL-8, IL-12p70, IL-1 β , TNF- α and TNF- β were observed consistent with a T_h1 and T_h2 cytokine pattern. Furthermore, using the LDH assay, a lack of cytotoxic responses was seen following NKT cell stimulation with the HP derived Ags.

In the final chapter, gene expression profiling of RNA isolated from gastric EP and LP derived CD2⁺ cells from HP infected and uninfected gastric mucosa was undertaken using the Affymetrix system in conjunction with the Karolinska Institute in Sweden. The findings from these studies generated a large database of novel genes differentially expressed in HP infection in key immunological areas such as cytotoxicity, cancer progression, inflammation, cytokine production and others. Moreover, we categorised the top 20 most significantly up-regulated and down-regulated genes in the EP and the LP in HP infection relative to controls. Together, this work contributed to gaining further knowledge about HP pathogenesis and immunity and gave important insights into the role of unconventional T cells in HP infection.

Chapter 1

General Introduction

1.1 INTRODUCTION TO THE IMMUNE SYSTEM

Historically, Edward Jenner is accredited with the initial roots of immunology for his discovery that vaccination with cowpox disease prevented smallpox (Jenner, 1798; Smith, 2005; Thiery, 2007). Next it was the turn of Robert Koch who made a significant contribution to the world of immunology through his work on Tuberculosis (Koch, 1882, reviewed by Murray, 2004). Upon discovering microorganisms were the cause of disease, he designed a set of postulates which were a set of criteria to be followed in order to establish disease etiology (Friedrich Krause, 1899; Petrie, 1943). Since then, microbiologists have been adding to our knowledge of the concepts of immunology with significant discoveries being made in the last 200 years in all areas of immunology. Today, we have a much wider understanding of the body's immune responses to infection with microorganisms.

Immunity is the method by which the body protects itself against infectious diseases. When the body encounters an infectious agent, the immune response occurs in three forms: the innate reaction, the early induced innate response and the adaptive immune response (Janeway *et al.*, 2005). Innate immunity is non-specific and forms the first defensive barrier against invading organisms. These non-specific innate components are present before the onset of disease and will attempt to rid the body of any foreign invaders such as bacteria, viruses or parasites. Adaptive immunity on the other hand mounts a highly specific response to antigens (Ags) recognised as foreign about 5-6 days after initial antigenic exposure. Reacting with a high degree of diversity, it is capable of recognising billions of components over a wide range of Ags. Both innate and adaptive arms of immunity have their own functions which are both very different but equally important for protecting the body against disease (Iwasaki &

Medzhitov, 2010). Innate and adaptive immunity will be discussed in detail in the forthcoming sections.

1.2 INNATE IMMUNITY

The human body is constantly being exposed to a variety of microorganisms. Innate or natural immunity distinguishes between self and non-self, is non-specific, and forms the first defensive barrier against invading organisms. Therefore cells of the innate immune system can recognise organisms deemed as foreign and attempt to eliminate them. Innate immune defences respond quickly to infection and are existent in all multi-cellular organisms (Jimenez-Valera & Ruiz-Bravo, 2011).

Physiological barriers include production of mucus, lysosyme and saliva in the nasopharynx, lacrimation or tears in the eyes, gastric acid, bile salts, enzymes, peristalsis and indigenous flora in the gastrointestinal tract (GIT) (Walker, 1976). The epithelium which is a tissue lining various cavities and surfaces throughout the body, is an important physiological component forming a physical barrier between external and internal surfaces of the body and forms the first barrier that microorganisms encounter to prevent them from becoming established (Magalhaes *et al.*, 2007). If bacteria do breach the epithelia, there are phagocytes in the underlying tissues that function to engulf and destroy invading pathogens. Complement system is also a component of the innate immune system. The complement system utilises serum molecules which play a part in controlling inflammation, clearing away immune complexes that have been formed between host and bacteria and also partake in pathogen killing (Carroll & Sim, 2011).

Bacteria possess structures known as pathogen associated molecular patterns (PAMPs) that induce innate immune responses. Innate immunity utilises germline

encoded receptors such as pathogen recognition receptors (PRRs) to identify pathogenic commonalities, hence its ability to distinguish between self and non-self (Kumar *et al.*, 2011; Janeway *et al.*, 2008; Uematsu & Fujimoto, 2010). PRRs such as Toll-like receptors (TLRs) are found on the surface of macrophages and dendritic cells (DCs) and recognise a limited set of PAMPs and components not usually found in non-infected vertebrates (Medzhitov, 2007). Other PRRs include nod-like receptors (NLRs), dectins, NALP, NAIP, retinoic acid inducible gene 1 (RIG-1), melanoma differentiation-associated gene 5 (MDA5) along with others that are not yet fully determined (Kumar *et al.*, 2011; Loo *et al.*, 2008). Antigenic stimulation of receptors on cells of the innate immune triggers the adaptive immune response (see section 1.3).

1.2.1 Cells of the innate immune system

Cells involved in innate immune responses include cytotoxic lymphocytes such as Natural Killer (NK) cells and innate T cells (see section 1.4), other white blood cells such as eosinophils and basophils, toxin secreting cells such as mast cells, phagocytic cells such as macrophages and neutrophils and DCs (Fig. 1.1A).

1.2.1.1 Macrophages

Macrophages are tissue resident cells that mature from peripheral monocytes and partake in non-specific phagocytosis of micro-organisms and function to produce cytokines and chemokines, which are proteins responsible for initiating an inflammatory response. They do this through the recruitment of neutrophils, monocytes and plasma cells to the site of infection (Janeway *et al.*, 2005). Macrophages also possess lysosomes which are involved in eliminating micro-organisms (Sansone, 2004).

1.2.1.2 Neutrophils

Neutrophils are another group of phagocytic cells which function to engulf invading organisms. Neutrophils stem from the bone marrow in response to infection and are abundantly found in the periphery (Janeway *et al.*, 2005). Neutrophils have the ability to summon additional leucocytes and lymphocytes to injured tissues during infection and thereby trigger a cascade of immune responses (Mantovani *et al.*, 2011). Neutrophils contain granules such as lysosomes important for killing micro-organisms such as bacteria.

1.2.1.3 Eosinophils

Eosinophils are tissue leukocytes which play key roles in protection against helminth infections and allergy. Cytokines and chemokines secreted by eosinophils include interleukin (IL)-1, IL-4, IL-8, IL-10 and Regulated on Activation, Normal T Expressed and Secreted (RANTES) (Gleich, 2000).

1.2.1.4 Basophils

Basophils which make up approximately 0.2% of white blood cells are found primarily in peripheral blood which are involved in defence against parasites and in allergy induced inflammation and in immunity against pathogens. Basophils are also capable of secreting anti-inflammatory cytokines such as IL-4 and IL-13 (Falcone *et al.*, 2000).

1.2.1.5 Mast cells

Mast cells are tissue resident cells that are involved in innate immunity. Their functions include cytokine and chemokine production, tissue repair and a role in host defence against micro-organisms and particularly against parasites (Metcalf *et al.*, 1997).

1.2.1.6 Dendritic cells

DCs are professional antigen presenting cells (APCs) capable of capturing and processing large quantities of Ags. DCs arise in the bone marrow and then relocate to the periphery as precursors before accumulating in tissues as immature DCs. Here, tissue resident DCs are drawn from tissues to site of Ag following chemotaxis at inflammation sites and partake in capturing Ags such as bacteria during infection and presenting to T cells (Banchereau & Steinman, 1998).

1.2.1.7 Natural Killer cells

NK cells are lymphocytes which arise in the bone marrow and play key roles in recognition of virus infected and tumour infected cells with altered major histocompatibility complex (MHC) class I expression (Lanier, 2008). NK cells are involved in cytotoxicity and have been shown to lyse target cells both *in vitro* and *in vivo*. NK cells also secrete cytokines such as interferon (IFN)- γ (Lanier, 1998). NK cell function is regulated by stimulatory and inhibitory signals *via* cytokines and inhibitory receptors. There exists several families of stimulatory and inhibitory receptors that control NK cells. For instance, NK1.1 is a member of the C-type lectin family located on the surface of murine NK cells. The human equivalent of NK1.1 is CD161 marker and this co-stimulatory molecule functions to regulate the activity of CD1d-restricted T cells (Exley *et al.*, 1998).

1.3 ADAPTIVE IMMUNITY

The adaptive immune response is composed of humoral and cell-mediated responses. B and T lymphocytes are the principal orchestrators of the adaptive immunity and these

cells have evolved to recognise a wide range of Ags from bacteria, viruses or other disease causing microorganisms (Janeway *et al.*, 2005) (Fig. 1.1B).

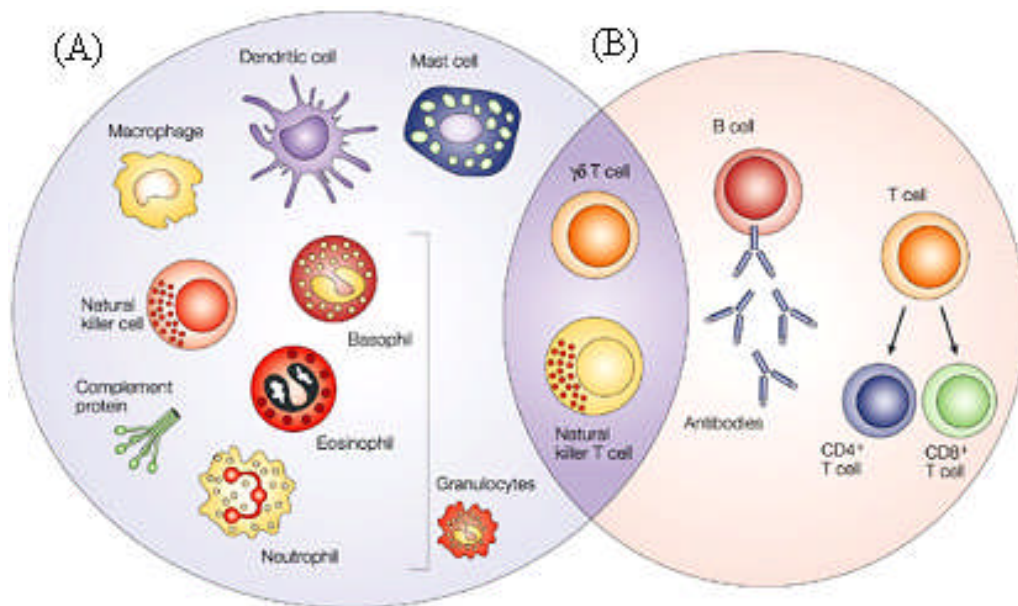


Fig. 1.1 Venn diagram illustrating cells involved in (A) innate and (B) adaptive immunity. Image taken from Dranoff. (2004). Permission to reproduce this image has been granted by Glenn Dranoff.

Humoral immunity

B lymphocytes arise in the bone marrow from hematopoietic stem cells (HSCs). There are many steps in their development from HSCs to immature B cells which include the generation of pre progenitor B, early pro-B, late pro-B, large pre-B, small pre-B and immature B cells (Hardy & Hayakawa, 2001). It is only when they become immature B cells that they are first capable of recognizing Ags. From the bone marrow, immature B cells congregate in the spleen to complete their development where they pass through transitional stages before becoming either follicular B cells, marginal zone B cells or B-1b B cells (Samitas *et al.*, 2010). B cells have several functions but primarily play a central role in the humoral immunity by producing antibodies (Abs) in response to soluble Ags (**Antibody generators**). Each B cell possesses an immunoglobulin (Ig) receptor protein, called a B cell receptor that is an Ab capable of recognizing one Ag

(Gonzalez *et al.*, 2011). B cells binds to Ag and receive a signal, in the form of cytokines or cell surface molecules *e.g.* CD40L (MacLeod *et al.*, 2009), from a T-helper (T_h) cell. Once antigenic stimulation has taken place, B cells divide and differentiate into either plasma cells which are responsible for Ab production or memory B cells are specific to that particular Ag during the immune response and are long lived enabling them to respond rapidly following a subsequent encounter with that Ag (Janeway *et al.*, 2008).

Cell-mediated Immunity

The thymus is a primary lymphoid organ and it is here the components of cell mediated immunity known as the T cells are formed. During the developmental process, cells can be positively selected through MHC interaction on thymic epithelial cells, or can be negatively selected upon recognition of a self Ag presented by MHC on an APCs (Goldrath & Bevan, 1999). A total of six types of T cells are known to form in the thymus prior to migration including $\alpha\beta^+$ $CD4^+$ and $CD8^+$ T cells, $\gamma\delta^+$ T cells, regulatory T cells (T_{reg}) cells, natural killer T (NKT) cells and intraepithelial lymphocytes (IELs) (Weinreich & Hogquist, 2008). A distinguishing feature of T cells is the presence of a T cell receptor (TCR) on their surface which functions to recognise Ags bound to MHC molecules on APCs. Structurally, several proteins form the TCR complex, of these $\alpha\beta$ heterodimeric glycoprotein is most commonly expressed but $\gamma\delta$ -TCR occurs in approximately 5% of T cells (Brenner *et al.*, 1986). The TCR alone is capable of recognising Ags, yet activation is dependant on association with other proteins which together form the TCR/CD3 complex. The CD3 complex which can be $CD3\epsilon\gamma$ and $CD3\epsilon\delta$ hetrodimers or a $CD3\zeta$ homodimer (Clevers *et al.*, 1988; Punt *et al.*, 1994; Rojo *et al.*, 2008) and functions to transduce signals created through the TCR into the

cytoplasm of the T cell and also to facilitate the expression of the TCR/CD3 complex (Abbas *et al.*, 1996). In addition to antigenic recognition by the TCR, activation requires several different signals which function to promote T cell activation (reviewed by Geppert *et al.*, 1990). Unlike the specific interaction between TCR/CD3 complex and Ag, accessory molecules are non-polymorphic and bind ligands expressed on a number of different cells. These molecules cannot specifically take part in Ag recognition, but ligation strengthens T and APC cell-cell interaction. The expression of accessory molecules varies with state of activation and differentiation, hence these molecules may influence the way in which T cells respond to an antigenic challenge (Abbas *et al.*, 1996). As shown in Fig. 1.2, other associated co-receptors and accessory molecules typically found on the surface of a T cells include CD4 or CD8 surface glycoproteins as well as CD28, CD45 and CD2 which will be discussed in more detail in chapter 5.

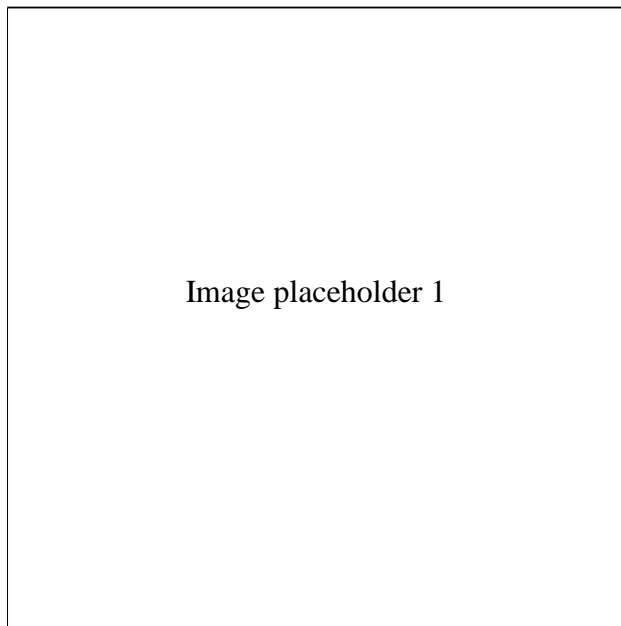


Fig.1.2 Diagram illustrating a T cell expressing some of the typical cell surface molecules. Adapted from Boes & Ploegh (2004).
Figure has been removed due to copyright restrictions

1.3.1 T cells of the adaptive immune system

1.3.1.1 $CD4^+$ and $CD8^+$ T cells

$CD4^+$ T cells or T_h cells become activated when large peptide molecules are presented to them by MHC class II molecules (Cresswell, 1994; Kaye *et al.*, 1989) expressed on the surface of specialised APCs such as DCs, B cells or macrophages (Muhlethaler-Mottet *et al.*, 1997). Once activated, $CD4^+$ T cells divide and activate other cells of the immune system such as B cells which subsequently produce Abs against the pathogen (Abbas *et al.*, 1996; Romagnani, 1992). Other functions include secretion of a wide range of cytokines which function to regulate the immune response. For instance, $CD4^+$ T cells can be further sub-classified into T_h1 helper cells which produce inflammatory cytokines such as $IFN-\gamma$ and T_h2 helper cells secreting anti-inflammatory cytokines such as IL-4 predominantly (Kaye *et al.*, 1989).

$CD8^+$ T cells or cytotoxic T cells (T_c) become activated when small sized protein Ags are presented by MHC class I molecules, expressed on the surface of nucleated cells (Townsend *et al.*, 1989). Upon activation, $CD8^+$ T cells have a cytotoxic effect on cells by secreting molecules such as granzyme, granulysin and perforin that induce a killing response (Chávez-Galán *et al.*, 2009).

In recent years, several groups reported the discovery of a distinct T cell lineage defined by their production of the cytokine IL-17 (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005; Park *et al.*, 2005). These T_h17 cells are IL-17A producing $CD4^+$ T cells. IL-6 and tumour growth factor- β (TGF- β) along with IL-21 are factors required for T_h17 polarisation to occur while IL-23 is vital for T_h17 cell expansion and sustainment (Zhu & Paul, 2008).

1.3.1.2 Regulatory T cells

In addition to the classical CD4⁺ and CD8⁺ T cells, other T cell populations play a role in cell mediated immunity. T_{reg}, originally known as suppressor T cells, function by contributing to controlling adaptive immune responses by shutting down effector T cell responses after an immune response has been elicited and exerting a suppressive effect on other lymphocyte populations such as autoreactive T cells (Izcue & Powrie, 2008). T_{reg} cells comprise approximately 5% of the total T cell population and are important in autoimmune diseases, infections, transplantation, allergic reactions and in cancer (Belkaid, 2007). Other subsets of T cells have been identified on the basis of their phenotype and function. These will be discussed in the following section.

1.4 UNCONVENTIONAL OR INNATE T CELLS

While classical CD4⁺ and CD8⁺ T cells are known as primary orchestrators of the adaptive immune response, there are a diverse array of overlooked unconventional or innate T cell populations mentioned briefly earlier in section 1.3 that also play central roles in immunity and primarily in infection. Unconventional T cells are innate-like lymphocytes in that they bridge innate and adaptive immunity by responding quickly to many infectious agents in addition to triggering an adaptive response (Bendelac *et al.*, 2001). Included in this group are NKT cells, Natural Killer Receptor⁺ T cells (NKR⁺ T cells), $\gamma\delta^+$ T cells and mucosal associated invariant T (MAIT) cells which have been classified as a subset of NKT cells.

1.4.1 Natural Killer T cells

NKT cells are a distinct T cell population formed in the thymus bearing an invariant V α 24J α 18 TCR α -chain paired with V β 11 chain in addition to NK cell surface markers

(V α 14J α 18 in mice) (Porcelli *et al.*, 1993), this restricted TCR repertoire enables NKT cells to recognise glycolipid Ags and respond quickly to these infectious agents to produce an array of cytokines such as IFN- γ and IL-4 (reviewed by Bendelac, 2007; Godfrey *et al.*, 2000; Kronenberg & Gapin, 2002; Matsuda *et al.*, 2008). Early studies on NKT cells were carried out on mice which defined NKT cells as a subset of T cells that shared similar characteristics with that of NK cells (Makino *et al.*, 1995). Further studies identified a unique subset of murine T cells that expressed an intermediate instead of a high TCR expression level and were CD4⁻CD8⁻ double negative (DN) (Budd *et al.*, 1987; Ceredig *et al.*, 1987; Fowlkes *et al.*, 1987). It was then discovered these murine cells were capable of potent cytokine production, mainly IL-4 and IFN- γ (Zlotnik *et al.*, 1992). Around the same time, other researchers reported findings around the same time of a subset of murine T cells that expressed an $\alpha\beta$ -TCR along with NK1.1. (Levitsky *et al.*, 1991; Sykes, 1990). This was significant as it was previously thought NK1.1 was solely expressed on mice NK cells. These NK1.1 expressing T cells also had intermediate TCR levels, were either CD4⁻CD8⁻ or CD4⁺ and were capable of potent cytokine production. Other groups similarly reported cytokine production by a mouse CD4⁺ T cell sub-population (Hayakawa *et al.*, 1992). Taken together, the early studies identified a novel T cell population, defined by NK1.1 expression and potent cytokine production. Next, it was discovered that NKT cells were positively selected (Bix *et al.*, 1993), required β_2m (Ohteki & MacDonald, 1994) and were CD1d reactive (Bendelac *et al.*, 1995; Godfrey & Berzins, 2007). Investigators working on CD1d showed that NKT cells were activated by CD1 molecules presenting glycolipids instead of the protein associated with classical MHC molecules (Beckman *et al.*, 1994). In particular, α -galactosylceramide (α -GalCer), a glycosphingolipid isolated from the marine sponge *Agelas mauritanus* was shown to be a potent NKT cell stimulator

(Kawano *et al.*, 1997). To date, α -GalCer is still the most potent activator of NKT cells. Collectively, the literature from 1987 through to the mid 1990's indicated that a unique population of murine T cells called NKT cells which were characterised by an invariant V α 24J α 18⁺ TCR, expression of CD4 or lack of CD4/CD8 and possessing CD1d reactivity (Godfrey *et al.*, 2004). Other ligands have subsequently been shown to activate NKT cells and these include *Streptococcus pneumonia* (Kawakami *et al.*, 2003), *Pseudomonas aeruginosa* (Nieuwenhuis *et al.*, 2002) and *Borrelia burgdorferi* (Kumar *et al.*, 2000).

NKT cell numbers are much higher in mice than in humans and their distribution differs in parts of the body. In mice, the numbers are more abundant in liver (5%) and spleen with smaller numbers found circulating in peripheral blood (Wingender & Kronenberg, 2008). In humans, NKT cells account for 0.1% of T cells in the spleen and in the periphery while they constitute approximately 1% of liver T cells (Karadimitris *et al.*, 2001; Kenna *et al.*, 2003; Kronenberg, 2005) and small numbers in the intestine and colon (O'Keeffe *et al.*, 2004). Interestingly, the human omentum or stomach peritonerm has recorded the highest NKT cell numbers to date with NKT cells constituting 30-50% of omental T cells (Lynch *et al.*, 2009). A high degree of person to person variability has been shown (Lee *et al.*, 2002b).

1.4.1.1 Subsets of NKT cells

NKT cells generally display an activated or memory phenotype expressing CD69⁺, CD62L^{low}, CD44^{high} and CD122^{high} and are positive for the expression of NK cell surface markers such as NK1.1 in mice/CD161 in humans, NKG2D and Ly49 markers (Ishihara *et al.*, 1999). While mice NKT cells are characterised by an invariant V α 24J α 18 TCR, being CD4⁺ or CD4⁻CD8⁻ DN, humans can also express the CD8⁺

glycoprotein (Ishihara *et al.*, 1999). Like with classical T cells, there are various subsets of NKT cells. As shown in Fig. 1.3, the expression of the TCR on NKT cells identifies three subsets defined by the following criteria:

- a) the expression or lack of an invariant $V\alpha 14J\alpha 18$ in mice/ $V\alpha 24J\alpha 18$ in humans TCR
- b) with/or NK1.1 in mice/CD161 in humans (Godfrey *et al.*, 2004.)

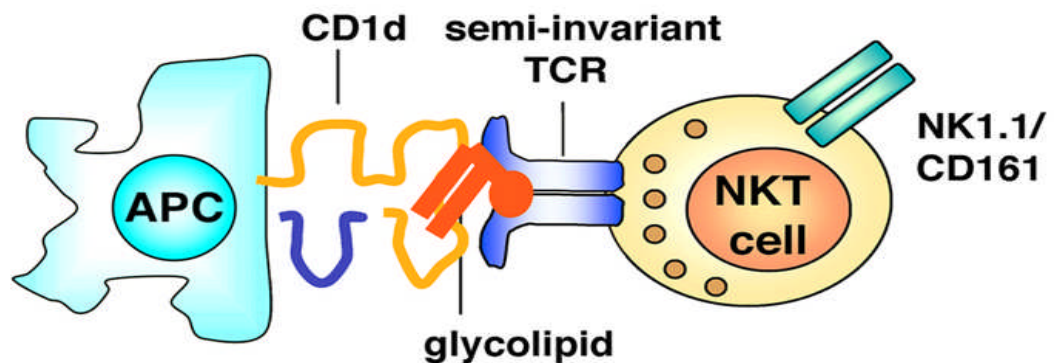


Fig. 1.3 Illustration showing an NKT cell which possesses an invariant $V\alpha 14J\alpha 18$ in mice/ $V\alpha 24J\alpha 18$ in humans TCR α chain in addition to NK receptor NK1.1 in mice/CD161 in humans. NKT cell is recognising glycolipid Ags being presented by CD1d on an APC. Adapted from Wu & Van Kaer, (2011). Permission to reproduce this image has been granted by Luc Van Kaer.

- (1) Type I $V\alpha 14J\alpha 18^+NK1.1^+CD1d$ -dependent NKT cells in mice/
 $V\alpha 24J\alpha 18^+CD161^+CD1d$ -dependent NKT cells in humans

Since conventional T cells can express NK receptors but may not be CD1 reactive and since some CD1d-restricted NKT cells may possess a diverse TCR repertoire and may be NK1.1⁻, the term invariant NKT cells was coined to describe this first subset (Godfrey *et al.*, 2004a). In mice, this subset is either CD4⁺ or DN. In contrast to murine populations, in humans $V\alpha 24J\alpha 18^+CD161^+$ NKT cells, CD8 can be expressed in up to 50% of NKT cells, occurring following intrathymic selection (Gumperz *et al.*, 2002). Therefore as regards the human subset, $V\alpha 24J\alpha 18^+$ NKT cells can be CD4⁺, CD8⁺ or DN which raises the possibility that each subset has a different function (O'Reilly *et al.*,

2011). It is known for example that in mice, CD4⁺ NKT cells produce increased levels of IL-4 *in vitro* following stimulation with CD3-specific Abs (Hammond *et al.*, 1999). There are also studies suggesting that the different populations of NKT cells reside in different tissues (Godfrey *et al.*, 2000; Hammond *et al.*, 1999).

(2) Type I V α 14J α 18⁺NK1.1⁻CD1d-dependent NKT cells in mice/

V α 24J α 18⁺CD161⁻CD1d-dependent NKT cells in humans

This subset is generally CD4⁺ in humans and can be CD4⁺ or DN in mice (Lee *et al.*, 2002a). There are other differences between the mouse subsets in that thymic NK1.1⁻CD1d-dependent NKT cells produce increased IL-4 levels and decreased levels of IFN- γ compared to those cells that are NK1.1⁺ (Gadue & Stein, 2002).

(3) Type II V α 14J α 18⁺NK1.1⁻CD1d-independent NKT cells in mice/

V α 24J α 18⁺CD161⁻CD1d-independent NKT cells in humans

The third NKT cell subset possesses a diverse TCR α -chain and is independent of CD1d. That said, expression of a V α 3J α 9 α -chain combination is common (Godfrey *et al.*, 2004a). This subset does not recognise glycolipids bound to CD1d as studies showed a lack of requirement for endosomal CD1d targeting (Chiu *et al.*, 2002). Fig. 1.4 summarises the main features of the different NKT cell subsets.

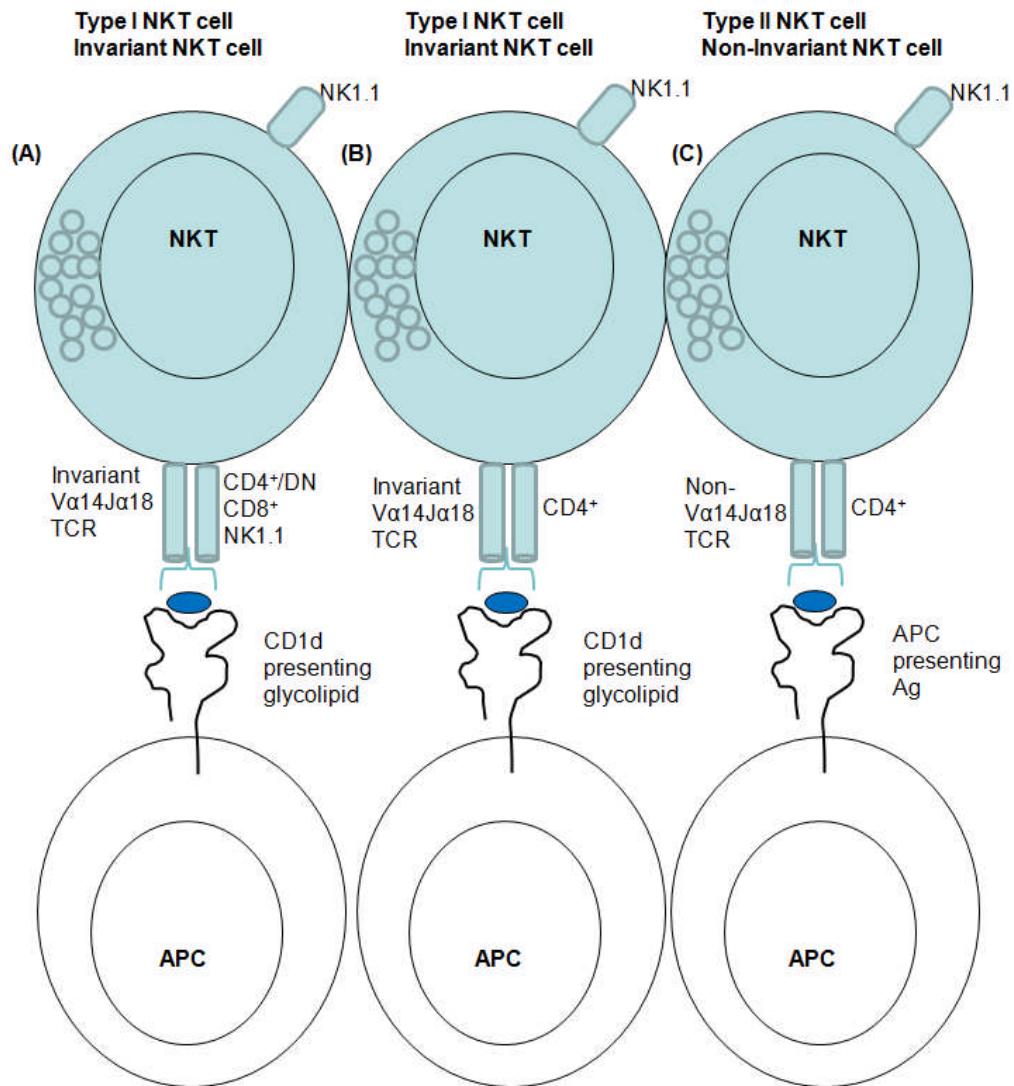


Fig. 1.4 Schematic diagram summarising the different NKT cell subsets. (A) Type I/Invariant NKT cell (NK1.1⁺); (B) Type I/Invariant NKT cell (NK1.1⁻); (C) Type II/non-invariant NKT cells

1.4.1.2 Function of NKT cells

NKT cells recognise glycolipid Ags bound to CD1d molecules present on APCs such as B cells and DCs. This pathway enables NKT cells to react to numerous infectious pathogens and therefore play crucial roles in the immune response. NKT cell activation can result in either stimulation or suppression of the hosts' immune responses as NKT cells are capable of inducing different responses depending on exposure to different circumstances (Matsuda *et al.*, 2008). Following stimulation, rapid cytokine production occurs with the first cytokines seen within a few hours following activation. These

cytokines are essential to trigger Ab production by B cells and development and proliferation of T_c cell for target killing (Bendelac *et al.*, 2007). NKT cells are now known to produce IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF- α , TGF- β and GM-CSF in addition to IL-4 and IFN- γ (Coquet *et al.*, 2007; Coquet *et al.*, 2008; Michel *et al.*, 2007; Sakuishi *et al.*, 2007). Moreover, NKT cells have the capacity to induce killing of other cells as their name suggests and evidence also shows NKT cells have an ability to influence other immune cells (Matsuda *et al.*, 2008). Overall, in disease states, NKT cells have been shown to respond quickly to infection. Through cytokine production, NKT cells can regulate other immune cells such as NK cells, CD4⁺ T cells, CD8⁺ T cells, B cells and macrophages, partake in tumour surveillance and rejection along with bacterial clearance of infectious agents (Matsuda *et al.*, 2008).

1.4.1.3 CD1d family and their antigens

CD1 molecules are a family of glycoproteins expressed on white blood cells such as B cells that present lipid Ags rather than protein Ags to T cells (reviewed Barral & Brenner, 2007; Brigl & Brenner, 2004; Dascher, 2007). CD1 isoforms are made in the T cell's endoplasmic reticulum and are then transported to the surface. Unlike MHC molecules, CD1 molecules are internalised for a second time into various cellular compartments where lipid binding takes place (Cohen *et al.*, 2009). It is now well established that T cell recognition of lipid Ags is crucial in pathogen detection and elimination. While mice and rats possess CD1d only, humans possess five isoforms (CD1a-e) (Calabi *et al.*, 1989), four of which present Ags to T cells (CD1a-d). CD1 molecules can recognise a range of lipids including sphingolipids, mycolates, diacylglycerolipids and phosphomycoketides. CD1 molecules have also been shown to present microbial lipids from mycobacteria (Fischer *et al.*, 2004). CD1a, b and c are

mainly expressed by APCs and have been shown to present mycolic acids, lipopeptides and polyisoprenoid lipids (Brigl & Brenner, 2004; Porcelli & Modlin, 1999), while CD1d is mainly expressed by epithelial, parenchymal, myeloid and vascular smooth muscle cells and has been shown to present α -GalCer (see section 1.4.1.4 below) as well as a small number of bacterial lipids (Kawano *et al.*, 1997; Venkataswamy & Porcelli, 2010). When presented by CD1d, NKT cell Ags are generally composed of a hydrophobic lipid tail that is immersed into CD1d and a hydrophilic sugar head region sticking out of CD1d molecule that interacts with the NKT TCR. NKT cell Ags such as α -GalCer contains an α -linked hexose sugar connected to the lipid backbone in contrast to the β -linked structures that most mammalian glycolipids are composed of (Godfrey *et al.*, 2010a). NKT cells are capable of recognising both exogenous and endogenous Ags (Mattner *et al.*, 2005). An exception is the NKT cell agonist isoglobotrihexosylceramide (iGb3) which harbours a β -linked galactose molecule (Zhou *et al.*, 2004). iGb3 as a natural ligand for NKT cell activation has proven controversial as it is not needed for the positive selection of NKT cells. iGb3 has not been found to be located in thymocytes and DCs of both mice and humans using high-performance liquid chromatography HPLC (Speak *et al.*, 2007). Moreover, NKT cells developed normally in mice that did not possess the enzyme needed for iGb3 synthesis (Porubsky *et al.*, 2007).

1.4.1.4 α -Galactosylceramide

The first Ag recognised by V α 14 NKT cells in mice was α -GalCer (Kawano *et al.*, 1997). α -GalCer was initially isolated from the marine sponge *Agelas mauritianus* when research was being undertaken to test compounds with anti-cancer properties (Natori *et al.*, 1993; Natori *et al.*, 1994; Zajonc & Kronenberg, 2007). Structurally, α -GalCer

possesses an α -linked hexose sugar to the lipid backbone as shown in Fig. 1.5. This is unusual seeing as mammals do not have the ability to generate α -linked glycolipids. For NKT cell activation to occur, the lipid must first be loaded into the CD1 binding groove. When α -GalCer is binding to CD1d, the lipid portion fits tightly and with high affinity into the binding groove of CD1d with the sphingosine chain embedded in the F' pocket while the acyl chain is embedded in the A' pocket. For NKT cell activation to occur, the C26 acyl chain of α -GalCer binds to A' while the sphingosine base is embedded in F' of CD1d binding groove with high affinity (Koch *et al.*, 2005). The α -linkage of the hexose sugar ensures the ring of the sugar is positioned parallel to the top of the binding groove of the CD1d molecule, an action needed for activation of NKT-TCR to occur (Zajonc & Kronenberg, 2007). The high specificity for the galactose monosaccharide has also been deciphered. Due to the unusual isolation and structure of α -GalCer, it is thought not to be a naturally occurring ligand for NKT cell activation.

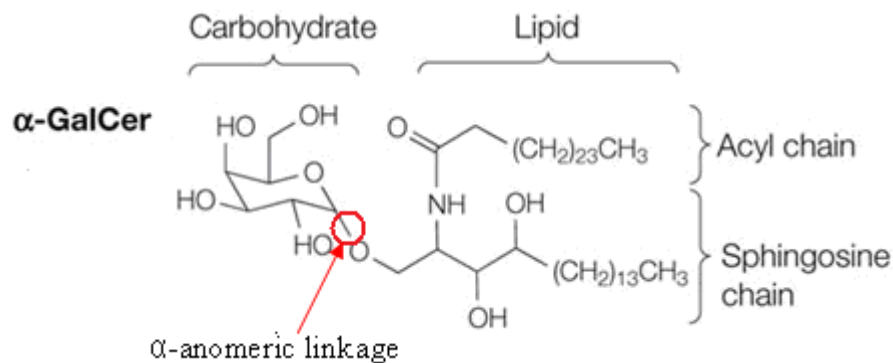


Fig. 1.5 Structure of α -GalCer highlighting the unusual α -linkage of the hexose sugar to the lipid. Image adapted from Van Kaer, (2005). Permission to reproduce this image has been granted by Luc Van Kaer.

1.4.1.5 Natural Killer T cell Antigens

Much interest has been shown in finding naturally occurring ligands that stimulate NKT cells and research is ongoing. To date, *Sphingomonas* and *Borrelia burgdorferi* have been identified as naturally occurring ligands for NKT cell stimulation *via* the production of IFN- γ and IL-4 cytokines (Amprey *et al.*, 2004; Kinjo *et al.*, 2005; Kinjo

et al., 2006; Sriram *et al.*, 2005). Other bacteria such as *Escherichia* and *Salmonella* have demonstrated a capability of NKT cell activation; however this process occurs indirectly through APC interaction with TLRs on host cells (Brigl *et al.*, 2003).

1.4.2 $\gamma\delta^+$ T cells

The majority of mature T cells are composed of an $\alpha\beta$ -TCR which recognises protein Ags presented by APCs and bound to MHC class I molecules to CD8⁺ cells or MHC class II molecules to CD4⁺ T cells. $\gamma\delta^+$ T cells are the second T cell lineage and these cells make up approximately 2-6% of human CD3⁺ peripheral blood lymphocytes, present in much higher numbers in the epithelium and the small intestine, (reviewed by Carding & Egan, 2002; Witherden & Havran, 2011). The heterodimer is made up of γ and δ chains can have several chain variations (Kabelitz, 2011; Bonneville *et al.*, 2010). Moreover, different subsets have been shown to display different functions as discussed in section 1.4.2.1. As shown in Fig. 1.6, other surface molecules typically expressed by $\gamma\delta^+$ T cells include NKG2D, TLRs and Dectin 1 (Bonneville *et al.*, 2010). $\gamma\delta^+$ T cells are the first population of cells to emigrate from the thymus to their intended locations functioning to monitor the surrounding environment for external stresses, infection and cell transformation (Witherden & Havran, 2011).

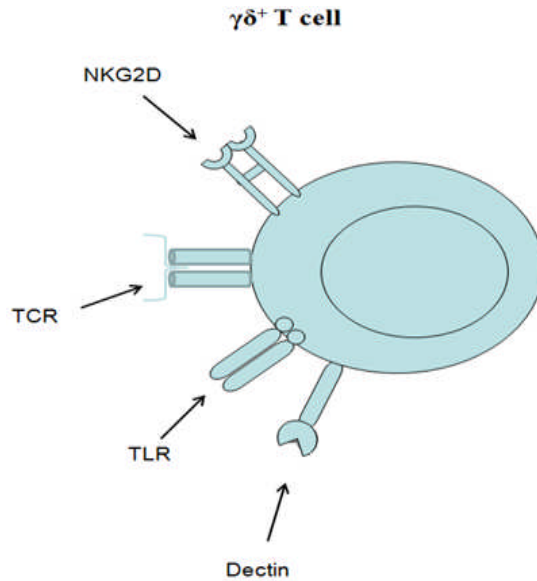


Fig. 1.6 Image showing surface molecules typically expressed by $\gamma\delta^+$ T cells including NKG2D, TLRs and Dectin 1 in addition to the TCR. Image adapted from Bonneville *et al.*, (2010). Permission to reproduce this image has been granted by Marc Bonneville.

1.4.2.1 Subsets of $\gamma\delta^+$ T cells

Not unlike B cells and $\alpha\beta^+$ T cells, the TCR of $\gamma\delta^+$ T cells is constructed with V, D and J gene segments. Variability in $\gamma\delta^+$ T cell subsets arises through different TCR canonical arrangements. In mice, $V\gamma 1$ and $V\gamma 4$ T cell subsets are located in the spleen, $V\gamma 5V\delta 1$ are located in the epidermis while $V\gamma 1V\delta 6.3$, $V\gamma 4$ and $V\gamma 6$ are present in the liver and $V\gamma 7V\delta 4$, $V\gamma 7V\delta 5$, $V\gamma 7V\delta 6$ subsets are found in gut epithelial tissues (Bonneville *et al.*, 2010). In humans, the $V\gamma 9V\delta 2$ subset predominates in peripheral blood, the $V\delta 1$ subset is located in the thymus, spleen, liver, gut epithelium and dermis while the $V\delta 3$ subset is primarily found in liver and gut epithelial tissues (Bonneville *et al.*, 2010). Many of these subsets possess varying antigenic specificities (Carding & Egan, 2002).

1.4.2.2 Function of $\gamma\delta^+$ T cells

Not unlike NKT cells, $\gamma\delta^+$ T cells have been shown to have multiple functions including pro-inflammatory cytokine production, potent killing effects following encounter with unconventional Ags (phosphorylated metabolite Ags or mycobacterial ligands), regulation of immune response and triggering of adaptive immune responses through

Ag processing and presentation to classical T cells (Bonneville *et al.*, 2010; Kabelitz, 2011). Therefore, $\gamma\delta^+$ T cells differ from their NKT cell counterparts in their location, their incidence in epithelial and mucosal tissues, the Ags they recognise and their ability to present Ags to T cells. Dendritic epidermal T cells (DETCs) are $\gamma\delta^+$ T cells which reside in the skin are known to play a role in wound repair and homeostasis in murine tissues (MacLeod & Havran, 2011; Sharp *et al.*, 2005). DETCs are important as epidermal cells need to be constantly replenished to form a protective barrier from external factors that challenge the body. It has also been shown that $\gamma\delta^+$ IELs present in the gut epithelium (see section 1.5) have a function in epithelial homeostasis and as mice deficient in $\gamma\delta^+$ IELs develop inflammatory bowel disease in animal disease models (Chen *et al.*, 2002; Inagaki-Ohara *et al.*, 2004). $\gamma\delta^+$ T cells are involved in regulation of inflammation through their involvement in renewal of epithelial cells following tissue injury. For example, studies have revealed that keratinocyte growth factor-1 production by DETCs causes hyaluronan generation by keratinocytes and that macrophage recruitment to wound sites is hindered in mice deficient in $\gamma\delta^+$ T cell expression (Jameson *et al.*, 2005). In keeping with inflammatory properties, activated $\gamma\delta^+$ T cells are also capable of chemokine and cytokine production. Pro-inflammatory cytokines produced by $\gamma\delta^+$ T cells include IFN- γ and TNF- α (Wang *et al.*, 2001) while secretion of the anti-inflammatory cytokine IL-4 by $\gamma\delta^+$ T cells has also been demonstrated (Dobmeyer *et al.*, 2002; Ferrick *et al.*, 1995). Finally $\gamma\delta^+$ T cells participate in the immune response to infectious pathogens and tumours. For instance, $\gamma\delta^+$ T cells are implicated in tumour surveillance by playing a role in cell lysis of melanomas and epithelial cells deemed cancerous (Dieli *et al.*, 2001; Koizumi *et al.*, 1991; Oliaro *et al.*, 2005).

1.4.2.3 $\gamma\delta^+$ T cell antigens

Since $\gamma\delta^+$ T cells are $CD4^-$ and $CD8^-$ they do not recognise conventional protein Ags presented by MHC class II or class I molecules respectively. Instead $\gamma\delta^+$ T cells recognise unconventional Ags such as phosphorylated microbial metabolites and lipid Ags rather than Ags processed through APCs (Constant *et al.*, 1994; Pfeffer *et al.*, 1990). An interesting finding is that murine $\gamma\delta$ -TCR becomes activated by non-classical class I molecules T10 and T22 (Schild *et al.*, 1994; Weintraub *et al.*, 1994) while human $\gamma\delta^+$ T cells can interact with CD1c (Porcelli *et al.*, 1989). In addition to self-reactive $\gamma\delta^+$ T cells, $V\gamma9V\delta2^+$ T cell TCR is stimulated by phosphoantigens which are of bacterial or tumour cell origin (Correia *et al.*, 2009). This process is strengthened further by interaction with TLR ligands directly and by dendritic cell activation by TLR ligands which occurs indirectly (Beetz *et al.*, 2008). Activating and inhibitory NK receptors also influence the $\gamma\delta^+$ T cell immune defence against infection and tumours. For example, $\gamma\delta^+$ T cells can kill tumour cells following NKG2D interaction with the mouse version of stress proteins MICA and MICB (Ferrarini *et al.*, 2002). Therefore $\gamma\delta^+$ T cells form part of the innate immune response and this clearly differentiates them as an unconventional T cell population from the classical $\alpha\beta^+$ T cells (He *et al.*, 2010).

Human $\gamma\delta^+$ T cells may be activated by <1 nM of phosphoantigen, secreted *via* the isoprenoid pathway functional in several microorganisms. The $V\gamma9V\delta2^+$ TCR recognises pyrophosphate Ags such as isopentyl pyrophosphate (IPP) and [(E)-1-hydroxy-2-methyl-but-2-enyl 4 diphosphate or HMB-PP] *via* the isoprenoid synthetic pathway (Constant *et al.*, 1994; Hintz *et al.*, 2001; Tanaka *et al.*, 1995). HMB-PP can stimulate $\gamma\delta^+$ T cells at picomolar and nanomolar ranges while the concentration of IPP needed to stimulate $\gamma\delta^+$ T cells is in the micromolar range (Kabelitz, 2011). IPP, produced *via* the mevalonate pathway, is present in healthy cells but concentration

increases in tumour or infected cells, which can consequently be detected by $\gamma\delta^+$ T cells (Gober *et al.*, 2003).

V δ 1 and V δ 3 subsets predominately residing in intestinal and mucosal tissues display high diverse repertoire in their TCR and this suggests their ability to recognise a large number of different Ags (Bonneville *et al.*, 2010; Hayday, 2000). Antigenic ligands for these V δ 1 and V δ 3 $\gamma\delta^+$ T cell subsets are still poorly understood. It has been hypothesised that MHC class I chain-related gene A (MICA) is a ligand for V δ 1 cells as this Ag which is present in healthy colonic epithelium, is up-regulated in tumour infected epithelial cells (Groh *et al.*, 1998). However, the V δ 1-MICA complex binds with a very low affinity (Zhao *et al.*, 2006). The location of $\gamma\delta^+$ T cell subsets in the epithelium of mucosal sites, their ability to rapidly produce cytokines, to exert cytotoxic effects and to induce cell-mediated responses implies a role for them in the early stages of immunity to infectious agents. At the later stages of infection, $\gamma\delta^+$ T cells display anti-inflammatory responses by participating in cessation of the immune response (Boismenu & Havran, 1994; Carding & Egan, 2002).

Regarding the role of $\gamma\delta^+$ T cells in microbial immunity, *M. tuberculosis* (Li *et al.*, 1996), *Listeria monocytogenes* (*L. momocytogenes*)(Mombaerts *et al.*, 1993) and HIV (Kabelitz & Wesch, 2001) have been shown to induce $\gamma\delta^+$ T cell activation. Key functions such as proliferation and apoptosis of $\gamma\delta^+$ T cells were hampered in HIV-1 seropositive patients when compared with controls. A reduction in the $\gamma\delta^+$ T cell subset V γ V δ 2 was observed in the peripheral blood and bronchoalveolar lavage of patients suffering from *M. tuberculosis*, implying a function for these cells in *M. tuberculosis* mediated immunity (Carding & Egan, 2002). Regarding *L. monocytogenes*, $\gamma\delta^+$ T cells respond during both early and late stages of infection and therefore contribute to both innate and adaptive immune responses to infection (Carding & Egan, 2002).

1.4.3 Natural killer receptor⁺ T cells

The term NKR⁺ T cell describes any T cell bearing a NK receptor and is independent of expression of variant/invariant TCR- α chain (Doherty *et al.*, 1999; O' Keeffe *et al.*, 2008). NKR⁺ T cells respond rapidly following stimulation and produce an array of cytokines including IFN- γ , TNF- α , IL-2 and IL-4 (McMahon & Raulet, 2001; Satoh *et al.*, 1996). T cells which are also CD56⁺ are known for their potent cytokine secretion, in addition to displaying NK-like and T cell restricted cytotoxicity in *in vitro* studies (Kelly-Rogers *et al.*, 2006; Loza *et al.*, 2002). The CD161 marker is known to be a co-stimulatory molecule that also plays a part in the regulation of T cells that are CD1d restricted (Exley *et al.*, 1998). T cells expressing CD94 are involved in the body's protection against viruses and autoimmunity (Moser *et al.*, 2002). When stimulated, NKR⁺ T cells become activated and respond quickly by secreting a range of cytokines that include IFN- γ , IL-2 and IL-4. Moreover, NKR⁺ T cells can display cytotoxic effects that are not restricted by MHC molecules (Doherty *et al.*, 1999) and have been shown to have an impact on bacterial colonisation (Nieuwenhuis *et al.*, 2002). Therefore NKR⁺ T cells can be viewed as innate like lymphocytes.

1.4.4 Mucosal associated invariant T cells

Recently, studies have identified the presence of another population of unconventional T cells which reside in mucosal tissues; these are aptly named MAIT cells. MAIT cells reside mainly in the gut lamina propria of humans and mice but are also found in human blood where they can make up 15% of DN T cell population (Treiner *et al.*, 2005). MAIT cells are generally not present in the intestinal epithelium but can be found in Peyer's patches. MAIT cells are not characterised well to date due to their rarity but evidence has shown commensal flora is necessary for their expansion in the lamina

propria as MAIT cells are not present in germ free mice (Treiner *et al.*, 2003). The existence of MAIT cells was first discovered when Porcelli *et al.*, (1993) who at the time were working on invariant V α 24J α 18⁺ NKT cells, noted the expression of a different invariant TCR α -chain; that of V α 7.2J α 33 (Porcelli *et al.*, 1993). MAIT cells are predominantly either CD8⁺ or DN in humans with only 1/5000 V α 7.2⁺ T cells being CD4⁺ T cells (Treiner *et al.*, 2005). MAIT cell selection and development is dependent on MHC, class I-related (MR1) molecule. Structurally MR1 consists of a heterodimer of a MR1 heavy chain and β 2 microglobulin (Kawachi *et al.*, 2006). The ligands required for MR1-MAIT interaction are currently unknown but any new evidence would provide clues to the MAIT cell function (Chua & Hansen, 2010). Currently, it is hypothesised MAIT cells have a role to play in gut defence against pathogens entering the body through the mouth and may have a part to play alongside DCs in the lamina propria in their role as an APC to T cells (Godfrey *et al.*, 2010b; Le Bourhis *et al.*, 2011). Le Bourhis *et al.*, (2010) reported stimulation of V α 7.2⁺T cells from peripheral blood by monocytes infected with *E. coli* (Le Bourhis *et al.*, 2010). Moreover, a proportion of T cells known to be MAIT cells due to their interaction with MR1 were capable of recognizing DCs infected with *M. tuberculosis* (Gold *et al.*, 2010). To date, MAIT cells have been found to be activated by bacterial and fungal but not virally infected cells (Le Bourhis *et al.*, 2010).

1.5 MUCOSAL IMMUNITY

1.5.1 Introduction to the mucosal immune system

The mucosal immune system consists of the gastrointestinal tract (GIT), the urogenital tract, the upper and lower respiratory tracts along with the exocrine glands that are associated with the above tracts. Mucosal immunity functions to protect the body's

internal surfaces; it constitutes the largest portion of immune tissues (Janeway *et al.*, 2005). The mucosal immune system harbours in excess of 40% of all lymphocytes, and is responsible for production of the majority of Ig in non-infected individuals (Lackner *et al.*, 2009).

The GIT portion of the mucosal immune system is composed of the oral cavity, oesophagus, stomach and intestine. The mucosal tissues of the GIT within the stomach form a physical barrier to invading external pathogens and are also a port of entry for other foreign Ags such as food and commensal bacteria (Kaiserlian *et al.*, 2005). This physical barrier comprises a single layer of epithelial cells constituting adsorptive cells, goblet cells, enteroendocrine cells, Paneth cells, M cells (Nochi & Kiyono, 2006) and IELs (see section 1.5.3) which separates the core from the external environment. Epithelial cells are tightly bound together by adhesion molecules such as occludin, claudin and E-cadherin (Nochi & Kiyono, 2006). Hence, the efferent epithelium forms a formidable first line of defence against organisms such as toxins, enzymes, food Ags and pathogens (Turner, 2009). The intestinal tract contains both non-immunological and local immunological host defence mechanisms. In addition to the epithelium, non-specific host defences include commensal flora, secretions such as mucins, antibacterial substances such as antimicrobial peptides, lysosyme, bile salts, Abs and the movement of intestinal peristalsis which encourages movement along the small intestine thereby controlling proliferation (Mahida *et al.*, 1997; Walker, 1976). The liver also plays a role in defence as Kupffer cells are suggested to act as a filter. Therefore, Kupffer cells are selective in the molecules they allow into the periphery. Specific immunity includes production of Igs and the Ag induced cell mediated immune response (Walker, 1976).

The GIT harbours up to 10^{14} microorganisms which co-exist peacefully with the local mucosal immune system (Ley *et al.*, 2006). Symbiotic microbes function to protect the host from pathogenic bacterial infections by outcompeting pathogenic invaders for nutrients and space, provide nutrients for the host such as vitamin B and folate and are important in establishing the profile of the mucosal immune system (Macpherson & Harris, 2004; O'Hara & Shanahan, 2006). Although gut microflora reside peacefully in mucosal tissues, just like pathogenic bacteria, they too possess PAMPs and therefore are capable of activating the innate immune response through PRRs on host cells. In order to prevent unnecessary and overactive mucosal immune responses such as uncontrolled inflammation and tissue destruction, two strategies are adopted including the presence of an intestinal barrier in the form of the epithelium (Fig. 1.7) and the mucus layer which physically separates indigenous flora from the host and regulation of immune responses (Cheroutre & Kronenberg, 2005; Goto & Kiyono, 2012). To protect underlying tissues from pathogenic microorganisms, the GIT has adopted a series of non-specific host defence mechanisms including secretions such as mucins to minimise adherence of potential infectious agents to the epithelium surface thereby preventing proliferation. Gastric acid and pepsin also function to minimise bacterial colonisation in the GIT. Lysosyme which possesses bactericidal properties has been found in the GIT; hence this enzyme may also be acting as a protective barrier. Natural Abs have also been isolated from the intestine (Walker, 1976). A study by Gibbons & Houte, (1975) suggests these Abs target specific intestinal pathogens but do not react against the normal gut flora.

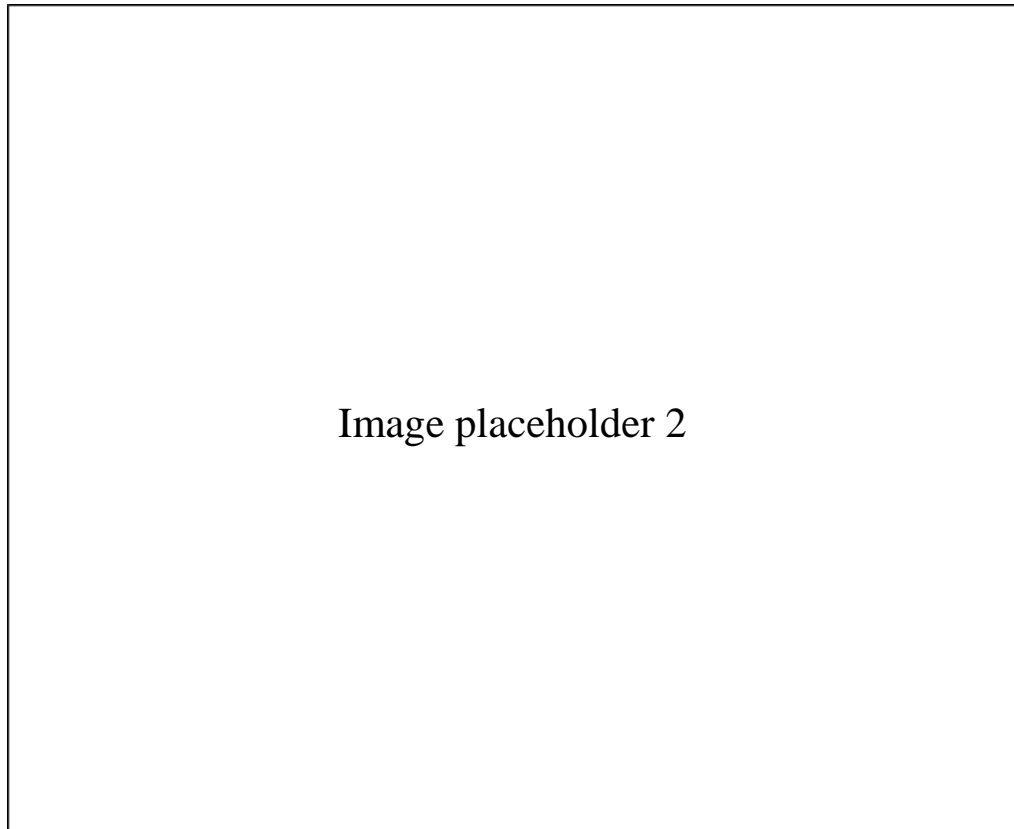


Fig. 1.7 Diagram illustrating mucosal exposure to Ags and the ensuing immune response. Taken from Smith *et al.*, (2011). Figure has been removed due to copyright restrictions

1.5.2 The gastric mucosa

The gastric antrum portion of the GIT or stomach is made up of the epithelium, the lamina propria and a layer of muscle called the muscularis mucosae (Janeway *et al.*, 2005). The epithelium is a continuous sheet of non-keratinised cells one cell thick and is covered by a layer of mucus. This mucus layer is produced by epithelial cells and is composed of 95% water, the remaining 5% contains lipids and glycoproteins and functions to prevent translocation of molecules such as Ags and ions into the gastric mucosa (Springer, 1970; Walker, 1976). The mucus gel exposed to the stomach lumen has a pH of 1-2, however the pH changes to that of 6-7 along the epithelial surface when not exposed to gastric juices. Not unlike intestinal tissues, cells residing in the gastric mucosa include T cells, macrophages, DCs, mast cells and plasma cells. The numbers of

lymphocytes residing in mucosal tissues are high with approximately 5-20% of lymphocytes located in the gut (Ganusov & De Boer, 2007). The epithelial monolayer mainly harbours epithelial cells and a substantial number of lymphocytes; in particular CD8⁺ T cells (O'Keeffe *et al.*, 2008). As stated previously, epithelial cells are in contact directly with the external environment and therefore provide the first line of defence against foreign Ags. These Ags may be in the form of infectious microorganisms, Ags from foods products, toxins and enzymes, all of which trigger a localised immune response. The underlying lamina propria provides important support for the epithelium and is highly vascular. The lamina propria layer is composed of connective tissue found beneath the mucosal epithelium in the GIT (Janeway *et al.*, 2005; Wu *et al.*, 1999). Blood and lymphoid vessels can cross the lamina propria and this layer contains cells involved in innate and adaptive immunity. The lamina propria contains lymphocytes, mainly a small number of T cells, predominantly CD4⁺, plasma cells, macrophages, mast cells, DCs, neutrophils and eosinophils which infiltrate into tissues during infection (Hatz *et al.*, 1996; Lundgren *et al.*, 2005).

1.5.3 Intestinal Intraepithelial lymphocytes and lamina propria layer lymphocytes

Much research has been undertaken in the areas of intestinal immunity and the cells residing in the intestinal compartments. The intestine is not only involved in absorption of minerals from foods, digestion and elements of secretion but also has a role to play in host defence (Cheroutre & Kronenberg, 2005). As stated above, not all Ags are noxious substances as some are dietary Ags and commensal flora and therefore, the cells of the mucosa must be able to distinguish between harmless and potential pathogens.

There exists two separate compartments in the intestine where immune cells are contained. The afferent compartment, where Ags are firstly recognised and presented to

adaptive cells, and which incorporates gut associated lymphoid tissues, mesenteric lymph nodes, the Peyer's patches and intestinal lymphoid follicles, and the efferent compartment which encompasses the epithelium and the lamina propria (Cheroutre, 2004; Cheroutre & Madakamutil, 2004) (Fig. 1.8). In the afferent compartment, DCs present Ags and provide co-stimulation needed for activation of naïve T cells. DCs also function to activate membrane receptors which allow attachment of cells to mucosal endothelium and this is signature for gut homing of immune cells. Gut homing triggers initiation of effector or T_{reg} cells (Meresse & Cerf-Bensussan, 2009).

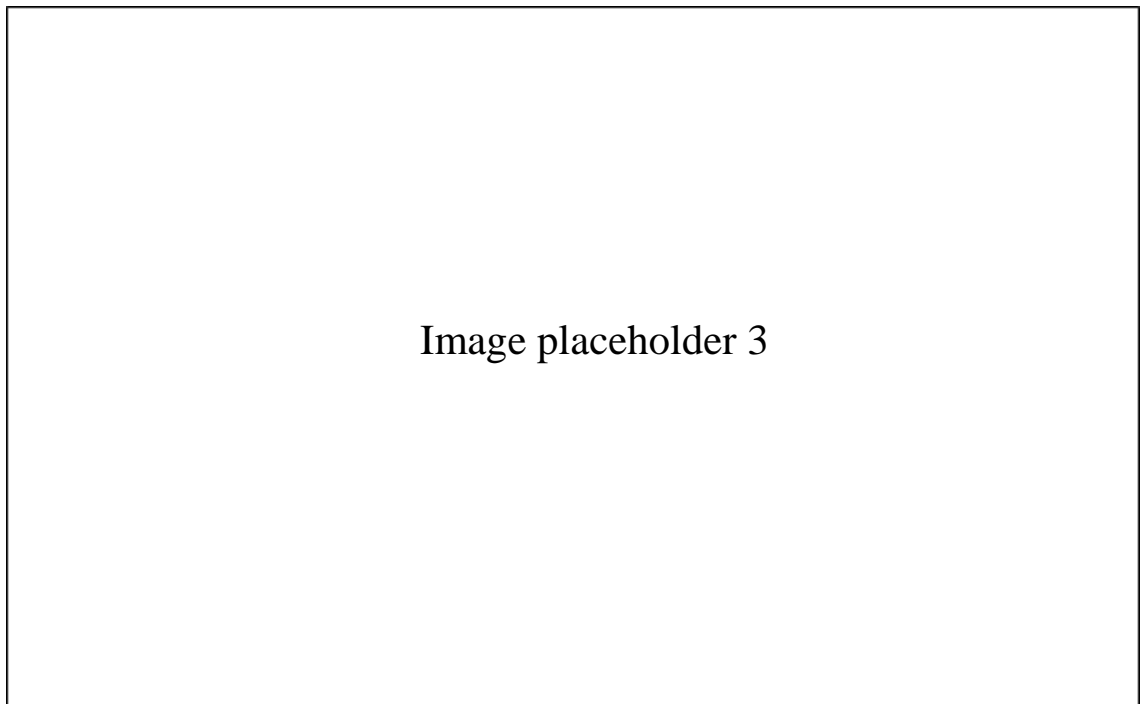


Fig. 1.8 Diagram of the epithelial and lamina propria layer showing IELs, macrophages, DCs, M cells and lymphoid follicles. Image taken from Gewirtz & Madara, (2001). Figure has been removed due to copyright restrictions

Intraepithelial lymphocytes

Mucosal T cells located in epithelial and lamina propria layer compartments are distinctive regarding their initial differentiation into effector T cells, the Ags they

recognise, the way in which they recognise these Ags and the functions carried out following antigenic interaction (Cheroutre & Kronenberg, 2005; Meresse & Cerf-Bensussan, 2009). The intestine is rich in lymphocytes with the epithelium harbouring one T cell per five to ten epithelial cells (Cheroutre & Kronenberg, 2005). IELs which reside in intestinal epithelium are the front line defence against infectious agents. IELs are primarily T lymphocytes and most possess cytoplasmic molecules exerting cytotoxic activity (Corazza *et al.*, 2000; Guy-Grand *et al.*, 1991; Lundqvist *et al.*, 1996). IELs are capable of secreting cytokines such as IL-2, IL-4, IL-17 and IFN- γ (Darlington & Rogers, 1966; Müller *et al.*, 2000; Roberts *et al.*, 1993). IELs express the CD103 molecule which is important for regulating intestinal immune responses (Annacker *et al.*, 2005). IELs also express CD8 $\alpha\alpha$ homodimers in conjunction with CD4 or CD8 co-receptors (Denning *et al.*, 2007; Kilshaw & Murant, 1990; Leishman *et al.*, 2002), CD8⁺ T cells are enriched amongst IELs while CD4⁺T cells are more commonly found in lamina propria tissues (Cheroutre *et al.*, 2011; Lefrancois, 1991; O'Keeffe *et al.*, 2004). IELs can be subclassed into natural and induced IELs.

Natural IELs are T cells expressing either a $\alpha\beta$ - or $\gamma\delta$ -TCR and may be either CD8 $\alpha\alpha$ ⁺ or CD8 $\alpha\alpha$ ⁻ while they are CD4⁻CD8⁻CD2⁻CD5⁻CD28⁻LFA1⁻. Natural IELs undergo an alternative maturation process and subsequently migrate directly to the intestine (Cheroutre *et al.*, 2011). Induced IELs on the other hand are either CD4⁺ or CD8⁺ T cells with an $\alpha\beta$ -TCR canonical arrangement and display a memory like phenotypic arrangement. Induced IELs are formed in the thymus, following development they migrate to the periphery and reach full maturation following interaction with Ags (Cheroutre *et al.*, 2011).

Because of their location, natural IELs are assumed to be directed against self Ags whereas induced IELs encounter invading Ags deemed as non-self due to their

original location in peripheral blood (Cheroutre, 2005). Therefore, induced IELs increase according to increasing antigenic encounters and have the advantage of creating a personalised mucosal immune response to specific invading pathogens. Both natural and induced IELs are affected by the surrounding environment of the mucosa as studies have shown that both commensal indigenous microflora and dietary Ags influence IELs (Cheroutre *et al.*, 2011; Hashimoto *et al.*, 1978). IELs respond *via* a localised immune response against invading pathogens to prevent colonisation and protect the body. IELs also serve as regulators as they prevent heightened inflammatory responses triggered by commensal bacteria which could hinder epithelium integrity (Cheroutre & Madakamutil, 2005).

Lamina propria lymphocytes

In the lamina propria compartment, in addition to DCs and macrophages, a high frequency of CD4⁺ T cells can be found amongst lamina propria lymphocytes (Meresse & Cerf-Bensussan, 2009). Lamina propria lymphocytes are known to induce cytotoxicity and produce cytokines. This is illustrated by Braunstein *et al.*, (1997) who showed a high level of IL-10 production by lamina propria lymphocytes suggested to control inflammation in the intestine. More recently, Asigbetse *et al.*, (2010) demonstrated that intestinal CD8⁺ lamina propria lymphocytes expressing a $\gamma\delta$ -TCR produce IL-10 and IL-17 in mice. Moreover, several papers reported IFN- γ production by intestinal lamina propria lymphocytes during Crohn's disease (Fais *et al.*, 1991; Fuss *et al.*, 1996). Included in lamina propria lymphocytes are the unconventional T cell population MAIT cells. As stated above in section 1.4.4, MAIT cells are an unconventional T cell population believed to be important in mucosal infections due to their resident location (Gold & Lewinsohn, 2011).

1.6 MUCOSAL INFECTIONS

Mucosal infections are prevalent worldwide with an annual death rate ranging from 12,000 due to roundworm and hookworm infections to 4 million due to acute respiratory infections. Other mucosal infections causing significant worldwide deaths include diarrheal diseases (1.8 million annual deaths), tuberculosis (1.5 million annual deaths), HIV (2.9 million annual deaths) and hepatitis B (103,000 annual deaths) (Janeway *et al.*, 2008). Mucosal infections are so prevalent worldwide because mucosal tissues throughout the body are constantly encountering a large range of Ags varying from harmless commensal bacteria and foods to harmful pathogens (Holmgren & Czerkinsky, 2005). Defence is needed against infectious bacterial, virus and parasitic species that can invade mucosal tissues; however the mucosal immune system also must distinguish between these infectious agents and harmless commensal bacteria that form part of the indigenous gut flora. Therefore forming a balance between protective immunity and homeostasis is essential (Nochi & Kiyono, 2006). *Helicobacter pylori* (*H. pylori*) is one such gastroduodenal pathogen that triggers the mucosal immune system and induces chronic gastritis, peptic ulceration and the development of gastric cancer in some cases. *H. pylori* is discussed in detail in the forthcoming sections.

1.7 HELICOBACTER SPECIES

1.7.1 The other *Helicobacters*

With increasing numbers of *Helicobacters* being isolated from symptomatic humans, there is speculation that some species play significant roles in many serious human conditions. Species of *Helicobacter* have been reported in 142 different vertebrates to date (Schrenzel *et al.*, 2010; Smet *et al.*, 2011). Stacy & Wellehan, (2010) recently reported the potential discovery of a new *Helicobacter* species (*spp.*) isolated from the

pancake tortoise *Malacochersus tornieri* (Stacy & Wellehan Jr, 2010). Gastric *Helicobacter spp.* includes *Helicobacter felis* (*H. felis*), *Helicobacter mustelae* (*H. mustelae*), *Helicobacter acinonychis* and *Helicobacter heilmannii* (*H. heilmannii*). *H. felis* has been isolated from the stomach of cats (Lee *et al.*, 1988) and dogs and has the ability to induce gastritis, apoptosis and proliferation of epithelial cells in mouse models (Ferrero *et al.*, 2000). *H. suis*, normally associated with pigs, is implicated with proliferative epithelial cell responses, infiltration of lymphocytes, severe inflammation and identification of lymphoid tissue using Mongolian gerbils as a model of infection, highlighting its potential factor in human gastric disease (Flahou *et al.*, 2010). However, *H. felis* and *H. suis* do not possess a cytotoxin-associated gene pathogenicity island (*cag* PAI) and lack virulence genes such as vacuolating toxin A (VacA), blood group antigen-binding adhesion (BabA) and sialic acid-binding adhesin (SabA), that said, both species do contain their own unique properties implicated with colonisation and virulence (Arnold *et al.*, 2011; Vermoote *et al.*, 2011). Gastric *Helicobacter spp.* are characterised by sheathed flagella used as a form of protection in the acidic stomach environment, their production of oxidase and catalase, their sensitivity to bile and bile salts in comparison with enterohepatic *Helicobacters* (Whary & Fox, 2004). Chronic *H. heilmannii* infection induces thickness of the mouse stomach as a result of proliferative responses in the epithelium. In addition, lymphoid molecules were detected in the mouse mucosa and cytokine production was observed (Park *et al.*, 2008). Since *H. heilmannii* is the second most common inducer of gastric infection aside from *H. pylori*, these findings provide further insights into gastric *Helicobacters'* pathogenesis (Park *et al.*, 2008). While *H. heilmannii* generally manifests itself in dogs, cats, pigs and non-human primates, *H. heilmannii* has been shown to infect humans with its prevalence being 0.5% in the general population (Dieterich *et al.*, 1998; Morgner *et al.*, 2000;

Solnick & Schauer, 2001). Moreover, Morgner *et al.*, (2000), reported an association between *H. heilmannii* infection and mucosal associated lymphoid tissue (MALT) lymphoma. Interestingly, *Helicobacter spp.* do not seem to be limited to the gastric tissues, *Helicobacter* DNA has been identified in the pancreas of cats (Sjödin *et al.*, 2011) and in the liver of aged rhesus monkeys (Marini *et al.*, 2010). Enterohepatic *Helicobacters* differ morphologically and biochemically from gastric *Helicobacters* as they contain unsheathed flagella, have no requirement for urease production and possess a tolerance for bile and bile salts which facilitates survival in the bile rich intestinal environment (Solnick & Schauer, 2001). Enterohepatic *Helicobacter spp.* are located in the lower GIT of humans and mammals, colonizing areas such as the colon, ileum and the biliary tree resulting in hepatobiliary disease (Pandey & Shukla, 2009). The first enterohepatic species discovered in humans were *H. cinaedi* and *H. fennelliae* which were initially cultured from homosexual men with proctitis, and later in cases of gastroenteritis and bacteraemia. *H. hepaticus* is the best documented enterohepatic *Helicobacter spp.* (Ward *et al.*, 1994). This species colonises the intestinal tract of mice and leads to chronic hepatitis, hepatic tumours and typhlocolitis in immunocompromised individuals (Ward *et al.*, 1994). Advancements have been made in the last year as Fox *et al.*, (2010) showed how *H. hepaticus* influenced the growth of liver tumours in transgenic mice suffering from hepatitis C (Fox *et al.*, 2010). Interestingly, new revelations have emerged regarding the type IV secretion system (TFSS) of *H. hepaticus* promoting homeostasis in the host. TFSS minimised epithelium host responses and induced a polarised anti-inflammatory response thereby allowing this invading pathogen to form a symbiotic relationship with its host (Chow & Mazmanian, 2010). Taken together, these studies highlight infection with *Helicobacter spp.* seem to be complex and can lead to serious pathologies if left untreated. Therefore,

there is a possibility that *Helicobacter spp.* other than *H. pylori* may be important risk factors in disease.

1.7.2 The discovery of *Helicobacter pylori* (*H. pylori*)

In the late 19th century, the existence of spiral microorganisms in the stomachs of animals was reported by the German scientist Giulio Bizzozero (Bizzozero, 1893). Shortly afterwards, the presence of these organisms was noted in humans. (Krienitz, 1906; Luger, 1917; Pel, 1899; Kusters *et al.*, 2006). Initially, bacteria were thought to be contaminants from ingested foods. Since all efforts to culture bacteria were unsuccessful, these findings were dismissed and forgotten about (Blaser, 1996). It wasn't until the early 1980's when Barry Marshall and Robin Warren carried out breakthrough experiments on *H. pylori* and discovered its presence consecutively in 58 of 100 patients. Through self-ingestion experiments by Marshall and another volunteer, both of whom were healthy to begin with, Marshall and Warren showed these bacteria were capable of colonizing the human stomach, and hence, can induce gastric inflammation. Next, following elimination of bacteria, the pair showed inflammation also disappeared and recurred again with subsequent infection (Marshall & Warren, 1983; Marshall & Warren, 1984). In follow up experiments, *H. pylori* (formerly *Campylobacter pylori*) were shown to be the causative agents of gastrointestinal conditions such as chronic gastritis, peptic ulcers and gastric cancers (Fig. 1.9) (Marshall *et al.*, 1985; Morris & Nicholson, 1987). These findings were significant for other chronic infectious cases, as the discovery of a bacterium surviving in the harsh environment of the gastric mucosa and persisting to induce chronic infection highlighted the importance of understanding bacterial pathogenesis. As a result, since the 1980's there has been great interest in researching all aspects of *H. pylori* infection.

A great deal is now known regarding diagnosis, epidemiology, immune responses and treatment as a result of extensive worldwide studies. However, there are still many areas that to date are poorly understood and require further investigation to provide a comprehensive insight into pathogenesis of this gastric pathogen. For example, use of an effective vaccine and whether *H. pylori* eradication can decrease the risk of cancer, why the bacterium causes ulcers in some patients and not others as well as the role of unconventional T cells in *H. pylori* infection.

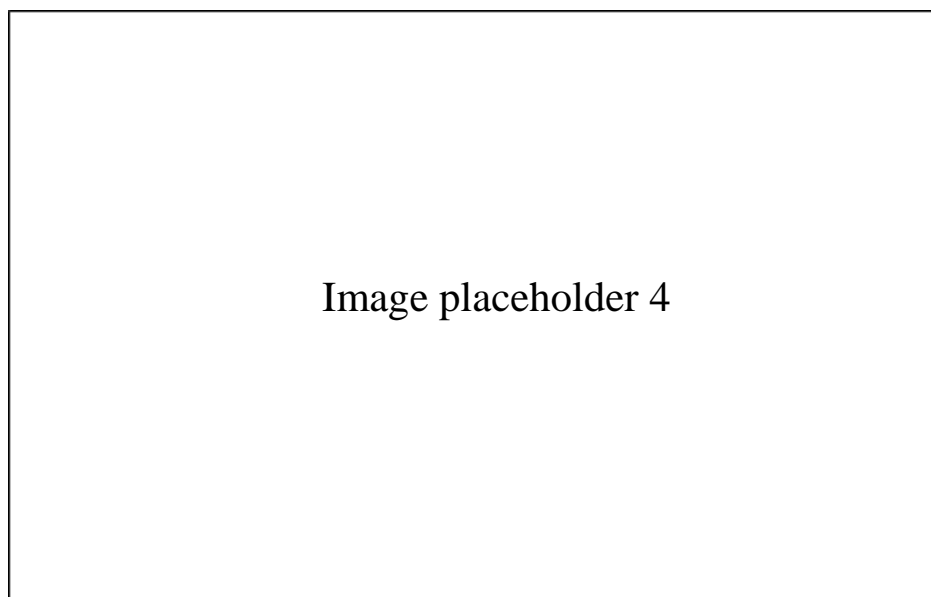


Fig. 1.9 Images of the various clinical outcomes associated with *H. pylori* infection. Image taken from Kim *et al.*, (2011a). Figure has been removed due to copyright restrictions

1.7.3 Taxonomy and characteristics of *Helicobacter pylori*

H. pylori is a member of the *Helicobacteraceae* family, a subdivision of the *Proteobacteria*, together belonging to the order *Campylobacterales*. *H. pylori* is a spiral shaped gram negative bacterium measuring 2-4 μm in length and 0.5-1 μm in width (Kusters *et al.*, 2006). *H. pylori* possesses 2-6 flagella which measure 3 μm in length (Fig. 1.10), these flagella assist in motility and allow the bacteria to swim and burrow into the protective gastric mucus layer overlying the gastric epithelium, a mechanism

later discovered to be important in the initial colonisation step (Lertsethtakarn *et al.*, 2011; O' Toole *et al.*, 2000). *H. pylori* is a microaerophilic organism, preferring oxygen levels of 2-5% and carbon dioxide levels of 5-10% at 37°C for optimal growth. Upon infection, the host exposes *H. pylori* to oxidative stress as an immune response mechanism, although *H. pylori* has adapted mechanisms to counteract this. For example, *H. pylori* produce large levels of urease to facilitate its survival and growth in the acidic gastric mucosa. Urease converts urea present in the gastric juices to bicarbonate and ammonia which are basic substances thereby neutralising the acidic gastric juices and allowing for survival in the gastric mucosa (Stingl *et al.*, 2002).

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Fig. 1.10 Electron micrograph showing morphology of *H. pylori*. Bar is equivalent to 1µm. Image taken from Yoshiyama & Nakazawa, (2000). Figure has been removed due to copyright restrictions

1.7.4 Structure of *Helicobacter pylori*

As shown in Fig. 1.11, the cell envelope of *H. pylori* is composed of an inner membrane, a periplasm containing peptidoglycan and an outer membrane. The outer membrane harbours lipopolysaccharide (LPS) and phospholipids. Interestingly, this phospholipid portion contains cholesterol glucosides, a feature not common in bacteria. *H. pylori* also contains a large number of outer membrane proteins (OMPs) such as

BabA and SabA (Haque *et al.*, 1996) which are important molecules in *H. pylori* infection (see section 1.7.4.2).

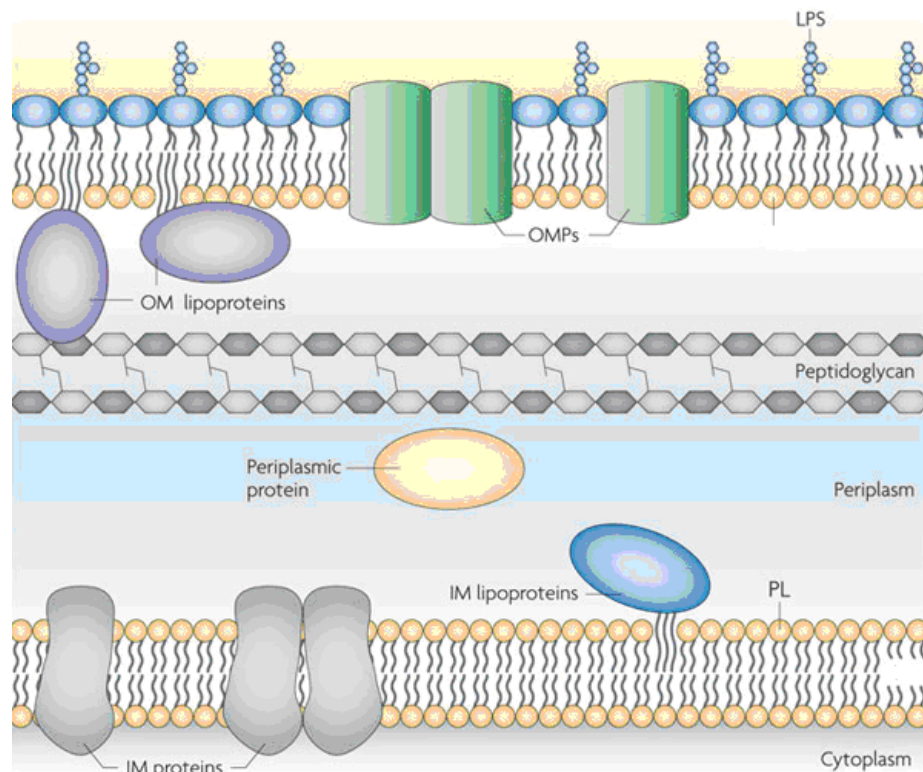


Fig. 1.11 Diagram illustrating the cell envelope of gram negative bacteria. Image taken from Ruiz *et al.*, (2009). Permission to reproduce this image has been granted by Thomas Silhavy.

1.7.4.1 Lipopolysaccharides

LPS was first discovered in 1965 when Westphal & Jann successfully isolated biologically active endotoxin from gram negative bacteria using the now widely used hot phenol-water extraction method (Westphal & Jann, 1965). Structurally, LPS is composed of a lipid portion known as lipid A, a core oligosaccharide and a polymeric sugar chain (Erridge *et al.*, 2002). The lipid A portion is responsible for LPS immunogenicity (Moran, 1995) as this PAMP is capable of activating both innate (Nielsen *et al.*, 1994) and complement immune systems (Morrison & Kline, 1977) ultimately leading to alterations in white blood cell counts, fever, shock and lethal

toxicity with high LPS dose (Lüderitz *et al.*, 1982). LPS is discussed in greater detail in chapter 4.

1.7.4.2 Outer Membrane of *H. pylori*

The bacterial OM forms a barrier to hydrolytic enzymes, detergents and hydrophobic antimicrobials (Tokuda, 2009). The OM is semi-permeable and size dependant regarding the materials it allows to pass through. Structurally, it consists of a 7nm thick asymmetric bilayer with an inner monolayer of phospholipid and an OM of LPS attached by lipoproteins to a peptidoglycan cell wall. Embedded in the OM are OMPs (Nikaido, 1996). The outer surface of OM is enveloped with surface proteins which vary between different bacterial *spp.* and strains can function as enzymes, adhesins, invasins or may prevent phagocytic destruction (Anderson & Wadström, 2001; Wilson *et al.*, 2008).

H. pylori possesses a number of types of OMPs functioning to aid virulence and pathogenesis (Radosz-Komoniewska *et al.*, 2005). *H. pylori* became the third bacterial genome to be sequenced (Tomb *et al.*, 1997) and since then a further 6 *H. pylori* strains have been sequenced. This genome sequence data revealed 32 OMP. Five OMP families exist including porins (Exner *et al.*, 1995) and adhesins which incorporates Hop2 proteins, while the remaining OMP families encompass the Hof4 family, Hom5 family, iron regulated OMPs and efflux pump OMPs. Together, *H. pylori* OMPs functions are vital for the bacterium's colonisation and persistence within the host (Odenbreit *et al.*, 2009). For example, Hop2 is a vital surface protein and loss of this protein incurs negative effects on *H. pylori* colonisation and cell density while AlpA and AlpB act as adhesions for gastric epithelial cells (Peyrol *et al.*, 1998). Interestingly, in a tactic to optimize bacterial colonisation and growth, bacteria are

capable of altering both OMP and LPS structure in response to its surrounding environmental conditions (Bishop, 2008; Gayet *et al.*, 2003).

1.7.5 Epidemiology of *H. pylori*

While it is known that *H. pylori* infects approximately half of the world's population, *H. pylori* prevalence varies throughout the world between differing geographical regions and ethnic groups. The incidence of *H. pylori* infection in developed countries is constantly declining, while in developing countries *H. pylori* affects up to 90% of the population (Bardhan, 1997). For example, recent studies carried out in African regions reported incidence rates of up to 91.3% (Aje *et al.*, 2010) while Epplein *et al.*, (2011) showed increased sero-positivity for *H. pylori* proteins among a population of African ancestry when compared to whites suggesting a link between *H. pylori* prevalence and ethnicity (reviewed in Goh *et al.*, 2011). Higher incidences of *H. pylori* infection have also been reported amongst individuals living in poor areas (Sarker *et al.*, 1997). Therefore, it can be deduced that there is a link between *H. pylori* prevalence and socioeconomic status. Moreover, epidemiological studies have shown that transmission rates were also found to be higher between infected siblings (Cervantes *et al.*, 2010) and between individuals living in close proximity (Fialho *et al.*, 2010). As living conditions have improved and antimicrobial treatment is available in the western world, it is less likely that *H. pylori* will be acquired. Triple therapy treatment also reduces the rate of carriers (see section 1.7.9). There is evidence to suggest that age is implicated in the incidence of *H. pylori* disease. In developing countries *H. pylori* infection increases in the early years of life remaining constantly high in latter years while in developed countries, the rate of infection is low in childhood and increases with age. Among children, there is an average incidence rate of 5% (Muhsen *et al.*, 2010).

New studies emerging suggest a correlation between *H. pylori* prevalence and other disease states. For instance the incidence of *H. pylori* disease was 85.5% among morbidly obese individuals requiring bariatric surgery (Al-Akwaa, 2010) and 75.3% in patients suffering from myelodysplasia (Diamantidis *et al.*, 2010). *H. pylori* transmission can occur either through the oral-oral route as *H. pylori* has been found in the oral cavity or *via* the oral-faecal route with *H. pylori* bacteria being isolated from drinking water in areas with poor hygiene living conditions (Ahmed *et al.*, 2007; Dattoli *et al.*, 2010). Eradication is not only proven to be cost efficient but also beneficial long term to community welfare. Regarding public health, *H. pylori* disease has also declined in recent years according to the United States Nationwide Inpatient Sample database. Data analysed from 1993-2007 recorded a steady decline in the incidence of *H. pylori* infection from 1998 onwards (Bashinskaya *et al.*, 2011).

1.7.6 Disease Transmission

H. pylori infection is transmitted from person to person and this process occurs through the oral-oral, gastric-oral through gastric reflux of bacteria or faecal-oral routes (reviewed in Kusters *et al.*, 2006). Transmission generally occurs in childhood with the earliest accounts reporting infection in children as young as 10 months old (reviewed in Amieva & El Omar., 2008). Increased transmission rates usually occur in crowded living spaces and sibling and/or maternal transmission are common (Goh *et al.*, 2011). Under conditions of stress, *H. pylori* bacteria change from the free-swimming helical form to the inactive coccoid form. It had been hypothesised that a possible transmission mechanism of *H. pylori* bacteria is to exist as a viable coccoid spore outside of the body and change into an infectious helical form once in the body. However, there is no evidence as of yet suggesting that *H. pylori* can revert back to the

helical form once this change occurs (Amieva & El Omar., 2008). It is more likely that infection occurs from direct contact with an infected person when *H. pylori* is temporarily out of the body as the bacterium is sensitive to high oxygen and light. *H. pylori* bacteria is not easily cultured from faecal matter from a healthy individual. However, when diarrhea is induced in an *H. pylori* infected person, bacteria can be cultured from up to 20% of patient samples. When vomitus was induced, viable *H. pylori* bacteria was culturable from almost all patients (reviewed in Amieva & El Omar., 2008).

1.7.7 Diagnosis of *H. pylori*

H. pylori infection can be diagnosed *via* invasive and non-invasive methods (Cutler *et al.*, 1995; Mégraud, 2005). Invasive methods include extraction of gastric biopsies for subsequent histology, biopsy culturing and the *Campylobacter* Like Organism or CLO[®] test which uses an indicator such as phenol red to detect a change in pH in biopsies from *H. pylori* infected individuals due to increased urease levels (Marshall *et al.*, 1987; McNulty & Wise, 1985). The urea breath test, serum analysis and the faecal Ag test are also among the non-invasive methods utilising bloods, breath, urine, stool or saliva for diagnosis. Recent advancements in methods for detection of *H. pylori* have been developed. Regarding invasive testing, Narrow Band Imaging (NBI) has recently been used for observation of mucosal and capillary patterns on the surface of the gastric tissues. Using this technique, Hidaka *et al.*, (2010) demonstrated a link between change in the pattern of collecting venules at two particular sites and the presence of *H. pylori* following endoscopy. This technique seems quite promising as high sensitivity and specificity values were reported for NBI (Hidaka *et al.*, 2010). NBI has now also been used to examine gastric mucosal tissues for changes following treatment and eradication

of infection (Okubo *et al.*, 2011). Confocal laser endomicroscopy uses an immunohistochemistry technique which allows an in depth analysis of the gastric mucosa (Kiesslich *et al.*, 2005) and recent studies have successfully identified *H. pylori* related gastritis utilising this method (Ji *et al.*, 2010). New methods of improving the sensitivity and the prevention of false negative results of the rapid urease test are currently being investigated (Ozaslan *et al.*, 2010; Vaira *et al.*, 2010). Using culturing as a method of detecting the presence of *H. pylori* in patients is still used, however culturing *H. pylori* from patient samples is reported to be laborious, hence this may not be the most effective method of diagnosis (see review by McNulty *et al.*, 2011).

Upon evaluation of the non-invasive methods of *H. pylori* diagnosis, new findings are emerging (reviewed in Calvet *et al.*, 2011) that stool Ag tests (SAT) for *H. pylori* detection are improving (Prell *et al.*, 2009), however, it is still debatable which SAT is the most efficient and SAT do not seem to be more effective than the current established methods (Calvet *et al.*, 2010). A limited number of studies incorporating saliva and dental plaque to detect *H. pylori* have been undertaken (Cellini *et al.*, 2010; Morales-Espinosa *et al.*, 2009; Namiot *et al.*, 2010). Additional experiments need to be undertaken to demonstrate their efficiency as diagnostic tools. While histology remains the gold standard method of *H. pylori* diagnosis utilised in clinical practice, factors such as location, hospital settings, age etc all affect and determine which method will be used (Kusters *et al.*, 2006).

1.7.8 Virulence and pathogenesis of *H. pylori*

Infection with *H. pylori* elicits chronic gastritis in nearly all patients (Ernst & Gold, 2000). However the symptoms, severity and associated conditions vary depending on factors such as the bacterial strain, host immune responses, host genetics and environmental factors such as diet (Wroblewski *et al.*, 2010). As shown in Fig. 1.12, bacterial components linked to pathogenicity include the cytotoxin associated gene product A (CagA) protein, the type IV secretion system (TFSS) encoded by the *cag* pathogenicity island (*cag*-PAI), VacA, LPS, bacterial adhesins such as BabA and SabA, peptidoglycans and other OMPs, protease HtrA, among others (Yamaoka, 2010). Infection with *H. pylori* can lead to severe gastritis, peptic ulcers and gastric cancers such as gastric adenocarcinoma and MALT lymphoma (Ernst & Gold, 2000). It has been deduced that some *H. pylori* strains are more virulent than others (Blaser & Atherton, 2004). The *cagA* gene is present in 50-70% of strains (Ching *et al.*, 1996; Covacci *et al.*, 1993) with CagA⁺ *H. pylori* strains have been shown to induce an increased inflammatory response with an increased risk of developing more severe forms of disease such as severe gastritis, gastric ulcers and gastric cancer (Blaser & Crabtree, 1996). The *cagA* gene forms part of the *cag*-PAI. Proteins encoded on this *cag*-PAI enable the construction of a TFSS which creates a syringe like structure penetrating the gastric epithelium thereby providing a pathway for CagA, peptidoglycans and other bacterial fractions to be internalised into host cells (Christie & Vogel, 2000; Terradot & Waksman, 2011). When inside epithelial cells, CagA is phosphorylated by Src family kinases, this phosphorylated CagA is involved with a range of host signalling events resulting in morphological changes in the gastric mucosa. *Cag* PAI-positive strains can induce apoptosis of host T cells and the TFSS can

trigger the production of a range of proinflammatory cytokines. It is thought that bacterial factors such as peptidoglycans induce this cytokine response (Tegtmeyer *et al.*, 2011).

It has been deduced that half of *H. pylori* strains harbour the vacuolating cytotoxin VacA. VacA is a 95kDa protein that causes pores to form in epithelial cell membranes thereby leaving the epithelium barrier vulnerable. This vacuolisation is instigated when *H. pylori* comes into contact with epithelial cells following direct delivery to the cell membrane surface. Other functions carried out by VacA include, induction of a proinflammatory response when bound to cell surface receptors, initiation of apoptosis when internalised and transported to cell mitochondria as well as inhibition of adaptive immune responses such as T cell proliferation (Cover & Blanke, 2005; Gebert *et al.*, 2003).

Another factor involved in *H. pylori* pathogenesis is urease, an enzyme produced by *H. pylori* implicated in converting urea into less harmful products ammonia and carbon dioxide (McGee & Mobley, 1999; Weeks *et al.*, 2000). Once *H. pylori* enters the gastric mucosa, it must progress to an area with a more neutral pH as the bacterium has been shown to lose its motility under exposure to the high acid environment of the gastric lumen (Schreiber *et al.*, 2005). The ammonia-induced increase in pH is believed to exert cytotoxicity on epithelial cells while the carbon dioxide has been shown to lower the effectiveness of the bactericidal nitric oxide metabolites produced by the host (Clyne *et al.*, 2007).

Bacterial flagellins are also implicated in *H. pylori* pathogenesis. *H. pylori* possesses 2-6 flagella encoded by two genes *FlaA* and *FlaB*. Flagellins assist in motility and allow the bacteria to swim and burrow into the protective and less acidic gastric

mucus layer overlying the epithelium. This mechanism forms part of the initial colonisation step (O' Toole *et al.*, 2000; Suerbaum, 1995).

Adhesins have been also shown to contribute to *H. pylori* pathogenesis. BabA is a 78kDa protein that assists binding of *H. pylori* to Lewis^b blood group Ags on host epithelial cells (Ishijima *et al.*, 2011; Yamaoka, 2008b), is believed to have an influential role in bacterial colonisation. SabA, another adhesin molecule contributes to *H. pylori* pathogenesis by assisting binding to sialic acid glycoconjugates, thus altered sialylated Lewis^a and Lewis^x play a role in *H. pylori* associated inflammation and gastric cancer (Ohno *et al.*, 2011; Yamaoka, 2008a). *H. pylori* can partake in molecular mimicry of host blood group Lewis Ags and this is another key factor in *H. pylori* pathogenesis. The O-chains of LPS can contain Lewis Ags along with blood groups A and B. The ability of *H. pylori* to express Lewis Ags that mimic self-epitopes has been implicated in bacterial colonisation and adhesion. Moreover, molecular mimicry can exert effects on the host by inducing inflammatory response in addition to polarisation of T cell subsets (Moran, 2008).

Overall, *H. pylori* employs a wide range of mechanisms from the moment it enters the gastric mucosa to aid successful colonisation and persistence (reviewed in Backert & Clyne, 2011). It does this *via* physical properties such as flagella for motility to penetrate the thick gastric mucus layer, adhesin proteins and production of enzymes such as urease to allow survival in harsh pH conditions (Atherton & Blaser, 2009). Virulence factors such as VacA and CagA along with factors which minimise host immune responses such as the bacterium's location are important for pathogenesis as host phagocytic cells do not appear to traverse the membrane; these in addition to molecular mimicry of host structures all play roles in *H. pylori* evading host immune responses and enhance colonisation (Fischer *et al.*, 2009; Molnar *et al.*, 2010).

Collectively, these factors contribute to *H. pylori* pathogenesis and chronic infection of the host.

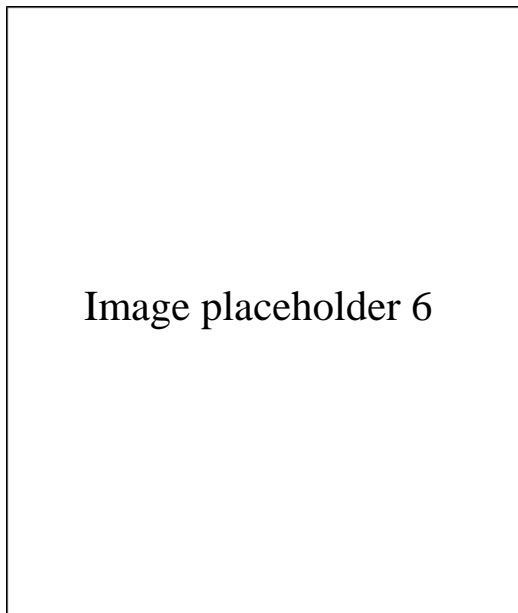


Fig. 1.12 Diagram showing *H. pylori* virulence factors. Image reproduced from Amieva & El-Omar, (2007). Figure has been removed due to copyright restrictions

1.7.9 Immunity to *H. pylori*

1.7.9.1 Innate and inflammatory immune responses to *H. pylori*

H. pylori colonisation of the gastric tissues triggers a complex immunological response initiating both innate and adaptive immune systems (for reviews see Algood & Cover, 2006; Kusters *et al.*, 2006; O’Keeffe & Moran, 2008; Sundquist & Quiding-Järbrink, 2010). Infection triggers an acute inflammatory response which involves the influx of immune cells such as neutrophils, lymphocytes, plasma cells and eosinophils. (Papadimitriou *et al.*, 1988) This occurs upon interaction of immune cells such as epithelial cells, DC’s and macrophages with PAMPs (Robinson *et al.*, 2007; Sobala *et al.*, 1991). However, this acute inflammatory response rarely leads to successful eradication of infection (Valle *et al.*, 1996). Host factors such as genetic predisposition and immune response, bacterial factors such as virulence factors along with environmental factors such as smoking and diet all contribute to disease outcome which

can result in chronic gastritis or in some cases leads to the onset of more severe pathologies such as peptic ulcers and gastric cancer (Blaser & Atherton, 2004; Montecucco & Rappuoli, 2001).

Innate and inflammatory immune responses occur early on in infection (Fig. 1.13). *H. pylori* induces the production of cytokines and chemokines such as IL-8, RANTES, Gro- α , MIP-1 α , IL-1, IL-6 and TNF- α (Bodger & Crabtree, 1998). The production of inflammatory chemokines is regulated by the transcription factor nuclear factor-kappaB (NF- κ B) (Baeuerle & Henkel, 1994). Initial nuclear factor kappa B (NF- κ B) activation is believed to be caused by epithelial cells (Baeuerle & Henkel, 1994; Münzenmaier *et al.*, 1997) and bacterial proteins within the *cag*-PAI (Glocker *et al.*, 1998). *CagA* is present in approximately 50-70% of *H. pylori* strains and is known to lead to a more exacerbated inflammatory response and patients infected with a *CagA*⁺ *H. pylori* strain are more likely to develop severe forms of disease such as peptic ulcers and gastric cancer (Blaser & Berg, 2001; Christie & Vogel, 2000; Covacci *et al.*, 1993). The number of *H. pylori* derived products that successfully induce a mucosal inflammatory response is constantly increasing with urease molecules, adhesion proteins, *H. pylori* derived heat shock proteins and VacA, all capable of causing damage to the gastric mucosa, inducing gastritis or triggering the secretion of pro-inflammatory cytokines (Hoffman & Garduno, 1999; Lamarque *et al.*, 2000) (Clyne *et al.*, 2007; Ibraghimov & Pappo, 2000).

H. pylori structures referred to as PAMPs, interacts with pathogen recognition molecules such as toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain-containing (NOD) proteins on innate cells such as epithelial cells, macrophages and DCs (Takeuchi & Akira, 2010). TLR and *H. pylori* interaction leads to the production of pro-inflammatory cytokines such as IFN- γ , IL-1 β , IL-6 and TNF- α

(Portal-Celhay & Perez-Perez, 2006). However, *H. pylori* is a weak agonist even with its virulent and pathogenic properties (Muotiala *et al.*, 1992). Gastric epithelial cells express TLR2, TLR4, TLR5 and TLR9 (Schmaußer *et al.*, 2004) which interact with PAMPs such as LPS, peptidoglycan, flagellin, lipoprotein, double stranded RNA and zymosan (Mogensen, 2009). While TLR4 and TLR5 are the TLRs implicated in most bacterial infections, *H. pylori* bacteria seem to interact with TLR2 (Mandell *et al.*, 2004). Therefore, TLR2 receptors in particular are important in the mucosal immune response to *H. pylori* infection. TLR2 interaction with the *H. pylori* lipopeptide neutrophil-activating protein (HP-NAP) leads to IL-12 and IL-23 production by APCs (Amedei *et al.*, 2006). TLR4 interacts with bacterial LPS (Park *et al.*, 2009). However, it is now known *H. pylori* LPS bioactivity is 500-1000 lower than that of LPS derived from other bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Bliss *et al.*, 1998; Perez-Perez *et al.*, 1995). TLR4 is present on gastric epithelial cells, and its function depends on the expression of the co-factor CD14 believed to be important for successful loading of LPS onto TLR4 (Delude *et al.*, 1995). However, studies suggest the absence of CD14 expression on gastric epithelial cells (Bliss *et al.*, 1998). *H. pylori* LPS activates NF- κ B, however this interaction occurs between NF- κ B and TLR2 instead of TLR4 (Smith Jr *et al.*, 2003). TLR5, expressed in the adult stomach (Schmaußer *et al.*, 2004), is implicated with the recognition of bacterial flagellin (Hayashi *et al.*, 2001). However the amino acid composition of *H. pylori* flagellins FlaA and FlaB are different from other bacteria, (Leying *et al.*, 1992; Suerbaum *et al.*, 1993) and so studies suggest that as a result, *H. pylori* may evade TLR5 recognition in this way (Fischer *et al.*, 2009). TLR4 and TLR5 activity is quite weak and *H. pylori* is a poor activator of these pathogen associated receptors (Lee *et al.*, 2003), consequently leading to survival of the bacteria and colonisation of the GIT. Moreover, *H. pylori*

evades TLR9 interaction (Suarez *et al.*, 2006) which recognises bacterial un-methylated CpG oligodeoxynucleotide (Rutz *et al.*, 2004). It has been suggested this is because of the high methylation rate of *H. pylori* DNA (Suarez *et al.*, 2006).

Another family of PRRs known as NOD proteins, have a crucial role in the immune response to *H. pylori* (Müller *et al.*, 2011). NOD1 is the receptor for bacterial peptidoglycans such as muramyl tripeptide (Chamaillard *et al.*, 2003), hence NOD1 is vital for innate mucosal response against *H. pylori* gastric invasion. NOD2, another member of this family recognises a muramyl dipeptide (Inohara *et al.*, 2003). IL-8, along with NF- κ B is induced by epithelial cells following NOD1 interaction with muropeptides from *H. pylori* peptidoglycan, triggering an inflammatory response (Viala *et al.*, 2004). Therefore, it could be argued that since innate recognition of *H. pylori* by epithelial cells occurs through NOD1, these proteins have a more central role to play than TLRs.

Another protein known as surfactant protein D (SP-D) is a PRR involved in innate immune response to *H. pylori* infection. SP-D is a collagenous protein expressed within gastric pits and topically at the gastric lumen. Increased SP-D expression has been recorded during *H. pylori* infection. *In vitro* studies have demonstrated that SP-D binds *H. pylori* bacterial cells in a lectin-like way, resulting in a decrease in *H. pylori* motility (Moran *et al.*, 2005; Murray *et al.*, 2002). Less understood are innate immune responses of innate T cells in *H. pylori* infection.

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Fig. 1.13 Illustration of *H. pylori* bacteria infecting gastric tissues and the ensuing localised immune responses. Image reproduced from Monack *et al.*, (2004). Figure has been removed due to copyright restrictions

1.7.9.2 Adaptive immune responses to *H. pylori*

It was first thought the adaptive immune response to *H. pylori* infection would primarily involve Abs as is the case with other mucosal infections (Kusters *et al.*, 2006) and also because Abs have a part to play in *H. pylori* colonisation in animals (Nomura *et al.*, 2005). However, it can be concluded from the literature that humoral immunity has minimal influence against *H. pylori* (Kusters *et al.*, 2006) and it is now widely known that adaptive immune responses as orchestrated by classical T cells has the greatest effect (Pappo *et al.*, 1999; Sutton *et al.*, 2000), albeit ineffective at times during *H. pylori* infection.

H. pylori infection initiates a polarised T_h1 response dominated by pro-inflammatory cytokines such as IFN- γ (Bamford *et al.*, 1998); however this seldom results in eradication of infection. In fact, the adaptive host immune response can play an important role in *H. pylori* pathogenesis (D'Elios *et al.*, 2003). T cells function to promote inflammation in infected gastric tissues, regulate the immune response and may also play a protective role (Akhiani *et al.*, 2002; Bodger & Crabtree, 1998; Kandulski *et al.*, 2010). Early studies investigating immune responses to *H. pylori* identified peripheral blood derived T cells from both healthy and *H. pylori* positive subjects proliferated in response to *H. pylori* antigenic stimulation (Karttunen *et al.*, 1990). Other studies recorded a reduction in responsiveness of memory T cells from peripheral blood of *H. pylori* positive subjects. It was then discovered that proliferative T cell responses returned following elimination of T_{reg} cells (Lundgren *et al.*, 2003). While both CD4⁺ and CD8⁺ T cells are recruited to the gastric mucosa upon infection, studies have shown a polarised T_h1 immune response dominates with the induction of IFN- γ and not IL-4 being observed. IL-12 is an important cytokine in *H. pylori* infection as it is associated with development of naïve T cells into mature T_h1 cells (Trinchieri, 1995). *H. pylori* infection induces the production of proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α (Fig 1.13) (Karttunen *et al.*, 1997; Meyer *et al.*, 2000). In addition, an increase in IL-17 and IL-18 has been implicated in *H. pylori* infected mucosa (Luzza *et al.*, 2000; Tomita *et al.*, 2001). In the last few years, several publications have highlighted the importance of the T_h17 response in *H. pylori* inflammatory and immune responses (Shi *et al.*, 2010). It is now believed to be as important as the T_h1 response (For more discussion on T cells in *H. pylori* infection, see section 4.1). T_h17 cells are a population of T cells that are induced in the presence of IL-23, IL-6 and tumour growth factor beta. T_h1 cells require the transcription factors

STAT1, STAT4 and T-bet (Szabo *et al.*, 2000) and T_h2 cells requires the transcription factors STAT6 and GATA-3 (Zheng & Flavell, 1997) for lineage differentiation. Unlike T_h1 and T_h2 cells, T_h17 cells instead require ROR γ T for T_h17 cell differentiation (Ivanov *et al.*, 2006). IL-17 production by T_h17 cells triggers chemokine production by immune cells such as fibroblasts, epithelial cells and macrophages resulting in the accumulation of polymorphs such as neutrophils (Ye *et al.*, 2001). A role for IL-17 in the onset of inflammation has been shown in other clinical conditions (bacterial infections) (Knauer *et al.*, 2007). Since chronic gastritis associated with inflammation and neutrophil recruitment is signature for *H. pylori* infection, it is likely that T_h17 cells are implicated in the development of gastric inflammation (see section 4.1.1). A study by Zulquernain *et al.*, (2012) (paper submitted) from our group found significant increases in IL-17 levels in sera and in gastric biopsies from *H. pylori* positive patients when compared with uninfected individuals.

In addition to the induction of T_h1 pro-inflammatory cytokines such as IFN- γ during the localised immune response to *H. pylori* infection, are the secretion of regulatory cytokines such as TGF- β (Lindholm *et al.*, 1998) by T_{reg} cells. T_{reg} cells function to maintain homeostasis in the body, control infection, and to prevent autoimmune disease development through suppression of B, T and NK cell activity (see reviews Belkaid, 2007; Izcue & Powrie, 2008; Mills, 2004). In *H. pylori* infection, CD4⁺CD25⁺ T_{reg} cells are infiltrated in *H. pylori* infected tissues (Lundgren *et al.*, 2005) especially when tumours are present (Enarsson *et al.*, 2006). Therefore while T_{reg} cells function to control inflammation by down-regulating protective immune responses (Lundgren *et al.*, 2003), their presence may actually be contributing to chronicity of infection. Less understood are the roles of unconventional T cells in the adaptive immune responses to *H. pylori* infection.

1.7.10 Treatment of *H. pylori* infection

It is generally believed that successful eradication of *H. pylori* following treatment has declined in recent years due to antibiotic resistance (Graham & Fischbach, 2010). The efficiency of dual, triple, quadruple and sequential therapies has been tested using a combination of different antibiotics to attempt to discover the treatment with the highest eradication rate. Dual therapies use a combination of proton pump inhibitor (PPI) with an antibiotic while triple therapy primarily uses a course of PPI with two different antibiotics. Triple therapy is most commonly used worldwide and due to development of resistance, quadruple therapy combining PPI, two antibiotics in addition to bismuth has been used. Combinations of clarithromycin, levofloxacin, ameprazole, amoxicillin and methronidazole have been tried and tested (Molina-Infante *et al.*, 2010) to find the highest success rate of first line eradication. Levofloxacin has been receiving attention lately for its success rate and is being used following bacterial resistance to clarithromycin, however, it is debatable whether levofloxacin should be used as first line therapy or second line therapy (Liou *et al.*, 2010). Bismuth has been introduced to triple and quadruple therapy in recent years (Malfertheiner *et al.*, 2011). Moreover, longer treatment appears to increase eradication rates (Sun *et al.*, 2010). Sequential therapy is also utilised to overcome clarithromycin resistance, whereby PPI is taken along with amoxicillin to compromise the cell wall of *H. pylori* thereby hindering channels being formed that prevent interaction between clarithromycin and *H. pylori*. A study by Gao *et al.*, (2010) provides evidence to support the efficacy of sequential therapy as they reported an eradication rate of 89% in comparison to 81% for triple therapy and 83% when the complex quadruple therapy using bismuth was used (Gao *et al.*, 2010). Probiotics have also been implicated with treatment of *H. pylori* infection. Probiotic bacteria compete with invading pathogenic bacteria and also trigger the host

immune response thereby preventing infection. Szajewska *et al.*, (2010) reported the use of *Saccharomyces boulardii* as an aid for eradication of *H. pylori* infection as this probiotic yeast significantly increased eradication rates when used as an adjunct during treatment (Szajewska *et al.*, 2010). As of yet it is debatable which form of therapy is the most efficient and which should be used as first line defence; clinical trials are ongoing to tailor the best treatment for patients.

1.7.11 Vaccines

While triple and quadruple therapies are used worldwide to combat *H. pylori* infection, efforts to develop a vaccine for *H. pylori* eradication have been ongoing since the 1990's (reviewed in Muller & Solnick, 2011). Since infection with *H. pylori* elicits a polarised T_h1 immune response (Portal-Celhay & Perez-Perez, 2006), it was initially thought that a vaccine tailored towards inducing a T_h2 immune response would eradicate infection (Saldinger *et al.*, 1998). More recently, *H. pylori* Ags such as urease, Omp18 and protein-conjugated LPS among others have been analysed as potential targets of vaccine development (Monteiro *et al.*, 2011; Talebkhan *et al.*, 2010; Wang *et al.*, 2010). Since *H. pylori* is more prevalent in areas with poor living conditions, poor hygiene and in low socioeconomic areas, improvements in standards of living in such areas would be a preventative method. Since infection rates are declining in the Western world, the need for a vaccine has been debated. However *H. pylori* is still a Class I Carcinogen and therefore, it is likely that methods to either reduce bacterial load or alter the immune response to *H. pylori* thereby decreasing the inflammatory response and lowering the threat of more severe disease forms would be welcomed.

1.8 HYPOTHESIS AND AIMS OF THIS THESIS

1.8.1 Hypothesis

H. pylori is established as an important and common bacterial pathogen of the gastroduodenal mucosa of humans, infecting about 50-60% of the world's population. This gram-negative bacterium is a cause of gastritis, is implicated in the development of peptic ulcers and is a co-factor in gastric cancer development. Infection in the mucosa elicits a complex immunological response involving neutrophils, plasma cells, eosinophils and lymphocytes, of which T cells are the principal orchestrators of immunity. There exists a diverse array of so-called unconventional T cells which may be central to the ensuing immune response. These include CD1d-restricted NKT cells and T cells bearing the $\gamma\delta$ -TCR. NKT cells are known to exert antimicrobial properties and are currently understood to have a major role in tumour surveillance. $\gamma\delta^+$ T cells display a broad range of functions including rapid cytokine production, potent killing activity, Ag processing and presentation and also possess regulatory functions. The hypothesis to be investigated in this study is that certain components of *H. pylori* stimulate the functional response of specific unconventional T cell populations *in vivo*, and variations in this response can determine the development of immunity or infection outcome in the gastric mucosa. Since NKT cells are known to rapidly secrete cytokines that can influence the adaptive immune response against tumours, overall, this new knowledge may potentially be important for therapeutic activation of NKT cells to prevent or reverse progression of *H. pylori* infection from asymptomatic form to the disease state. Thus, the extent and the effectiveness of NKT cells and $\gamma\delta^+$ T cells within the gastric mucosa and their interplay and responses to *H. pylori* is likely to play an important role in disease outcome.

1.8.2 Aims of present investigation

The specific objectives are:

1. To quantify and characterise T cells, NKT cells and $\gamma\delta^+$ T cells with respect to surface marker expression, cytokine production, proliferation and cytotoxicity in normal and *H. pylori* infected tissues
2. To correlate NKT and $\gamma\delta^+$ T cell phenotypes with detailed clinical parameters and disease associated with *H. pylori* infection. Ethical permission for these studies was obtained from the University College Hospital, Galway.
3. To evaluate the stimulatory capacity of *H. pylori* components and products on function of gastric epithelial and lamina propria cells, NKT cells and $\gamma\delta^+$ T cells. For stimulation, fractionated *H. pylori* preparations derived from bacterial membranes were tested along with purified LPS.

To undertake gene expression profiling on purified CD2⁺ cells isolated from the epithelial and lamina propria layer of gastric biopsies from patients infected with *H. pylori* to obtain detailed insights into host responses to bacterial infection and immunogenesis.

Chapter 2

Materials and Methods

2.1 SUBJECTS

This prospective study involved the enrollment of patients in an open access endoscopy unit attending for oesophago-gastro-deudenoscopy. The subjects used for this study were chosen by a gastroenterologist from the University College Hospital, Galway. Patients receiving antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressant's, or presenting with alcohol abuse, with previous gastric surgery or with other chronic inflammatory diseases and co-morbidities were excluded from the study. Clinical data including age, background history, medication history, symptom record with alarm score, smoking history, brief physical examination and endoscopy results were recorded. Of the 84 patients whose biopsies were utilised in this study; 32 were female. The patients ranged from 17-85 years of age (mean age was 46). Following CLO testing it was revealed that 25 patients were *H. pylori* positive while the remaining 59 tested negative. Specified ethical approval was obtained in conjunction with the University College Hospital Galway and the National University of Ireland, Galway for use of fresh gastric biopsies. Voluntary written consents were signed by all patients taking part in the study and their confidentiality was strictly maintained. Most of the patients included in the study had dyspeptic symptoms. Two biopsy samples from the gastric antrum portion of the stomach (see Fig. 2.2) were kept for diagnosis in University College Hospital Galway and the remaining biopsy samples were transported to the laboratory in calcium- and magnesium-free HBSS (Gibco-BRL, Paisley, UK) for gastric lymphocyte isolation and subsequent analyses. All of the *H. pylori* positive patients were diagnosed on the basis of *Campylobacter* like organism (CLO[®]) testing. CLO[®] testing was also utilised for histological analysis (Sydney Classification) of biopsy samples. A list of patients included in this study along with their clinical information is listed in Table 2.1. Peripheral blood mononuclear cells (PBMCs) samples

(n = 15) utilised in this study were obtained from healthy subjects (HS) from both University College Hospital, Galway and *via* the Student Health Clinic, National University of Ireland, Galway and also from University College Hospital, Galway. Serum is liquid separated from clotted blood (Fig. 2.14) and contains proteins (other than clotting factors), Abs, Ags, exogenous molecules and hormones. Serum was isolated from the peripheral blood of *H. pylori* positive (n = 12) and *H. pylori* negative (n = 12) patients by Dr. Syed Akbar Zulquernain in University College Hospital, Galway. Serum was stored at -80°C prior to use. Samples were collected from October 2007-March 2009.

Table 2.1 List of patients used in this study

Clinical status						
Total patients (n)	84					
Female (n)	32					
Male (n)	52					
Age (mean)	46.36					
Age (range)	17-85					
	n	Hiatus hernia	Ulcer	Antral gastritis	Fundal gastritis	Duodenitis
<i>H. pylori</i> positive	25	13	4	20	7	9
<i>H. pylori</i> negative	59	21	3	14	5	5

n = sample size; *H. pylori* = *Helicobacter pylori*

2.2 ISOLATION AND ANALYSIS OF IMMUNE CELLS

2.2.1 Separation of PBMCs from whole blood by density gradient centrifugation

A vacutainer (BD Vacutainer, BD Biosciences, Belgium) lined with lithium heparin to stop coagulation of blood was used to collect blood samples. For separation of PBMCs from whole blood, an equal volume of Hanks Balanced Salt Solution (HBSS) (Gibco, Paisley, UK) was added to the blood. This blood-HBSS mixture was layered over a volume of Histopaque-1077[®] (Sigma Chemical Co., St. Louis, MO) equivalent of the initial volumes so that a 1:1:1 ratio was obtained. The function of Histopaque is that of a

density gradient medium, its density was modified so that it was supplied at 1.077 g/ml. The blood sample was centrifuged at $373 \times g$ for 25 min. This led to the separation of the sample mixture to four different layers: the red blood cells gathered at the bottom, the histopaque layer rested on top of this layer, next the buffy coat which contained the PBMCs was evident, and finally a layer of HBSS was situated at the top (Fig. 2.1). The buffy coat which was the layer of interest was collected *via* a Pasteur pipette and PBMCs were washed with twice its volume of HBSS and the solution was centrifuged at $373 \times g$ for 10 min. The supernatant was disposed of and the pellet containing the PBMCs was re-suspended in the appropriate amount of RPMI-1640 medium (Gibco, Paisley, UK) which contained L-glutamine, 10% foetal calf serum (FCS) (Sigma Chemical Co., St. Louis, MO) and 5,000 units (U) of penicillin/5 mg streptomycin/ ml (Sigma Chemical Co., St. Louis, MO).

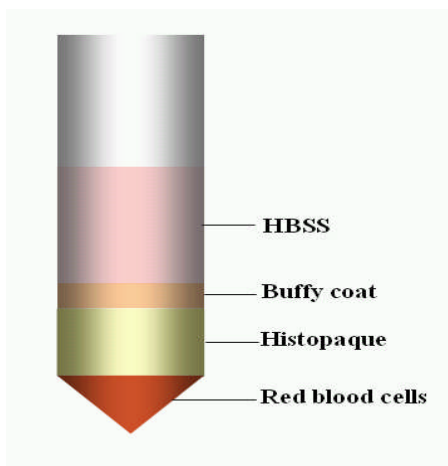


Fig. 2.1 Diagram showing the location of the lymphocyte population in the buffy coat following density gradient centrifugation of whole peripheral blood. Image supplied by Dr. Carol Gately

2.2.2 Preparation of epithelial and lamina propria layer single-cell suspensions from gastric biopsies

Single-cell suspensions of the epithelial layer and lamina propria (Fig. 2.2) were prepared from gastric biopsy specimens as described by Madrigal *et al.*, (1993). This involved rotating biopsy samples at 37°C for 1 h in calcium- and magnesium-free HBSS supplemented with 5% FCS, 1 mM dithiothritol (DTT) and 1 mM ethylenediamine tetraacetic acid (EDTA) (all from Sigma Chemical Co, St, Louis, MO) to remove the epithelial layer leaving the lamina propria and the basement membrane intact. The epithelial layer single-cell suspension was then washed in RPMI-1640 medium (Gibco-BRL) supplemented with 10% FCS and penicillin/streptomycin. The remaining lamina propria layer tissue was incubated in 5 ml of supplemented RPMI-1640 medium containing 130 U/ml collagenase (Type 1A, Sigma) and rotated at 37°C for 3 h to isolate the lamina propria layer cells. This suspension was washed in RPMI-1640 medium.

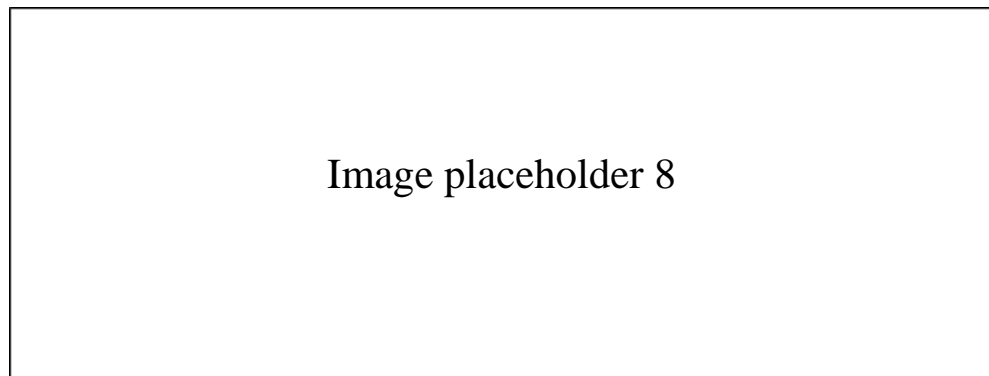


Fig. 2.2 Illustration of the gastric mucosa showing the epithelial and lamina propria layer tissues from which biopsies were extracted prior to analysis. Image adapted from Tortora & Grebowska, (1996).

This technique has previously been optimised by our group to ensure no cross contamination of epithelial and lamina propria layer cells occurred during the separation process (Fig. 2.3).

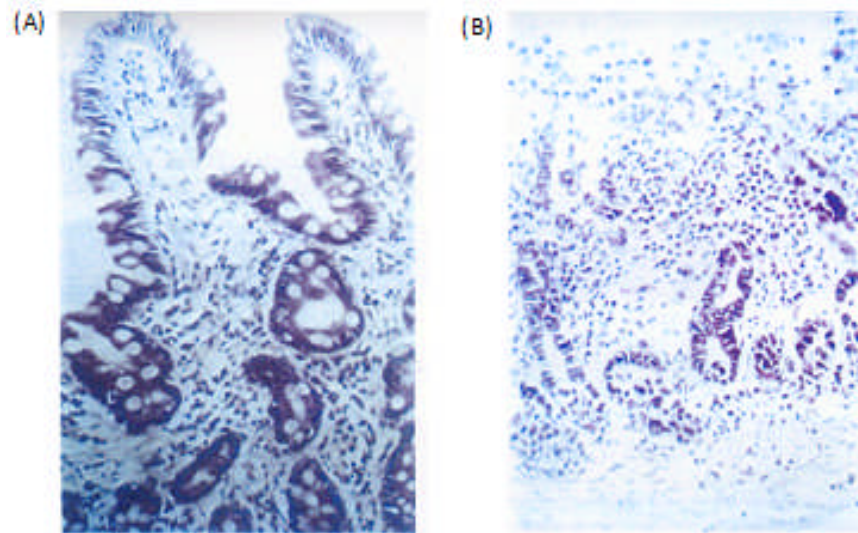


Fig. 2.3 Duodenal mucosa (A) before and (B) after EDTA/DTT treatment. The surface epithelium is removed while the lamina propria remains intact. Image supplied by Dr. Joan O' Keeffe.

2.2.3 Cell counting using a haemocytometer

For each blood sample, 10 μ l of freshly isolated PBMCs were placed onto a haemocytometer. This was then inserted under a light microscope and, accurate cell counts were performed by counting cells in all four chambers of the hemacytometer and calculating the average (Fig. 2.4). Cell numbers were then adjusted to 1×10^6 /ml using RPMI-1640 on the basis of the average cell numbers obtained.

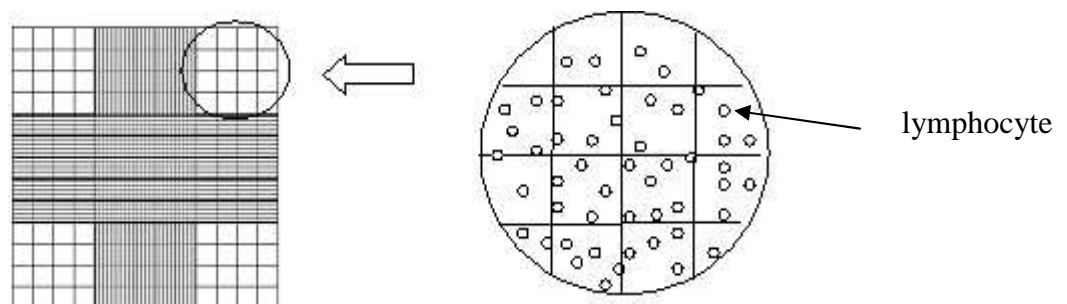


Fig. 2.4 Counting grid showing four quadrants of hemacytometer. One chamber has been enlarged to mimic the image seen under a light microscope. Cell counts were performed on all lymphocytes within this chamber and this action was repeated for each of the four chambers and the average number of cells was obtained.

2.2.4 Trypan blue staining of PBMC to determine cell viability

Trypan blue (Sigma Chemical Co, St, Louis, MO) was added to cells at a ratio of 1:10 for 5 min at room temperature (RT) before 10 μ l of this mixture was placed onto the hemacytometer. Cell viability was measured based on dye retention. When viewed under the microscope, live cells were unstained, whereas dead cells appeared blue due to the trypan blue stain entering the damaged cell walls of non-viable cells (Fig. 2.5). Cell numbers of live and dead cells were counted, and percentage of cell viability was determined $(\text{live cells} / (\text{live cells} + \text{dead cells})) * 100$.

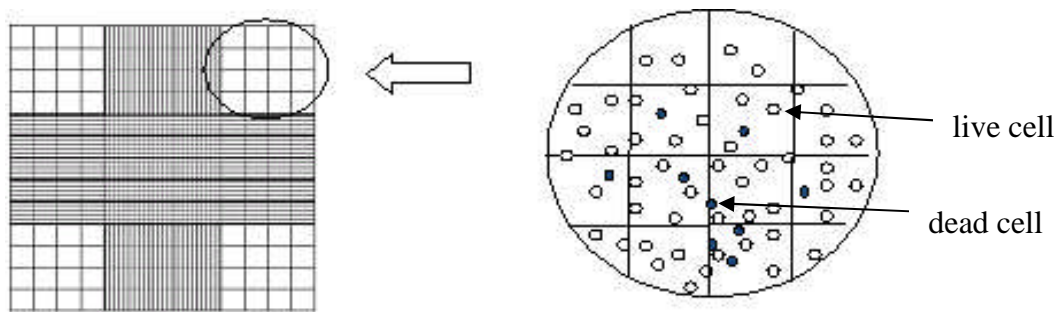


Fig. 2.5 Counting grid showing four quadrants of hemacytometer. One chamber has been enlarged to mimic the image seen under a light microscope. Cell counts were performed on all both live lymphocytes seen in white and dead lymphocytes seen in blue to calculate the percentage of viable cells.

2.2.5 Immunophenotyping using fluorescently labelled Antibodies

Immunophenotyping is a technique of identifying cells using cell surface Ags and markers such as proteins, glycoproteins, glycolipids and carbohydrates. This technique incorporates firstly labeling cell populations of interest with monoclonal Abs (mAb) marked with fluorescent labels directed against these membrane bound molecules before phenotypically characterising them by flow cytometry.

In order to detect the presence of a cell-Ab interaction, the Ab must first be fluorescently labeled either directly or indirectly (Stewart & Stewart, 1995).

Fluorescence is achieved using fluorochromes. Fluorochromes are dyes that emit light at specific wavelengths once excited by a beam of light at a particular wavelength. The light source used is usually an argon laser or an arc lamp and there are many types of fluorochromes for fluorescently labeling Abs such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), alexa flour® 488, PerCP™, Texas Red™, Cy3, Cy5, Cy7, CasB, CasY, TR and propidium iodide (PI) (Stewart & Stewart, 2004). Fluorochromes must ideally fulfill specific criteria before choosing the most suitable combinations for experimental analysis. Fluorochromes should:

- be biologically inert in that they bind to components of the cells but do not alter the cells in any way
- have high fluorescence intensities meaning a difference between stained and unstained cells should be clearly observed
- possess minimal spectral overlap between each other. This means limiting the signal from each fluorochrome to one specific intended detector. A technique known as compensation alleviates this problem which arises with increasing dye numbers as there are some overlaps in the emission spectra.
- be readily bound to mAb (Baumgarth & Roederer, 2000).

A small number of fluorescent dyes were used in this study. FITC is a derivative of fluorescein and is the most commonly used fluorescent compound (The & Feltkamp, 1970). Excitation of the FITC dye occurs at the 488 nm line (Fig. 2.6A) of an argon laser and this fluorochrome emits a green colour that has a maximum wavelength intensity of 520 nm. PE is a red protein typically found in cyanobacteria (Oi *et al.*, 1982), red algae (Rossano *et al.*, 2003) and cryptomonads (Ludwig & Gibbs, 1989). Only one PE molecule is conjugated to an Ab due to the large nature of the protein as it contains 25 fluors (Roederer, 1997). The PE dye is excited at 495 nm (Fig. 2.6B) and

once excitation occurs, an orange-red dye is emitted that is at its most intense at 578 nm (Winkelstein & Donnenberg, 1997). The third dye we used in this study was the cyanine (Cy) Cy5-PE dye, which is excited at 488 nm and has emission spectra of 670 nm and this fluorochrome emits a purple colour. PE-Cy5 is a tandem dye which prior to its generation, required *optimisation* of the two dyes. Once completed, the dye was equally successful for use as a mAb conjugant. Tandem dyes operate by utilizing fluorescence resonance energy transfer. In this case, the excited PE donor fluorochrome is capable of transferring its energy over a 1-6 nm distance to a Cy5 acceptor fluorochrome, which then fluoresces at a different and longer wavelength. When Cy5 was coupled with PE, little compensation was needed, as Cy5 has an emission peak at 667 nm (Fig. 2.6C) and therefore is already distanced from PE.

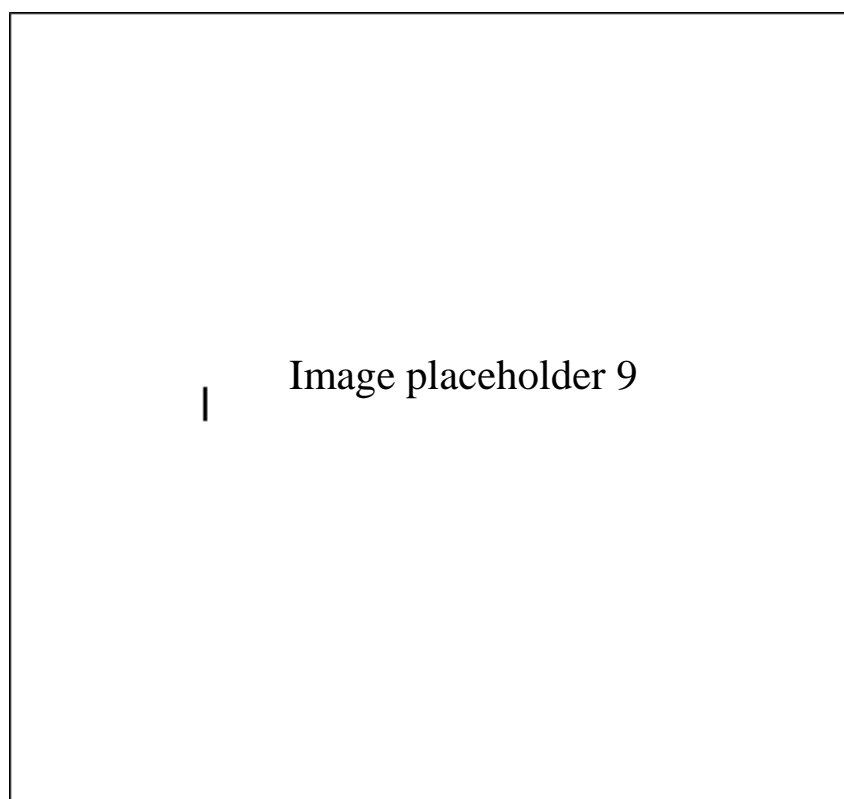


Fig. 2.6. Diagram showing absorption spectra of the fluorescent compounds used in this study (A) FITC spectra; (B) PE spectra; (C) PE-Cy5 spectra. Images taken from www.bdbiosciences.com/colors/spectra/ Figure has been removed due to copyright restrictions

The majority of fluorochrome dyes have a saturation rate of excitement at 100-150 mW so therefore it is ideal for each laser to possess 50 mW of power. These fluorescent labels were chosen as they were compatible with the single 488nm argon ion laser. Therefore the flow cytometer we used to analyse these fluorescently labelled cells accommodated the acquisition of tri-colour fluorescence.

2.2.6 Antibodies used for immunophenotyping and flow cytometry

CD3 was the cell surface marker used to identify T cells in this study. Other markers such as CD2, CD5 and CD7 are found on T cells but are also expressed on other cell populations and therefore one would not be presented with a pure T cell population. For instance, CD2 is found on both T and NK cells (Bolhuis *et al.*, 1986; Moretta *et al.*, 2000), CD7 marker can be found on both platelets and NK cells in addition to T cells (Leta *et al.*, 1996; Rabinowich *et al.*, 1994) and CD5 is expressed on B cells (Berland & Wortis, 2002). Therefore, CD3 whether being expressed on the cell surface membrane or inside the cell is the most specific marker for T cells (Campana *et al.*, 1989). The CD4 mAb was used to identify T_h cells while a CD8 mAb was used as a tool for T cytotoxic cell identification.

NKT cells express NK cell surface markers in addition to a V α 24J α 18 TCR- α chain canonical arrangement (Porcelli *et al.*, 1993). The marker used to identify human NKT cells was the V β 11 Ab directed against the conserved CDR3 region of the canonical V α 24J α 18 TCR (Table 2.2). Since human V α 24 NKT cells are paired only with V β 11, 6B11 alone or in combination with anti-V β 11 or anti-CD3 is an efficient way of analysing of NKT cell harbouring the invariant TCR α chain in humans.

An Ab directed against the $\alpha\beta$ -TCR was used to examine cells expressing the conventional $\alpha\beta$ -TCR canonical arrangement, which constitutes the majority of T cells,

while the remaining T cells express a $\gamma\delta$ -TCR and these cells were identified using an Ab directed against the $\gamma\delta$ -TCR. In humans, V γ 9V δ 2 subset predominates in peripheral blood while V δ 1⁺ $\gamma\delta$ ⁺ T cells are mainly found in intestinal and epithelial tissues. Since our Ab was directed against the $\gamma\delta$ -TCR, all subsets should be accounted for. These mAbs allowed us to determine the frequencies of T cells and in particular unconventional T cells in gastric tissues.

Table 2.2 List of cell surface markers used in this study, their fluoro-chrome labels and their concentrations.

Cell type	Cell marker	Fluorochrome -labelled	Stock conc. (mg/ml)	Final conc. (μ g/ml)	Source
T cell	CD3	Cy5	0.2	0.8	Serotec, Oxford, UK
	CD4	FITC	0.2	0.8	Serotec, Oxford, UK
	CD8	PE	0.2	0.8	Serotec, Oxford, UK
	$\alpha\beta$ -TCR	FITC	0.2	0.8	Serotec, Oxford, UK
	$\gamma\delta$ -TCR	FITC	0.2	0.8	Serotec, Oxford, UK
NK cell	CD56	PE	0.02	0.08	Serotec, Oxford, UK
		FITC	0.1	0.4	Serotec, Oxford, UK
	CD161	PE	0.1	0.4	Serotec, Oxford, UK
		FITC	0.1	0.4	Serotec, Oxford, UK
	CD94	PE	0.1	0.4	Serotec, Oxford, UK
NKT cell	V α 24	PE	0.002	0.008	Immunotec, Marseilles, France
	iV α 24J α 18	PE	0.002	0.008	BD Pharmingen, Oxford, UK

Cy5 = cyanine; PE = phycoerythrin; FITC = fluorescein isothiocyanate; NK cell = natural killer cell; NKT cell = natural killer T cell

2.2.7 Labelling cells with mAbs prior to flow cytometric analysis

Polystyrene round-bottom tubes (5 ml) were obtained for flow cytometry (BD Falcon, Oxford, UK). Isolated cells (1×10^5) were stained with 4 μ l of fluoro-chrome-labelled mAbs that were designed for humans at their optimised concentrations (Table 2.2). Multiple staining of the same samples was carried out using a combination of compatible Abs for cell characterisation (CD3, CD4, CD8 CD56, CD161, $\alpha\beta$ -TCR, $\gamma\delta$ -TCR, and V α 24J α 18). The cells were placed in the dark at 4 °C for 30 min and then 2 ml

of phosphate saline buffer (PBS) (Oxoid Limited, Hampshire, England) was added and solution was centrifuged for 7 min at $871 \times g$ to wash cells. The unwanted supernatant was discarded and the cell pellet was resuspended in 300 μ l of PBS if cells were being analysed within 24 h. Cell fix (300 μ l of 1X solution) (BD Biosciences, Oxford, UK) was added to those cells being analysed after 24 h. Cells were stored at 4°C prior to use. Cells were then acquired using a fluorescent assisted cell sorter (FACS) Calibur® (Becton Dickinson, Oxford, UK) flow cytometer and analysis was carried out using CellQuest software.

2.2.8 Flow cytometry as a method of immunophenotyping cells

Flow cytometry is a technology used to analyse and immunophenotype a range of specimens from cells and supernatants to cell surface and cytoplasmic Ags (Cyto Ags) in addition to DNA analysis and functional characterisation (see chapter 3). Recent advancements now means that many samples can be analysed at once and an increasing number of parameters can be simultaneously analysed (Jain *et al.*, 2011). It is also worth noting that compensation, validation and quality control are essential in flow cytometric immunophenotyping to ensure the highest quality results (Owens *et al.*, 2000) especially with increasing availability of the numbers of parameters being simultaneously measured (De Rosa *et al.*, 2001) and more and more clinical uses for flow cytometry constantly being discovered (Tang *et al.*, 2011).

2.2.8.1 Principle of flow cytometry

The general principles of flow cytometry are that it measures optical and fluorescence characteristics of a single cell. Flow cytometry incorporates firstly staining biological cells with mAbs of interest before injection into the vibrating flow chamber of the flow

cytometer. The cells are streamed into single file before emerging from the chamber immersed in a sheath of buffer. Each cell/bead in the stream is illuminated by light from a laser and each cell is scattered according to its size (forward scatter), granularity (side scatter) and cellular markers of interest (fluorescence), thereby providing information about the samples' properties. These parameters equate the number of cells which display a certain feature or cell surface marker. Parameters are measured in events. Events are formed when the vibration in the cell stream causes the cells to be charged one by one. Using deflection plates in conjunction with the computer analysis, these events are grouped accordingly. Once cells have been identified, they can be selected or gated on for subsequent analysis (see section 2.2.7) (Mandy *et al.*, 1995; Stewart & Stewart, 2004).

For this part of the study which involved isolating and analysing immune cells, flow cytometry was undertaken to phenotypically characterise immune cells from PBMCs, gastric epithelial and lamina propria layer tissues using immune cell surface markers (Fig. 2.7).

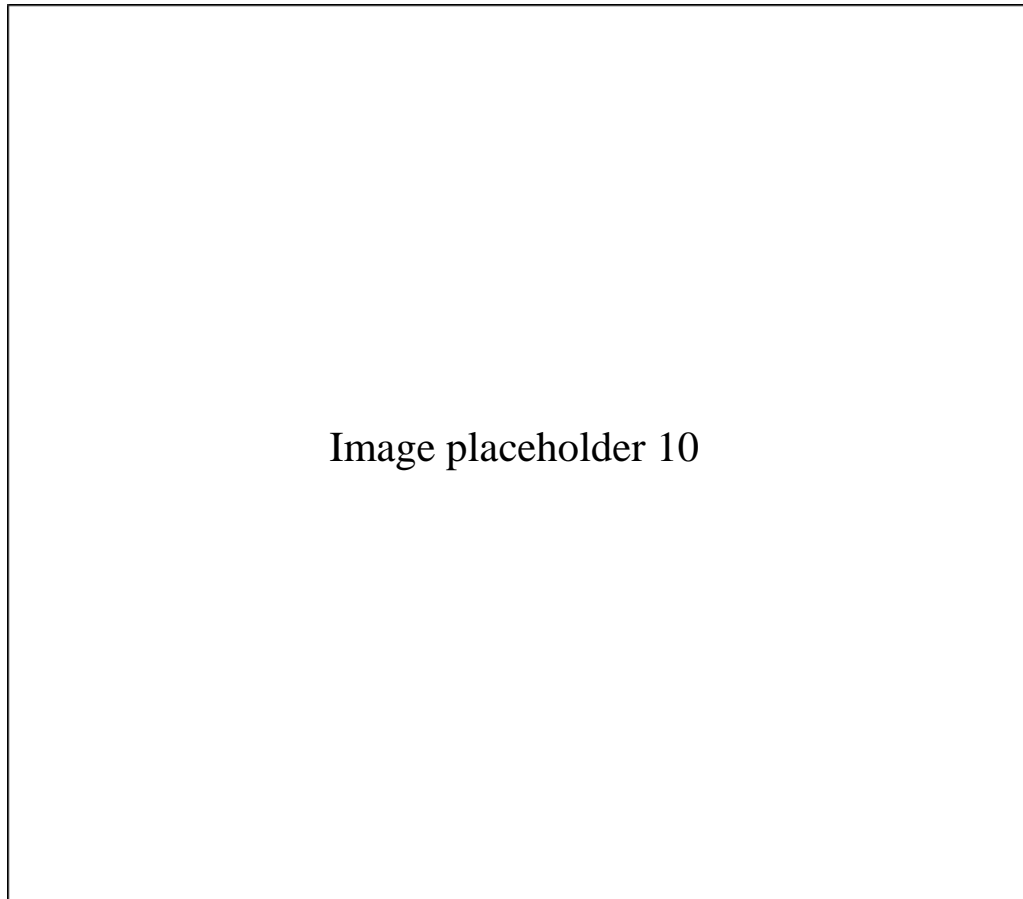


Fig. 2.7 Illustration showing the method of analysis of cell surface markers using flow cytometry. Image taken from http://www.medscape.com/viewarticle/532108_sidebar2. Figure has been removed due to copyright restrictions

2.2.8.2 Flow cytometry and analysis

FL1 (FITC), FL2 (PE) and FL3 (Cy5) were the channels for the fluorescently labelled Abs. Cells of interest were then differentiated based on their fluorescence by constructing dot plots in the following combinations (i) FL1 vs. FL2; (ii) FL1 vs. FL3; and (iii) FL2 vs. FL3. Data analysis was undertaken using CellQuest[®] lysis software (Becton Dickinson, Oxford, UK).

For phenotypic characterisation of human gastric epithelial and lamina propria layer T cell subsets in *H. pylori* positive and *H. pylori* negative patients (chapter 3), a population of 43 patients including *H. pylori* CLO positive (n = 9) and *H. pylori* CLO

negative ($n = 34$) individuals were used for comparing numbers of various lymphocyte subsets from *H. pylori* infected and uninfected gastric tissues.

In order to achieve this information, to begin with, the control tube which contained cells only was utilised to call up the FSC-SSC dot plot which was used to gate on lymphocytes (Fig. 2.8A).

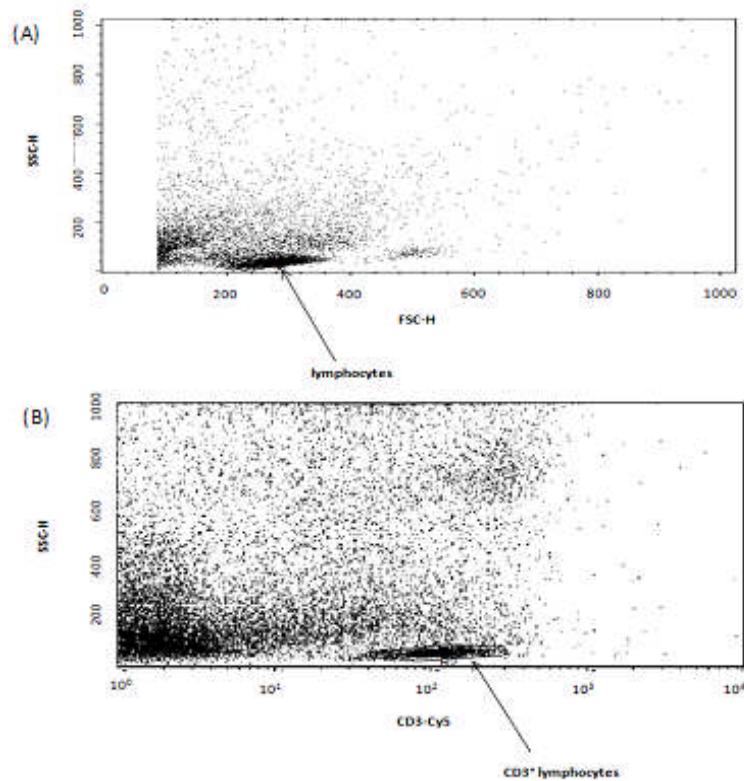


Fig. 2.8 (A) Representative FSC-SSC dot plot of gastric epithelial layer cells from a *H. pylori* negative patient showing the lymphocyte cell population. Since the lymphocyte population is the cell subset of interest for this particular study, a gate was formed around the lymphocytes prior to subsequent analysis and (B) representative CD3-SSC dot plot from the lamina propria layer of a *H. pylori* positive individual showing the gated CD3⁺T cell population. From this lymphocyte population, dot plots for analysis of other T cell populations were generated.

Next, since this study was focused on T lymphocytes, each sample tube contained CD3 in addition to test mAb. Therefore a CD3-SSC dot plot was generated since CD3 is a cell surface marker found on T cells (Fig. 2.8B). Then, dot plots showing each channel

of interest was generated from this data to uncover information regarding frequencies of lymphocytes and T cells paying particular attention to unconventional T cells in the gastric epithelium and lamina propria

Dot plots were called up to analyse data of interest which present a two-parameter display of data. Suitable IgG isotype controls were used in conjunction with each Ab to compensate for any non-specific binding that may have occurred (Fig. 2.9a).

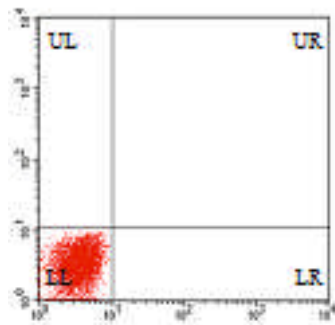


Fig. 2.9a Representative dot plot showing gated IgG isotype control

For instance, when analysing the below FL1-FL2 dot plot, the lower left (LL) quadrant is negative for both FL1 and FL2 cell surface markers, in the lower right (LR) quadrant is single positive for cell surface markers in the FL1 channel while in the upper left (UL) quadrant events are single positive for cell surface markers in the FL2 channel and finally, the upper right (UR) quadrant is DP for both FL1 and FL2 channel cell surface markers (Fig 2.9b).

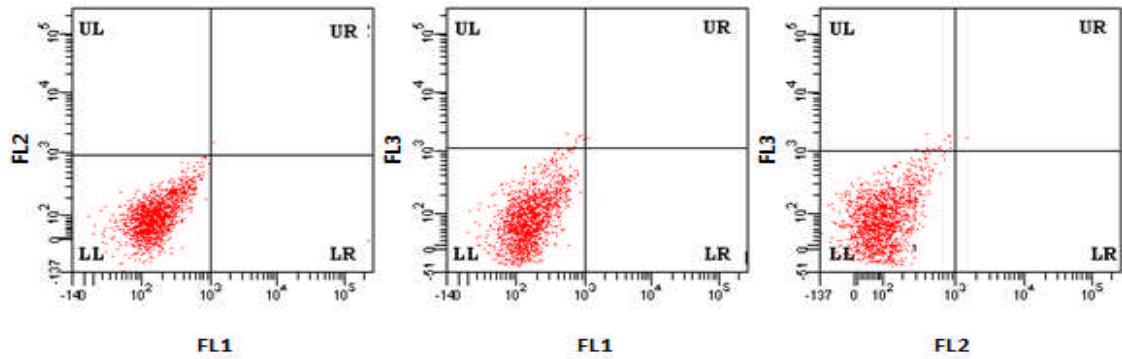


Fig. 2.9b Dot plots showing the three combinations of channels from which dot plots can be generated. The dot plots are segmented into quadrants therefore distinguishing cell populations. Cell populations can be analysed from the lower left (LL), lower right (LR), upper left (UL) and upper right (UR) quadrants.

2.2.9 Statistical analysis

GraphPad InStat[®] software (GraphPad Software, Inc., California, U.S.A.) was used to undertake statistical analysis on cells thought to be significantly different in epithelial and lamina propria layers during *H. pylori* infection. The Mann-Whitney U test was used to determine significance of data points that were non-parametric. Significance was examined at $p < 0.05$.

2.3 FUNCTIONAL CHARACTERISATION OF IMMUNE CELLS

The proliferative, cytokine and cytotoxic responses of immune cells to *H. pylori* and *H. pylori* derived Ags were tested in this part of the study (chapter 4).

2.3.1 Preparation and maintenance of samples for immunological assays

2.3.1.1 Cell line maintenance

The cell lines used in this study included C1R CD1d, C1R mock, HeLa CD1d, HeLa mock, NKT cell clones BC19 and BC20 (Fig.2.10) and $\gamma\delta^+$ T cell clones (all cell lines were a gift from Dr. Derek Doherty, St. James Hospital, Dublin). C1R cell lines were

transfected with individual CD1 isoforms (Exley *et al.*, 1997). HeLA cell lines were transfected with CD1d (CD1d-HeLa). Mock transfected HeLa (HeLa-mock) and C1R (C1R-mock) cell lines were used as controls. Caco2 cell line derived from human colon carcinoma cells resemble intestinal epithelial cells and were also used in this study (Sigma).

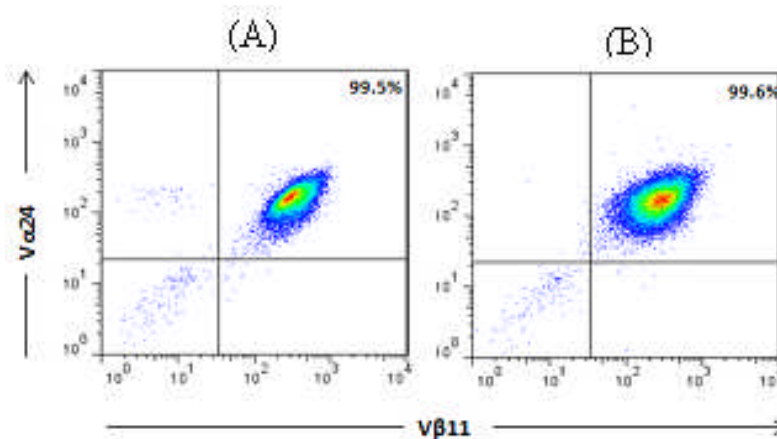


Fig. 2.10 Dot plots showing purity of NKT cell clones (A) BC19 and (B) BC20. Percentages of purity are displayed in upper right quadrant. Image supplied by Dr. Derek Doherty, St. James Hospital, Dublin.

Reconstituting cells

Cell lines were stored at -80°C for short term storage and liquid nitrogen for long term storage prior to reconstitution. RPMI supplemented with 10% FCS and antibiotics was pre-warmed at 37°C for 10 min. A small aliquot of RPMI (2 ml) was then added drop wise to a half thawed stock vial of cells. This was added to the remaining warmed RPMI in a tissue culture flask which was sprayed with ethanol and left overnight at 37°C . When the colour of the cell suspension began to change from orange to yellow indicating nutrient depletion, an additional 20 ml of pre-warmed RPMI was added to give a final volume of 40 ml of cell line suspension to work with.

Splitting and feeding cells

Cells were generally maintained in 40 ml aliquots in T75 flasks (Eppendorf, Stevenage, UK) and so to split and feed them, 20 ml of the cell suspension was removed and discarded into Virkon[®] (Day-Impex Ltd. Colchester, Essex, UK). Pre-warmed RPMI supplemented with 10% FCS and antibiotics (20 ml) was added to the remaining cells. The flasks were sprayed with ethanol to sterilise and placed in an incubator set at 37°C with 5% CO₂.

Freezing stocks

A freezing mix which consisted of 90% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) was prepared one hour prior to freezing stocks. This was incubated at 4°C to allow the heat to subside from this exothermic reaction. A quantity of cells (10 ml) was removed from the tissue culture flask and was placed in a sterile 15 ml tube (Sarstedt, Germany). The suspension was centrifuged at 300 *x g* for 10 min and the supernatant was discarded. The cells were counted and adjusted to 2 x 10⁶ cells/ml. The cell pellet was resuspended in 1 ml of freezing mix and stored at -80°C for 24 h before being transferred to liquid nitrogen for long term storage.

2.3.1.2 Culture and growth media for bacterial preparations

Blood agar culture media

Blood agar was used for cultivation of *H. pylori* for this study. The agar was made by dissolving 19.5 g of Columbia agar base (Oxoid, Hampshire, UK) and 1.3 g of bacteriological agar (Sigma Chemical Co., St. Louis, MO, U.S.A) in 500 ml of distilled water and autoclaving at 121°C for 15 min at 15 lb in⁻² to sterilise. The sterilised agar was left in an oven at 50°C overnight to cool to required temperature. Once this

temperature was reached, 30 ml of defibrinated horse blood (Charles River Laboratories, Ballina, Co. Mayo, Ireland), 1.7 ml isoVitaleX (BBL Becton Dickinson Microbiology, Sparks, MD, U.S.A.) and the antibiotic selective supplement DENT (Oxoid, Hampshire, U.K., L21) were aseptically added to the agar. The medium was quickly poured into sterile petri dishes and left to solidify.

Components of the nutrient supplement IsoVitaleX

IsoVitaleX was provided in dehydrated powder form with a diluents included separately. The additional diluent was added aseptically to the powder contained 10% (w/v) dextrose and 1.7 ml of this rehydrated solution was added to 500 ml of sterile medium. The constituents of 10 ml of IsoVitateX are outlined in Table 2.3.

Table 2.3 Constituents of IsoVitaleX nutrient supplement

Constituent	per litre
Vitamin B12	10 mg
L-glutamine	10 g
Adenine	1 g
L-Cysteine hydrochloride	25.9 g
L-Cysteine	1.1 g
Dextrose	100 g
Guanine HCL	30 mg
P-Aminobenzoic acid	13 mg
Diphosphopyridine Nucleotide, oxidised (Coenzyme 1)	250 mg
Coccarboxylase	100 mg
Ferric Nitrate	20 mg
Thiamine HCL	3 mg

Components of the antibiotic selective supplement DENT

Initially DENT's selective medium was created for the isolation of *H. pylori* from gastric biopsies (Dent and McNulty, 1988). The antibiotic selective supplement aroused

from this. DENT was commercially available as a dehydrated powder (Oxoid SR0147). A single vial of DENT was dissolved in 2 ml of sterile distilled water before being added to 500 ml of sterile medium. The components of DENT are outlined in Table 2.4.

Table 2.4 Constituents of rehydrated DENT

Constituent	per litre
Trimethoprimlactate	5 mg
Cefsulodin	5 mg
Amphotericin	5 mg
Vancomycin	10 mg

Storage medium

Stocks of *H. pylori* cultures were stored at -70°C in a semi-solid maintenance medium. The storage medium was made by adding together the constituents outlined in Table 2.5 and shaking them sufficiently to ensure a homogenous solution. The storage medium was aliquoted into 7 ml volumes in glass vials, solutions were sterilised at 121°C for 15 min at 15 lb in^{-2} . The sterile medium was stored at 4°C for use after cooling.

Table 2.5 Constituents of storage media, used for storing stocks of *H. pylori*

Constituent	per 100 ml
Tryptone soya broth (Lab M)	3 g
Distilled water	80 ml
Yeast extract (Oxoid)	0.3 g
Bacteriological agar	50 mg
Glycerol (BDH, Poole, U.K.)	15% (v/v)

2.3.1.3 *H. pylori* isolation and cultivation

H. pylori bacteria [supplied by Prof. Anthony Moran (R.I.P)] were cultured on Columbia blood agar plates to grow biomass for Blaser outer membrane protein extraction (see below). *H. pylori* bacteria were isolated from fresh gastric biopsies that had been placed in saline solution using a pestle and mortar, placed on pre-warmed

blood agar plates and incubated for three to five days at 37°C in a microaerobic environment. *H. pylori* stock cultures were removed from -80°C freezer. Once thawed, 200-500 µl of stock was aseptically pipetted onto blood plates which had previously been warmed up in a 37°C incubator (Carbolite). Inoculated plates along with a gas pack were placed in a gas jar and incubated for three days at 37°C. *H. pylori* bacteria were subcultured onto blood plates using sterile cotton swabs (Technical Service Consultants Ltd., Lancashire, England) after three day incubation. From one plate of growth, approximately five plates were subcultured; these were incubated for two days at 37°C. *H. pylori* biomass was extracted from blood plates for isolation of LPS (undertaken from stock cultures in the Laboratory of Molecular Biochemistry using a hot phenol extraction method) and protein fractions by adding 1 ml of sterile PBS and collecting the growth to one area using a sterile spreader (Sarstedt, Germany). This was collected in a sterile tube using a pipette and was frozen at -80°C until required. Stock cultures were made by adding 1-2 ml of storage medium to biomass, collecting the growth to one area of the plate using a sterile spreader and pipetting it into a cryo vial (Starstedt, Germany) which was stored at -80°C until required. To create an atmosphere composed of 5% oxygen, 10% carbon dioxide and 85% nitrogen, *H. pylori* was incubated in anaerobic gas jars by BBL Microbiology Systems along with disposable gas generating kits (Oxoid BR56, Hamshire, UK). Since *H. pylori* favours a moist atmosphere, an additional petri dish minus its lid that contained distilled water was placed on top of growth plates. The presence of *H. pylori* was confirmed through identification of small translucent colonies that grew under microaerophilic conditions and whose cells stained negative after gram staining. Spiral, u-shaped or curved rods was indicative of a positive result for the presence of *Helicobacters* (Marshall &

Warren, 1984). This morphology was examined using gram staining and examination of this with light microscopy.

Gram staining

The gram staining procedure arose from an amendment of that of Ogg (Ogg, 1962). Gram staining was undertaken by taking a small quantity of biomass from a growth plate using a sterile cotton swab and dispersing it on a glass slide (BDH). The sample was heat fixed by momentarily passing the slide with the growth side up through a Bunsen flame three-four times. Next, methyl violet was placed on sample for 1 min; this was followed by washing the slide in water and adding iodine (Sigma Chemical Co., St. Louis, MO) for 3 min whose purpose was to fix the methyl violet in the cells. Another washing step followed and alcohol (Merck, Germany) was added for 20 s. Methyl violet-iodine is removed when the bacteria in question is gram negative as the complex is soluble in alcohol and so sample is left colourless. Cells were again washed in water and counter-stained using carbol fuchsin (Sigma Chemical Co., St. Louis, MO) for a total of 1 min, washed and left to dry by the Bunsen flame. *H. pylori* along with all other gram negative bacteria stained pink following the counter stain and this was visualised using light microscopy with an Olympus CH20 microscope (Olympus Optical Co., Tokyo, Japan) under a magnification of x 100.

The Blaser outer membrane extraction method

The extraction of OMPs was developed and published by Blaser *et al.*, in 1983. Solutions of Tris-HCL and Sarkosyl (BDH Laboratory Supplies, Poole, England) were prepared before the Blaser experiment was carried out. A 0.01 M Tris-HCL buffer was needed at pH 7.4; this was made by dissolving 0.12 g of Trizma® base (Sigma

Chemical Co., St. Louis, MO) in 50 ml distilled water. The pH of 7.4 was reached using 8 M HCL and a final volume of 100 ml was reached using distilled water. Regarding the Sarkosyl solution, a 1% sarkosyl in 7 mM EDTA was required. This was prepared by dissolving 0.204 g of EDTA in 50 ml of water; 3.33 ml of a 30% Sarkosyl solution was then added and a final volume of 100 ml was reached using distilled water. Harvested *H. pylori* biomass was washed x 2 in sterile water of analytical grade and centrifuged at 5,000 \times g for a period of 10 min at 4°C, this was followed by suspension in Tris buffer. Using Tris buffer the O.D was adjusted to 26% transmission at 450 nm. Next, sonication was carried out with the suspension on ice for 30 s periods with 30 s intervals for 10 min; the suspension was then centrifuged at 5,000 \times g for 10 min to ensure eradication of whole cells and debris. The supernatant was ultracentrifuged at 100,000 \times g for 1 h at 4°C in plastic ultracentrifugation tubes (Beckman Instruments Inc., Spinco Division, Palo Alto, CA, U.S.A.). The pellet containing the cellular membrane was placed in 3 ml of sterile water with 200 μ l of this being stored for experimental analysis. The remaining 2.8 ml was dissolved in 20 ml of Sarkosyl solution which had been pre-warmed to 37°C, solution was incubated at 37°C for 20 min. Solution was shaken intermittently during this time. Next, another ultracentrifugation step was carried out on the suspension at 100,000 \times g for 2 h at 4°C, with the purpose being to form a pellet of the insoluble OMP containing Sarkosyl fraction. The remaining supernatant was kept for analysis while the pellet was added to 2 ml Tris and ultracentrifuged at 100,000 \times g for a further 2 h at 4°C to wash it. The pellet was then added to 1 ml sterile distilled water and kept at 4°C for a short period before being transferred to -20°C for long term storage.

Hartree-Lowry assay for protein determination

The Hartree-Lowry assay is a modification of the original Lowry assay used for quantification of total protein. This assay is a colorimetric assay based on cupric ions and Folin-Ciocalteu reagent for phenolic groups. This modified version uses fewer reagents, has shown improved sensitivity with certain proteins, maintains a more linear response over a wider range of concentrations and is less likely to become saturated. The following reagents were prepared and stored at RT for 3 months (Table 2.6).

Samples were prepared and tested in 1 cm plastic spectrophotometry cuvettes. A dilution series of BSA (Sigma-Aldrich, Steinheim, Germany) standards ranging from 10 $\mu\text{g/ml}$ to 700 $\mu\text{g/ml}$ were prepared with a 2 mg/ml stock BSA solution and sterile distilled water. A blank containing no protein was also included. A volume (1 ml) of each standard/protein sample was added to 0.9 ml of Reagent A. Solutions were mixed and incubated for 10 min in a 50°C water bath. The tubes were removed and cooled to RT. Next, 0.1 ml of Reagent B was added to each tube for 10 min at RT. Rapidly, 3 ml of Reagent C was added to each tube; solutions were mixed thoroughly and incubated for 10 min at 50°C. Solutions were then cooled to RT before their absorbance at 650 nm was read. A calibration plot of the BSA standards was prepared by plotting net A₆₅₀ nm standard values versus protein concentration. The unknown protein concentrations were then determined through interpolation of the standard plot.

Table 2.6 Constituents of reagents for Hartree-Lowry assay for protein determination

Hartree-Lowry Reagent	Concentration	Source
Reagent A		
Sodium carbonate (Na ₂ CO ₃)	100 g	Sigma
1 M Sodium hydroxide (NaOH)	500 ml	AnalaR
Sodium potassium tartrate tetrahydrate (COOK.CHOH.CHOH.CooNa.4H ₂ O)	2 g	AnalaR
Water	Up to 1 l	
Reagent B		
Copper sulphate pentahydrate (CuSO ₄ .5H ₂ O)	1 g	BDH
Sodium potassium tartrate tetrahydrate (COOK.CHOH.CHOH.CooNa.4H ₂ O)	2 g	AnalaR
1 M Sodium hydroxide (NaOH)	10 ml	AnalaR
Water	90 ml	
Reagent C		
Folin-Ciocalteu's phenol Reagent	1:15	Sigma

H. pylori Ags

The LPS molecules from *H. pylori* strains National Collection of Type Cultures (NCTC11637) and Culture Collection, University of Göteborg, Sweden (CCUG17874) were utilised in this study. Bacterial strains whose immune responses have not been extensively studied were chosen for this study. Both strains were prepared in the laboratory of Molecular Biochemistry by Prof. Anthony P. Moran. *E. coli* clinical isolate LPS, known for being a more potent stimulator of immune cells than *H. pylori* LPS were used in conjunction with *H. pylori* test LPS for cell culture analysis. Additional *H. pylori* bacterial Ags were isolated following fractionation of *H. pylori* strains 26695 and J99 using Blaser Outer Membrane Protein protocol outlined earlier in chapter 2. All *H. pylori* bacterial Ags utilised in this study are listed in Table 2.7 and were used at concentrations ranging from 1 ng – 10 µg/ml.

Table 2.7 List of bacterial fractions used for stimulating immune cells in this study

Bacterial type	Strain	Bacterial fractions	Rough/Smooth LPS
<i>H. pylori</i>	26695 J99	WCE,	
		Cyto Ags	
		C.M. Ags	
		OMP	
		IMP	
	NCTC11637	LPS	Rough
	CCUG17874	LPS	Rough
<i>E. coli</i>	Clinical isolate	LPS	Smooth

NCTC = National Collection of Type Cultures; CCUG = Culture Collection University of Göteborg; WCE = whole cell extract; Ags = antigens; Cyto Ags = cytoplasmic antigens; C.M Ags = crude membrane antigens; OMP = outer membrane protein; IMP = inner membrane protein; LPS = lipopolysaccharide.

Chemical cleaning of glass bottles for LPS samples

Since autoclaving alone does not eliminate LPS, another method to prepare LPS-free bottles for LPS preparation was devised. The alternative method involved immersing glass bottles without their caps in a glass beaker of acetone (BDH AnalaR) for a period of 20 min. The bottles were then transferred to a beaker containing chloroform (Merck, Germany) for 20 min. The chloroform was removed and the bottles were incubated at 160°C for 2 h, this was followed by repetition of the acetone and chloroform cleaning steps. The bottles were then placed at 160°C overnight to ensure removal of any contaminating LPS present. The glass bottle caps were rinsed in methanol followed by acetone and then dried before use.

Reconstitution of LPS

LPS preparations (supplied by Prof. Anthony Moran) were dissolved in sterile Millipore water at a concentration of 1 mg/ml.

2.3.1.4 Reconstitution of other glycolipids

α -GalCer, a glycosphingolipid isolated from the marine sponge *Agelas mauritianus* was supplied by Alexis Biochemicals (San Diego, USA) [now Enzo Life Sciences, (UK) LTD, Exeter, UK]. α -GalCer was used as a positive control for NKT cell studies as it is a known potent activator of NKT cells. It was dissolved in 10% DMSO in 1X PBS at a concentration of 1 mg/ml. Before use, stocks of glycolipids were first vortexed for 1 min, heated for 2 min at 80°C followed by sonication for 10 min. Working stocks containing 100 μ M glycolipids were made by further dilution in DMEM media before vortexing for a further minute followed by heating for 2 min at 80°C and a final 5 min sonication step. These stocks were stored at 4°C before being added to cell cultures.

2.3.2 Stimulation of immune cells

2.3.2.1 Stimulation of PBMCs, gastric epithelial or lamina propria layer cells with *H. pylori* LPS.

Freshly isolated epithelial, lamina proprial or PBMCs (1×10^5) were co-incubated with different concentrations of LPS (10 ng-10 μ g) at different time points in 24 well tissue culture plates (Sarstedt, Germany) in a 5% CO₂ atmosphere. The plates were then centrifuged at 300 $\times g$ in a Becton Dickenson Coulter centrifuge for ten min. Cell pellets were discarded and supernatants were either analysed immediately for cytokine production *via* (enzyme linked immunosorbent assay) ELISA or T_h1/T_h2 11plex FlowCytomix kit (BenderMed Systems, Vienna, Austria) or stored at -80°C until required.

2.3.2.2 Stimulation of human invariant NKT cell clones with *H. pylori* derived bacterial fractions

NKT cells were co-incubated *in vitro* with mock-transfected or CD1d-transfected HeLa cells pulsed with either vehicle or glycolipid. The HeLa cells were maintained at a density of 0.5×10^6 cells/ml in DMEM media (Gibco, Paisley, UK) supplemented with fetal calf serum (FCS) and antibiotics at 37°C and 5% CO₂. HeLa mock and CD1d transfected cells were cultured in 96 well round bottomed tissue culture plates (1×10^5) and were first co-incubated with 100 ng/ml glycolipid or vehicle (0.1% DMSO) for 24 h before an equal number of NKT cells were added for a further 24 h. Following stimulation, supernatants were assayed for cytokine production.

2.3.2.3 Stimulation of human $\gamma\delta^+$ T cell clones with *H. pylori* derived bacterial fractions

The $\gamma\delta^+$ T cell clones were maintained at a density of 0.5×10^6 cells/ml in DMEM media supplemented with FCS and antibiotics at 37°C and 5% CO₂. The $\gamma\delta^+$ T cell clones were cultured in 96 well round bottomed tissue culture plates (2×10^5) and were first co-incubated with 100 ng/ml *H. pylori* derived Ags for 24 h. Following stimulation, supernatants were assayed for cytokine production.

2.3.2.4 Control stimuli

Mitogens

For the complete activation of T cells as a positive control stimulus (Chmiela *et al.*, 1996) the following mitogens were used in this study. PHA is a lectin isolated from the red kidney bean *Phaseolus vulgaris* containing potent mitogenic properties. PHA is composed of an erythroagglutinin (PHA-E) which has low mitogenic and high erythroagglutinin activity, and leucoagglutinin (PHA-L) possessing high mitogenic and

leucoagglutinating activity, but minimal erythroagglutinating activity (Allen *et al.*, 1969; Weber, 1969; Yachnin *et al.*, 1972). It is believed the combination of these structures determines its lymphocyte stimulating activities (Leavitt *et al.*, 1977). PHA was used at 5 – 10 µg/ml. Other mitogens used for T lymphocyte stimulation were phorbol 12-myristate 13-acetate (PMA), a polyfunctional diterpene phorbol ester. Phorbol was first isolated from the croton plant, native to Southeast Asia and has uses in cancer research as a tumour promoter (Goel *et al.*, 2007). For cytokine induction by T cells, PMA was used in conjunction with ionomycin (I) which is isolated from *Streptomyces globatus* (Meyers, 1975). PMA is activated through the TCR/CD3 complex while I provides co-stimulatory effects. The combination provides complete T cell activation. Cells were stimulated with 25 ng/ml PMA and 1 µg/ml I.

α-GalCer

One of the best-documented glycolipids shown to stimulate and activate NKT cells is a marine sponge-derived glycolipid α-GalCer (Kronenberg & Gapin, 2002; Zajonc & Kronenberg, 2009). In this study, 5 -25 µg/ml of α-GalCer was used as a positive control for NKT cell stimulation (see section 1.4.1.3).

(E)-1-hydroxy-2-methyl-but-2-enyl 4-diphosphate (HMB-PP)

For stimulation of $\gamma\delta^+$ T cells, [HMB-PP] was used as the positive control (10nM) in this study (a gift from Dr. Derek Doherty). HMB-PP is a metabolite in the 2-C-methyl-D-erythritol-4 phosphate (MEP) pathway for isoprenoid biosynthesis (Kabelitz, 2011; Puan *et al.*, 2007). HMB-PP has been shown as a ligand for V γ 9V δ 2 T cell stimulation for several bacterial species including *E. coli* and *M. tuberculosis* (Puan *et al.*, 2007) and is the most potent stimulus known to date for $\gamma\delta^+$ T cells.

2.3.3 Immunological assays

2.3.3.1 Cell proliferation ELISA, BrdU (colorimetric)

BrdU is an analogue of thymidine (Fig. 2.11) used to determine proliferation.

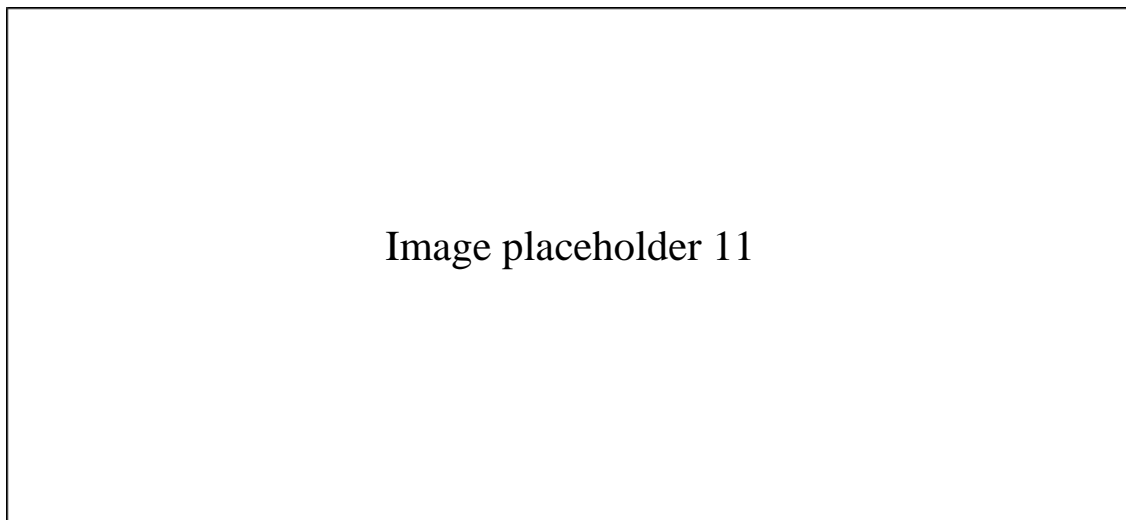


Fig. 2.11 The molecular structure of thymidine and 5-bromo-2'-deoxyuridine. Taken from Apoptosis, Cell Death and Proliferation Manual, 3rd edition, Roche Applied Science, http://www.roche-applied-science.com/sis/apoptosis/docs/manual_apoptosis.pdf. Figure has been removed due to copyright restrictions

The BrdU ELISA (Roche Diagnostics, Germany) technique measures DNA synthesis as a marker of proliferation. Cells undergo DNA synthesis during the S phase of the cell cycle and its genome replicates. Dividing cells will integrate a labelled DNA precursor such as BrdU into their DNA if added to the cell culture. BrdU quantity incorporated into DNA is measured by a cellular enzyme immunoassay using mAbs against BrdU. This determines the sum of labelled DNA in a given population. BrdU integration into DNA is directly proportional to the amount of cell division in the cell culture.

Each kit came with 10 mM BrdU in PBS, pH 7.4, a FixDenat solution to denature DNA, an anti-BrdU-POD mAb conjugated with peroxidase (POD), an Ab dilution solution, wash buffer and a substrate solution of 3,3',5,5'-tetramethyl-benzidine (TMB).

Optimisation experiments were carried out initially to discover the most suitable time points, cell numbers and mitogen concentration phytohaemagglutinin (PHA) to use. PHA is a lectin found in plants and is considered a positive control because it is a potent mitogen for cells. Briefly, a 96-well plate (Sarstedt, Germany) was set up with 20,000 cells/well. Cells were incubated in triplicate with 5 µg/ml of PHA. A negative control consisting of cells only was also used.

For PBMCs, cells were cultured for 6 days at 37°C in 5% CO₂. On day 6, the 10mM BrdU labelling solution was diluted 1:100 to 100µM. To each well, 10 µl of the diluted BrdU labelling solution was added and cells were cultured for a further 24 h. The BrdU labelling solution was removed by centrifugation of the plate at 300 g for 10 min followed by tapping. Next, the cells were dried for 1 h at 60°C. FixDenat solution (200 µl) was added for 30 min at RT and then removed by tapping the plate. Anti-BrdU-POD solution was added to plate. It was first diluted 1:100 with the Ab diluting solution and 100 µl was added to each well which was then incubated for 90 min at RT. The plate was washed 3 times with the washing solution. Finally, 100 µl of TMB substrate solution was added and incubated for 5-30min at RT. H₂SO₄ stop solution (25 µl of 1M) was added to cease the reaction and plate analysis followed on a photo spectrometer (Thermo Spectronic) at 450 nm. For epithelial or lamina propria mononuclear cell cultures, 7, 5, 4 and 3 day cultures were tested while for NKT cell clones, cells were stimulated for 24 and 48 h prior to BrdU ELISA.

2.3.3.2 Multiplexing kit for cytokine analysis

BenderMed Systems (Vienna, Austria, now supplied by eBioscience, Hatfield, AL10 9NA United Kingdom) was the supplier of the T_h1/T_h2 11 plex FlowCytomix kit which allows simultaneous analysis of IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF- α and TNF- β whilst using only 25 μ l of sample. The human T_h1/T_h2 11 plex kit is a bead-based analyte detection system for quantitative measurement of cytokine production. There are two bead sizes, A and B, which are dyed internally with fluorescent dyes of varying intensities. Setup beads, fluorescent beads coated with specific Abs, standards for each of the cytokines, biotin-conjugate specific for each Ab, assay buffer, reagent dilution buffer and streptavidin-phycoerythrin are provided with each kit.

Firstly, 25 μ l of test supernatants were added to polystyrene round bottom tubes and eight standard tubes ranging from 400 ng/ml to 0 ng/ml were prepared as per protocol instructions. Bead mix (25 μ l) was then added to each tube. The bead mix contained beads for each cytokine, which allowed for their individual detection and the beads were coated with an Ab that reacted with the cytokines of interest. Biotin-conjugate solution (50 μ l) was then added to each tube. The tubes were vortexed to ensure homogenous solution and were incubated for 2 h at RT. The tubes were washed twice with 1 ml of assay buffer. Streptavidin-PE solution (50 μ l) was then added to each tube before being incubated for 1 h. The function of streptavidin-PE was to bind the biotin-conjugate and hence emit a fluorescent signal. The tubes were then washed as above. Finally, 500 μ l of assay buffer was added to each tube which was stored at 4°C until analysis by flow cytometry.

Flow cytometric analysis involved separation and identification of the two bead populations, A (R2) and B (R1). Setup beads were used as a method of experimental

standardisation prior to test analysis (Fig. 2.12). Once set-up was completed, the standards were analysed, bead populations were analysed from right to left as their standard concentrations reduced. The data files were transferred from the flow cytometer to the computer software, FlowCytomixPro 2.2 that was supplied with the kit for data analysis (Fig. 2.13).

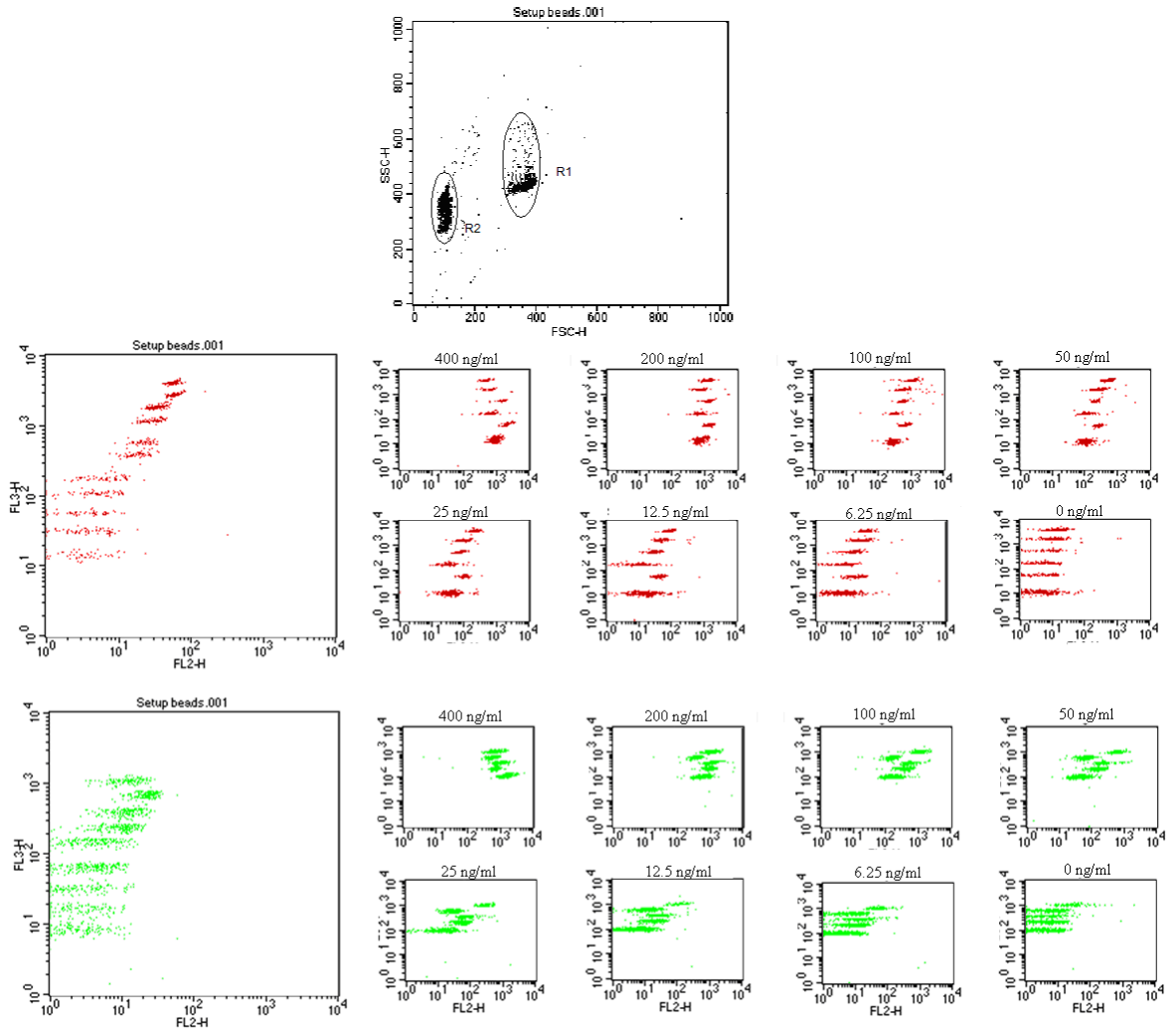


Fig. 2.12 FlowCytomixPro 2.2 analysis of Human T_{h1}/T_{h2} 11plex kit. Software image of bead populations A and B. Using setup beads, it is possible to adjust instrument settings on the flow cytometer.

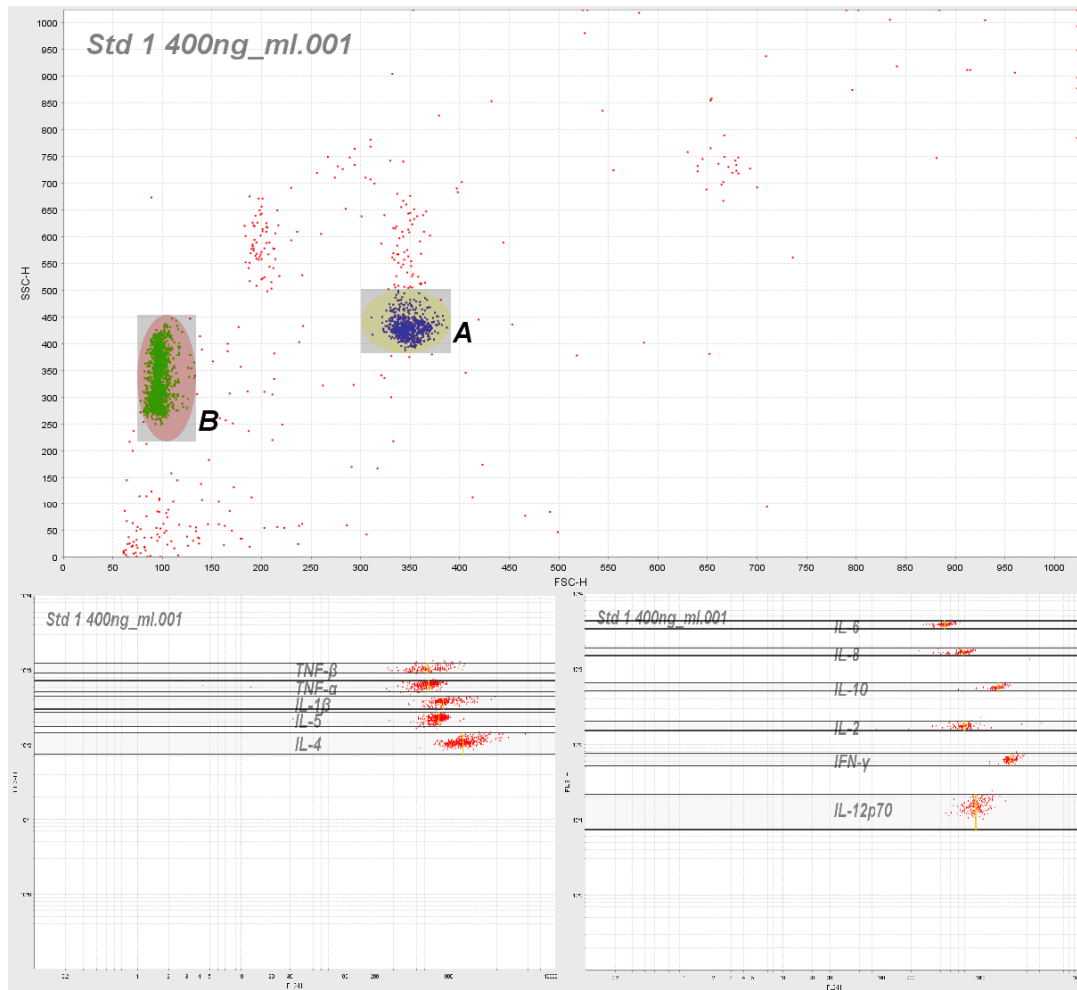


Fig. 2.13 FlowCytomixPro 2.2 analysis of Human T_h1/T_h2 11plex kit showing image of cytokines in the relative bead populations A and B from a standard sample. The two bead sizes are dyed internally with fluorescent dyes of varying intensities corresponding to each cytokine.

2.3.3.3 Lactate dehydrogenase assay as a measure of *in vitro* cell lysis of immune cells

LDH enzymatic activity is present in all eukaryotic cells. CytoTox-ONE™ (Promega UK, Southampton, UK) was a fluorometric method used to detect the levels of LDH in target immune cells (Caco2) through measurement of lactate dehydrogenase (LDH) release from cells whose membrane has been compromised following antigenic stimulation. NKT cells were used as effector cells and a range of Ags derived from *H. pylori* were tested. Briefly, a lyophilized substrate mix and assay buffer were combined to reconstitute CytoTox-ONE reagent of which a quantity (50 μ l) was added to each

sample for 30 min. Stop solution (25 μ l of 1M H₂SO₄, Merck, Darmstadt, Germany) was added and the fluorescent signal was determined. The amount of fluorescence seen was proportional to level of cell lysis. A similar experiment was set up without NKT cells as a control.

2.4 RNA PREPARATION FOR MICROARRAY

Gastric epithelial and lamina propria layer cells were firstly isolated from fresh gastric biopsies taken at endoscopy from *H. pylori* positive and *H. pylori* negative patients (see section 2.2.2). A cell count was performed and viability was tested. CD2⁺ cells were then isolated (see below) from each of the layers separately. This was followed by RNA extraction.

2.4.1 Isolation of CD2⁺ cells using CD2 MicroBeads

CD2 Microbeads (Miltenyi Biotec, Gladbach, Germany) were used for the isolation of human cells that expressed the CD2 cell surface Ag. The CD2⁺ cells were positively selected by firstly magnetically labelling them with CD2 MicroBeads and then loading the cells onto a column attached to a MACS[®] Separator (Miltenyi Biotec, Gladbach, Germany) magnetic field whereby the CD2⁺ cells only remained in the column. The isolated cell population were then eluted before experimental analysis is carried out (Fig. 2.14).



Image placeholder 11

Fig. 2.14 Image of the OctoMACS separator magnetic field where magnetically labelled cells are loaded onto the MS column. Image taken from

http://www.miltenyibiotec.com/en/PG_1109_181_OctoMACS_Separator.aspx Figure has been removed due to copyright restrictions

Reagents were prepared and instruments were obtained according to the manufacturer's instructions prior to the experimental procedure. Cell samples had previously been prepared through gastric lymphocyte isolation as previously explained. Cells were counted and viability was tested. Cells were firstly passed through pre-separation filters (Miltenyi Biotec, Gladbach, Germany) to ensure removal of cell aggregates or any other large particles which may hinder positive selection. Next, the cells were magnetically labelled. This was carried out by counting cells, centrifuging them at $300 \times g$ for 10 min and discarding the supernatant. The cell pellet was then resuspended in 80 μ l of pre-made buffer and this was followed by the addition of 20 μ l of CD2 MicroBeads. The solution was mixed thoroughly and incubated for 15 min at 4°C. Cells were then washed through addition of 1-2 ml of buffer and centrifuging them at $300 \times g$ for 10 min. The supernatant was then removed and cells were resuspended in 500 μ l of buffer. Finally the cells were magnetically separated using MS Columns. This was achieved by first placing an MS column (Miltenyi Biotec, Gladbach, Germany) in the magnetic field of an OctoMACS separator (Miltenyi Biotec, Gladbach, Germany) and washing it with 500 μ l of buffer. The cells were then added to the column. Once the unlabeled cells had completely passed through the column they were washed with 500 μ l of buffer and this was repeated three times. The flow through was collected each time and was pooled to form the unlabeled cell fraction. To isolate the CD2⁺ cells, the column was removed from the magnetic separator to a collection tube where the fraction was flushed with 1 ml of buffer using a plunger given with the column. The purity of epithelial and lamina propria layer CD2⁺ cells was then checked using flow cytometry. If purity was below 95%, the samples were discarded.

2.4.2 Isolation of total RNA from CD2 positive cells

Total RNA was isolated from gastric epithelial and lamina propria layers of fresh gastric biopsies using the RNeasy Protect Mini kit (Qiagen® West Sussex, UK RH10 9NQ). Briefly, after the cells were harvested and counted, they were pelleted by centrifugation for 5 min at $300 \times g$. The cells were then disrupted by adding 350 μ l of buffer RLT and pipetting up and down to mix the solution. The lysate was then homogenised by passing it a minimum of five times through a blunt 20 gauge needle (BD Emerald, NJ, USA) fitted onto an RNase syringe and one volume of 70% ethanol (Merck, Darmstadt, Germany) was added to this, the solution was mixed well by pipetting. A volume of 700 μ l of each sample was transferred to an RNeasy spin column that was resting in a 2 ml collection tube. The samples were centrifuged at $>8000 \times g$ for 15 s. The eluted portion was discarded and the collection tube was reused. Next, buffer RWI (700 μ l) was added to the RNeasy spin column. The lid was carefully closed and the solution was centrifuged at $>8000 \times g$ for 15 s as a washing step. The flow through was again discarded. Buffer RPE (500 μ l) was then added to the column and the solution was centrifuged at $>8000 \times g$ for 15 s to wash the membrane of the spin column. The flow through was once again discarded. Buffer RPE (500 μ l) was once again added to the column and the solution was centrifuged at $>8000 \times g$ for 2 min as a washing step for the spin column membrane. The column was then inserted into a new 2 ml collection tube and was centrifuged at $>8000 \times g$ for 1 min. Finally, the spin column was inserted into a 1.5 ml collection tube where 20 μ l of RNase free water was added. The column was centrifuged at $>8000 \times g$ for 1 min to elute the RNA. This final step was repeated with the eluate to increase the yield and purity of RNA.

2.4.3 Nanodrop technology

RNA quantity and purity was measured using Nanodrop technology (Thermo Fisher Scientific, Dublin, Ireland). Nanodrop technology is a computer controlled spectrophotometer based on a sample retention system which entails a combination of surface tension and fiber optics to retain and measure small amounts of protein and nucleic samples. Briefly, 1 μ l of RNA was loaded on top of the optical pedestal. The RNA was then sucked into a column where its quantity and purity was measured and read off the computer screen (Fig. 2.15). Only RNA with a 260/280 ratio of >2 was considered for this part of the study.

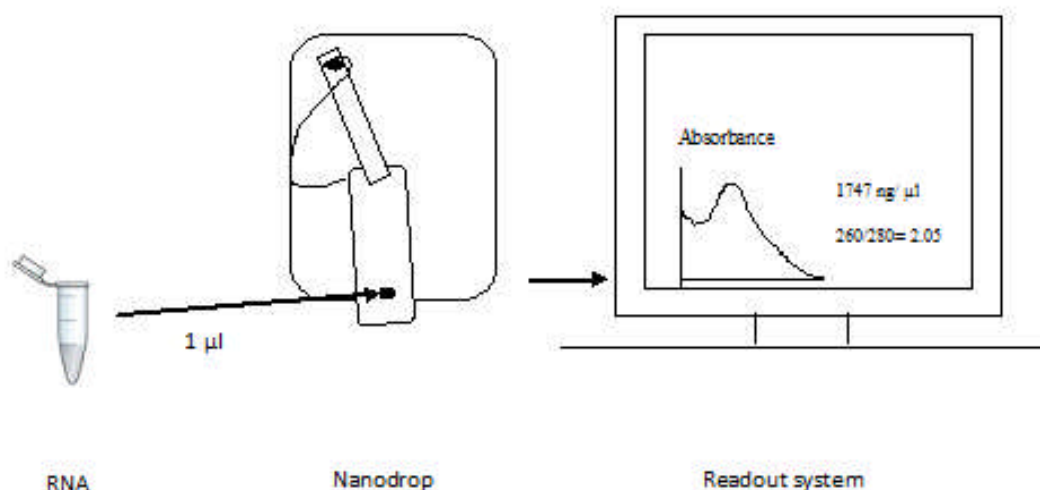


Fig. 2.15 Diagram illustrating the measurement of the quality of RNA samples using Nanodrop technology

2.4.4 Preparation of RNA for Affymetrix microarray using TURBO DNase

TURBO DNase (Ambion, Applied Biosystems) was utilized to remove any contaminating DNA from gastric epithelial and lamina propria RNA samples. A volume (0.1%) of 10X TURBO DNase buffer and 1 μ l of TURBO DNase was added to RNA (typically 20 μ l), the solution was mixed gently and incubated at 37°C for 20-30 min. Next, a volume of DNase inactivation reagent (0.1%) was added to RNA solution

before incubating at RT for 5 min. Solution was mixed by vortexing every 30 s. The RNA was then centrifuged at $10,000 \times g$ for 90 s and the RNA only was transferred to a clean tube.

2.4.5 Pooling of samples for microarray study

RNA was isolated from 35 patients in total for transcriptomics:

- 8 from *H. pylori* positive patients with severe gastritis were included in study
- 14 from *H. pylori* negative patients which were used in Microarray study (these patients displayed minimal gastritis and gastric inflammation)

RNA was isolated from a further 13 *H. pylori* negative patients, however this RNA was not included in the microarray study as patients showed symptoms of severe gastritis and/or inflammation. Pools of RNA were collected from the 8 *H. pylori* positive and 14 *H. pylori* negative subjects over a six month period and were stored at -80°C prior to microarray. There were four pools in total comprising of RNA collected from the epithelial and lamina propria layers from both *H. pylori* positive and *H. pylori* negative subjects (Table 2.8).

Table 2.8 Quantity of RNA collected from each pool for microarray project.

	µg of RNA	
	Epithelium	Lamina propria
<i>H. pylori</i> positive	2.7	11.5
<i>H. pylori</i> negative	4.6	27

When approximately 2 µg of RNA was obtained from each group, the RNA was sent to the Karolinska Institute in Sweden for Affymetrix analysis.

2.4.6 Microarray target preparation, labeling, cRNA generation, hybridization, washing, staining and scanning

This part of the study was undertaken in the Karolinska Institute, Sweden. A quantity (2 µg) of total RNA was converted to cDNA, cleanup was undertaken and the biotin labelled cRNA was synthesized. Unincorporated NTPs were then removed and the quality was assessed using Agilent 2100 bioanalyzer. Total RNA (100 ng) was used for cDNA synthesis, this resulted in unlabelled cRNA which was cleaned up before being reverse transcribed in the second cycle first strand cDNA synthesis, the double-stranded cDNA was cleaned up, amplified and labelled. The newly synthesized biotin labelled cRNA was cleaned thus removing unincorporated NTPs. Its quality was then determined. cRNA (25 µg) was generated in the *in vitro* transcription (IVT) reaction and fragmented using 5X fragmentation buffer and RNase-free water. The fragmentation reaction was carried out at 94°C for 35 min to generate 35-200 base fragments for hybridization. The quality was once again assessed. Prior to hybridization the adjusted cRNA yield was calculated for total RNA carryover in the IVT reaction. Fragmented cRNA (15 µg) was made up into a hybridization cocktail before being added to HG-U133 Plus 2.0 array and this was hybridized for 16 h at 45°C, washed and stained on fluidics station. Once completed the samples were scanned using GeneChip® Scanner 3000.

2.5 MICROARRAY DATA INTERPRETATION

2.5.1 Bioinformatics and data analysis methodology

This portion of the study was undertaken by Dr. Kaveh Mashayekhi. The GeneChip, HG-U133 Plus 2.0 (Homo sapiens) in situ oligonucleotide array (Affymetrix. Inc., Santa

Clara, CA) covered the transcribed human genome, comprised of 54,675 probe sets, ESTs and well-substantiated human genes. The CEL file, which stored the results of the intensity calculations on the pixel values of probes were pre-processed as the first order data analysis for the background correction using Robust Multichip Averaging (RMA) algorithm using Affymetrix GeneChip® Operating Software (GCOS). Non linear polynomial curve fitting, using convergence of three replicates (OriginPro 8 SRO computer software v8.0724, OriginLab Corporation, One Roundhouse Plaza, Northampton MA 01060) with a general formula of $Y = a_0 + a_1 X_1 + a_2 X_2 + \dots + a_{n-1} X^{n-1}$ was used to create a fourth replicate as a mean for estimation of overall average of the gene expression and estimation of fold change later in the analysis.

The data points of replicates were scaled to the polynomial curve fitted value for each treatment separately. The background corrected data were normalized by per-chip, per-gene and the global median polishing normalization method using GeneSpring bioinformatics software (GeneSpring GX 7.3.1, Silicon Genetics, Redwood City, CA). Statistical analysis was based on significance analysis of microarrays (SAM, Tibshirani R[X]) algorithm implementing multiple t-test, considering false discovery rate (FDR) estimate using R-Bioconductor integrated module [Y]. Estimated Q-values, as a measure of significance then converted to *p*-values for the ease of reading. Gene filtering on normalized intensity followed by fold changes >2-fold and *p*-value of < 0.05 was used to generate list of genes for expression profiles.

2.5.2 Microarray data analysis

The microarray data was presented in four groups after bioinformatics:

- epithelium of *H. pylori* positive patients vs. *H. pylori* negative patients (EP-HP⁺/HP⁻)

- lamina propria of *H. pylori* positive patients vs. *H. pylori* negative patients (LP-HP⁺/HP⁻)
- epithelium vs. lamina propria of *H. pylori* positive patients (HP⁺ EP/LP)
- epithelium vs. lamina propria of *H. pylori* negative patients (HP⁻ EP/LP)

To begin experimental analysis, genes differentially expressed in epithelial and lamina propria layers during *H. pylori* infection were identified; the data in each of the eight groups was organised according to the highest gene fold change. Genes with a fold change of two or greater were considered to be significant. The overall percentage of genes with a significant fold change was calculated for each group to get a general overview of the number of significantly up-regulated and down-regulated genes in the gastric mucosa following *H. pylori* infection. Due to the large amount of data generated two strategies were adopted. Firstly, the top twenty genes with the highest fold change was then scrutinised in each group to analyse the highest changes in gene expression in the epithelial and lamina propria layer of *H. pylori* infection. Secondly, the data in each group was alphabetically ordered. Scientific sources such as papers and books were then scanned for genes known to be of relevance in CD2⁺ cell immunity. The microarray data was then searched to see were any of these genes up-regulated or down-regulated with regards to CD2⁺ cells and *H. pylori* infection and genes of interest that were altered in relevant functional categories were subsequently grouped:

- Cytokines
- Cytotoxicity
- Proliferation
- Receptors
- Signal Transduction

A form of bioinformatics was then carried out to manipulate the huge resource of data. This entailed the generation of a heat map in triplicate as a graphical representation of the data whereby changes in gene expression was represented in a 2D manner by a colour change. Up-regulated genes in each group were represented by a red colour and down-regulated genes were coloured blue. Next, ANOVA analysis of genes with statistically significant differences among the groups based on values of epithelial and lamina propria layer of *H. pylori* infected and non-infected gastric mucosa was undertaken and represented in a matrix form. Then, fold change comparison matrix of differentially expressed genes among the four groups based on genes significantly expressed two-fold and greater in the epithelial and lamina propria layer of *H. pylori* positive and negative mucosa were constructed. Finally, 2D bar charts representing the degree of up-regulation and down-regulation of genes in healthy and infected gastric mucosa with a view to key functional areas such as receptors, signal transduction, proliferation, cytotoxicity and cytokines were also generated.

2.6 PCR AND AGAROSE GEL ELECTROPHORESIS

2.6.1 Reverse Transcription-PCR

Polymerase chain reaction (PCR) is a molecular technique developed in 1983 by Kary Mullis (Mullis & Faloona, 1987) used to amplify minute amounts of DNA sequences. Reverse-Transcription-PCR (RT-PCR) is a variant of this whereby RNA is first transcribed into its DNA complement using an enzyme called reverse transcriptase before being amplified. RT-PCR works by utilizing a pair of primers that are complementary to a particular sequence of the two cDNA strands. DNA polymerase is then used to extend the primers and thereby make copies of the DNA strands; this is one cycle which is repeated to form exponential DNA amplification.

The reaction mix for one reverse transcription (RT) part of a RT-PCR experiment was made up of the following. In a sterile RNase-Free Thin-walled Frosted Lid 0.5 ml PCR reaction tube (Ambion, life technologies, Dun Laoghaire, Dublin), 10 μ l of a 2X RT Buffer (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, California, USA), 1 μ l of 20X Enzyme Mix (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, California, USA), 4 μ l of RNA sample and 5 μ l of Nuclease-free water (Ambion, life technologies, Dun Laoghaire, Dublin) were mixed so that a final volume of 20 μ l was reached for the RT reaction. The lid was then placed on the tube which was briefly centrifuged to spin down the solution and to ensure the solution was free of any air bubbles. The tube was then placed on ice while being transferred to the thermal cycler (Eppendorf mastercycler gradient, Stevenage, UK) for the reverse transcription reaction. The tube was incubated in a thermal cycler at 37°C for 59 min and then reaction was ceased by heating it to 95°C for 5 min before being held at 4°C. The cDNA was then ready for the PCR reaction stage. The reaction mix for one PCR contained the following (ice cold): a quantity (1 μ l) of PCR Buffer

(Biosciences, Dun Laoghaire, Dublin, Ireland), 0.2 μ l of 10mM dNTP mix (10 μ l of each dNTP (Biosciences, Dun Laoghaire, Dublin, Ireland), 0.3 μ l MgCL₂ (Biosciences, Dun Laoghaire, Dublin, Ireland), 1 μ l of forward primer of choice, 1 μ l of the reverse primer of choice, 1 μ l Taq polymerase enzyme (Biosciences, Dun Laoghaire, Dublin), 10.5 μ l of RNase free water was added to a sterile 0.5 ml PCR tube make a PCR mastermix. An aliquot of this PCR mastermix (15 μ l) was incubated in a new PCR tube with 5 μ l of RT reaction so that a final volume of 20 μ l was reached. Immediately, this reaction mastermix was loaded into a PCR block. The reaction cycle was as follows:

94°C for 2 min
94°C x 1
55°C x 1
72°C x 2
72°C for 15 min
4°C Hold

} x 30 repeat cycles

The PCR cycle described above was used for all reactions except those involving the Kir6.1 gene which employed the following cycle conditions.

95°C for 5 min
95°C for 45 s
52°C for 45 s
72°C for 75 s
72°C for 10 min for elongation

} x 30 repeat cycles

All primers that were used for these experiments were at a working concentration of 25 μ M and are listed in Table 2.9. Primers were reconstituted with nuclease free water (Ambion, life technologies, Biosciences, Dun Laoghaire, Dublin, Ireland) according to manufacturer's instructions (Eurofins MWG, Ebersberg, Germany).

Table 2.9 List of primers used for RT-PCR experiments

Primer Name	Gene	Sequence (5' to 3')	Source
REG4 Forward	Regenerating islet 4	GTCTGATGCCGAGCTCGAGTG	MWG
REG4 Reverse	Regenerating islet 4	CAGGAAGTGTGGCGCTTGT	MWG
PRSS7 Forward	Protease serine 7	TTCTTGTCAGGGGGATTTCAG	MWG
PRSS7 Reverse	Protease serine 7	AGGCACGGCACACTTGTATC	MWG
CTLA4 Forward	Cytotoxic T lymphocyte antigen 4	CACAAGGCTCAGCTGAACCT	MWG
CTLA4 Reverse	Cytotoxic T lymphocyte antigen 4	AGGTGCCCGTGCAGATGGAA	MWG
GZMA Forward	Granzyme 1	TCAAATACCATCTGTGC TGG	MWG
GZMA Reverse	Granzyme 1	AGAGGGAGCTGACTTATTGC	MWG
GKN1 Forward	Gastrokine 1	ATGGAAATGGCTTTGCTGCAACC	MWG
GKN1 Reverse	Gastrokine 1	TCCCACGACACATGTTTGCAATG	MWG
ITGA6 Forward	Integrin alpha 6	GCTGGTTATAATCCTTCAATATCAATTGT	MWG
ITGA6 Reverse	Integrin alpha 6	TTGGGCTCAGAACCTTGGTTT	MWG
REG3A Forward	Regenerating islet 3	GGTGAAGAACCCCAGAGGGA	MWG
REG3A Reverse	Regenerating islet 3	CTAGTCAGTGAACCTGCAGA	MWG
CPB1 Forward	Carboxypeptidase B1	CGTTGTCTTGGTCATGTCAC	MWG
CPB1 Reverse	Carboxypeptidase B1	CCATGAAAACGGCAGGCTTA	MWG
CD160 Forward	Cluster of differentiation 160	TGCAGGATGCTGTTGGAACCC	MWG
CD160 Reverse	Cluster of differentiation 160	CCTGTGCCCTGTTGCATTCTTG	MWG
CXCL14 Forward	Chemokine CXC ligand 14	AAGCTGGAAATGAAGCCAAA	MWG
CXCL14 Reverse	Chemokine CXC ligand 14	TTCCAGGCGTTGTACCACTT	MWG
ELA2A Forward	Elastase 2A	CATGTGCCCCCGTCGTGTGA	MWG
ELA2A Reverse	Elastase 2A	CAAGGGGAGCGGGGTGGGAGTA	MWG
TP73L Forward	Tumour protein 73L	TGCCATCAGGAATGGTTGTA	MWG
TP73L Reverse	Tumour protein 73L	TGAAACGTACAGGCAACAGC	MWG
FUT6 Forward	Fucosyltransferase 6	AATGGGTCCCGCTTCCCAGACAG	MWG
FUT6 Reverse	Fucosyltransferase 6	GCGTCCGTACACGTCCACCTTG	MWG
IL-7R Forward	Interleukin-7 Receptor	TCGCAGCACTCACTGACC	MWG
IL-7R Reverse	Interleukin-7 Receptor	CGGGAAGGAGCCAATGAC	MWG
IL-8 Forward	Interleukin-8	ATGACTTCCAAGCTGGCCGTGGCT	MWG
IL-8 Reverse	Interleukin-8	TCTCAGCCCTCTCAAAAACCTTCTC	MWG
Kir6.1 Forward	KCNJ8	TGGCTGCTCTTCGCTATC	MWG
Kir6.1 Reverse	KCNJ8	GGGCTACGCTTGTCAATC	MWG

2.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to separate and analyse DNA and RNA fragments. Ethidium bromide was used to visualise gels. Agarose gels (1%) were prepared by dissolving 1 g of agarose (Sigma-Aldrich, Steinheim, Germany) in 100 ml of 10% Tris borate EDTA (TBE) buffer and microwaving for 1 min. Solution was left to cool slightly and ethidium bromide (50 µl) was then added before being poured into gel tank. Any remaining bubbles were removed and comb was inserted. The gel was left

to set for 1 h. The gel was then immersed in tank containing 1% TBE running buffer. When loading the gel, a molecular weight hyperladder I marker (Bioline, MyBio Ltd. Republic of Ireland) was loaded into lane 1. A mixture of DNA samples (10 μ l) and loading dye (2 μ l) (Bioline, MyBio Ltd. Republic of Ireland) were loaded into subsequent wells. The gel tank was closed, the power source was switched on (BioRad, Hertfordshire, HP27DX) and the gel was run at 80 V for 1 h. Gels were then analysed under UV light.

2.6.3 Real time RT-PCR

RNA used for real time RT-PCR was diluted 1 in 50 before use. Real time PCR was undertaken using a RealTime Ready custom RT-qPCR assay (Roche Diagnostics, Mannheim, Germany) on a Lightcycler 480. Since RNA was a limiting factor, custom plates were used to minimise optimisation of RealTime PCR. Custom plates were designed using the Roche RealTime Ready Configurator in conjunction with Roche Diagnostics (Mannheim, Germany) that were compatible with the Lightcycler480 system. Customising a panel enabled the quantification of gene expression of targets of interest by designing a panel of custom targets on Lightcycler 480 multiwall plates (Table 2.10). When received, assays were pre-plated and dried down for a reaction volume of 20 μ l on 96-well plates. Sample cDNA and LightCycler® 480 Probes Master was added to each well. LightCycler® 480 Probes Master contained all necessary components for the reaction: Two reference genes were used as controls (GAPDH and B2M). To each well, 2 μ l of the appropriate template cDNA, 10 μ l of 1 X Probes Master solution and 8 μ l of RNase-free water was added. The plate was sealed with optical adhesive covers (Roche Diagnostics, Mannheim, Germany) and loaded into the LightCycler. The cycle involved three steps. Firstly, pre-incubation took place at 95°C

for 10 min. This was followed by amplification incorporating 45 cycles of 10 sec at 95°C, 30 sec at 60°C and 1 sec at 72°C. The final step was cooling at 40°C for 30 sec. Relative quantification of gene expression analysis of samples was then undertaken.

Table 2.10 Custom panel of genes on a 96 multiwell plate designed for real-time PCR

	1	2	3	4	5	6	7	8	9	10	11	12
A	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>	REG4 <i>H. sapiens</i>	PRSS7 <i>H. sapiens</i>
B	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>	CTLA4 <i>H. sapiens</i>	GZMA <i>H. sapiens</i>
C	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>	GKN1 <i>H. sapiens</i>	ITGA6 <i>H. sapiens</i>
D	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>	REG3A <i>H. sapiens</i>	CD160 <i>H. sapiens</i>
E	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>	CXCL14 <i>H. sapiens</i>	CELA2A <i>H. sapiens</i>
F	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>	TP63 <i>H. sapiens</i>	IL7R <i>H. sapiens</i>
G	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>	IL8 <i>H. sapiens</i>	KCNJ8 <i>H. sapiens</i>
H	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>	GAPDH <i>H. sapiens</i>	B2M <i>H. sapiens</i>

2.7 BUFFERS

Analytical grade chemicals and diluents were utilised for the generation of buffers.

2.7.1 Phosphate buffered saline

Phosphate buffered saline (PBS) was made by dissolving one PBS tablet (Oxoid BR14a, Poole, England) per 100 ml distilled water. Solution was then sterilised by autoclaving at 121°C for 15 min at 15 lb in-2. A pH of 7.3 was reached when the solution was cooled.

2.7.2 Tris buffered saline

Tris buffered saline (TBS) was made by preparing a 1:10 dilution of a 10X TBS solution that is available to buy from Bio-Rad Laboratories, Inc., C.A., U.S.A. The resulting solution consisted of 500 mM sodium chloride and 20 mM Tris at a pH of 7.5.

2.8 GENERAL PROCEDURES

2.8.1 Spectrophotometry

Analysis of the optical density (O.D) of bacterial suspensions along with colorimetric assays was undertaken using a ThermoScientific Helios Epsilon spectrophotometer (MA, USA). A Shimadzu UV-160 spectrophotometer from Shimadzu Corporation, Shinjuku-Ku, Tokyo, Japan was used for measurement of UV/visible scanning spectroscopy of LPS.

2.8.2 Centrifugation

Centrifugation of bacterial suspensions and small volumes was performed in an Eppendorf 5417C bench-top centrifuge (Eppendorf AG, Hamburg, Germany). Larger volumes including those up to 50 ml were placed in either 15 ml or 50 ml plastic centrifuge tubes (Sarstedt, AG & Co., Numbrecht, Germany) and centrifuged in a Beckman Coulter Avanti J-20 XP centrifuge containing a JLA 16.250 rotor. Centrifugation of tissue culture samples was undertaken in an Eppendorf 5804 bench-top centrifuge (Eppendorf AG, Hamburg, Germany). Multiwell plates were centrifuged in a Beckman Coulter Allegra X-12R centrifuge. Ultracentrifugation of bacterial fractions was carried out in a Beckman Coulter Optima L-90K ultracentrifuge.

2.8.3 Measurement of mass

A B204-S electronic balance by Mettler Toledo was used to determine the mass of matter that was 1 g or less. This balance was supplied by Mason Technology, Dublin, Ireland. For matter that weighed 1 g or more, a Satorius BL160 (Sartorius, Göttingen, Germany) balance was used for measurement.

2.8.4 Sonication

Those samples which needed further solubilisation were sonicated in a 2510 Branson sonication bath (Branson Ultrasonics Corp., Danbury, CT, U.S.A.) which had a heating option. Sonication times were limited to 30 min and the maximum temperature reached was 60°C. For isolation of proteins and LPS from *H. pylori* biomass using the Blaser Outer membrane Extraction protocol, sonication was undertaken on ice using MSE Soniprep 150.

2.8.5 Determination of pH

The pH of solutions was determined using either Hydrion Microfine pH test paper set (Sigma-Aldrich) or an Orion A⁺ pH metre (Orion Research Inc., Beverly, MA, U.S.A.).

2.8.6 Autoclaving

Sterilisation was carried out via autoclaving using a HICLAVE HV-85L autoclave (HMC Europe GmbH, Hafing, Germany).

2.9 STATISTICS

Where sufficient numbers of patient samples were available, statistical analysis was carried out using GraphPad InStat[®] software (GraphPad Software, Inc., California, USA). The Mann-Whitney U test was used to calculate significance between non-parametric data points. The degree of significance was considered as $P < 0.05$.

Chapter 3

Phenotypic Characterisation of Human Gastric Epithelial and Lamina Propria Layer Lymphocytes in *H. pylori* Positive and *H. pylori* Negative Patients

3.1 INTRODUCTION

3.1.1 Mucosal immunity

The location of the mucosa separating internal and external contents of the gut lumen implies a constant exposure to a large range of luminal Ags including food products, enzymes and microbes. The mucosa is protected by a variety of factors such as the epithelium, which itself forms a physical barrier limiting the entry of noxious substances along with the mucus layer, secretory IgA and proteolytic enzymes (Kaiserlian *et al.*, 2005). In addition, there exists a highly sophisticated immune system known as the MALT responsible for the initiation of the body's immune responses to infectious micro-organisms on mucosal surfaces. The MALT can be divided into two different subgroups namely the inductive and effector sites (Cesta, 2006). Inductive sites harbour secondary lymphoid tissues where B lymphocyte clonal expansion takes place following T cell stimulation with antigenic peptides (Woof & Mestecky, 2005). After T cell activation has occurred, both T and B cells relocate to effector sites found in epithelium and lamina propria mucosal tissues. In these effector sites, CD4⁺ T cells, IgA, IgG, IgM, plasma cells, B cells, macrophages and DCs can be found along with IELs and lamina propria lymphocytes (Cesta, 2006).

Intraepithelial lymphocytes

IELs are the first lymphocyte population coming into contact with external environmental Ags within the gut and therefore make up a large portion of the body's lymphocytes. IELs are primarily located in the basal area of adsorptive enterocytes in the gut epithelium and virtually all are T cells. In fact, greater than 70% of IELs residing in the human small intestine are CD8⁺ T cells with an $\alpha\beta$ -TCR (Cheroutre *et al.*, 2011; Jarry *et al.*, 1990; Moens & Veldhoen, 2012), however, a significant proportion of IELs

express a $\gamma\delta$ -TCR (Cheroutre *et al.*, 2011). Higher numbers of $\gamma\delta^+$ T cells are present here in epithelial tissues than in mouse or human peripheral blood (Hayday *et al.*, 2001; Hayday, 2000). A significant proportion of IELs are equipped with a CD8 $\alpha\alpha$ homodimer, a characteristic not usually attributed to systemic T cells (Jarry *et al.*, 1990). In humans, CD4 $^+$ T cells are not found as frequently in the intestinal epithelium (Lefrancois, 1991) with only 5-15% of CD4 $^+$ T cells constitute the IEL population (Spencer *et al.*, 1989). DN CD4 $^-$ CD8 $^-$ T cells are quite abundant in murine intestinal tissues comprising approximately 10% of IELs in the mouse small intestine and a significant proportion of other intestinal compartments (Hayday *et al.*, 2001). The majority of IELs are activated through the CD69 and $\alpha E\beta 7$ surface markers (Cerf-Bensussan *et al.*, 1987). A high percentage (80%) of IELs are CD45RO $^+$ which is a memory-associated cell surface marker (Jarry *et al.*, 1990). Human IELs do not express cell surface markers associated with cytotoxicity such as CD16, while there is evidence of CD5 and CD28 expression, but expression is at lower levels than that of T cells circulating in the periphery suggesting minimal involvement of IELs with B cells (Ebert, 1989).

Lamina propria T cells

Within the underlying lamina propria, T cells comprise 25-40% of the leukocyte population (Ullrich *et al.*, 1990). Higher numbers of CD4 $^+$ T cells than CD8 $^+$ T cells have been reported in lamina propria tissues (Abuzakouk *et al.*, 1998; Lefrancois, 1991). The vast majority of lamina propria T cells express the $\alpha\beta$ -TCR canonical arrangement with 3% expressing the alternative $\gamma\delta$ -TCR (Ullrich *et al.*, 1990). Lamina propria T cells contain similar numbers of CD4 $^+$ and CD8 $^+$ T cells to that in the peripheral blood and are CD45RO $^+$ /CD45RBlo, CD44hi with 10% positive for the

expression of CD45RA (Schieferdecker *et al.*, 1992). Taken together, this constitutes a memory phenotype. Lamina propria T cells also express markers of activation with 18% expressing human leukocyte (HLA)-DR Ags and 10% of lamina propria T cells positive for IL-2R expression (Senju *et al.*, 1991).

Mucosal immunity in infection and autoimmune disease

Infection with enteric bacteria induces alterations in the composition of cells residing in intestinal tissues. For instance, infection may cause the infiltration of certain cell populations to intestinal tissues from other areas of the body or alternatively may induce the migration of resident cells away from intestinal tissues. Quantifying changes in cell numbers is vital to understanding the pathogenesis of infectious organisms as immune cells have an impact on the ensuing immunological host response (Eckmann & Kagnoff, 2005). Regarding IEL and lamina propria lymphocyte cell numbers in autoimmune diseases and infection, a study by Kutlu *et al.*, (1993) revealed a significant decrease in $\alpha\beta^+$ T cells in the epithelial layer of patients reporting previous coeliac disease whose mucosa had returned to normal after strict diet in comparison to active coeliac sufferers. As a result, the authors suggest that $\alpha\beta^+$ T cells have a role to play in pathogenesis of villous atrophy due to responsiveness to gliadin in coeliac disease (Kutlu *et al.*, 1993). Senju *et al.*, (1991) investigated CD4⁺ and CD8⁺ co-expression in PBMCs and lamina propria lymphocytes of individuals suffering from Crohn's disease and ulcerative colitis and compared their incidence with uninfected controls. The authors reported increased CD4⁺CD8⁺ expression in PBMCs in inflammatory bowel disease (IBD) when compared with healthy controls. Moreover, in healthy controls and in patients with Crohn's disease, there was a higher incidence of CD4⁺CD8⁺ cells in the lamina propria than in PBMC samples while lamina propria CD4⁻CD8⁺ T cells were reduced during ulcerative

colitis. Together, data suggests activation of immune cells occurs during remission in IBD patients, while the high numbers of CD4⁺CD8⁺ double positive (DP) cells in the lamina propria implies the lamina propria is being constantly stimulated (Senju *et al.*, 1991). T cell numbers are also expanded during viral infections. Barnaba *et al.*, (1994) reported an increase in cytotoxic CD4⁺CD56⁺ T cells in the liver of individuals implicated with chronic Hepatitis B infection.

Mucosal immunity and *H. pylori* infection

In *H. pylori* infected individuals, the bacteria normally resides within or underneath the gastric antral mucus (Lee, 1994) or in the duodenum (Steer, 1984) and in close proximation with the epithelial layer. Upon *H. pylori* infection, an innate mucosal inflammatory immune response is firstly triggered following activation of host PRRs such as TLRs and NLRs by *H. pylori* derived PAMPs such as LPS and flagella. This interaction leads to the infiltration of polymorphonuclear and mononuclear inflammatory cells (Goodwin *et al.*, 1986) such as plasma cells, macrophages, neutrophils, B and T lymphocytes to gastric tissues (Ernst & Gold, 2000). Both CD4⁺ T cells and CD8⁺ T cells are recruited to gastric tissues (see section 1.8.8). While viruses induce a CD8⁺ cytotoxic T cell immune response, *H. pylori* is generally endocytosed by APCs, where Ags are processed and presented to CD4⁺ T cells. Therefore, only CD4⁺ T cells are activated by *H. pylori*. This action triggers a T_h1 mediated immune response (Pellicanò *et al.*, 2007; Wilson & Crabtree, 2007) which occurs after differentiation of naïve CD4⁺ T cells as a result of IL-12 production by APCs and arises following activation of APC and T cell derived transcription factors (Szabo *et al.*, 2002). CD4⁺ T cells display an activated or a memory phenotype and therefore are CD45RO⁺ (Hatz *et al.*, 1996). D'Elis *et al.*, (2003) studied T cell clones isolated from the gastric antrum

of *H. pylori* infection with chronic gastritis, peptic ulcers, gastric adenocarcinoma and low grade B-cell lymphoma and found that the majority of these cells displayed CD4⁺ phenotype while CD8⁺ T cells were present in much lower numbers (D'Elia *et al.*, 2003). Another consequence of a polarised T_h1 immune response is that T_{reg} cells are induced during *H. pylori* infection. There is an up-regulation of the activity of T_{reg} cells during infection, marked by an increase in CD25 and Foxp3 expression, contributing towards the onset of clinical pathologies (Rad *et al.*, 2006). The production of anti-inflammatory cytokines IL-10 and TNF-β occurs at this time. T_{reg} cells function to down-regulate inflammation in gastric tissues by suppressing the ensuing adaptive immune response (Kandulski *et al.*, 2010). The majority of immune cells infiltrated in gastric tissues during *H. pylori* infection are T_h1 driven, however it is suggested a small percentage of may be anergic (Lundgren *et al.*, 2003). This anergic response is believed to occur following the interaction of T cells with an APC co-stimulatory molecule harbouring suppressive properties present on gastric epithelial cells called B7-H1 (Beswick *et al.*, 2007; Das *et al.*, 2006). *H. pylori* infection is also associated with large titres of localised and circulating Abs (Mattsson *et al.*, 1998) suggesting a role for monocytes or macrophages in *H. pylori* antigenic presentation to immune cells. Higher numbers of CD4⁺ T cells than CD8⁺ T cells have been reported in lamina propria tissues of *H. pylori* infected patients. Moreover, CD4⁺ T cells express elevated levels of L-selectin, a homing receptor and CCR4 expression, known for their presence on tissue infiltrating memory CD4⁺CD25^{high} T_{reg} cells (Eric *et al.*, 2002; Iellem *et al.*, 2001; Lundgren *et al.*, 2005). Overall, it is known that *H. pylori* infection elicits a complex immunologic response in the gastric mucosa which initiates both innate and adaptive responses and involves cell subsets such as neutrophils, plasma cells, eosinophils and lymphocytes, of which classical T cells are the primary orchestrators (O'Keeffe &

Moran, 2008). On the other hand, the role of mucosal unconventional T cells in *H. pylori* infection is relatively unknown.

3.1.2 NKT cells in mucosal immunity

In mice, $V\alpha 14^+$ T cells constitute approximately 10% of cells in liver and bone marrow while high numbers are also recorded in spleen, thymus and peripheral blood (1-3%). In humans, the numbers of $V\alpha 24J\alpha 18^+$ T cells are much lower constituting < 0.1% of peripheral and bone marrow T cells and <1% of liver T cells (Gumperz *et al.*, 2002; Karadimitris *et al.*, 2001; Kenna *et al.*, 2003; Lee *et al.*, 2002). Interestingly, Lynch *et al.*, (2009) recorded the highest known NKT cell numbers in the body in the omentum, a fold of the peritoneum that surrounds the body's organs, with up to 30-50% of T cells expressing the $V\alpha 24J\alpha 18$ chain. While NKT cells are known to mainly populate the liver, spleen and bone marrow, both invariant and variant NKT cell populations also reside in intestinal tissues as they constitute a portion of both IEL and LPL populations.

In intestinal tissues of mice, $CD3^+$ T cells with NK receptors account for 4% of small intestinal IELs, 8-10% of large intestinal IELs and constitute 7% of lamina propria lymphocytes (Wingender & Kronenberg, 2007). Using CD1d loaded tetramers containing α -GalCer, it was shown that 1% of NKT cells reside in small intestinal tissues while NKT cells constitute 2% of mouse lamina propria lymphocytes. In humans, $CD3^+CD161^+$ NKT cells lacking the invariant chain account for 50-70% of small intestinal IELs, 40-45% of large intestinal IELs and 9% of large intestinal lamina propria lymphocytes. Using CD1d loaded tetramers containing α -GalCer, it is estimated invariant $CD3^+V\alpha 24J\alpha 18^+$ NKT cells make up <0.4% of human intestinal T cells with the majority residing in lamina propria layer tissues (O'Keeffe *et al.*, 2004; Wingender & Kronenberg, 2007).

Differences in NKT cell numbers have been recorded in several intestinal infections. For example, in mice, CD1d⁺NK1.1⁺ T cells and invariant CD1d restricted T cell numbers are increased during infection with *Salmonella typhimurium*. This is accompanied by the up-regulation of CD69 cell surface marker expression and IFN- γ cytokine production (Berntman *et al.*, 2005). In humans, a study involving 120 cancer patients has shown a 50% decrease in the incidence of circulating V α 24⁺V β 11⁺ NKT cells in patients with cancer when compared with age and gender matched healthy individuals. Moreover, this lower incidence occurs regardless of tumour type and tumour load (Molling *et al.*, 2005). In intestinal tissues, in mice, NKT cells have been linked with an involvement in the onset of colitis (Liao *et al.*, 2012).

Functionally, it is hypothesised that intestinal NKT cells play a role in mucosal immunity against both indigenous microflora and invading pathogens (Middendorp & Nieuwenhuis, 2009). Their location in mucosal tissues along with their innate ability to rapidly produce cytokines, display cytotoxicity and their ability to trigger adaptive immune responses making NKT cells an ideal candidate for localised immune response. Upon activation with α -GalCer, NKT cells can secrete a wide range of cytokines and growth factors that lead to activation and polarisation of an adaptive immune response (Brigl & Brenner, 2004; Goto *et al.*, 2009; Rachitskaya *et al.*, 2008). In mice, NKT cell activation can lead to prevention of tumour growth, provide protection against microbial pathogens and help alleviate autoimmune diseases (Behar & Porcelli, 2007; Crowe *et al.*, 2005; Wilson & Van Kaer, 2003). In humans, following activation, NKT cells respond quickly and produce a range of cytokines, possess potent killing properties and regulate other immune cells (Matsuda *et al.*, 2008).

NKT cell numbers gastric tissues have yet to be elucidated and their incidence in *H. pylori* infection has not yet been explored. Moreover, the different NKT cell subsets

have not been assessed in the gastric mucosa, important as NKT cell phenotype has been shown to determine their repertoires. O'Reilly *et al.*, (2011) recently analysed phenotypic and functional properties of CD4⁺, CD8 α ⁺ and DN NKT cell subsets and recorded that CD4⁺ NKT cells expanded more rapidly than CD8 α ⁺ and DN counterparts, while CD8 α ⁺ and DN subsets predominantly expressed CD56, CD161 and NKG2D markers. Moreover, the authors recorded varying cytokine production amongst subsets; therefore discerning the phenotype of NKT cell subsets is a crucial factor in NKT cellular function.

3.1.3 $\gamma\delta$ ⁺ T cells in mucosal immunity

Cells bearing a $\gamma\delta$ -TCR are enriched in intestinal tissues. In mice, 35-65% of small intestinal IELs as well as the vast majority of DETCs and vaginal IELs harbour the $\gamma\delta$ -TCR canonical arrangement (Hayday, 2000). In humans, $\gamma\delta$ ⁺ T cells constitute 15% of small intestinal IELs, 40% of colonic IELs, 5% of lamina propria layer lymphocytes and peripheral blood T lymphocytes (reviewed by Meresse & Cerf-Bensussan, 2009). $\gamma\delta$ ⁺ T cells are the first cells to migrate from the thymus following the maturation process to their final destination within residential epithelial tissues and in the periphery. Different subsets can be found in various locations around the body. In mice, V γ 1 and V γ 4 subsets can be found in the spleen, V γ 5V δ 1 reside in the epidermis, V γ 4, V γ 1V δ 6, V γ 1V δ 6.3 predominate in liver, V γ 7 paired with V δ 4,5 or 6 arrangements are located in gut epithelial tissues while V γ 4 and V γ 6 are localised in the lung epithelium (Bonneville *et al.*, 2010; Meresse & Cerf-Bensussan, 2009). In humans, V γ 9V δ 2 cells are generally found circulating in the peripheral blood of adults and these account for 80-90% of the $\gamma\delta$ ⁺ T cell population (Meresse & Cerf-Bensussan, 2009), V δ 1 subset can be found in the thymus, spleen, dermis, liver and gut epithelium while V δ 3 subset also

resides in liver and gut epithelial tissues (Bonneville *et al.*, 2010; Komori *et al.*, 2006; O'Brien *et al.*, 2007). In human epithelia, $\gamma\delta^+$ T cells constitute 10% of the entire T cell population and this number is higher in mice where $\gamma\delta^+$ T cell frequency can be between 50-100% of T cells (Witherden & Havran, 2011). $\gamma\delta^+$ T cells populations have distinct functions according to their location and different subsets of are believed to participate in different immune functions. For example, differences in both the numbers and the subset have been observed during infection. In disease state, in mice infected with influenza A virus, Augustin *et al.*, (1989) demonstrated an increase in $V\gamma 4^+$ T cells 10 days after infection while Carding *et al.*, (1990) reported an infiltration of $V\gamma 1^+$ T cells 13 days following infection in the lung of mice. In humans, in *H. pylori* infection, Engstrand *et al.*, (1991), examined gastric biopsies in patients suffering from chronic gastritis as a result of *H. pylori* infection and reported an increase in $\gamma\delta^+$ T cells in the epithelium. Moreover Futagami *et al.*, (2006) investigated $\gamma\delta^+$ and $CD45RO^+$ T cell numbers in the gastric mucosa of patients suffering varying severities of gastritis using immunohistochemistry. The authors found that the $V\delta 1$ subset is predominantly located in the gastric tissues of infected patients and observed an increase in $\gamma\delta^+$ T cell numbers in grade III gastritis patients which was associated with potent Ig responses to *H. pylori* urease. Moreover, after *H. pylori* eradication, a decrease in $\gamma\delta^+$ T cell numbers in grade III gastritis patients was recorded (Futagami *et al.*, 2006).

Notwithstanding this, studies showed varying cytokine profiles associated with the two distinct $\gamma\delta^+$ T cell subsets which would further suggest different functions for each subset. $V\delta 1$ T cells have been shown to kill tumour cells in the epithelium (Groh, 1999) and their locality in mucosal tissues suggests a role in immuno-surveillance. Known functions of $\gamma\delta^+$ T cells include tissue homeostasis, pro-inflammatory cytokine production, potent killing effects following encounter with phosphorylated metabolite

Ags or mycobacterial ligands, regulation of immune response and triggering of adaptive immune responses through Ag processing and presentation to classical T cells (see section 1.4.2) (Bendelac *et al.*, 2001; Brandes *et al.*, 2005; Hayday, 2009; Treiner *et al.*, 2003). For instance, small intestinal (SI) $\gamma\delta^+$ T cells produce antimicrobial molecules in response to a bacterial encounter thereby helping to maintain tissue homeostasis (Ismail *et al.*, 2011). Intestinal $\gamma\delta^+$ T cells have been shown to regulate inflammation in infection in chemically induced colitis (Inagaki-Ohara *et al.*, 2004) and $\gamma\delta^+$ T cells are suggested to play a part in controlling infection as murine DETCs are activated by LPS from gram negative bacteria (Leclercq & Plum, 1995; Reardon *et al.*, 1995).

3.1.4 Aims

Given the unique roles of T cells in mucosal immunity and in particular the roles of NKT and $\gamma\delta^+$ T cells, the aim of this chapter was to study the cellular response of these immune cells in *H. pylori* infected human gastric mucosa. This was accomplished by using fluorescently labelled mAbs and flow cytometry to identify their surface marker expression:

1. To determine the frequencies of:
 - T cells ($\alpha\beta^+$ T cells, CD4⁺ T cells, CD8⁺ T cells and CD4⁺CD8⁺ T cells) and NK cells (CD3⁻CD56⁺),
 - NKT cell subsets using mAb directed against V α 24J α 18:
 1. CD3⁺ V α 24J α 18⁺
 2. CD3⁺ V α 24J α 18⁺CD4⁺
 3. CD3⁺ V α 24J α 18⁺CD8⁺
 4. CD3⁺ V α 24J α 18⁺CD56⁺
 5. CD3⁺ V α 24J α 18⁺CD161⁺
 - $\gamma\delta^+$ T cells using a mAb directed against $\gamma\delta$ -TCR in normal gastric epithelial and lamina propria layers.
2. To compare the frequencies of each population in *H. pylori* positive and negative individuals.

3.2 RESULTS

3.2.1 Quantification of conventional T cell subsets in *H. pylori* infected and uninfected gastric mucosa

A population of 43 patients incorporating both *H. pylori* positive (n = 9) and negative (n = 34) individuals were used across this part of the study. After separating fresh antral gastric biopsies into their epithelial and lamina propria layers (see section 2.2), flow cytometry was used to determine the frequency of T cell populations in *H. pylori* infected and uninfected gastric tissues.

$\alpha\beta^+$ T cell numbers in *H. pylori* infected and uninfected gastric mucosa

$\alpha\beta^+$ T cells were quantified using an $\alpha\beta$ -TCR mAb. As shown in Table 3.1 and Fig. 3.1, in normal epithelium and lamina propria layers from *H. pylori* negative subjects, the numbers of CD3⁺TCR $\alpha\beta^+$ were not significantly different in the two layers (medians, 89.41% vs. 86.94%; $p = 0.7$; n = 13). Likewise, in the epithelial and lamina propria layer of *H. pylori* positive subjects, large numbers of $\alpha\beta^+$ T cells were found (medians, 79.02 % vs. 83.3%; n = 5).

CD4⁺ T cells and CD8⁺ T cells

As shown in Table 3.1 and Fig. 3.1, in normal epithelium from *H. pylori* negative subjects, the numbers of CD8⁺ T cells (n = 15) were more abundant than CD4⁺ T cells (n = 19) (medians, 85.7% vs. 15.8%). In the lamina propria, likewise, the numbers of CD8⁺ T cells were higher (median, 55.5 %) compared with CD4⁺ T cells (median, 37.9%), although not significant. In the lamina propria layer of *H. pylori* positive subjects, the numbers of CD4⁺ T cells and CD8⁺ T cells were significantly higher (median, 61%; $p = 0.02$) and lower (median, 39.48%; $p = 0.02$) respectively compared

with *H. pylori* negative control subjects (n = 9). Among epithelial layer lymphocytes, there were no significant differences in the numbers of CD4⁺ or CD8⁺ T cells when compared with control subjects (n = 9).

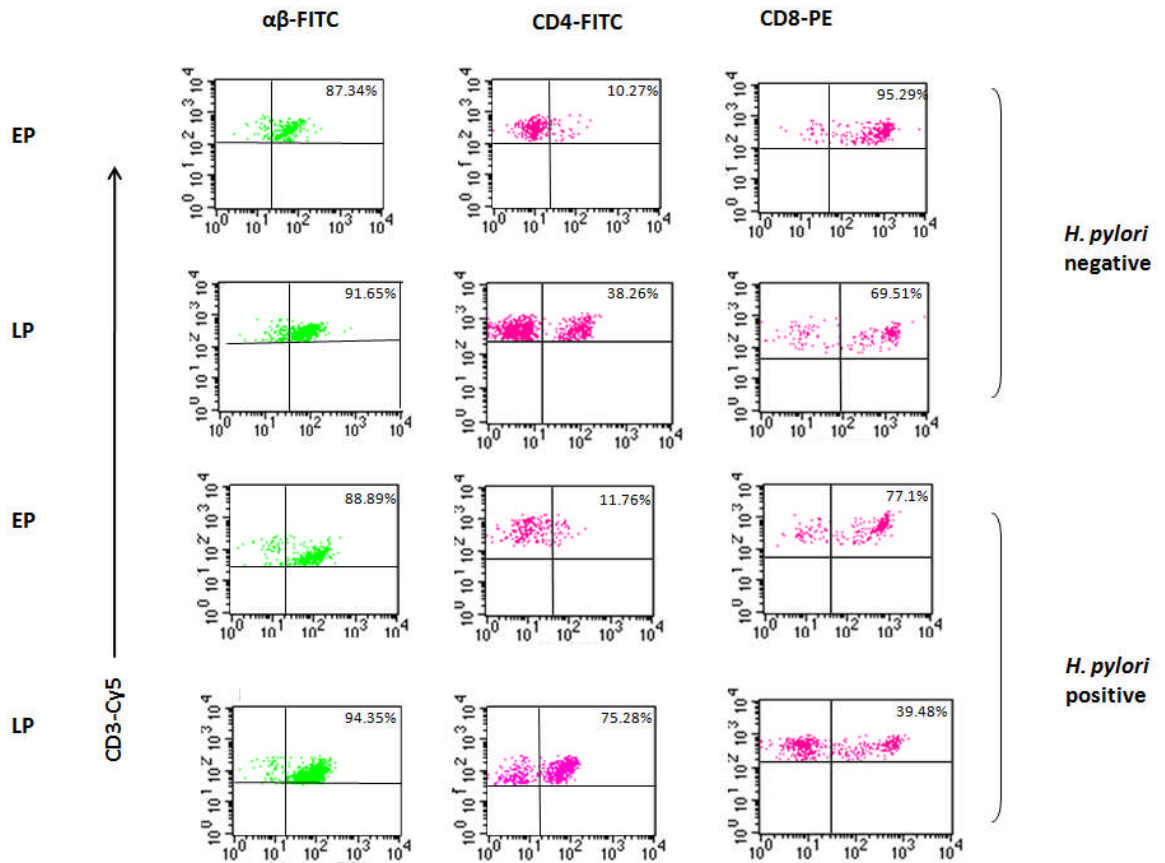


Fig. 3.1 Representative dot plots comparing CD4⁺, CD8⁺ and $\alpha\beta$ ⁺ T cells in the gastric epithelial and lamina propria of *H. pylori* positive and *H. pylori* negative patients. Percentages of positive cells are displayed in the upper right quadrants. Cells were quantified using double staining and gated on the basis of FSC/SSC and CD3 expression and quadrants were set on the basis of isotype matched control Abs (see section 2.2).

CD4⁺CD8⁺ double positive T cells

CD4⁺CD8⁺ DP T cells were quantified by triple staining using the CD3 mAb in conjunction with CD4 and CD8 mAbs. As shown in Table 3.1 and Fig. 3.2, in normal epithelium and lamina propria from *H. pylori* control subjects; similar numbers of DP T cells were found (medians, 5.87% vs. 4.79%; n = 8). Interestingly, in the two *H. pylori*

positive subjects studied, the numbers of DP T cells were markedly higher in both the epithelium (median, 14.13%) and in the lamina propria (median, 10.12%) when compared with controls, although the differences did not reach significance ($p = 0.71$, epithelium; $p = 0.99$, lamina propria).

In summary, upon analysis of gastric T cells, when comparing healthy versus infected subjects, in the epithelial and lamina propria layer of *H. pylori* positive subjects, large numbers of $\alpha\beta^+$ T cells were recorded in each compartment of both infected and uninfected individuals. In the lamina propria layer of *H. pylori* positive subjects, the numbers of $CD4^+$ T cells and $CD8^+$ T cells were significantly higher and lower respectively compared with *H. pylori* negative control subjects. Regarding $CD4^+CD8^+$ T cells, in the two *H. pylori* positive subjects studied, the numbers of DP T cells were markedly higher in the epithelium and in the lamina propria when compared with controls. An increase in subject numbers would reveal more.

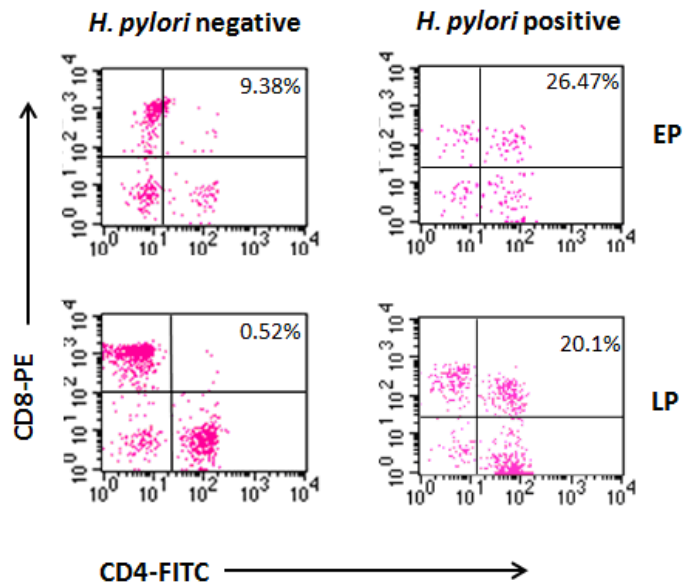


Fig. 3.2 Representative dot plots comparing $CD4^+CD8^+$ T cells in the gastric epithelial and lamina propria of *H. pylori* positive and *H. pylori* negative patients. Percentages of positive cells are displayed in the upper right quadrants. Cells were quantified using triple staining and gated on the basis of FSC/SSC and CD3 expression and quadrants were set on the basis of isotype matched control Abs (see section 2.2).

Table 3.1 Comparison of T cells subsets in the gastric epithelial and lamina propria layer of *H. pylori* - positive and -negative individuals.

		<i>HP</i> -negative (%)		<i>HP</i> -positive (%)	
		Epithelium	Lamina propria	Epithelium	Lamina propria
CD3 ⁺ αβ ⁺	Median ± SEM	89.41 ± 1.38	86.94 ± 5	79.02 ± 7.78	83.3 ± 2.87
	Range	(79.6-92.7)	(54.55-97.25)	(46.98-95.78)	(77.39-94.35)
	<i>p</i> -value	NS	NS	NS	NS
	n	13	13	5	5
CD3 ⁺ CD4 ⁺	Median ± SEM	15.8 ± 2.3	37.9 ± 4.3*	14.3 ± 9	61 ± 8.2*
	Range	(2.5-37)	(17.6-63.9)	(2.2-88.6)	(8-95.3)
	<i>p</i> -value	NS		NS	<i>p</i> = 0.02
	n	19	19	9	9
CD3 ⁺ CD8 ⁺	Median ± SEM	85.7 ± 4.9	55.5 ± 4.8*	79.4 ± 4.7	39.48 ± 3.2*
	Range	(36.6-98.1)	(32.2-78.1)	(51.1-90.1)	(23.2-55)
	<i>p</i> -value	NS		NS	<i>p</i> = 0.02
	n	15	15	9	9
CD3 ⁺ CD4 ⁺ CD8	Median ± SEM	5.87 ± 1.17	4.79 ± 1.46	14.13 ± 12.3	10.12 ± 9.98
	Range	(0.18-9.38)	(0.49-12.97)	(1.79-26.47)	(0.14-20.1)
	<i>p</i> -value	NS	NS	NS	NS
	n	8	8	2	2

HP = *H. pylori*; NS = Not significant; SEM = standard error of the mean; * indicates significance where *p* is comparing the lamina propria layer of *H. pylori* positive and negative patients. No other comparisons revealed significance

3.2.2 Quantification of NK cells in *H. pylori* infected and un-infected gastric epithelium and lamina propria

NK cells

NK cells were quantified by staining cells of the gastric epithelial and lamina propria layers on the basis of lack of CD3 expression and the expression of CD56. In *H. pylori* negative subjects (Fig. 3.3 and Table 3.2), there were no significant differences in the numbers of NK cells ($CD3^-CD56^+$) in the epithelial layer (median, 4.2%) compared with the lamina propria (median, 8.79%) ($n = 21$). In *H. pylori* positive subjects, NK cell numbers were significantly lower in the lamina propria layer (median, 4.43%) when compared with controls (median, 8.79%; $p = 0.03$), whereas there were no significant differences seen in NK cell numbers in the infected epithelial layer compared with controls (medians, 3.9% vs. 4.2%) ($n = 12$).

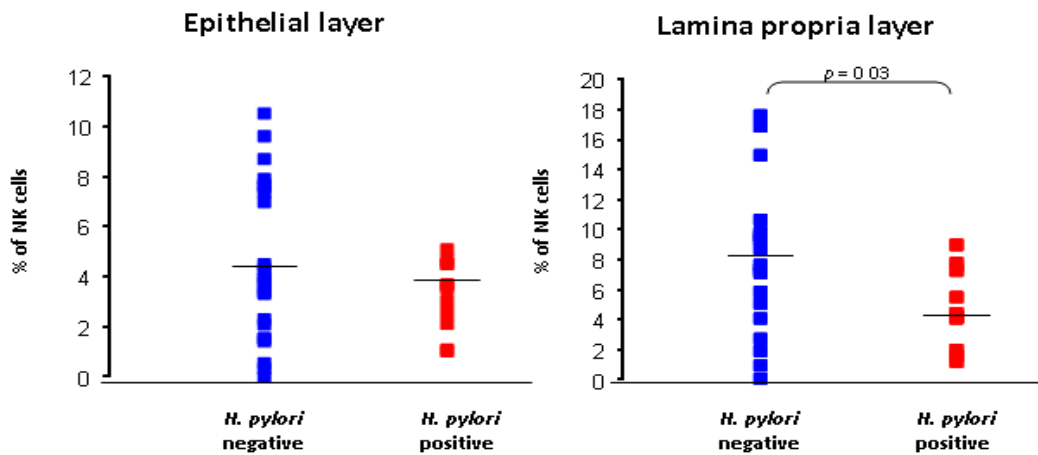


Fig. 3.3 Comparative XY scatter plots showing differences in percentages of NK cells amongst epithelial and lamina propria layer lymphocytes in *H. pylori* negative patients and *H. pylori* positive patients.

Table 3.2 NK cells in the gastric epithelial and lamina propria layer of *H. pylori* positive and negative individuals.

		<i>HP</i> -negative (%)		<i>HP</i> -positive (%)	
		Epithelium	Lamina propria	Epithelium	Lamina propria
CD3 ⁺ CD56 ⁺	Median ± SEM	4.2 ± 0.6	8.79 ± 1*	3.9 ± 0.4	4.43 ± 0.7*
	Range	(0.3-10.8)	(0.14-17.6)	(1.3-5.4)	(1.3-9.06)
	<i>p</i> -value	NS		NS	<i>p</i> = 0.03
	n	21	21	12	12

HP = *H. pylori*; NS = Not significant; SEM = standard error of the mean; **p*-values comparing lamina propria layer tissues of *H. pylori* infected and uninfected patients.

3.2.3 Quantification of Unconventional T cell populations in *H. pylori* infected and un-infected gastric epithelium and lamina propria

NKT cells

The expression of the V α 24J α 18⁺TCR defines the invariant CD1d-restricted NKT cell population in humans (Porcelli *et al.*, 1993). Flow cytometry was used here to quantify NKT cells among gastric epithelial and lamina propria layer lymphocytes. As shown in Fig. 3.4 and Table 3.3, a small percentage of T cells in both mucosal compartments of *H. pylori* negative patients expressed the V α 24J α 18⁺TCR (median, 1.88% epithelium; 0.81% lamina propria; *p* = 0.14; n = 23). In *H. pylori* positive subjects (n = 8), NKT cells were more abundant in the epithelium (median, 2.66%; *p* = 0.4) compared with controls. There were no changes in NKT cell numbers in the lamina propria layer in the *H. pylori* positive subjects compared with controls (medians, 0.86% vs. 0.81%).

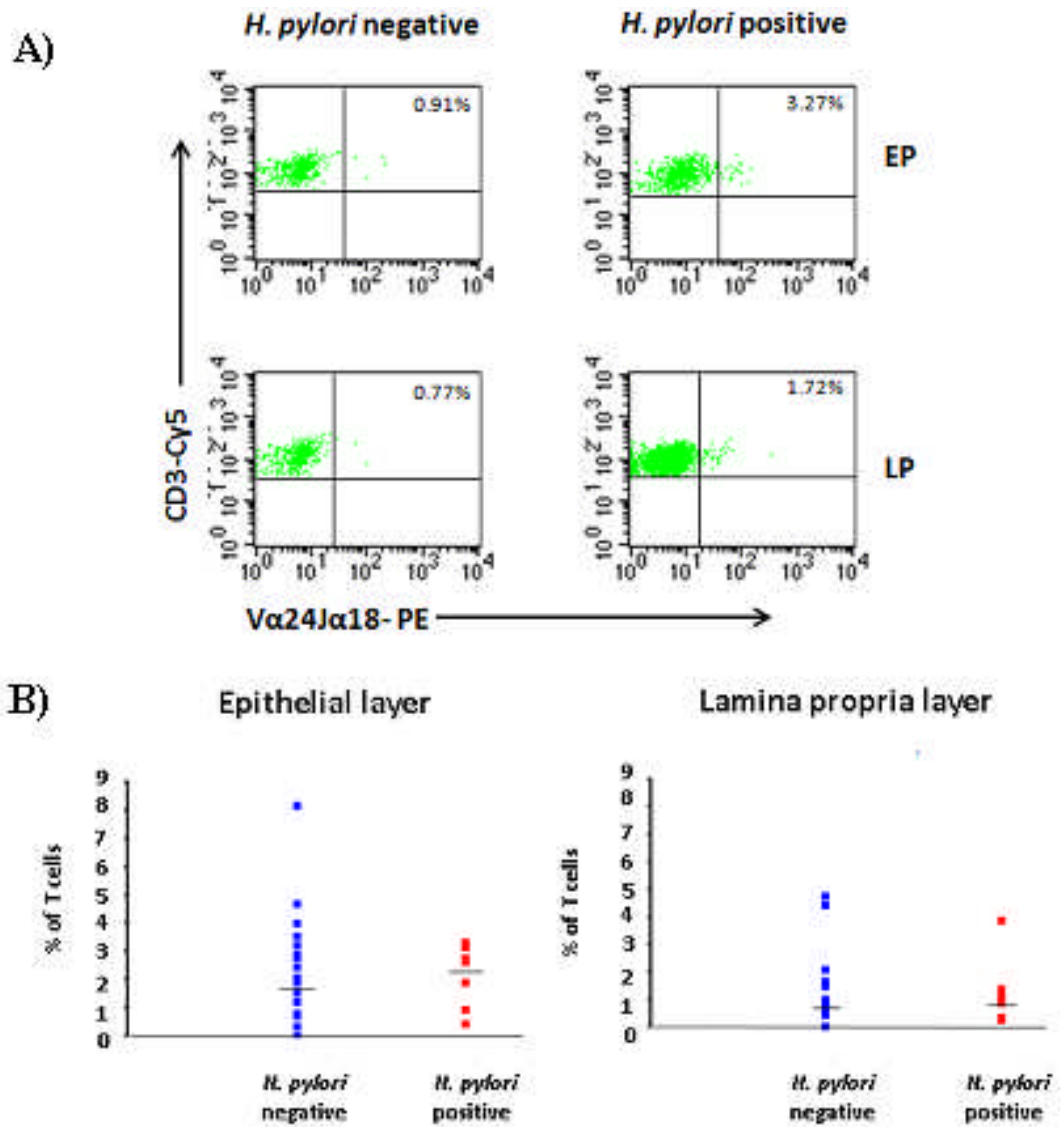


Fig. 3.4 Representative flow cytometric dot plots (A) showing invariant NKT cells in the gastric epithelial (EP) and lamina propria (LP) layer where percentages of NKT cells are displayed in the upper right quadrants. Cells were gated on the expression of CD3 where quadrants were set on the basis of isotype matched controls. Comparative XY scatter plots (B) showing differences in percentages of NKT cells amongst epithelial and lamina propria layer lymphocytes in *H. pylori* negative patients ($n = 23$) and *H. pylori* positive patients ($n = 8$). *H. pylori* negative samples are in blue and *H. pylori* positive samples are displayed in red. Horizontal lines indicate median values.

Further phenotypic characterisation of NKT cells

NKT cells were then further characterised using triple staining for the examination of NK receptors, CD56 and CD161 and for the expression of co-receptors CD4 and CD8.

The results are shown in Fig. 3.5 and Table 3.3.

CD3⁺V α 24J α 18⁺CD56⁺ NKT cells

CD3⁺V α 24J α 18⁺CD56⁺ NKT cells were quantified by triple staining using the CD3 mAb in conjunction with V α 24J α 18 and CD56 mAbs. As shown in Table 3.3, in the epithelial layer of *H. pylori* negative controls, CD3⁺V α 24J α 18⁺CD56⁺ NKT cells were more abundant but not significant (median, 1.16%) compared with the lamina propria (median, 0.13%; $p = 0.15$; $n = 7$). In *H. pylori* positive subjects ($n = 2$), there were no differences seen in CD3⁺V α 24J α 18⁺CD56⁺ NKT cell numbers in either epithelial or lamina propria layers (medians, 1.25% and 0.69%) compared with controls. An increase in patient numbers would reveal more.

CD3⁺V α 24J α 18⁺CD161⁺ NKT cells

CD3⁺V α 24J α 18⁺CD161⁺ NKT cells were quantified by triple staining using the CD3 mAb in conjunction with V α 24J α 18 and CD161 mAbs. As shown in Table 3.3, in the epithelial layer of *H. pylori* negative controls, CD3⁺V α 24J α 18⁺CD161⁺ NKT cells were more abundant but not significant (median, 0.59%) when compared with the lamina propria (median, 0.24%; $p = 0.53$; $n = 11$). In the one *H. pylori* positive subject studied, increases were seen in both the epithelial (median, 0.84%) and lamina propria layer (median, 0.68%) CD3⁺V α 24J α 18⁺CD161⁺ NKT cell numbers compared with controls, although none significant as there were not sufficient patient numbers available for

statistical analysis. A greater number of samples may lead to significant differences in cell numbers between the two gastric layers or during infection.

CD3⁺Vα24Jα18⁺CD4⁺ NKT cells

CD3⁺CD4⁺Vα24Jα18⁺ NKT cells were quantified by triple staining using the CD3 mAb in conjunction with Vα24Jα18 and CD4 mAbs. As shown in Table 3.3, in the epithelial layer of *H. pylori* negative controls, CD3⁺Vα24Jα18⁺CD4⁺ NKT cells were more abundant (median, 1.18%) but not significant when compared with the lamina propria (median, 0.61%; $p = 0.94$; $n = 4$). In the one *H. pylori* positive subject studied, decreases in CD3⁺Vα24Jα18⁺CD4⁺ NKT cell numbers were seen in both epithelial (median, 0.89%) and lamina propria layers (median, 0) compared with controls, although none significant due to insufficient numbers studied. Similar studies with higher numbers of subjects may reveal more interesting results.

CD3⁺Vα24Jα18⁺CD8⁺ NKT cells

CD3⁺Vα24Jα18⁺CD8⁺ NKT cells were quantified by triple staining using the CD3 mAb in conjunction with Vα24Jα18 and CD8 mAbs. As shown in Table 3.3, in the epithelial layer of *H. pylori* negative controls, CD3⁺Vα24Jα18⁺CD8⁺ NKT cell numbers were significantly higher ($p = 0.02$) in the epithelial layer (median, 2.96%) when compared with the lamina propria (median, 0.85%; $n = 8$). In *H. pylori* positive subjects ($n = 1$), once again CD3⁺Vα24Jα18⁺CD8⁺ NKT cells were more abundant in epithelial tissues (median, 2.18%) compared with lamina propria tissues (median, 0.23%). There were no differences seen in CD3⁺Vα24Jα18⁺CD8⁺ NKT cell numbers in infected tissues compared with controls. It is worth noting that an increase in patient samples may reveal significance.

In summary, CD3⁺Vα24Jα18⁺CD8⁺ NKT cell numbers were significantly higher in the epithelial layer than in the lamina propria layer of normal *H. pylori* negative mucosa. When NKT cells were compared in healthy vs. *H. pylori* infected tissues, in *H. pylori* positive subjects, there were no differences seen in CD3⁺Vα24Jα18⁺CD56⁺ or NKT CD3⁺Vα24Jα18⁺CD8⁺ NKT cell numbers in infected epithelial or lamina propria layer tissues compared with controls. In the one *H. pylori* positive subject studied, increases were seen in both the epithelial (median, 0.84%) and lamina propria layer (median, 0.68%) of CD3⁺Vα24Jα18⁺CD161⁺ NKT cell numbers compared with controls while decreases in CD3⁺Vα24Jα18⁺CD4⁺ NKT cell numbers were seen in both epithelial (median, 0.89%) and lamina propria layers (median, 0) compared with controls.

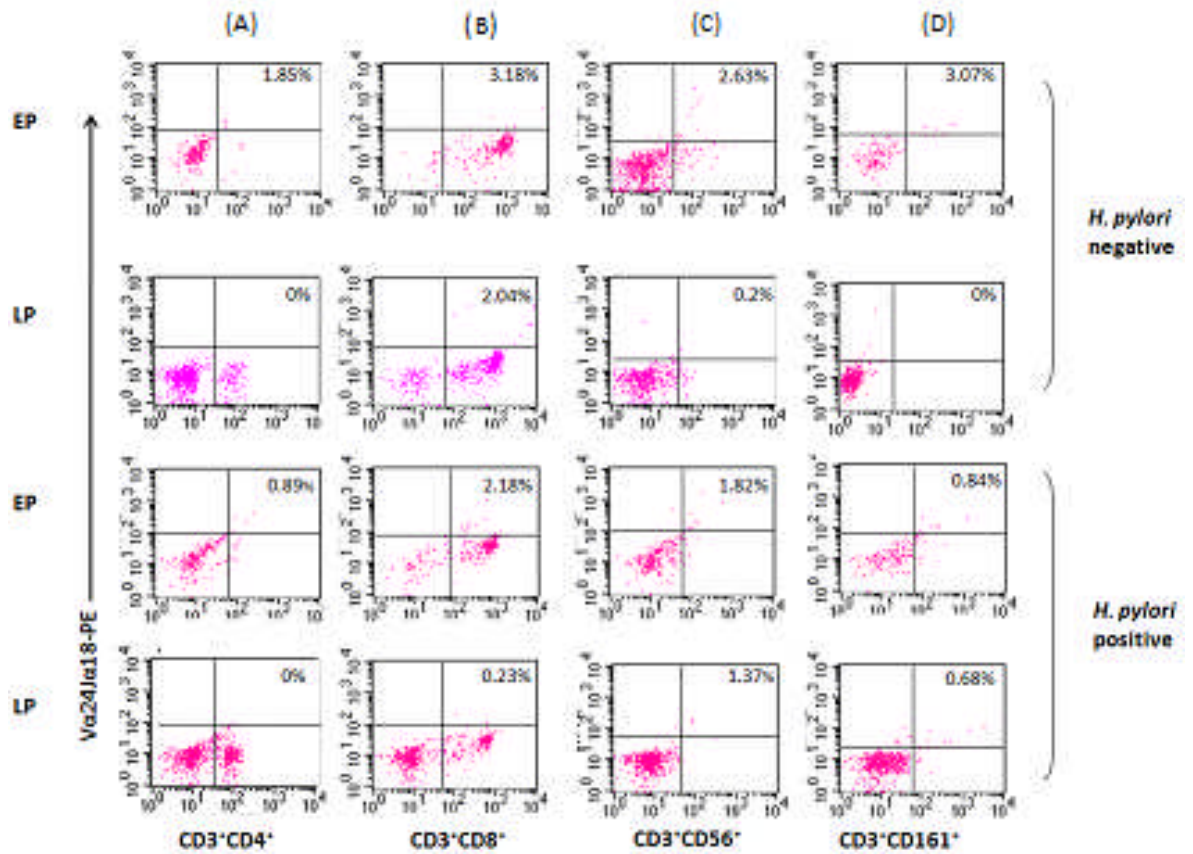


Fig. 3.5 Representative flow cytometry dot plots showing invariant NKT cell subsets including (A) $V\alpha 24J\alpha 18^+CD4^+$ T cells, (B) $V\alpha 24J\alpha 18^+CD8^+$ T cells, (C) $V\alpha 24J\alpha 18^+CD56^+$ T cells and (D) $V\alpha 24J\alpha 18^+CD161^+$ T cells in the gastric epithelial and lamina propria layer of *H. pylori* positive and *H. pylori* negative subjects. Cells were quantified using triple staining and gated on the expression of CD3 where quadrants were set on the basis of isotype matched controls. Percentages of invariant NKT cells are displayed in the upper right quadrants.

Table 3.3 NKT cells subsets in the epithelial and lamina propria of *H. pylori* positive and negative individuals.

		<i>HP</i> -negative (%)		<i>HP</i> -positive (%)	
		Epithelium	Lamina propria	Epithelium	Lamina propria
CD3 ⁺ Vα24Jα18 ⁺	Median ± SEM	1.88 ± 0.39	0.81 ± 0.26	2.66 ± 0.39	0.86 ± 0.47
	Range	(0-8.12)	(0-4.74)	(0.7-3.28)	(0-3.83)
	<i>p</i> -value	NS	NS	NS	NS
	n	23	23	8	8
CD3 ⁺ CD4 ⁺ Vα24Jα18 ⁺	Median ± SEM	1.18 ± 0.51	0.61 ± 0.71	0.89	0
	Range	(0-2.09)	(0-2.99)	(0.89)	(0)
	<i>p</i> -value	NS	NS	NS	NS
	n	4	4	1	1
CD3 ⁺ CD8 ⁺ Vα24Jα18 ⁺	Median ± SEM	2.96 ± 0.44*	0.85 ± 0.43*	2.18	0.23
	Range	(2.05-4.15)	(0-2.04)	(2.18)	(0.23)
	<i>p</i> -value	<i>p</i> = 0.014			
	n	8	8	1	1
CD3 ⁺ Vα24Jα18 ⁺ CD56 ⁺	Median ± SEM	1.16 ± 0.37	0.13 ± 0.13	1.25 ± 0.57	0.69 ± 0.69
	Range	(0-2.73)	(0-0.97)	(0.68-1.82)	(0-1.37)
	<i>p</i> -value	NS	NS	NS	NS
	n	7	7	2	2
CD3 ⁺ Vα24Jα18 ⁺ CD161 ⁺	Median ± SEM	0.59 ± 0.27	0.24 ± 0.19	0.84	0.68
	Range	(0-3.07)	(0-1.84)	(0.84)	(0.68)
	<i>p</i> -value	NS	NS	NS	NS
	n	11	11	1	1

HP = *H. pylori*; NS = not significant; SEM = standard error of the mean; **p* is comparing the epithelium and lamina propria from *H. pylori* negative patients

$\gamma\delta^+$ T cells

$\gamma\delta^+$ T cell numbers were quantified using a mAb directed against the $\gamma\delta$ -TCR. As shown in Table 3.4 and Fig. 3.6, in normal epithelium from *H. pylori* negative subjects, the numbers of $\gamma\delta^+$ T cells were significantly higher (median, 7.91%; $p = 0.0018$) than in the lamina propria layer of normal mucosa (median, 3.11%; $n = 12$). Likewise, in *H. pylori* positive subjects, $\gamma\delta^+$ T cells were higher in epithelial (median, 14.61%) compared with lamina propria tissues (median, 4.26%). However, this figure did not reach significance ($p = 0.11$; $n = 4$). Moreover, the numbers of $\gamma\delta^+$ T cells were markedly higher in *H. pylori* infected epithelium when compared with controls, although once again, the differences did not reach significance ($p = 0.17$).

Table 3.4 Comparing $\gamma\delta^+$ T cell numbers in the gastric epithelial and lamina propria of *H. pylori* positive and negative individuals.

		<i>HP</i> -negative (%)		<i>HP</i> -positive (%)	
		Epithelium	Lamina propria	Epithelium	Lamina propria
CD3 ⁺ $\gamma\delta^+$	Median \pm SEM	7.91 \pm 1.79*	3.11 \pm 2.79*	14.61 \pm 0.45	4.26 \pm 1.32
	Range	(2.39-25.44)	(1.07-5.51)	(4.36-16.77)	(1.07-6.88)
	p -value	$p = 0.0018$			
	n	12	12	4	4

HP = *H. pylori*; NS = not significant; SEM = standard error of the mean. * p -values comparing $\gamma\delta^+$ T cell numbers the epithelium and lamina propria of normal healthy mucosa.

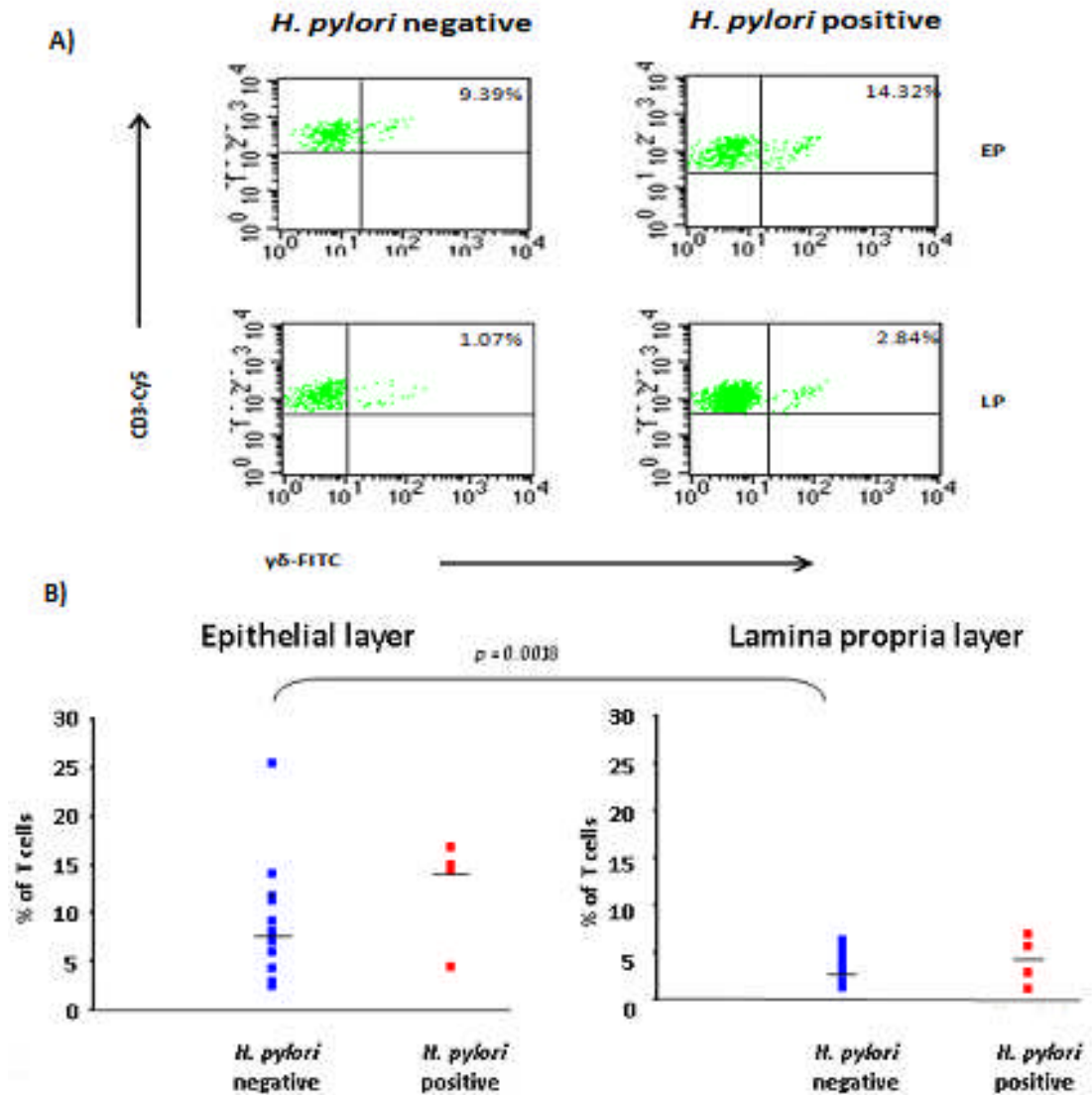


Fig. 3.6 Representative dot plots showing $\gamma\delta^+$ T cells expressing the $\gamma\delta$ -TCR in the epithelial layer and in the lamina propria layer of infected human gastric mucosa (A) where percentages of $\gamma\delta^+$ T cells are displayed in the upper right quadrants. Cells were gated on the expression of CD3 where quadrants were set on the basis of isotype matched controls. (B) XY scatter plots showing the range and percentages of $\gamma\delta^+$ cells in *H. pylori* negative patients (n = 12) and *H. pylori* positive patients (n = 4) in both the epithelial and lamina propria layers respectively *H. pylori* negative samples are in blue and *H. pylori* positive samples are displayed in red. Horizontal lines indicate median values.

3.2.4 NKT cell numbers in peripheral blood versus gastric tissues

A comparative analysis of NKT cell numbers in peripheral blood versus gastric tissues was undertaken in *H. pylori* negative subjects to analyse the frequency of these cells residing in different compartments of the body ($n = 5$). Differences in cell numbers may provide clues regarding their contribution to either a systemic or localised immune responses. As shown in Fig. 3.7, flow cytometric analysis of $CD3^+V\alpha24J\alpha18^+$ NKT cell numbers in gastric biopsies and amongst PBMCs revealed NKT cells are present in significantly higher numbers ($p = 0.0236$) in gastric epithelium (median, $1.86\% \pm 0.69$; range, 0.17-3.98) when compared with PBMCs (median, $0.1\% \pm 0.06\%$; range, 0.05-0.32) and gastric lamina propria layer tissues (median, $1\% \pm 0.4$; range, 0-2.07). The numbers of NKT cells were similar amongst PBMCs and lamina propria lymphocytes.

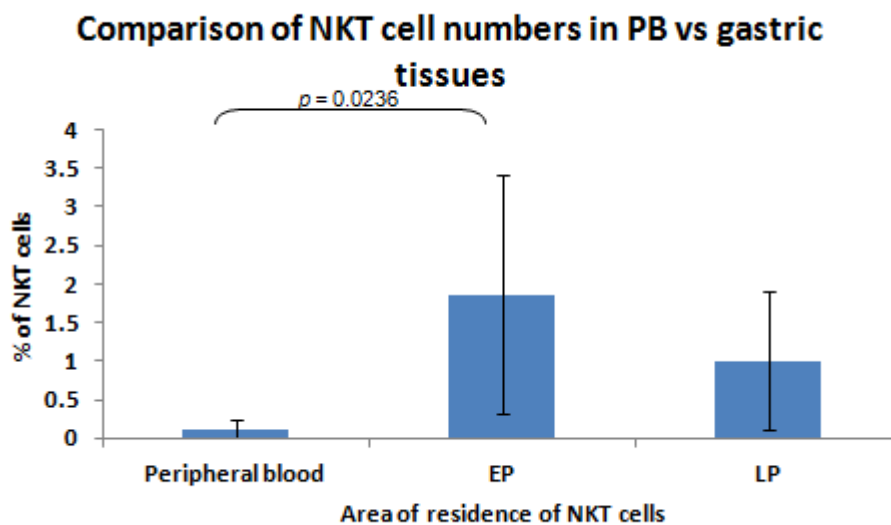


Fig. 3.7 Bar graph illustrating the comparison of $CD3^+ V\alpha24J\alpha18^+$ NKT cell numbers residing in peripheral blood, gastric epithelium and gastric lamina propria layer of the same patients ($n = 5$). The Y-error bars indicate median values \pm SEM.

3.3 DISCUSSION

The gastric mucosa is a site where distinct lymphocyte populations differentially distribute in health and in *H. pylori* associated disease state. Many cell populations have been implicated as immune cells involved in the pathophysiology of *H. pylori* infection. However, the present study, is the first to focus on phenotyping on populations of T cells, $\gamma\delta^+$ T cells, NKT cells and NK cells in the gastric epithelium and lamina propria of *H. pylori* infected and uninfected patients. Our results confirmed the presence of unconventional T cells including NKT cells and $\gamma\delta^+$ T cells in small numbers in the gastric mucosa, revealed differences in these cell numbers within the normal gastric epithelial and lamina propria layer compartments and discovered variations in these unconventional T cell numbers in gastric tissues during *H. pylori* infection.

While both innate and adaptive arms of immunity are implicated in *H. pylori* infection, classical T cells play a primary role (Kusters *et al.*, 2006; Müller *et al.*, 2011). Infection elicits a polarised T_H1 response with the induction of acute inflammatory cytokines and recruitment of immune cells such as lymphocytes, mast cells, plasma cells, macrophages, DCs and neutrophils to infected gastric tissues (Suzuki, 2002; Lundgren *et al.*, 2005). That said, unconventional T cells have been receiving attention of late as a result of their emerging importance in a broad spectrum of diseases (O’Keeffe & Moran, 2008) such as autoimmune diseases (Calleja *et al.*, 2011), inflammatory diseases (Grose *et al.*, 2007b), cancer (Kenna *et al.*, 2003; Kenna *et al.*, 2004), allergies (Lisbonne *et al.*, 2003), allograft rejection (Sonada *et al.*, 2002; Seino K.-I., *et al.*, 2001; Higuchi *et al.*, 2002) and bacterial infections (Tupin *et al.*, 2007). Included in these cell populations are NKT cells and $\gamma\delta^+$ T cells. The prevalence and importance of these unconventional T cell populations have previously been investigated in the liver, intestine and other epithelial tissues (Emoto & Kaufmann,

2003; Meresse & Cerf-Bensussan, 2009; O’Keeffe *et al.*, 2004) but their incidence has not yet been fully explored in healthy or *H. pylori* infected human gastric mucosa. We investigated lymphocyte including NKT and $\gamma\delta^+$ T cell frequencies in normal, healthy gastric epithelium and lamina propria and during *H. pylori* infection.

The present study expanded upon an investigation of gastric NKR⁺ T lymphocytes by our group where we quantified NKR⁺ T cells amongst the gastric epithelial and lamina propria layer T cell populations, and compared their incidence in normal and *H. pylori* infected human gastric mucosa (O’Keeffe *et al.*, 2008). Earlier, we reported an abundance of CD94⁺ T cells in the gastric epithelium with CD94⁺ T cells representing up to 40% of all T cells, and while there were no significant differences amongst this NKR⁺ T cell population in *H. pylori* infected and uninfected individuals. The results also revealed a significant increase in the numbers of CD161⁺ T cells among epithelial layer T cells when compared with controls. CD161⁺ T cells are mainly memory T cells, therefore further analysis needs to be undertaken to determine whether these cells were selectively expanded upon due to chronic *H. pylori* infection.

In the present study, CD56⁺ T cells and CD56⁺ NK cells were significantly decreased in the lamina propria layer of *H. pylori* positive subjects when compared with control subjects. Cells expressing CD56 marker display cytotoxic properties while CD56⁺ T cells have been shown to exert NK and T cell cytotoxicity and produce cytokines such as IFN- γ and TNF- α . Other studies have shown that gastric NK cells are activated during *H. pylori* infection and once activated, produce potent levels of IFN- γ . NK cells are lymphocytes that lack the expression of CD3 and have the ability to kill certain cellular targets of viral and tumour origin in a non-MHC restricted manner (Ramsdell & Golub, 1987; Trinchieri, 1989). Therefore, a decrease in NK cells in *H. pylori* infected subjects may be of clinical importance.

CD4⁺ and CD8⁺ classical T cells were significantly higher and lower respectively in the lamina propria of *H. pylori* infected individuals when compared with uninfected individuals (see Table 3.2). Since the immune response to *H. pylori* infection is primarily orchestrated by T_h1 and in particular CD4⁺ T cells which recognise Ags processed by MHC class II molecules, higher CD4⁺ T cells and lower CD8⁺ T cells could be explained by recruitment of cells primarily involved in immune response to infection site. There were far greater numbers of CD8⁺ T cells present in gastric epithelial tissues, however no significant differences in these classical T cell numbers during *H. pylori* infection were seen. Our findings are consistent with other studies as it is CD8⁺ T cells that are primarily found in intestinal epithelium while CD4⁺ T cell numbers are predominantly higher in the lamina propria (reviewed in Kunisawa *et al.*, 2007). Interestingly, in the two *H. pylori* positive subjects studied, the numbers of DP T cells were markedly higher in the epithelium and in the lamina propria when compared with controls. Expression of the CD4 marker on T_h cells and CD8 marker on T_c cells was once considered to be mutually exclusive. DP T cells are unique in that they display both CD4 and CD8 cell surface markers and therefore possess properties attributed to both. An increase in DP T cells have been linked to autoimmune diseases such as multiple sclerosis and kawasaki disease (Parel & Chizzolini, 2004), therefore, an increase in the DP T cell numbers in *H. pylori* infected tissues is an interesting find.

As in previous studies in human peripheral blood (Porcelli *et al.*, 1993) and the intestine (O'Keefe *et al.*, 2004), the results of the present investigation have shown that in freshly isolated gastric biopsy specimens, only a small minority of T cells expressed the V α 24V β 11 TCR, which identifies the invariant NKT cell population. When NKT cell numbers were compared between *H. pylori*-positive and -negative individuals, we found an increase in numbers in the *H. pylori* infected epithelium, although not

significant, while no changes in the lamina propria layer were found following infection. Moreover, we observed differences in the frequency of NKT cell subsets (as defined by the expression of CD4, CD8, CD56 or CD161 in addition to CD3 and $V\alpha 24J\alpha 18^+$) within the two gastric layers during *H. pylori* infection as significant differences in $CD8^+V\alpha 24J\alpha 18^+$ T cells were documented in epithelial layer when compared with lamina propria layer of normal mucosa. The importance of NKT cells in mounting an immune response against infectious agents has been highlighted in recent years with NKT cells shown to have anti-tumour properties and promote bacterial clearance and exacerbation of disease states have been linked with deficiencies in NKT cell numbers in animal models (Bendelac *et al.*, 2007; Kinjo *et al.*, 2011). Could NKT cells be playing a protective or cytotoxic role in the epithelium immunity as the epithelial layer interacts with large numbers of environmental Ags such as commensal bacteria, food Ags as well as pathogenic microbes in the GIT? Numerical differences in NKT cells have been reported in the literature in the intestine and also in several human diseases. For instance, NKT cells are mainly present in the liver and thymus and most of these are either $CD4^+$ or DN (Godfrey *et al.*, 2000). Bannai *et al.*, (2001) found an enrichment of numbers of $CD8^+$ or DN NKT cells with an $\alpha\beta$ -TCR canonical arrangement, which were not CD1d restricted and did not possess a $V\alpha 24J\alpha 18$ invariant chain at intraepithelial sites in the large intestine of mice. Grose *et al.*, (2007a) reported a reduction in $V\alpha 24^+$ T cells and NKT cells in the intestine and PB of patients suffering from coeliac disease using α -GalCer loaded CD1d tetramers. The authors hypothesise the overall decrease may have an impact on the activation of T cells that become sensitised as a result of coeliac disease. NKT cells were also defective in producing IL-4 which the authors speculate could be due to NKT cells varying in their thymic development to classical T cells. The same group recorded lower numbers of circulating

V α 24⁺ T cells and NKT cells in the peripheral blood of patients with Crohn's disease and ulcerative colitis cases while intestinal V α 24⁺ T cells alone were decreased in Crohn's disease (Grose *et al.*, 2007b). Molling *et al.*, (2005) reported a decline in peripheral blood derived V α 24⁺V β 11⁺ NKT cell numbers in individuals with cancer irrespective of the type of tumour when compared with age and gender matched controls. Due to the fact that NKT cells were shown to produce IFN- γ , the authors suggest this reduction could indicate a risk factor for the onset of cancer (Molling *et al.*, 2005). Since approximately 85% of *H. pylori* bacteria are known to reside in the gastric mucus on the surface of epithelial cells and since we are reporting differences in NKT cells in the epithelial layer during *H. pylori* infection, we hypothesise a potential role for these cells in *H. pylori* disease state.

Significant differences in $\gamma\delta^+$ T cell numbers were detected when the epithelium and lamina propria tissues of normal healthy mucosa were compared, in addition to marked differences seen between the infected and uninfected tissues. $\gamma\delta^+$ T cell numbers were significantly higher in the epithelium of uninfected mucosa while numbers were far lower in lamina propria tissues of disease and non-disease states. This is keeping with other areas of the body where $\gamma\delta^+$ T cells are enriched in the epithelia. For example, while $\gamma\delta^+$ T cells make up approximately 2-6% of CD3⁺ peripheral blood lymphocytes, they are present in much higher numbers in the epithelium and the small intestine constituting 15% of small intestinal IELs, 40% of colonic IELs, 5% of lamina propria lymphocytes and peripheral blood T lymphocytes (Meresse & Cerf-Bensussan, 2009). $\gamma\delta^+$ T cells are involved in regulation of inflammation and wound repair through renewal of epithelial cells following tissue injury and they participate in the immune response to infectious pathogens and tumours (Carding & Egan, 2002). $\gamma\delta^+$ T cells are also implicated in the processing and presentation of Ags to classical T cells thereby

triggering adaptive immune responses (Brandes *et al.*, 2005). While their incidence and functions have been investigated in the intestine, $\gamma\delta^+$ T cells prevalence and importance has not yet been fully explored in the gastric mucosa, therefore the marked increase in $\gamma\delta^+$ T cells in *H. pylori* infected gastric epithelium could be pathologically relevant. A possible theory for differences seen here could be that $\gamma\delta^+$ T cells produce anti-microbial molecules resulting from bacterial encounters thereby maintaining oral tolerance as antimicrobial peptides are produced by cells in the GIT such as epithelial cells in response to pathogenic invaders (Mahida *et al.*, 1997). IL-23 regulates IL-22 and IL-17 production and in turn IL-17 and IL-22 are involved in secretion of anti-microbial peptides (Kao *et al.*, 2004; Wolk *et al.*, 2004). Since $\gamma\delta^+$ T cells are potent producers of IL-17 (Roark *et al.*, 2008) and since IL-23 is needed for sustainment of IL-17 production in *H. pylori* infected mucosa (Caruso *et al.*, 2008), an increase in anti-microbial peptides may explain the hypothesis of an increase seen in $\gamma\delta^+$ T cells in *H. pylori* infected epithelium. Or indeed, like *Bordetella pertussis* infection (Zachariadis *et al.*, 2006), it could also be argued that $\gamma\delta^+$ T cells are regulating bacteria mediated inflammation thereby contributing to *H. pylori* persistence. $\gamma\delta^+$ T cells have already been linked to an involvement in *H. pylori* related gastritis. Romi *et al.*, (2011) have reported cytokine secretion, chemokine secretion and CD69 activation of T cells and primarily $\gamma\delta^+$ T cells by *H. pylori in vitro*. In other studies of infection, Li *et al.*, (2008) report decreases in V γ 2V δ 2 T cell subset during HIV infection and hypothesise a link between these $\gamma\delta^+$ T cells and progression of disease from a study of 146 patients, suggested following the observation of an inverse relationship between viral load and V γ 2V δ 2 T cell counts. Changes in cell numbers have been linked with other clinical conditions so it is reasonable to suggest that the changes in $\gamma\delta^+$ T cell numbers reported here may have an important role to play in the pathology of *H. pylori* infection.

A recent study by Calleja *et al.*, (2011) investigated NK cells, both variant and invariant NKT as well as $\gamma\delta^+$ IELs in Coeliac Disease (CD) with a view to age, diet and histopathology correlations and discovered that in patients with CD, there were significant increases in $CD4^+/CD103^+$ and $CD3^+\gamma\delta^+$ T cell numbers while NK, variant NKT and invariant NKT cell numbers were decreased significantly in disease state. Nonetheless, while expanded numbers of $\gamma\delta^+$ T cells may be implicated in *H. pylori* infection, especially at the gastric epithelium, analysis of a greater number of infected subjects is required, particularly since $\gamma\delta^+$ T cells represent a small proportion of the overall cellular population.

Clinically, of the *H. pylori* 34 negative patients included in this portion of the study, 10 subjects suffered from a hiatus hernia, 2 patients suffered from a duodenal ulcer, 9 subjects were implicated with antral gastritis, 4 with fundal gastritis and 3 with duodenitis. Out of the 9 *H. pylori* positive patients included in this part of the study, 3 patients suffered from a hiatus hernia, 2 patients were implicated with duodenal ulcers, 5 patients had antral gastritis, 1 patient suffered from fundal gastritis and 2 had duodenitis. This poses the question whether the increases in unconventional T cell populations could be linked to any particular pathology as most of the 8 *H. pylori* patients studied possessed additional inflammatory conditions? At present, it is difficult to postulate but further experimental investigation whereby cell numbers from a greater number of *H. pylori* infected patients were examined and graded according to severity of gastritis would reveal more.

In conclusion, the results of this study which was a continuation of a novel study undertaken by our group investigating numbers of gastric NKR⁺ T cells in *H. pylori* infected and uninfected mucosa, have shown that the adult human gastric mucosa is a site where, in addition to the resident known epithelial and lamina propria lymphocytes

subsets, populations of T cells, including unconventional T cells such as T cells bearing NK receptors (NKR⁺ T cells), various different NKT cell subsets and $\gamma\delta^+$ T cells are present in small numbers. Given their frequency in the mucosal tissues, the differences naturally occurring in these cell numbers in the two gastric layers and the observed differences in the numbers of these cell populations in *H. pylori*-positive and -negative individuals suggests an important role for unconventional T cells and in particular, $\gamma\delta^+$ T cells in the immune response to *H. pylori*. Further functional characterisation of these immune cells with a greater number of biopsy samples will reveal more.

Chapter 4

Analysis of Systemic and Localised Immune Responses to *H. pylori* and *H. pylori* Derived Products

4.1 INTRODUCTION

4.1.1 T cell responses in *H. pylori* infection

T_h1/T_h2 cells

It is widely known in *H. pylori* infection that T cells take part in local inflammatory responses, play protective roles and regulate the overall immune response (Ernst & Gold, 2000). In chapter 3 of this thesis we reported significant increases in CD4⁺ T cells and decrease in CD8⁺ T cell numbers in the lamina propria layer respectively of *H. pylori* infected patients when compared with normal healthy controls. We also previously reported that, in *H. pylori* –infection, the numbers of CD161⁺ T cells were significantly greater in the epithelium, whereas the numbers of CD56⁺ T cells were lower in the lamina propria (O’Keeffe *et al.*, 2008). A minor population of T cells in both mucosal layers of *H. pylori*-negative subjects constitute NKT cells and $\gamma\delta^+$ T cells, whose proportions were not significantly different to those in *H. pylori*- infected individuals. Regarding unconventional T cell subsets, there were significantly more CD8⁺V α 24J α 28⁺ T cells in epithelial tissues than in lamina propria layer tissues in normal gastric mucosa.

Since its discovery, great interest has been shown in *H. pylori* with 31,851 entries recorded in a 2012 PubMed search on this gastric pathogen. Significant advancements have been made so far regarding host-pathogen interactions. Karttunen & Niemela, (1990) contributed to early immunological studies by revealing *H. pylori* derived products in both healthy subjects and *H. pylori* infected patients activate lymphocytes in peripheral blood *in vitro*. In a separate paper, Karttunen *et al.*, (1990) further investigated T cell responses to *H. pylori* infection and reported *H. pylori* capability to stimulate mononuclear cells to secrete the T cell cytokine IFN- γ , proliferate and lead to the production of Ig producing B cells. Continuing on their work

of T cell responses to *H. pylori* infection, Karttunen, (1991) revealed IL-12R expression by T cells during secretion of IL-2, TNF- α and IL-4 in supernatants when analysing the host's immune responses in patients with and without anti-*H. pylori* Abs. Years later, the same group found *H. pylori* induces a T_h1 proinflammatory cytokine response dominated by increases in IFN- γ but not IL-4 and found in gastritis sufferers albeit in *H. pylori* positive or negative cases (Karttunen *et al.*, 1995). To date, it is known that the T_h1 cytokines IL-1 β , IL-8, IL-12, IL-18, IL-23 and TNF- α along with other proinflammatory mediators are also elevated in *H. pylori* infection (Moss *et al.*, 1994; Noach *et al.*, 1994; Tomita *et al.*, 2001).

The T_h1/T_h2 cell paradigm refers to a tendency of chronically stimulated T cells being polarised into T_h1 or T_h2 cytokine production (Del Prete, 1998). *H. pylori* induces a T_h1 pro-inflammatory cytokine response in mucosal tissues as IFN- γ but not IL-4 is predominantly produced (Bamford *et al.*, 1998). This is unusual as extracellular bacteria normally induces a T_h2 response while infection with intracellular pathogens usually induces a T_h1 driven immune response (D'Elcios & Del Prete, 1998). Therefore, in *H. pylori* infection, it would be expected that a T_h2 response would predominate with B cells having a major role to play in *H. pylori* mediated immunity; however this is not the case. D'Elcios *et al.*, (1997a) revealed it was possible to clone T_h1 cells specific for *H. pylori* from *H. pylori* infected gastric tissues and these cells were capable of lysing infected epithelial cells. Moreover, neutrophils and macrophages contribute to the T_h1 response by producing IL-12, IL-1, IL-6, IL-8 and TNF- α (Peek Jr *et al.*, 2010). T_h1 polarisation in *H. pylori* infection can lead to more severe forms of disease such as the onset of peptic ulcers and gastric cancer (Wroblewski *et al.*, 2010). Evidence to support this theory was provided by studies that discovered high levels of cytokines with a T_h1 phenotype only in *H. pylori* induced peptic ulcer patients while both T_h1 and T_h2

cytokines were secreted in patients suffering from gastritis not related to ulcers (D'Elcios *et al.*, 1997a; D'Elcios *et al.*, 1997b). Hence, findings indicate the T_h1 immune response actually leads to persistence of inflammation, disease and chronicity of *H. pylori* infection where as a T_h2 response exhibits anti-inflammatory immune effects (D'Elcios *et al.*, 2003).

Chronic infection occurs as a result of the inability of the polarised T_h1 inflammatory response to clear infection in addition to evasion mechanisms exerted by the bacteria (Wroblewski *et al.*, 2010). Interestingly, Fan *et al.*, (1994) reported an attenuated response in *H. pylori* positive patients which may be caused by recruitment of cells to infected gastric tissues and away from the periphery. Several other groups have documented suppressed lymphocyte responses in *H. pylori* infection (Grebowska *et al.*, 2010; Hybenova *et al.*, 2010) as a result of *H. pylori* virulence factors (Muller *et al.*, 2011). IL-10, a regulatory cytokine and T_{reg} cells are also believed to play a role in chronicity of *H. pylori* infection by dampening host immune responses (Kandulski *et al.*, 2010). Studies have shown that memory T cells do not respond as well to *H. pylori* antigenic stimulation (Quiding-Järbrink *et al.*, 2001). However, when T_{reg} cells were removed, the proliferative immune response to *H. pylori* was restored (Lundgren *et al.*, 2003).

T_h17 cells

The role of IL-17 in *H. pylori* infection is only recently being recognised as being of great importance. Increases in IL-17 and IL-17 RNA transcript production have been found amongst both gastric mucosal and mononuclear cells (Luzza *et al.*, 2000). Mizuno *et al.*, (2005) reported increases in IL-17 and IL-8 in the gastric antrum of patients suffering from gastric ulcers as well as non-ulcerated patients infected with *H.*

pylori. A study by Caruso *et al.*, (2008) has also shown an increase in IL-23 levels in patients with gastritis caused by *H. pylori*, this is relevant as IL-23 is involved in regulating IL-17 production *via* the transcription factor STAT3. Moreover, IL-17 may contribute to the onset of gastric cancer as increased numbers of T_h17 cells were found in circulating peripheral blood and in tumour-draining lymph nodes of patients with cancer while higher levels of both IL-17 and IL-23 were found in sera samples from patients suffering from gastric cancer (Kabir, 2011; Zhang *et al.*, 2008). Since *H. pylori* is one of the leading causes of gastric cancer this is an interesting finding. A recent study in our group has confirmed elevated serum levels of IL-17 in *H. pylori* infected patients and demonstrated increases in T_h17 cells amongst gastric epithelial and lamina propria T cells (Zulquernain *et al.*, 2012) (article submitted to *Helicobacter*).

4.1.2 Innate T cells and infection

NKT cells may also be involved in the immune response to *H. pylori* infection and to date, their role has not been widely explored. NKT cells display potent anti-tumour properties (Metelitsa *et al.*, 2001), rapidly release cytokines such as IFN- γ and IL-4 (Bendelac *et al.*, 2007) and promote the maturation of DCs into functioning APCs as explained in section 1.5.1 (Vincent *et al.*, 2002). In addition, the protective role of NKT cells has already been shown in several bacterial infections such as an acute pneumonia model following *Streptococcus pneumoniae* infection (Kawakami *et al.*, 2003), in pulmonary infection with *Pseudomonas aeruginosa* (Nieuwenhuis *et al.*, 2002) and in a Lyme disease model system which is caused by *B. burgdorferi* (Kumar *et al.*, 2000). The importance of NKT cells in the immune response against microbial pathogens has also been highlighted in a number of intestinal infections in mice such as *Salmonella*, *Listeria monocytogenes* and *Toxoplasma gondii* (Wingender & Kronenberg, 2007).

NKT cells promote microbial clearance and deficiencies in their numbers in animal models have resulted in disease exacerbation (Tupin *et al.*, 2007). Interestingly, regarding *H. pylori* infection, a recent paper has emerged whereby it has been reported a cholesterol derived lipid from *H. pylori* called P157 induced activation of NKT cells in both mice and humans (Chang *et al.*, 2011). Given that NKT cells have an important role in infection, despite this study, little is known about NKT cell location and function within the gastric epithelial and lamina propria layers (see chapter 3).

$\gamma\delta^+$ T cells are also implicated in many roles in intestinal immunity including cytokine production, oral tolerance, wound repair and Ag presentation (Bonnevillie *et al.*, 2010; Carding & Egan, 2002; Kabelitz, 2011). Human $\gamma\delta^+$ T cells expressing V γ 9V δ 2 chains secrete T_h1 cytokines IFN- γ and TNF- α following stimulation with viable a bacterial component called *iso*-butylamine (IBA) which is produced by pathogenic bacteria such as *S. typhimurium* and *L. monocytogenes* (Hara *et al.*, 1992; Jouen-Beades *et al.*, 1997). Interestingly, Wang *et al.*, (2001) also reported that cytokine production does not occur when $\gamma\delta^+$ T cells were stimulated with dead bacteria or *E.coli* derived LPS. Live *H. pylori* bacteria from strain G27 activates purified CD3⁺ T cells and in particular $\gamma\delta^+$ T cells *in vitro* to induce cytokine and chemokine production in addition to up-regulating CD69 expression thus promoting an inflammatory response which may favour the bacteria to persist and cause chronic disease state (Romi *et al.*, 2011). A separate study by Futagami *et al.*, (2006) revealed peripheral blood $\gamma\delta^+$ T cells were activated by *H. pylori* urease to secrete IL-10 and IFN- γ . The importance of $\gamma\delta^+$ T cells has also been highlighted in other mucosal infections where these cells formed the first line of defence against infectious agents and in tissue injury (Cheroutre, 2005; Komori *et al.*, 2006). Since $\gamma\delta^+$ T cells are present in gastric tissues and since we identified marked differences in *H. pylori* infected epithelial tissues (see chapter 3), it is

likely this unconventional T cell population has a role to play in *H. pylori* mediated pathogenesis but remains largely unexplained to date.

4.1.3 Bacterial fractions derived from *H. pylori*

Infection with *H. pylori* is associated with a range of clinical gastroduodenal diseases such as gastritis, peptic ulcers and gastric cancer (Bodger & Crabtree, 1998; Peek & Crabtree, 2006; Uemura *et al.*, 2001). Factors affecting the pathogenesis of this gastric pathogen include bacterial factors such as strain, host factors such as genetic predisposition and environmental factors such as diet and smoking (Wroblewski *et al.*, 2010). Since *H. pylori* is non-invasive, it is hypothesised that products secreted or proteins/lipids expressed on or in this bacterium contribute to overall pathogenesis. PAMPs such as lipoproteins, flagellins, nucleic acids, peptidoglycan, OMPs and LPSs all may stimulate the innate immune system and drive the onset of inflammation (Peek Jr *et al.*, 2010).

4.1.3.1 Cytoplasmic fractions

H. pylori bacteria are capable of secretion of proteins (Vanet & Labigne, 1998) and also release of cytoplasmic proteins following bacterial cell lysis (Phadnis *et al.*, 1996). Examples of Cyto Ags include urease and catalase among others. Flagellins, although not cytoplasmic, are cell associated and have a role to play in *H. pylori* colonisation and pathogenesis by facilitating the movement of *H. pylori* through the gastric mucosa and through the mucus layer where they reside or attach to epithelial cells (Lertsethtakarn *et al.*, 2011). FlgM is an anti-sigma factor is responsible for the expression of flagellar genes such as FlaA in *H. pylori* (Rust *et al.*, 2009). Urease B (UreB) is another well documented cytoplasmic protein essential for *H. pylori* survival and colonisation (Kim

et al., 2002). UreB buffers the pH of the surrounding gastric environment allowing *H. pylori* to survive in the now less acidic gastric mucosa. Catalase is an enzyme of interest found in the cytoplasm and the periplasm of *H. pylori* bacteria. Catalase is involved in a redox reaction whereby hydrogen peroxide is simultaneously oxidised into molecular oxygen and reduced to water therefore allowing persistent bacterial colonisation in the human stomach. KatA is the gene that encodes catalase (Harris & Hazell, 2003). Kim *et al.*, (2002) carried out *in vitro* analysis of proteins released by *H. pylori* strain 11637 and identified proteins selectively released from the bacteria. Following analysis of supernatant derived from strain 11637, 13 proteins were found, which the authors hypothesise may have been secreted intentionally by *H. pylori* and could be implicated in inflammation of gastric tissues or *H. pylori* mediated pathogenesis. Mortazavi *et al.*, (2011) carried out a study on proteins from the cytoplasmic fraction of spiral and coccoid *H. pylori* bacteria. Their results yielded the discovery of two immunosuppressive proteins from both spiral and coccoid bacteria which exerted anti-proliferative activity and one of these proteins also displayed urease activity. The second protein discovered possessed 63 and 57kDa molecular weight bands following SDS-PAGE analysis. The function of this protein is currently unknown but the authors suggest an importance for investigating the immune response to this protein as it could potentially have an involvement in *H. pylori* mediated chronic infection or indeed gastric cancer (Mortazavi *et al.*, 2011). Therefore, cytoplasmic fractions derived from *H. pylori* are of interest as they have been shown to play key roles in *H. pylori* mediated pathogenesis.

4.1.3.2 Outer membrane proteins

H. pylori possesses over 30 genes encoding the expression of OMP (Amieva & El-Omar, 2008). Examples of OMP include OipA, BabA, SabA, AlpA, AlpB and porins among others. *H. pylori* employs numerous tactics to successfully colonise the gastric mucosa by surviving the acidic gastric environment, penetrating the mucus lining, with some attaching to the surface of epithelial cells all whilst invoking minimal host immune responses (Kusters *et al.*, 2006). *H. pylori* adheres to the surface of IELs with the help of bacterial adhesins and the majority of these are OMPs (Parker & Keenan, 2012). Adhesion OMPs are interactive surface molecules which play a vital role in bacterial initial colonisation and survival (Juge, 2012). For instance, the SabA binds *H. pylori* bacteria to sialylated gangliosides (Mahdavi *et al.*, 2002; Roche *et al.*, 2004). Other OMPs include adhesin-associated proteins such as AlpA and AlpB (Lu *et al.*, 2007) and porin proteins which are involved in penetrating the host cell membrane through formation of water-filled channels allowing the translocation of products across the membrane (Odenbreit *et al.*, 1999). Moreover, Yamaoka *et al.*, (2006) found that a large proportion of CagA⁺ strains from individuals in East Asia possess OipA. When CagA⁺ strains were cultured *in vitro* with gastric epithelial cells, the authors observed neutrophil infiltration associated with the production of large amounts of IL-8 which decreased by 40% when OipA was inactivated. Therefore, an association with OipA and onset of gastroduodenal diseases is hypothesised. Taken together, one can speculate potential roles of OMP in *H. pylori* mediated pathogenesis.

4.1.3.3 Inner membrane proteins

Examples of IMP include urease I (UreI) and the *H. pylori* gene for membrane protein 1 (HP-MP1). The UreI gene is responsible for the enzyme urease altering the acidity in

the stomach once the pH drops below a certain level (Rektorschek *et al.*, 2000). UreI transports urea molecules to the cell membrane to catalyse urea hydrolysis which ultimately leads to bacterial survival in the highly acidic gastric mucosa. It does this using a proton pump which opens a urea channel allowing urease transport to take place (Kusters *et al.*, 2006; Weeks *et al.*, 2000). Interestingly, the presence of the virulence factor CagA at the inner membrane of *H. pylori* was detected under these acidic conditions and this was not the case in neutral pH (Wu *et al.*, 2005) implying that molecules other than urea molecules can be transferred in this manner. This may impact on aiding bacterial pathogenesis. Yoshida *et al.*, (1999) discovered a 16 kDa IMP called HP-MP1 which induced IL-1 α , TNF- α , IL-8 and macrophage inflammatory protein 1 α (MIP1 α) production from monocytes. These findings hypothesise a potential role for HP-MP1 as a membrane associated IMP in *H. pylori* pathogenesis through its potential involvement in the inflammation of gastric tissues (Yoshida *et al.*, 1999).

4.1.3.4 Lipopolysaccharides

LPS are a family of phosphorylated lipoglycans in the outer membrane of gram negative bacteria considered to possess potent immunomodulating and immunostimulatory effects (Alexander & Rietschel, 2001). As shown in Fig. 4.1, LPS is composed of a poly (oligosaccharide) O-chain and a lipid component known as lipid A (Alexander & Rietschel, 2001). It is widely accepted the lipid A portion is responsible for exerting LPS endotoxic effects. Structurally, lipid A consists of a β (1-6) linked D-hexosamine disaccharide backbone in addition to phosphate groups at positions 1' and 4' and (R)-3-hydroxy-and (R)-3-acyloxyacyl residues at positions 2, 3, 2' and 3'. *H. pylori* LPS has been shown to exhibit inhibitory effects on mucus glycosylation, to hinder mucus-receptor interactions, to compromise mucosal integrity and to promote the secretion of

pepsinogen (Moran, 1996; Young *et al.*, 1992). For instance, pepsinogen is a precursor of the enzyme pepsin that is thought to be mucolytic ultimately leading to duodenal ulcers. This facilitates attachment of *H. pylori* to the extracellular matrix glycoprotein laminin in the basement membrane which is located between epithelial and lamina propria tissues (Moran, 1996).

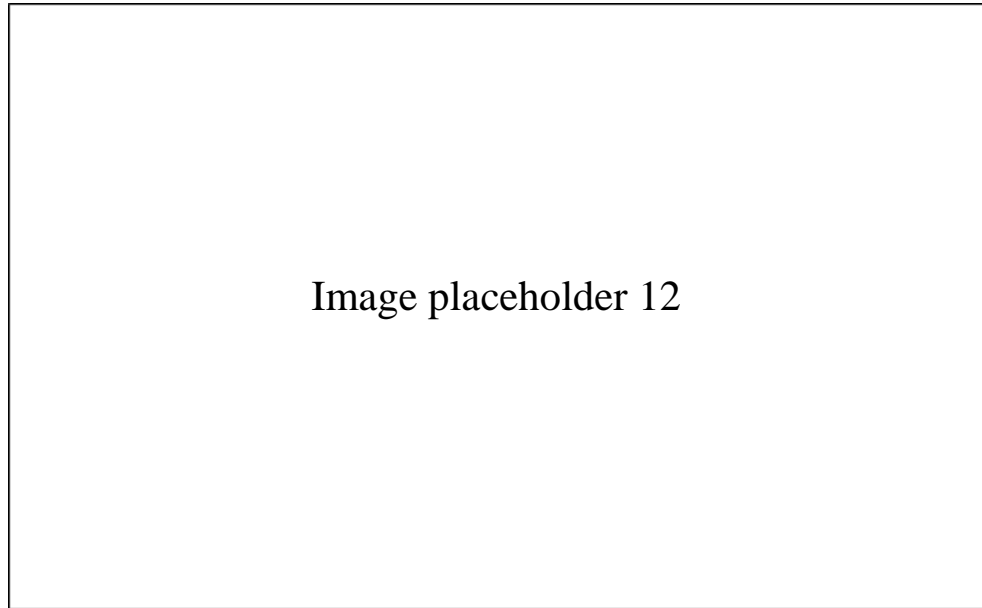


Fig. 4.1 This diagram illustrates the structure of *H. pylori* LPS consisting of the lipid A moiety, core oligosaccharide and the repeating sugars of the O-specific chain. Adapted from Holst *et al.*, (1996). Figure has been removed due to copyright restrictions

The stimulatory capacity of *H. pylori* LPS has been tested by a number of different groups and results have shown *H. pylori* LPS exerts low endotoxic activity (Muotiala *et al.*, 1992). It is believed that the composition of the lipid A fatty acid chains which are longer than those in enterobacterial lipid A in addition to the lipid A component being underphosphorylated are responsible for such low immunological activity in comparison to other LPS containing bacteria (Moran, 1995). For instance, *E. coli* LPS and *S. typhimurium* LPS exert bioactivity 1000 times higher when compared with that

of *H. pylori* LPS (Muotiala *et al.*, 1992). That said, *H. pylori* LPS is still considered an important factor in the bacterium's virulence and pathogenesis.

LPS interacts with PRRs such as TLRs on the surface of immune cells triggering innate immune responses (Takeuchi & Akira, 2010). While TLR4 and TLR5 are considered to be the TLRs typically responsible for LPS recognition, this is not the case with *H. pylori* LPS. *H. pylori* demonstrates low biological activity and while *H. pylori* LPS does activate TLR4 *via* the MD-2 complex, this has so far only been demonstrated *in vitro* where the TLR4/MD-2 complex was highly expressed (Muller *et al.*, 2011). It is TLR2 and not TLR4 has been shown to be the recognise *H. pylori* LPS (Ferrero, 2005). In this way, it is thought *H. pylori* LPS has evolved its structure and elicits chronic infection in the gastric mucosa by minimally interacting with mucosal immune cells thereby escaping early detection and subsequent elimination.

Moreover, *H. pylori* along with other gram negative bacteria alter the lipid A of its LPS as a tactic to evade the body's innate immune defences (Moran, 2007). To do this, the bacteria disguise the phosphate groups on the disaccharide backbone portion of the lipid A. Phosphate groups are normally negatively charged but through the insertion of positively charged molecules such as phosphoethanolamine, the negative charged is disguised, impacting on bacterial resistance to cationic antimicrobial peptides (CAMPs) (Cullen *et al.*, 2011). Another method of modifying bacterial lipid A used is the elimination of phosphate groups from the lipid A disaccharide backbone. This action also results in the removal of negative charges on the bacterial surface which once again results in the bacteria having more of a resistance to CAMPs. These positively charged CAMPs interact with the negatively charged bacterial components resulting in the formation of pores in the bacterial membrane leading to cell death. Hence, *H. pylori* can eliminate its lipid A phosphate groups from positions 1' and 4' from its disaccharide

backbone. The enzyme involved in this process has been recently discovered and is known as Jhp 1487 (LpxF) (Cullen *et al.*, 2011). Gram negative bacteria including *H. pylori* alter the lipid A chemical structure as a means of evading the innate immune system. For instance, through the possession of longer acyl chains whereby the bacteria harbours 16 or 18 in place of 12 or 14 carbons or by complete removal of acyl chains thereby reducing the original hexa-acylated lipid A form to a penta- or tetra-acylated lipid A structure (Moran, 1998; Moran & Aspinall, 1998) as seen in Fig. 4.2, this immune evasion can be achieved.

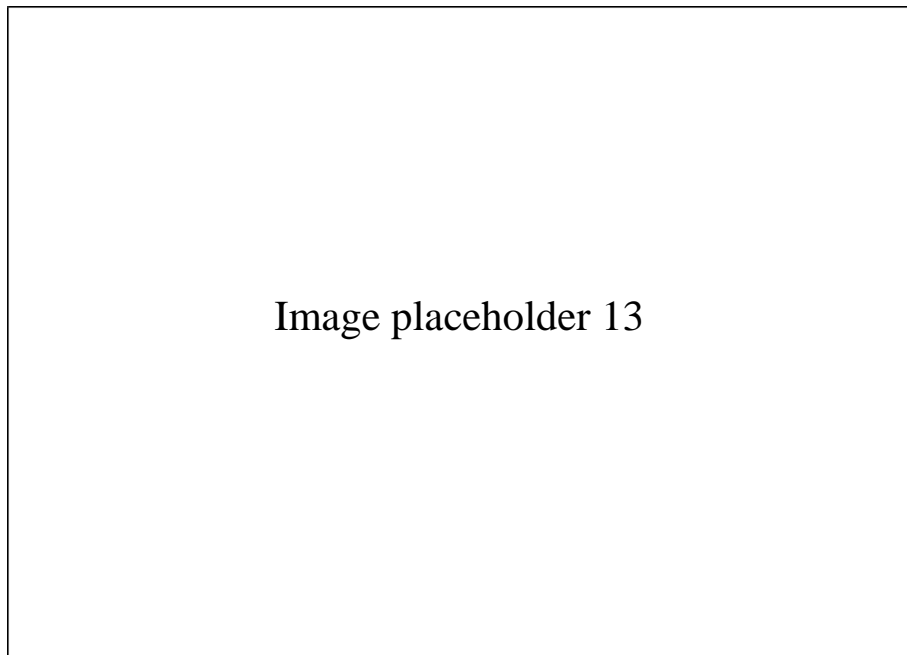


Fig. 4.2 Structure of LPS strain from (A) *H. pylori* LPS and (B) *E.coli* LPS adapted from Miller *et al.*, (2005). The varying structures of the two different LPS types can be observed from this diagram, notably the presence of fewer and longer acyl chains in *H. pylori* LPS as well as underphosphorylation occurring at positions 1' and 4' of the disaccharide backbone. Figure has been removed due to copyright restrictions

Fresh clinical isolates are structurally smooth in form as they contain O chain, core oligosaccharide and lipid A moieties. On the other hand, severely subcultured *H. pylori* LPS are rough in form meaning they produce low molecular weight LPS as they do not

contain an O chain (Nilsson *et al.*, 2008). O chain units of repeating sugars on certain LPS strains are known to partake in molecular mimicry as LPS can mimic the structure of host Lewis^x and Lewis^y blood group Ags that are present in healthy gastric mucosa, hence through this mimicry of self-epitopes, the bacteria may go undetected and persist in gastric tissues. This contributes to inflammation and gastric injury (Appelmelk *et al.*, 2000; Moran, 2007).

Regarding the *H. pylori* strains used in this study, both *H. pylori* 26695 and J99 are highly virulent expressing CagA⁺, CagE⁺, VacAS1M1 toxin, and outer membrane proteins babA2, OipA, icaA1 and SabA. While *H. pylori* 26695 expresses low molecular weight semi-rough LPS, J99 possesses a high molecular weight smooth LPS. There is approximately 6% genetic diversity between these two strains (Salama *et al.*, 2000). *H. pylori* NCTC11637 is also CagA⁺ and also possesses VacA, BabA and OipA (Monteiro *et al.*, 2000).

4.1.4 Aims

Having identified in chapter 3 that there are differences in the numbers of CD4⁺ and CD8⁺ T cells, NK cells, NKT cells and $\gamma\delta^+$ T cells in *H. pylori* infected mucosa compared with controls, the aims of this chapter were to further investigate the functional responses of both peripheral blood and gastric lymphocyte populations to *H. pylori* and to various *H. pylori* derived fractions. The following bacterial fractions derived from *H. pylori* were used to stimulate immune cells: WCE, Cyto Ags, C.M. Ags, OMPs and inner membrane proteins (IMPs) from *H. pylori* strains 26695 and J99 in addition to LPS from *H. pylori* strains NCTC11637 and CCUG17874.

The specific aims of this chapter were to:

- (i) Investigate the proliferative response of PBMCs, gastric epithelial and lamina propria layer cells and NKT cell clones to *H. pylori* antigenic stimulation using the BrdU ELISA kit (Roche)
- (ii) Analyse patterns of cytokine production produced by PBMCs, gastric epithelial and lamina propria layer cells following stimulation with the above *H. pylori* derived bacterial fractions. Assays for cytokine analysis included the T_h1/T_h2 11plex FlowCytomix kit (BenderMed)
- (iii) To investigate further the functional response of NKT cells and $\gamma\delta^+$ T cells to *H. pylori*. For these studies, NKT cell clones and $\gamma\delta^+$ T cell clones (see chapter 2) were stimulated with all of the *H. pylori* Ags above and proliferation of NKT cells and cytokine analysis of both NKT cells and $\gamma\delta^+$ T cell cytokine analysis was performed before and after stimulation.
- (iv) Examine cytotoxic response of effector NKT cell clones towards target Caco2 cells following with *H. pylori* fractions by measuring LDH activity (promega).

- (v) To evaluate the expression of CD1a, CD1b, CD1c and CD1d mRNA using RT-PCR in normal healthy mucosa.

4.2 RESULTS

4.2.1 Proliferative responses of PBMCs, NKT cell clones , gastric epithelial and lamina propria layer cells following stimulation with *H. pylori* derived products

4.2.1.1 Optimisation experiments

Initial experiments were carried out previously in our laboratory by Dr. Carol Gately using PBMCs in order to determine the optimum concentration and time point for measurement of proliferation using PHA as the stimulus and the BrdU ELISA kit. It was concluded the optimum concentration of PHA for proliferation was 5 µg/ml and time point 144 h using 20,000 cells/well. For all other stimuli used, a range of concentrations (from 100 ng/ml to 5 µg/ml) was studied using PBMCs and a 144 h time point. Finally, the optimum concentration of 10 µg/ml of α-GalCer for stimulation of PBMCs was determined.

4.2.1.2 PBMC proliferative responses to *H. pylori* stimulation using BrdU ELISA

Since *H. pylori* is known to induce systemic immune responses (Andersen *et al.*, 1996; Rathbone *et al.*, 1986), proliferation experiments were firstly performed using PBMCs following stimulation with *H. pylori* LPS preparations NCTC11637 and CCUG17874. A culture using cells and media was set up and used as a negative control (unstim), PBMCs were stimulated with the following: PHA, α-GalCer, *E. coli* clinical isolate as a LPS control and two test strains of *H. pylori* LPS including *H. pylori* NCTC11637 LPS and CCUG17874 LPS. A 7 day culture was set up after which time, the proliferative responses of these cells were analysed using BrdU ELISA. As shown in Fig. 4.3, PHA induced a significant proliferative response in PBMC samples from four healthy donors (Unstim OD = 0.13 ± 0.019; PHA OD = 1.96 ± 0.67). In contrast, none of the other stimuli tested (α-GalCer 25 µg/ml OD = 0.14 ± 0.02; α-GalCer 10 µg/ml OD = 0.15 ±

0.03; α -GalCer 5 $\mu\text{g/ml}$ OD = 0.16 ± 0.03 ; *E. coli* LPS 100 ng/ml OD = 0.13 ± 0.06 ; *H. pylori* 11637 LPS 100 ng/ml OD = 0.11 ± 0.04 ; *H. pylori* 17874 LPS 100 ng/ml OD = 0.17 ± 0.12) induced any significant proliferative responses in PBMCs (n = 4).

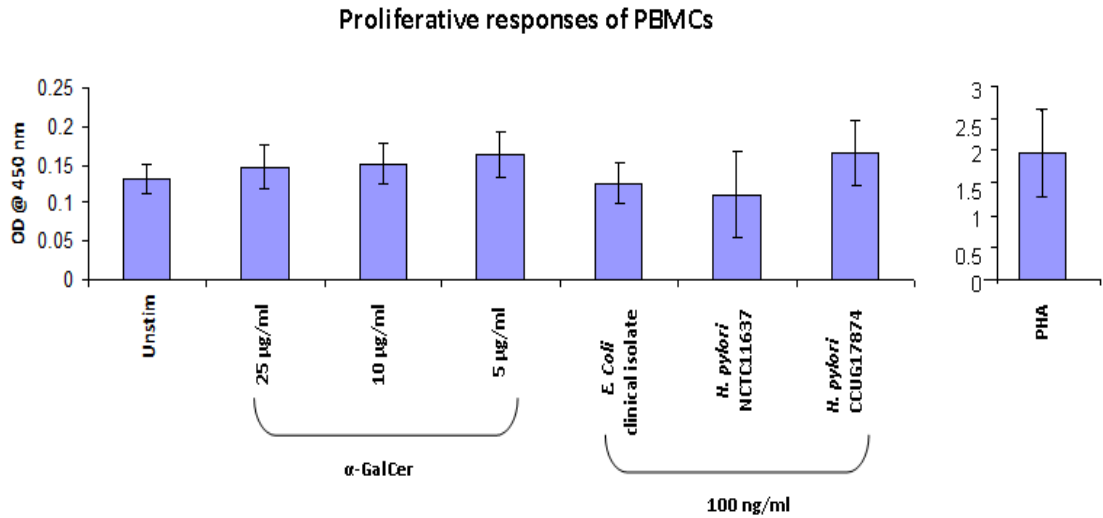


Fig. 4.3 Representative bar chart showing proliferation of PBMCs after stimulation with PHA, α -GalCer, *E. coli* clinical isolate, *H. pylori* NCTC11637 and CCUG17874 LPS (n = 4) for 7 days. Bars indicate mean values of 4 experiments performed in triplicate and the Y-error bars indicate standard error values.

4.2.1.3 Proliferative responses of gastric epithelial and lamina propria layer cells following *H. pylori* LPS stimulation.

Next, the proliferative capacity of the cells residing in the gastric epithelium and lamina propria layers were tested in response to stimulation with PHA, α -GalCer, *E. coli* LPS (as an LPS control), *H. pylori* NCTC11637 LPS and CCUG17874 LPS Ags since *H. pylori* resides in gastric tissues and induces localised immune responses (Robinson *et al.*, 2007). As before, a 7 day culture was set up stimulating total single cell suspensions from both epithelium and lamina propria layers with *H. pylori* LPS after which time proliferation was tested using BrdU ELISA. Results showed stimulation of gastric epithelial and lamina propria layer cells with PHA, α -GalCer, *E. coli* LPS, *H. pylori* NCTC11637 and CCUG17874 LPS failed to induce any significant proliferation using BrdU ELISA after 7 days in culture (n = 5 donors).

Given the lack of proliferation at seven days, to investigate further, proliferative responses of gastric epithelial and lamina propria layer cells were tested after 5, 4 and 3 days. Like after 7 days, neither epithelial nor lamina propria layer cells proliferated in response to stimulation with PHA, α -GalCer, *E. coli* LPS, *H. pylori* NCTC11637 LPS and CCUG17874 LPS after 5 days in culture. After 4 (PHA OD = 1.54 ± 0.01) (Fig. 4.4) and 3 (PHA OD = 0.9 ± 0.2) day stimulation, the only proliferative response seen was when gastric lamina propria layer cells were stimulated with the mitogenic control (PHA) while all other stimuli (α -GalCer, *E. coli* LPS, *H. pylori* NCTC11637 LPS and CCUG17874 LPS) failed to induce a proliferative response in either lamina propria or epithelial layer cells. Thus to summarise, neither epithelial nor lamina propria layer cells proliferated following stimulation with α -GalCer, *E. coli* and *H. pylori* LPS after 7 (n = 5), 5 (n = 3), 4 (n = 2) or 3 (n = 2) days. PHA induced proliferation by gastric lamina propria layer cells after 4 and 3 days only in culture. All data for the above experiment is shown in Appendix I.

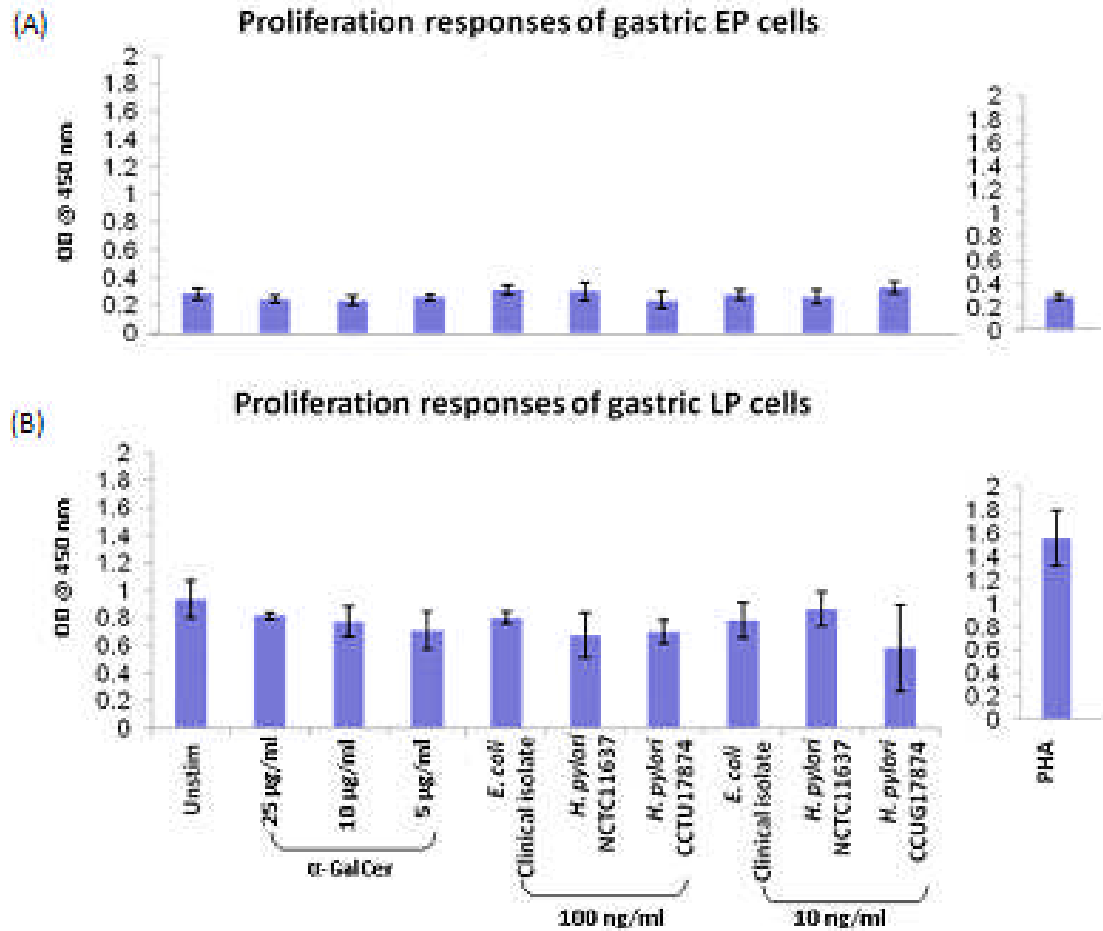


Fig. 4.4 Lack of proliferation of gastric (A) epithelial and (B) lamina propria layer cells observed following *H. pylori* LPS stimulation. Representative bar chart showing proliferative responses of gastric epithelial and lamina propria layer cells stimulated with α -GalCer, *E. coli* LPS, *H. pylori* NCTC11637 LPS and *H. pylori* CCUG17874 LPS after 4 day stimulation period (n = 3). Bars indicate mean values of 3 experiments performed in triplicate. The Y-error bars indicate standard error values.

4.2.1.4 Proliferative responses of NKT cell clones following stimulation with *H. pylori* derived Ags

In next experiments, instead of PBMCs or gastric lymphocyte cell suspensions, NKT cell clones were stimulated with PHA, *E. coli* clinical isolate LPS, *H. pylori* NCTC11637 LPS and *H. pylori* CCUG17874 LPS. In addition, to explore further the finer response to *H. pylori*, NKT cell clones were stimulated with the following: WCE, Cyto Ags, C.M. Ags, OMPs and IMPs of *H. pylori* strain J99. Since NKT cells have been shown to be activated quickly in response to stimulation, the proliferative responses of NKT cells are optimum between 24-48 h (personal communication with Dr. Derek Doherty). α -GalCer (5 μ g) was the positive control, 100 ng/ml of bacterial Ag was the concentration used for NKT cell stimulation and an unstimulated sample with NKT cells and media alone was also set up. In these experiments, NKT cell clones failed to proliferate in response to α -GalCer (OD = 2.3 ± 0.15); WCE (OD = 2.3 ± 0.3); Cyto Ags (OD = 2.1 ± 0.11); C.M Ag (OD = 2.03 ± 0.4); OMP (OD = 1.29 ± 0.33 ; $p = 0.011$); IMP (OD = 0.26 ± 0.02 ; $p = 0.0000026$); *H. pylori* 11637 (OD = 2.17 ± 0.19) and *H. pylori* 17874 (OD = 1.97 ± 0.37) beyond levels observed in unstimulated cultures (OD = 2.2 ± 0.3) (Fig. 4.5A) ($n = 3$) Similar results were obtained after 48 h ($n = 3$). (Unstim OD = 3.41 ± 0.49 ; α -GalCer OD = 3.5 ± 0.18 ; WCE OD = 3.39 ± 0.32 ; Cyto Ags OD = 2.97 ± 0.36 ; OMP OD = 3.37 ± 0.22 ; IMP OD = 0.15 ± 0.05 , $p = 0.0000099$; *H. pylori* 11637 OD = 3.65 ± 0.49 ; *H. pylori* 17874 OD = 3.32 ± 0.18) (Fig. 4.5B). Results show the mean values of 3 experiments carried out in triplicate. Further experimental analysis such as an MTT assay would need to be undertaken to confirm whether *H. pylori* IMP is evoking immunosuppressive responses by NKT cells.

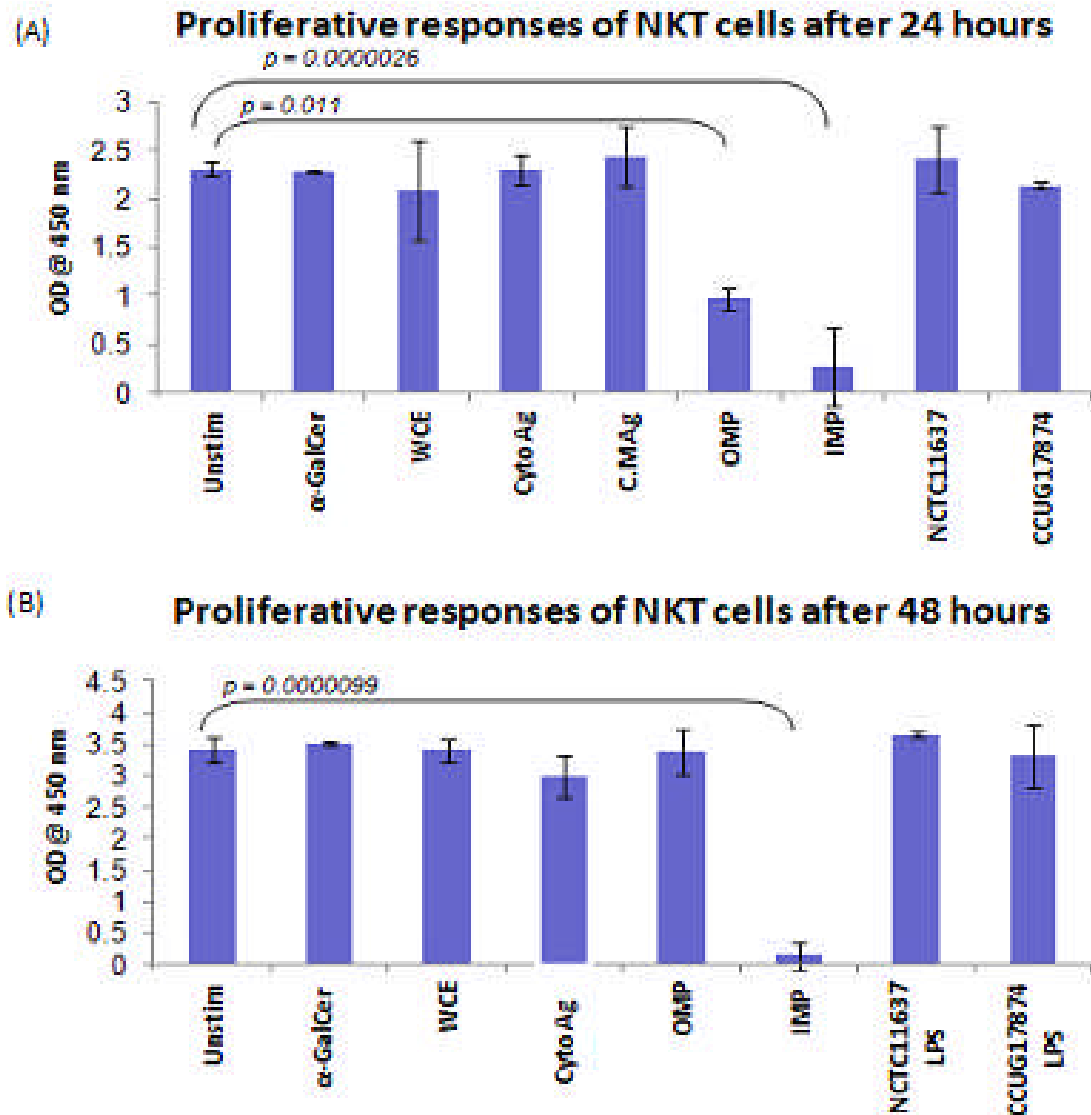


Fig. 4.5 Lack of proliferative responses of NKT cell clones following stimulation with *H. pylori* derived bacterial fractions (n = 3). Bar charts showing proliferative responses of NKT cells after stimulation with α -GalCer and a range of *H. pylori* Ags including WCE, Cyto Ags, OMP and IMP from J99, NCTC11637 LPS and CCUG17874 LPS for (A) 24 h and (B) 48 h. Bars indicate mean values of 3 experiments performed in triplicate. The Y-error bars indicate standard error values.

4.2.2 Cytokine responses

Cytokines are vital in controlling initial infection as well as promoting and maintaining the ensuing adaptive immune responses (Janeway *et al.*, 2005). Therefore, uncovering cytokine production patterns of immune cells in response to *H. pylori* is key to understanding *H. pylori* pathogenesis.

4.2.2.1 Optimisation experiments

Initial optimisation experiments were carried to determine optimum Ag concentration and time points for cytokine production. PBMCs from healthy subjects (HS) were stimulated with *H. pylori* NCTC11637 LPS with six different concentrations ranging from 10 ng/ml- 10 µg/ml at three different time points (24, 48 and 72 h), The T_h1/T_h2 11plex FlowCytomix kit was used as a means of analysis. While patterns in cytokine production were difficult to decipher as variations in cytokine production were seen at all time points, using IL-4 as a representative example, Fig. 4.6 shows the optimum time point for cytokine production after PBMC stimulation with LPS was 72 h. While cytokine production was observed at several different concentrations, it was determined that the optimum LPS concentration for cytokine production was 0.1 µg/ml as the highest levels of IL-1β, IL-4, IL-10 and TNF-β cytokine production were observed (Fig. 4.7). All data can be found in Appendix II.

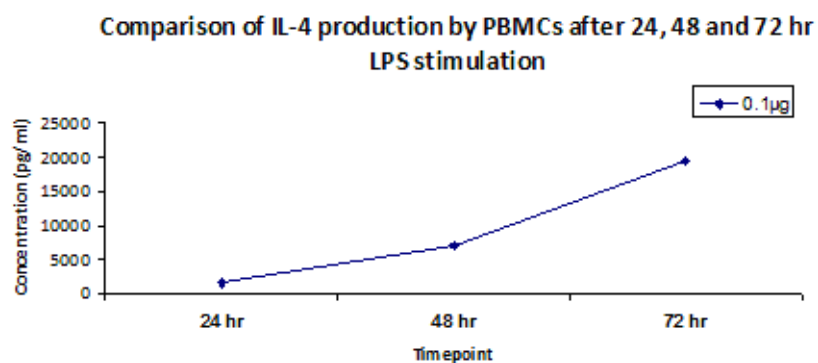


Fig. 4.6 Representative line graph showing the optimum time point and LPS concentration for cytokine production by PBMCs.

At 72 h, as shown in Fig. 4.8, *H. pylori* NCTC11637 LPS induced IL-4, IL-5, IL-10, IL-1 β , TNF- α and TNF- β beyond levels in unstimulated cultures at concentrations ranging from 1 ng/ml- 10 μ g/ml. There was no IFN- γ , IL-2, IL-8 or IL-13 produced.

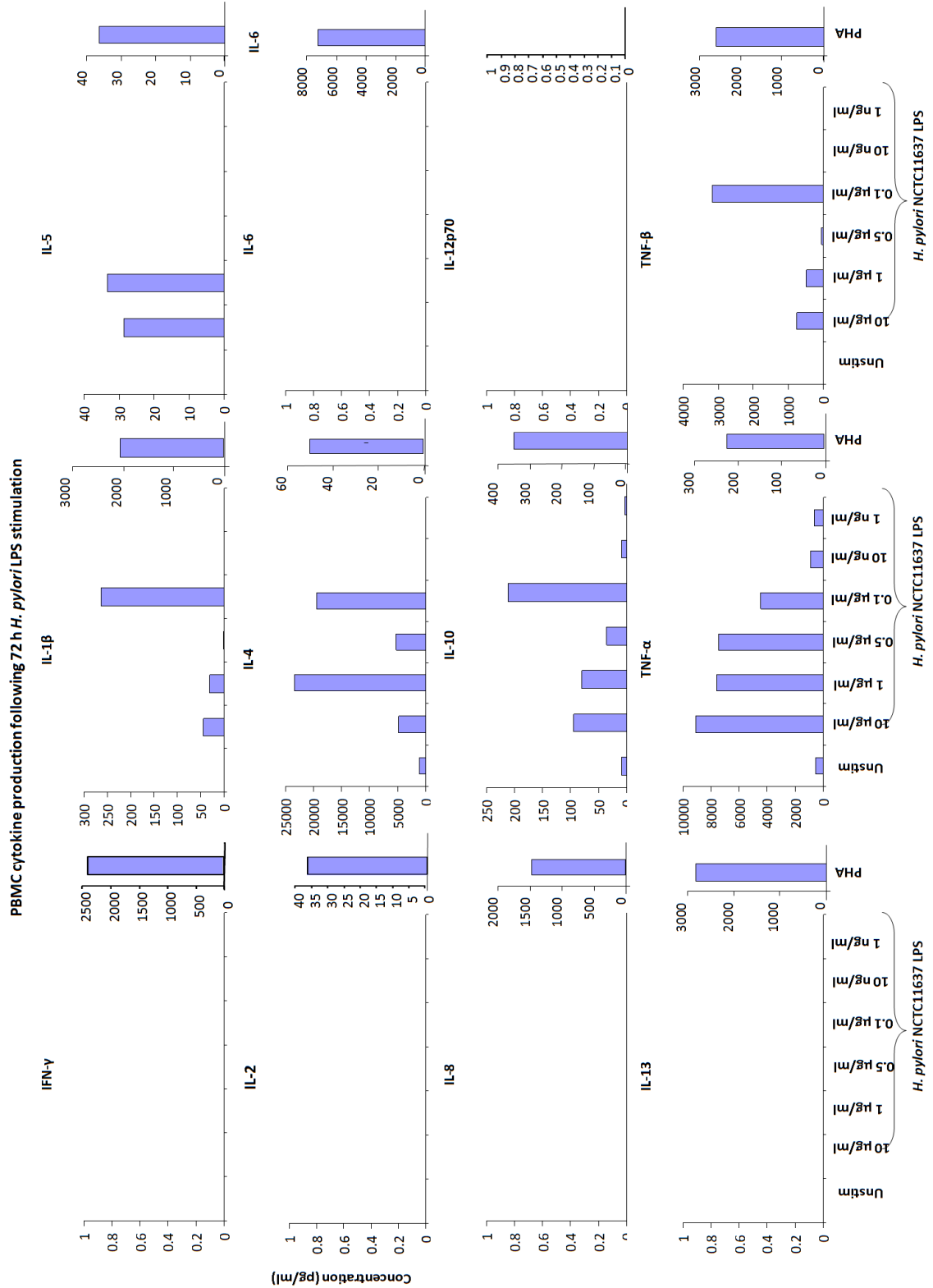


Fig. 4.7 Bar charts showing cytokine production by PBMCs after optimum 72 h stimulation with *H. pylori* LPS at six different concentrations ranging from 10 μ g - 10 ng (n = 1). Bars indicate levels of cytokine production measured in pg/ml.

4.2.2.2 Cytokine production by gastric epithelial and lamina propria layer cells following *H. pylori* LPS stimulation

In order to analyse localised *in vitro* immune responses to *H. pylori* LPS stimulation, isolated gastric epithelial layer cells and lamina propria layer cells from *H. pylori* negative patients which were either asymptomatic or suffered from mild gastritis were stimulated with PHA and *H. pylori* LPS from strains NCTC11637 and CCUG17874 for 72 h after which time cytokine production was examined using T_h1/T_h2 11plex FlowCytomix kit. As shown in Fig. 4.8, results showed IL-2 production by epithelial cells following stimulation with *H. pylori* CCUG17874 LPS (28.21 pg/ml) while levels of all other cytokines did not increase beyond unstimulated cells with either PHA or the two LPS strains shown. However, it must be noted no cellular response to PHA observed beyond control stimuli following epithelial layer analysis.

Secondly, isolated lamina propria layer cells from *H. pylori* negative patients which were either asymptomatic or suffered from mild gastritis were stimulated with *H. pylori* NCTC11673 and CCUG17874 LPS for 72 h after which time; cytokine production was recorded using T_h1/T_h2 11plex FlowCytomix kit and flow cytometry. As shown in Fig. 4.9, IL-2 (3.73 pg/ml) production was observed following stimulation of immune cells with *H. pylori* NCTC11637 LPS while no increase in any of the other cytokines beyond levels in unstimulated cell cultures was seen. This was true of PHA and both LPS strains.

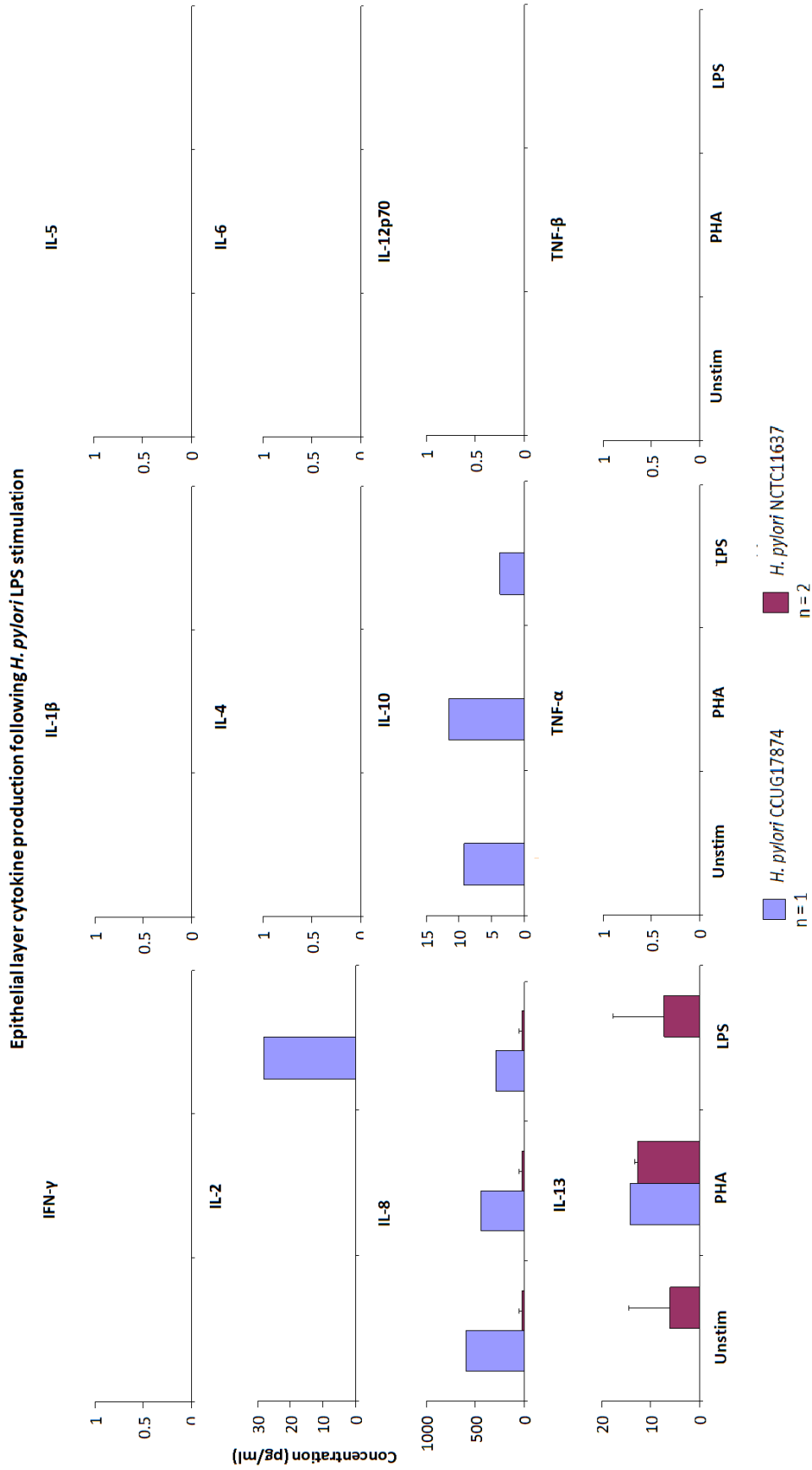


Fig. 4.8 Cytokine production in the gastric epithelial layer following *H. pylori* LPS stimulation. Illustration of cytokine production by gastric epithelial layer cells following stimulation with *H. pylori* NCTC11637 (n = 2) and CCUG17874 (n = 1) LPS for 72 h. Bars indicate cytokine production measured in pg/ml. The Y-error bars indicate standard error values.

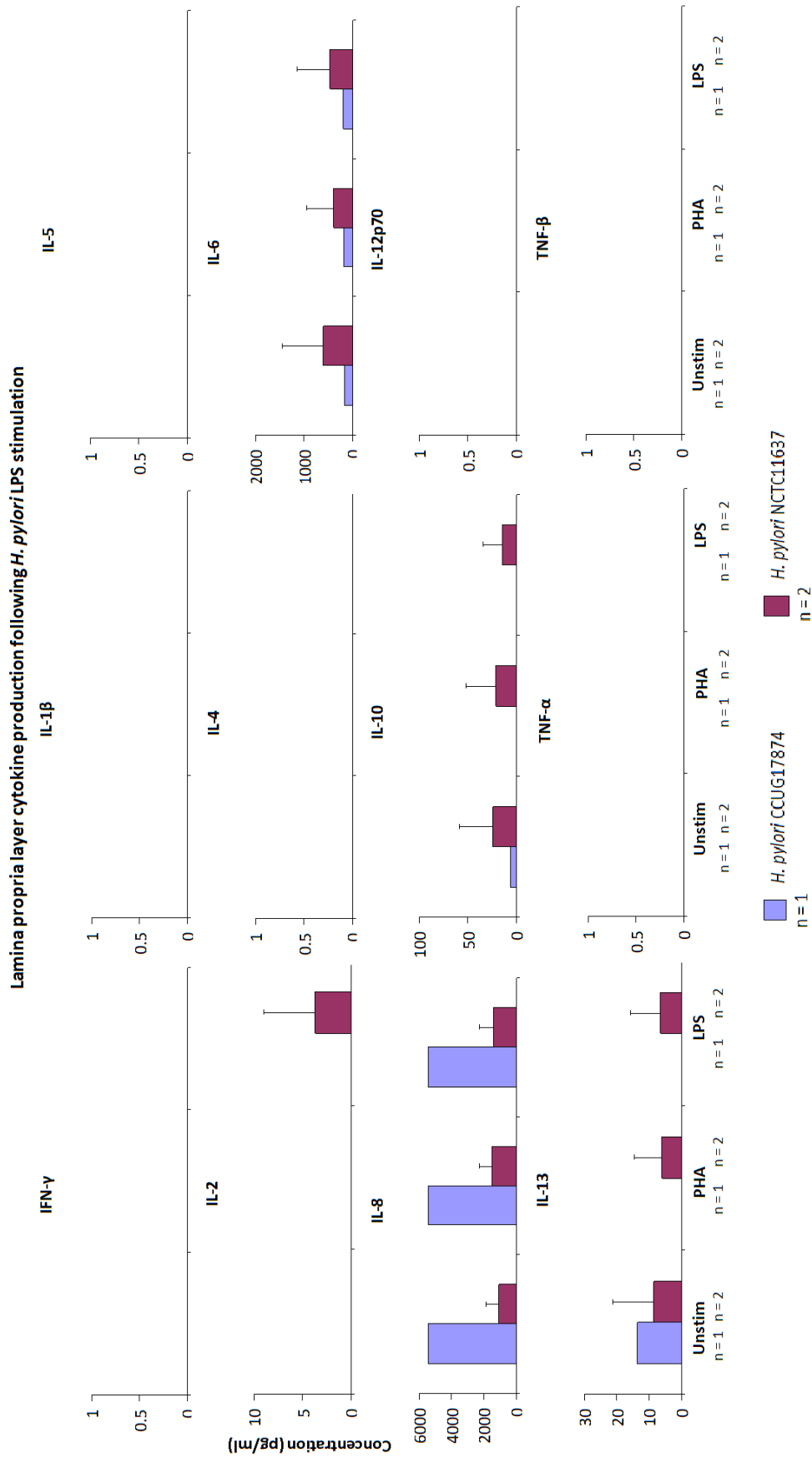


Fig. 4.9 Bar charts showing cytokine production in the gastric lamina propria layer following *H. pylori* NCTC11637LPS stimulation (n = 2) and CCUG17874 LPS stimulation (n = 1). Cytokine production was measured in pg/ml. The Y-error bars indicate standard error values.

4.2.2.3 Cytokine production of human NKT cell clones following stimulation with *H. pylori* derived Ags

Having determined that PBMCs and gastric epithelial and lamina propria layer cells fail to respond with any great conviction to either PHA or *H. pylori* LPS (NCTC11637 and CCUG17874), in next experiments, purified NKT cell clones were used to address the question of the stimulatory capacity of a range of *H. pylori* fractions *in vitro*. To date, NKT cells are known to be activated by α -GalCer as well as certain glycolipids from *Sphingomonas*, *Ehrlichia* and *B. burgdorferi* (Kinjo *et al.*, 2005; Kumar *et al.*, 2000; Mattner *et al.*, 2005). The aim of this study was to determine whether bacterial fractions derived from *H. pylori* strains NCTC11637, CCUG17874, 26695 and J99 were capable of stimulating NKT cells to produce cytokines. Two NKT cell lines (BC19 and BC20, a gift from Dr. Derek Doherty) were used for this part of the study (see chapter 2, sections 2.3.1). The NKT cell clones were firstly co-cultured along with CD1d HeLa cells as APCs, as it is known CD1d presents glycolipids to NKT cells. α -GalCer was a positive control as α -GalCer is a potent activator and inducer of cytokine production by NKT cells. Secondly, NKT cells were co-cultured for 24 h with CD1d HeLa cells and with 100 ng/ml of bacterial test Ags including WCE, Cyto Ags, C.M. Ags, OMP and IMP from *H. pylori* 26695 and J99 and with LPS derived from *H. pylori* 11637, *H. pylori* CCUG17874 and *E.coli* clinical isolate. An identical culture was set up using a mock cell line which did not possess the CD1d Ag presenting capability as a negative control. Other controls included unstimulated cells as well as a vehicle control where 0.1% DMSO replaced α -GalCer. Cytokine production was recorded using T_h1/T_h1 11plex FlowCytomix kit. Two experiments were performed using both BC19 and BC20 NKT cell clones. The average values of cytokine production of the two cell lines were taken.

A) Cytokine production observed by NKT cells after stimulation with α -GalCer

Upon analysis, results shown in Fig. 4.10 revealed α -GalCer, the positive control, induced NKT cells to produce higher levels of IFN- γ (3492 ± 493 pg/ml), IL-2 (3085 ± 1784 pg/ml), IL-10 (0.33 ± 0.4 pg/ml), IL-8 (4620 ± 351 pg/ml), IL-6 (2021 ± 163 pg/ml), IL-4 (4020 ± 3782 pg/ml), IL-5 (6165 ± 5085 pg/ml), IL-1 β (1.32 ± 1.86 pg/ml), TNF- α (802 ± 287 pg/ml), TNF- β (81 ± 41 pg/ml) when incubated with CD1d transfected cell line compared with the mock control cell line IFN- γ (0), IL-2 (53 ± 75 pg/ml), IL-10 (0), IL-8 (115 ± 57 pg/ml), IL-6 (251 ± 49 pg/ml), IL-4 (24 ± 15 pg/ml), IL-5 (82 ± 116 pg/ml), IL-1 β (0), TNF- α (0), TNF- β (0). IL-12p70 was the only cytokine not produced by NKT cells after α -GalCer stimulation when co-cultured with either mock or CD1d transfected cell line. All The results display values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants). All data is shown in Appendix II.

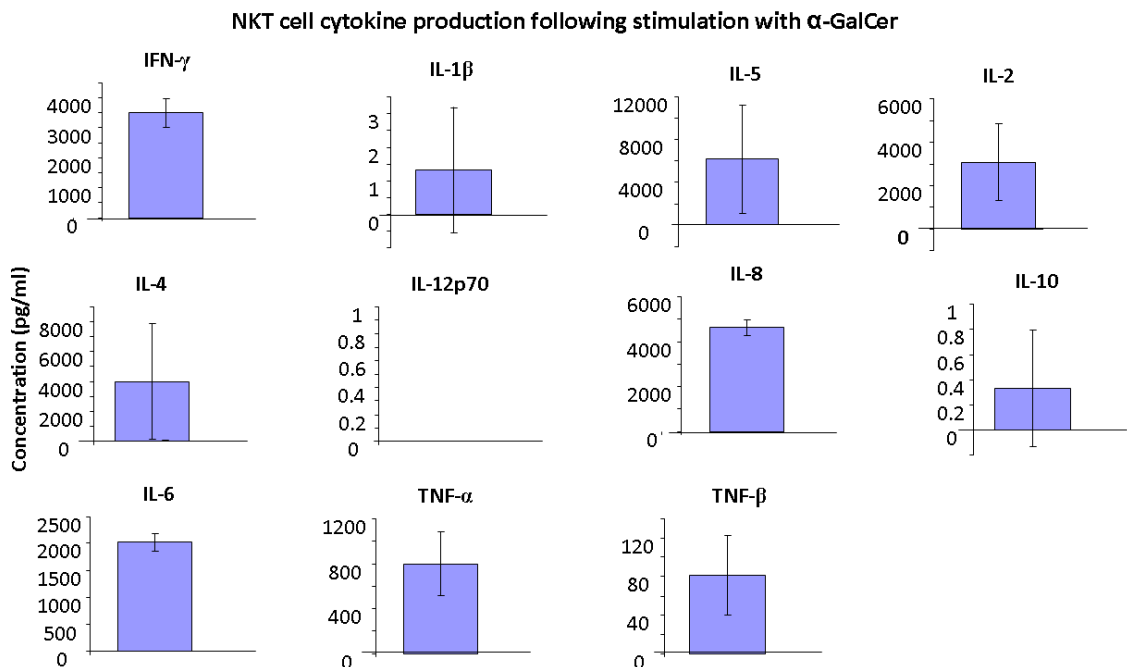


Fig. 4.10 Bar charts showing NKT cell cytokine production following stimulation with α -GalCer using CD1d transfectant cell lines as an APC. Bars represents cytokine increases beyond levels obtained in control wells (mock transfectants and NKT cells) ($n = 2$). The Y error bars indicate mean values \pm SD.

B) Cytokine production following NKT cell stimulation with Ags derived from *H. pylori* 26695

Secondly, NKT cell clones BC19 and BC20 were stimulated with the WCE, Cyto Ags, C.M Ags, OMP and IMP Ags from *H. pylori* 26695 for 24 h using mock or CD1d cell transfected cell lines as APCs. Supernatants were then analysed for cytokine production via T_h1/T_h2 11plex FlowCytomix kit. The results display values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants, the vehicle controls as well as unstimulated NKT cells have been subtracted from test samples). All data is shown in Appendix II. The results for all cytokines are shown in Fig. 4.11.

IFN- γ : When NKT cells were cultured with WCE or Cyto Ags in the presence of the CD1d transfected cell line, an increase in IFN- γ production (mean, 10.03 pg/ml and 6.45 pg/ml respectively) was found beyond levels in control wells, All other Ags (C.M Ag, OMP, IMP) failed to stimulate NKT cells to produce IFN- γ beyond controls.

IL-2: An increase in IL-2 production beyond that found in control wells was found when NKT cells were cultured in the presence of CD1d transfected cells with and the Cyto Ags (mean, 6.17 pg/ml) and the OMP (5.06 pg/ml). All other Ags failed to stimulate NKT cells to produce IL-2 beyond controls (WCE, C.M Ag, IMP).

IL-4: An increase in IL-4 production beyond that found in control wells was found only when NKT cells were cultured with IMP (mean, 9.81 pg/ml) in the presence of CD1d transfected cells. All other Ags failed to stimulate NKT cells to produce IL-4 beyond controls (WCE, Cyto Ags, C.M Ag, OMP)

IL-5: An increase in IL-5 production beyond that found in control wells was found when NKT cells were cultured with WCE (mean, 63 pg/ml), Cyto Ags (mean, 18.3 pg/ml) and the OMP (mean, 3.6 pg/ml) in the presence of CD1d transfected cells.

This was in contrast to the C.M Ag and IMP *H. pylori* Ags which failed induce IL-5 production by NKT cells beyond controls following 24 h stimulation.

IL-8: An increase in IL-8 production beyond that found in control wells was found when NKT cells were cultured with the WCE (mean, 22.5 pg/ml), Cyto Ags (mean, 10.9 pg/ml), C.M Ag (mean, 12.5 pg/ml) and the OMP (mean, 63.3 pg/ml) in the presence of CD1d transfected cells. While the IMP failed to stimulate NKT cells to produce IL-8 beyond controls following 24 h antigenic stimulation.

Beyond controls, no increase in **IL-1 β , IL-6, IL-10, IL-12p70, TNF- α or TNF- β** was observed upon NKT cell stimulation with WCE, Cyto Ags, C.M Ag, OMP and IMP Ags derived from *H. pylori* 26695. Table 4.1 summarises the cytokines produced by NKT cells upon stimulation with Ags derived from *H. pylori* 26695. As shown, firstly, the WCE induced production of IFN- γ , IL-5 and IL-8; secondly, the Cyto Ags induced IFN- γ , IL-2, IL-5 and IL-8. Thirdly, the C.M Ag induced production of IL-8 while fourthly, the OMP induced production of IL-2, IL-5 and IL-8 and finally, IMP induced IL-4 production alone by NKT cells. However, it is important to note that cytokine levels produced by NKT cells in response to *H. pylori* derived Ags are far below those values obtained with α -GalCer.

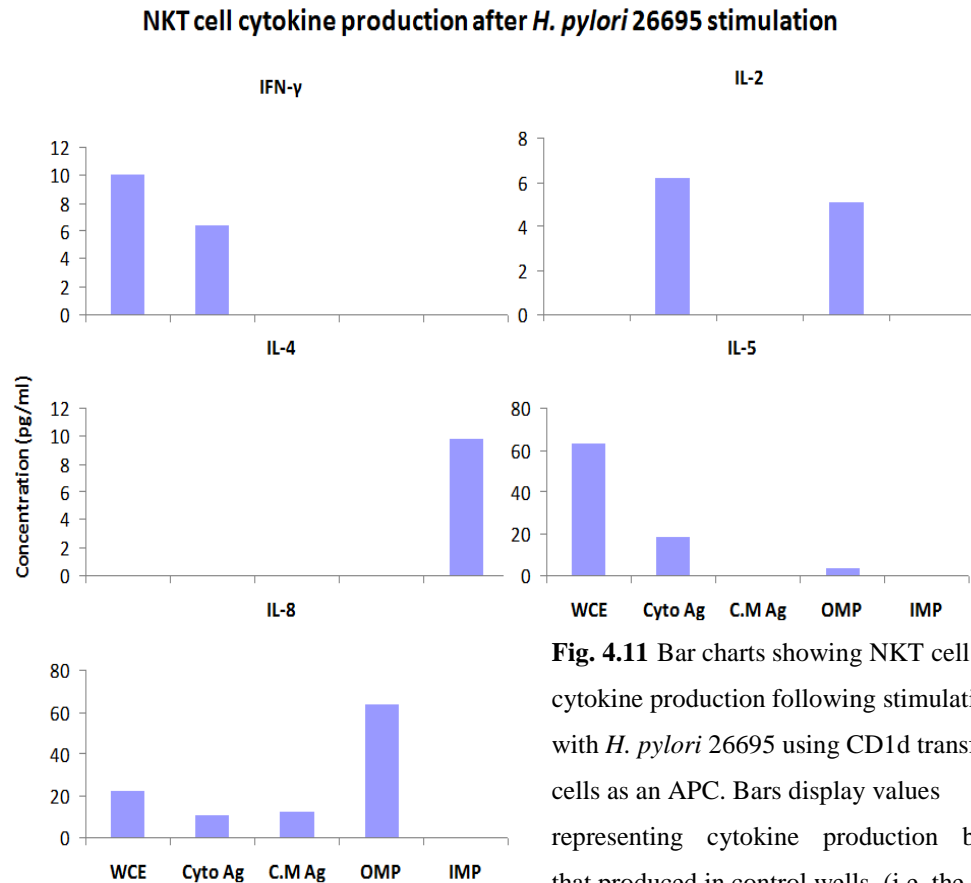


Fig. 4.11 Bar charts showing NKT cell cytokine production following stimulation with *H. pylori* 26695 using CD1d transfected cells as an APC. Bars display values representing cytokine production beyond that produced in control wells (i.e. the wells

containing the mock transfectants or the vehicle controls as well as unstimulated NKT cells). Experiment carried out in duplicate and values represent mean values of two experiments after control wells have been subtracted.

Table 4.1 Summary of cytokine production observed after NKT cell stimulation with Ags derived from *H. pylori* 26695 in the presence of CD1d transfected cells

	WCE	Cyto Ags	C.M Ag	OMP	IMP
IL-12p70	-	-	-	-	-
IFN-γ	+	+	-	-	-
IL-2	-	+	-	+	-
IL-10	-	-	-	-	-
IL-8	+	+	+	+	-
IL-6	-	-	-	-	-
IL-4	-	-	-	-	+
IL-5	+	+	-	+	-
IL-1β	-	-	-	-	-
TNF-α	-	-	-	-	-
TNF-β	-	-	-	-	-

NKT = Natural Killer T cells; WCE = whole cell extract, Cyto Ags = cytoplasmic antigens; C.M Ag = crude membrane antigens, OMP = outer membrane proteins; IMP = inner membrane proteins; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor. A + sign indicates cytokine production by NKT cells beyond that produced in control wells. A - sign indicates no significant cytokine production.

C) Cytokine production observed after NKT cell stimulation with Ags derived from *H. pylori* J99

NKT cell clones were then stimulated with the WCE, Cyto Ags, C.M Ag, OMP and IMPs Ags from *H. pylori* J99 for 24 h using mock or CD1d transfected cell lines as APCs. After this time, supernatants were analysed for cytokine production via T_h1/T_h2 11plex FlowCytomix kit. The results displayed values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants or the vehicle controls as well as unstimulated NKT cells). All data is shown in Appendix II. The results for all cytokines are shown in Fig. 4.12.

IFN- γ : When NKT cells were cultured with WCE and C.M Ag in the presence of the CD1d transfected cell line, an increase in IFN- γ production was found beyond levels in control wells, (mean, 7.4 pg/ml and 3.7 pg/ml respectively). All other Ags (Cyto Ags, OMP, IMP) failed to stimulate NKT cells to produce IFN- γ beyond controls.

IL-8: An increase in IL-8 production beyond that found in control wells was found when NKT cells were cultured in the presence of CD1d transfected cells with the WCE (mean, 23.5 pg/ml), Cyto Ags (mean, 22.2 pg/ml), C.M Ag (mean, 25.8 pg/ml), OMP (mean, 18.7 pg/ml) and IMP (mean, 14.8 pg/ml) of *H. pylori* J99. Therefore all Ags derived from *H. pylori* J99 induced IL-8 production by NKT cells following 24 h antigenic stimulation.

Beyond controls, no increase in **IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, TNF- α** or **TNF- β** was observed upon WCE, Cyto Ags, C.M Ag, OMP and IMP antigenic stimulation of NKT cells with *H. pylori* J99. Table 4.2 summarises the cytokines produced by NKT cells upon stimulation with Ags derived from *H. pylori* J99. As shown, WCE and C.M Ag induces production of IFN- γ and IL-8 while the OMP, Cyto Ags and IMP Ags additionally induce IL-8 production alone. However, it must be noted

that cytokine levels produced by NKT cells in response to *H. pylori* derived Ags are far below those values obtained with α -GalCer.

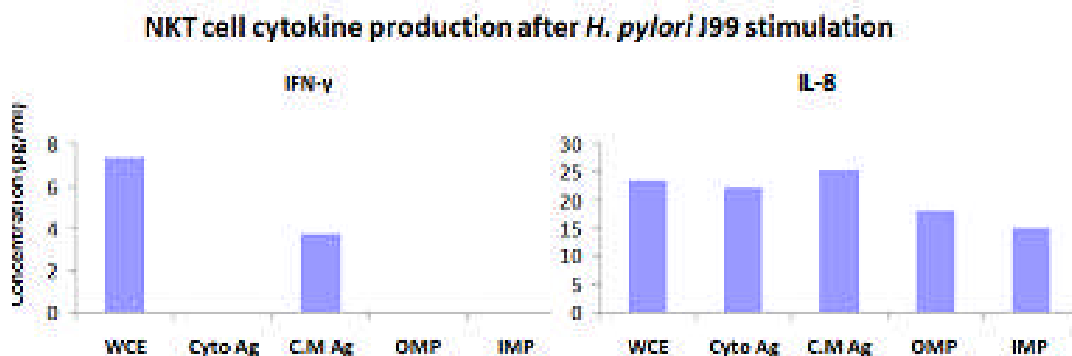


Fig. 4.12 Bar charts showing NKT cell cytokine production following stimulation with *H. pylori* J99 using CD1d transfected cell lines as an APC. Bars display values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants or the vehicle controls as well as unstimulated NKT cells.) Experiment carried out in duplicate and mean values were obtained.

Table 4.2 Summary of cytokine production observed after NKT cell stimulation with Ags derived from *H. pylori* J99 in the presence of CD1d transfected cells

	WCE	Cyto Ags	C.M Ag	OMP	IMP
IL-12p70	-	-	-	-	-
IFN- γ	+	-	+	-	-
IL-2	-	-	-	-	-
IL-10	-	-	-	-	-
IL-8	+	+	+	+	+
IL-6	-	-	-	-	-
IL-4	-	-	-	-	-
IL-5	-	-	-	-	-
IL-1 β	-	-	-	-	-
TNF- α	-	-	-	-	-
TNF- β	-	-	-	-	-

NKT = Natural Killer T cells; WCE = whole cell extract, Cyto Ags = cytoplasmic antigens; C.M Ag = crude membrane antigens, OMP = outer membrane proteins; IMP = inner membrane proteins; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor. A + sign indicates cytokine production by NKT cells beyond that produced in control wells. A – sign indicates no significant cytokine production

D) Cytokine production observed after NKT cell stimulation with *H. pylori* and *E. coli* LPS

Finally, NKT cell clones were stimulated with *E. coli* clinical isolate, *H. pylori* NCTC11637 and *H. pylori* CCUG17874 LPS for 24 h using mock or CD1d transfected HeLa cell lines as APCs. After this time, supernatants were analysed for cytokine production via T_h1/T_h2 11plex FlowCytomix kit. The results display values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants or the vehicle controls as well as unstimulated NKT cells). All data is displayed in Appendix II. The results for all cytokines are shown in Fig. 4.13.

IFN- γ : When NKT cells were cultured with *H. pylori* CCUG17874 LPS in the presence of the CD1d transfected cell line, IFN- γ production was observed (mean, 6.2 pg/ml). All other Ags failed to stimulate NKT cells to produce IFN- γ beyond controls (*H. pylori* NCTC11637 LPS, *E. coli* clinical isolate LPS).

IL-5: An increase in IL-5 production beyond that found in control wells was found when NKT cells were cultured in the presence of CD1d transfected cells with *E. coli* clinical isolate LPS (mean, 20.2 pg/ml). This was in contrast to *H. pylori* NCTC11637 and *H. pylori* CCUG17874, both of which failed to induce IL-5 production by NKT cells following 24 h stimulation.

IL-8: An increase in IL-8 production beyond that found in control wells was found when NKT cells were cultured with *E. coli* clinical isolate (mean, 38.4 pg/ml), *H. pylori* NCTC11637 (mean, 31.4 pg/ml) and *H. pylori* CCUG17874 (mean, 39.2 pg/ml) LPS in the presence of CD1d transfected cells. Therefore all LPS samples tested induced IL-8 production by NKT cells.

TNF- β : An increase in TNF- β production beyond that found in control wells was found only when NKT cells were cultured with *H. pylori* CCUG17874 (mean, 6.1

pg/ml) in the presence of CD1d transfected cells. All other Ags failed to stimulate NKT cells to produce TNF- α beyond controls (*E. coli* clinical isolate, *H. pylori* NCTC11637 LPS).

Beyond controls, no increase in **IL-1 β** , **IL-2**, **IL-4**, **IL-6**, **IL-10**, **IL-12p70** or **TNF- α** was observed upon antigenic stimulation of NKT cells with *H. pylori* NCTC11637, CCUG17874 and *E. coli* clinical isolate LPS. Table 4.3 summarises the cytokines produced by NKT cells upon stimulation with LPS. As shown, *H. pylori* NCTC11637 LPS induced production of IL-8. *H. pylori* CCUG17874 LPS induced IFN- γ , IL-4, IL-8 and TNF- β production by NKT cells while *E. coli* clinical isolate LPS activated NKT cells to produce IL-5 and IL-8. However, cytokine levels produced by NKT cells in response to *H. pylori* derived Ags are far below those values obtained with α -GalCer.

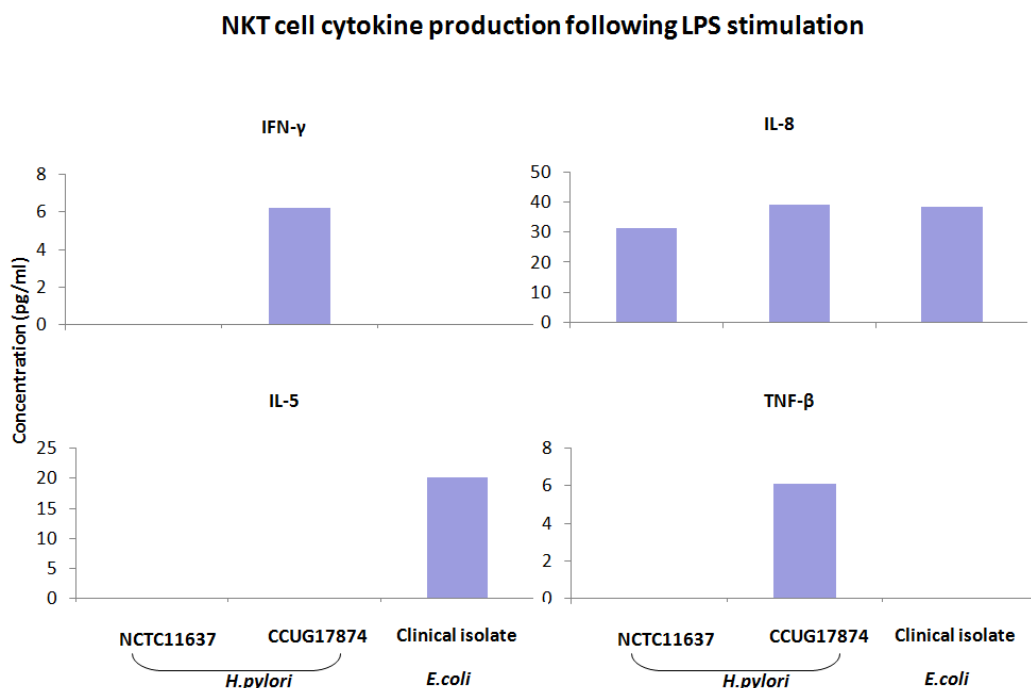


Fig. 4.13 Bar charts showing NKT cell cytokine production following stimulation with *H. pylori* and *E. coli* LPS using CD1d transfected cell lines as an APC. Bars display values representing cytokine production beyond that produced in control wells (i.e. the wells containing the mock transfectants or the vehicle controls as well as unstimulated NKT cells). Experiment carried out in duplicate and the mean value was obtained.

Table 4.3 Summary of cytokine production observed after NKT cell stimulation with *H. pylori* and *E. coli* LPS in the presence of CD1d transfected cells

	<i>H.</i> NCTC11637 LPS	<i>pylori</i> LPS	<i>H.</i> CCUG17874 LPS	<i>pylori</i> LPS	<i>E. coli</i> clinical isolate LPS
IL-12p70	-		-		-
IFN-γ	-		+		-
IL-2	-		-		-
IL-10	-		-		-
IL-8	+		+		+
IL-6	-		-		-
IL-4	-		-		-
IL-5	-		-		+
IL-1β	-		-		-
TNF-α	-		-		-
TNF-β	-		+		-

NKT = Natural Killer T cells; LPS = lipopolysaccharide; IFN = Interferon; IL = interleukin; TNF = tumour necrosis factor. A + sign indicates cytokine production by NKT cells beyond that produced in control wells. A – sign indicates no significant cytokine production.

4.2.2.4 Cytokine production of human $V\gamma 9V\delta 2$ T cell clones upon stimulation with *H. pylori* bacterial fractions

A study was next devised to determine whether *H. pylori* was capable of stimulating $\gamma\delta^+$ T cell clones to produce cytokines. To date, $\gamma\delta^+$ T cells are known to respond to stimulation with HMB-PP along with a small number of other bacterial fractions such as *Mycobacterium tuberculosis*, *Yersinia enterocolitica* and *E.coli* to produce IFN- γ and TNF- α (Puan *et al.*, 2007). Currently, there is not a lot known about the effect *H. pylori* has on this particular unconventional T cell population. Recently Romi *et al.*, (2011) have shown that *H. pylori* strain G27 stimulated peripheral CD3⁺ $\gamma\delta^+$ T cells to produce cytokines TNF- α and IFN- γ and chemokines MIP- β and RANTES *in vitro*. Therefore this study was undertaken to determine whether bacterial fractions derived from *H. pylori* strains 26695 and J99 were capable of stimulating $\gamma\delta^+$ T cell clones to induce cytokine production thereby giving an insight into the potential importance of this cell population in *H. pylori* infection. $\gamma\delta^+$ T cells are MHC unrestricted and therefore do not require presentation of Ags, therefore no APCs were needed for this part of the study.

$\gamma\delta^+$ T cell clones (a gift from Dr. Derek Doherty, see chapter 2) were co-cultured firstly with HMB-PP and secondly with 100 ng *H. pylori* derived bacterial Ags including the WCE, Cyto Ags, C.M Ags, OMP and IMP from *H. pylori* strains 26695 and J99 for 24 h, after which period T_h1/T_h2 11plex FlowCytomix kit (eBioscience) was carried out on supernatants for cytokine analysis. Miteogenic controls were also used in this portion of the study which included PMA/I and PHA. This was undertaken twice, firstly with addition of PMA to the cultures and secondly, in the absence of PMA. PMA was also additionally utilised in one of the cultures as a stimulant as studies previously undertaken by Dr. Derek Doherty on the $\gamma\delta^+$ T cell clones have shown a potential potent immune cell response when this mitogen was additionally used in combination with many Ags including HMB-PP (personal communication). All data is shown in Appendix II. The results for all cytokines display increases beyond levels in control wells and are shown in Fig. 4.14 and Fig. 4.15.

A) Cytokine production after $\gamma\delta^+$ T cell stimulation with *H. pylori* 26695

IL-12p70: As shown in Fig. 4.14, when PMA was added to culture, only the Cyto Ags (96 pg/ml) induced IL-12p70 production by $\gamma\delta^+$ T cells beyond levels in control wells. All other control stimuli and bacterial Ags failed to induce IL-12p70 production (Unstim, PHA, PMA/I, HMB-PP, WCE, C. M Ag, OMP, IMP). In the absence of PMA, all samples tested failed to induce IL-12p70 production by $\gamma\delta^+$ T cells beyond unstimulated controls (PHA, PMA/I, HMB-PP, WCE, Cyto Ags, C. M Ag, OMP, IMP).

IFN- γ : Firstly, the positive controls all induced IFN- γ production (PHA, 2009 pg/ml; PMA/I, 7254 pg/ml; HMB-PP, 431 pg/ml) when $\gamma\delta^+$ T cells were cultured with the WCE (18.5 pg/ml), Cyto Ags (49 pg/ml) in the presence of the PMA, an increase in IFN- γ was found beyond levels in control wells. All other Ags (C. M Ag, OMP and

IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond controls. Secondly, when $\gamma\delta^+$ T cells were cultured only with the WCE (19.8 pg/ml) in the absence of the PMA, an increase in IFN- γ was still recorded beyond levels in control wells. All other Ags (Cyto Ags, C. M Ag, OMP and IMP failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond controls.

IL-2: Firstly, the positive controls all induced IL-2 production (PHA, 3573 pg/ml; PMA/I, 24414 pg/ml; HMB-PP, 1138 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the OMP and IMP in the presence of the PMA, an increase in IL-2 was found beyond levels in unstimulated control wells (144 pg/ml and 144 pg/ml respectively). All other Ags (WCE, Cyto Ags, C. M Ag) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond controls. Finally, when $\gamma\delta^+$ T cells were cultured with the OMP (304 pg/ml) and the IMP (593 pg/ml) in the absence of the PMA, an increase in IL-2 was still recorded beyond levels in unstimulated control wells. All other Ags (WCE, Cyto Ags, C. M Ag) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond controls.

IL-10: Firstly, the positive controls all induced IL-10 production (PHA, 372 pg/ml; PMA/I, 401 pg/ml; HMB-PP, 152 pg/ml). Secondly, despite the presence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, and OMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-10 beyond unstimulated controls.

IL-8: Firstly, the positive controls all induced IL-8 production (PHA, 2052 pg/ml; PMA/I, 3658 pg/ml; HMB-PP, 597 pg/ml). When $\gamma\delta^+$ T cells were cultured with the WCE (131 pg/ml) and Cyto Ags (129 pg/ml) in the presence of PMA, an increase in IL-8 was recorded beyond levels in unstimulated control wells. All other Ags (C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond controls. Finally,

all Ags (WCE, Cyto Ags, C. M Ag, OMP, and IMP) failed to induce IL-8 production by $\gamma\delta^+$ T cells beyond unstimulated controls in the absence of PMA.

IL-6: Neither control nor test samples albeit in the presence nor absence of PMA, induced IL-6 production by $\gamma\delta^+$ T cells after stimulation with *H. pylori* 26695 (PHA, PMA/I, HMB-PP, Unstim, WCE, Cyto Ags, C. M Ag, OMP, IMP).

IL-4: Firstly, the positive controls all induced IL-4 production (PHA, 1234 pg/ml; PMA/I, 2814 pg/ml; HMB-PP, 139 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the WCE (118 pg/ml), Cyto Ags (7.5 pg/ml) and IMP (13 pg/ml) in the presence of PMA, an increase in IL-4 was recorded beyond levels in unstimulated control wells. All other Ags (C. M Ag, OMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond controls. Finally, in the absence of PMA, when $\gamma\delta^+$ T cells were cultured only with Cyto Ags (74 pg/ml), an increase in IL-4 was seen beyond levels in unstimulated control wells. All other Ags (WCE, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond controls

IL-5: To begin with, the positive controls all induced IL-5 production (PHA, 41591 pg/ml; PMA/I, 15550 pg/ml; HMB-PP, 5188 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the WCE (3024 pg/ml), Cyto Ags (7163 pg/ml) and C.M Ag (17.7 pg/ml) in the presence of PMA, an increase in IL-5 was recorded beyond levels in unstimulated control wells. All other Ags (OMP, IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond controls. Finally, in the absence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-5 beyond controls.

IL-1 β : Firstly, the positive controls all induced IL-1 β production (PHA, 12.6 pg/ml; PMA/I, 4.8 pg/ml; HMB-PP, 8.8 pg/ml). Secondly, in the presence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to

produce IL-1 β beyond unstimulated controls. Finally, when $\gamma\delta^+$ T cells were cultured with the Cyto Ags (26.68 pg/ml) in the absence of PMA, an increase in IL-1 β was observed beyond levels in unstimulated control wells. All other Ags (WCE, C. M Ag, OMP, IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- α beyond unstimulated controls.

TNF- α : Firstly, the positive controls all induced TNF- α production (PHA, 3566.2 pg/ml; PMA/I, 8095.14 pg/ml; HMB-PP, 62.68 pg/ml). However, in the presence and absence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- α beyond unstimulated controls.

TNF- β : Firstly, two of the three positive controls (PHA, 222.05 pg/ml; PMA/I, 49.21 pg/ml) induced TNF- β production by $\gamma\delta^+$ T cells while HMB-PP failed to induce TNF- β production by $\gamma\delta^+$ T cells. Secondly, when $\gamma\delta^+$ T cells were cultured with the WCE (34.5 pg/ml) and the OMP (9.61 pg/ml) in the presence of PMA, an increase in TNF- β was observed beyond levels in unstimulated control wells. All other Ags (Cyto Ags, C. M Ag, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- β beyond unstimulated controls. Finally, in the absence PMA, all Ags (WCE, Cyto Ags, C. M Ag, and OMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- β beyond unstimulated controls.

Table 4.4 summarises the cytokines produced by $\gamma\delta^+$ T cells upon stimulation with Ags derived from *H. pylori* 26695. As shown, in the presence of PMA, WCE induced production of IFN- γ , IL-4, IL-5, IL-8 and TNF- β ; Cyto Ags induced IL-12p70, IFN- γ , IL-4, IL-5 and IL-8 production; C.M Ag induced IL-5 production only; OMP induced IL-2 and TNF- β cytokine production while IMP induced only IL-2 production. In the absence of PMA, WCE induced production of IFN- γ ; Cyto Ags induced IL-4 and

IL-1 β production; C.M Ag does not induce cytokine production; OMP and IMP induced IL-2 production.

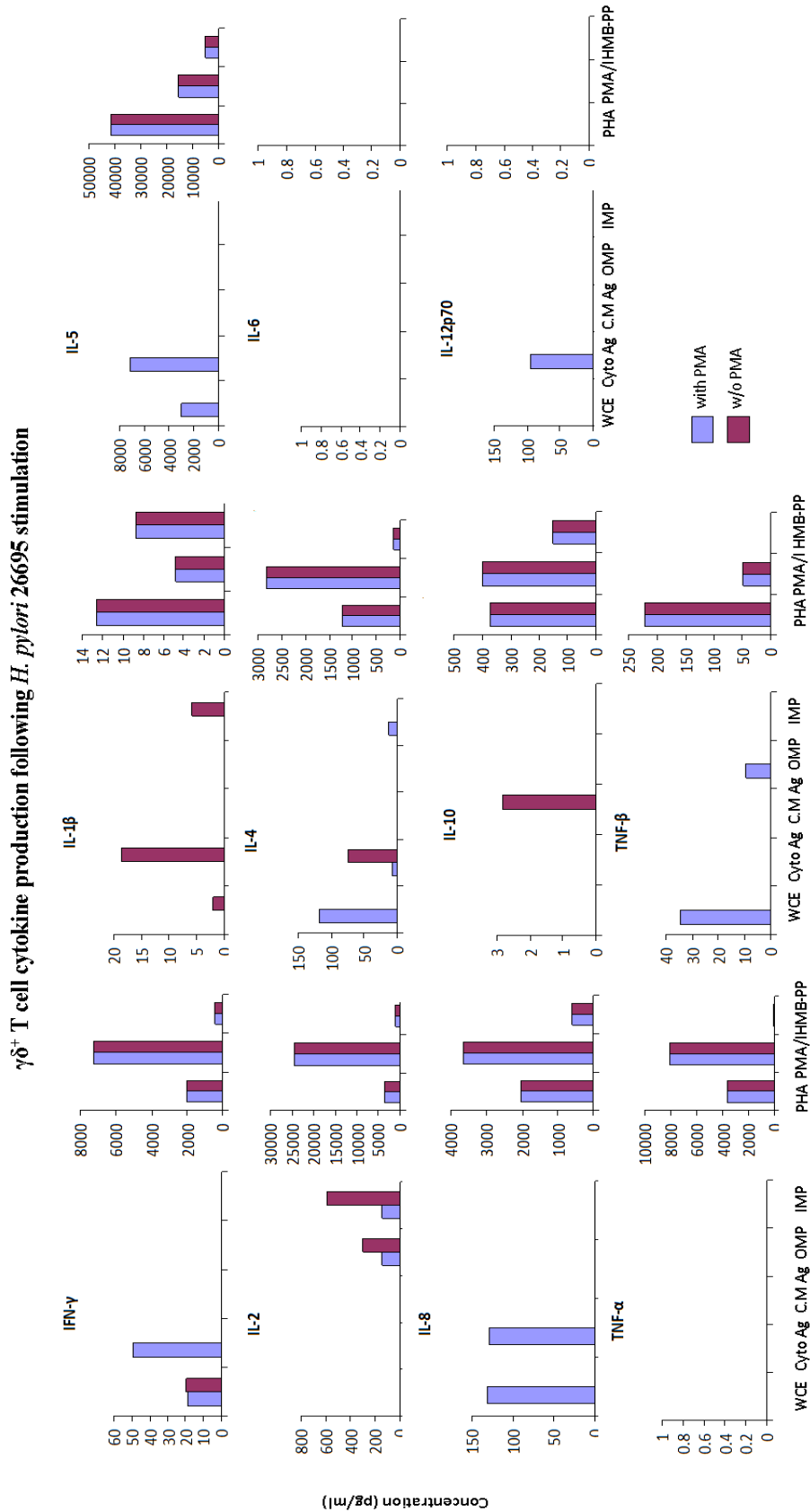


Fig. 4.14 Bar charts showing $\gamma\delta^+$ T cell cytokine production following stimulation with Ags derived from *H. pylori* 26695. Data shows values of cytokine increases beyond unstimulated control wells (n = 1).

Table 4.4 Summary of cytokine production by $\gamma\delta^+$ T cells following stimulation with Ags derived from *H. pylori* 26695

	WCE wPMA	WCE w/o PMA	Cyto Ags wPMA	Cyto Ags w/o PMA	C.M Ag wPMA	C.M Ag w/o PMA	OMP wPMA	OMP w/o PMA	IMP wPMA	IMP w/o PMA
IL12p70	-	-	+	-	-	-	-	-	-	-
IFN-γ	+	+	+	-	-	-	-	-	-	-
IL-2	-	-	-	-	-	-	+	+	+	+
IL-10	-	-	-	-	-	-	-	-	-	-
IL-8	+	-	+	-	-	-	-	-	-	-
IL-6	-	-	-	-	-	-	-	-	-	-
IL-4	+	-	+	+	-	-	-	-	+	-
IL-5	+	-	+	-	+	-	-	-	-	-
IL-1β	-	+	-	+	-	-	-	-	-	+
TNF-α	-	-	-	-	-	-	-	-	-	-
TNF-β	+	-	-	-	-	-	+	-	-	-

WCE = whole cell extract, Cyto Ags = cytoplasmic antigens; C.M Ag = crude membrane antigens, OMP = outer membrane proteins; IMP = inner membrane proteins; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor; wPMA = with the addition of PMA to cultures; w/o PMA = without the addition of PMA in cultures. A + sign indicates cytokine production by NKT cells beyond that produced in control wells. A - sign indicates no significant cytokine production.

B) Cytokine production after $\gamma\delta^+$ T cell stimulation with *H. pylori* J99

IL-12p70: As seen in Fig. 4.15, to begin with, all three positive controls (PHA, PMA/I, HMB-PP) failed to induce IL-12p70 production by $\gamma\delta^+$ T cells. Secondly, when $\gamma\delta^+$ T cells were cultured with the C.M Ag (1290 pg/ml) in the presence of PMA, an increase in IL-12p70 was observed beyond levels in unstimulated control wells. All other Ags (Cyto Ags, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-12p70. Finally, in the absence PMA, when $\gamma\delta^+$ T cells were cultured with the WCE and IMP, an increase in IL-12p70 production was seen (6724 pg/ml and 748 pg/ml respectively). All other Ags (Cyto Ags, C. M Ag and OMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-12p70.

IFN- γ : Firstly, all three positive controls induced IFN- γ production by $\gamma\delta^+$ T cells (PHA, 2010 pg/ml; PMA/I, 7255 pg/ml; HMB-PP, 431 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the Cyto Ags (31 pg/ml), C.M Ag (67 pg/ml) and OMP (16 pg/ml) in the presence of PMA, an increase in IFN- γ was observed beyond levels in

unstimulated control wells. All other Ags (WCE, IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond unstimulated controls. Finally, when $\gamma\delta^+$ T cells were cultured with the Cyto Ags (14 pg/ml) and OMP (36 pg/ml) bacterial fractions in the absence of PMA, an increase in IFN- γ was found beyond levels in unstimulated control wells. All other Ags (WCE, C.M Ag, IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond unstimulated controls.

IL-2: Firstly, all three positive controls induced IL-2 production by $\gamma\delta^+$ T cells (PHA, 3573 pg/ml; PMA/I, 24414 pg/ml; HMB-PP, 1138 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the C.M Ag (242 pg/ml) and OMP (64 pg/ml) Ags in the presence of PMA, an increase in IFN- γ was observed beyond levels in unstimulated control wells. All other Ags (WCE, Cyto Ags, IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IFN- γ beyond unstimulated controls. Finally, when $\gamma\delta^+$ T cells were cultured with the WCE (193 pg/ml), Cyto Ags (163 pg/ml) C.M Ag (96 pg/ml) and OMP (183 pg/ml) in the absence of PMA, an increase in IL-2 production was recorded. This was in contrast to the IMP which failed to induce IL-2 production by $\gamma\delta^+$ T cells.

IL-10: To begin with, all three positive controls induced IL-10 production by $\gamma\delta^+$ T cells (PHA, 373 pg/ml; PMA/I, 401 pg/ml; HMB-PP, 152 pg/ml). However, in both the presence and absence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- α beyond unstimulated controls.

IL-8: Firstly, all three positive controls induced IL-8 production by $\gamma\delta^+$ T cells (PHA, 2053 pg/ml; PMA/I, 3658 pg/ml; HMB-PP, 597 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the C.M Ag (137 pg/ml) and OMP (97 pg/ml) in the presence of PMA, an increase IL-8 production was recorded. All other Ags (WCE, Cyto Ags and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond unstimulated controls.

Finally, in the absence of PMA, all Ags (WCE, Cyto Ags, C. M Ag, OMP, and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-8 beyond unstimulated controls.

IL-6: Neither control nor test samples albeit in the presence nor absence of PHA, induced IL-6 production by $\gamma\delta^+$ T cells after stimulation with *H. pylori* 26695 (PHA, PMA/I, HMB-PP, Unstim, WCE, Cyto An, C. M An, OMP, IMP).

IL-4: Firstly, all three positive controls induced IL-4 production by $\gamma\delta^+$ T cells (PHA, 1234 pg/ml; PMA/I, 2814 pg/ml; HMB-PP, 139 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the Cyto Ags (83 pg/ml), C.M Ag (78 pg/ml) and OMP (54 pg/ml) in the presence of PMA, an increase in IL-4 production was recorded. All other Ags (WCE and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-4 beyond unstimulated controls. Finally, in the absence of PMA, when $\gamma\delta^+$ T cells were cultured with only the WCE (29 pg/ml), an increase in IL-4 production was seen. All other Ags (Cyto Ags, C.M Ag, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-4 beyond unstimulated controls.

IL-5: Firstly, all three positive controls induced IL-5 production by $\gamma\delta^+$ T cells (PHA, 41591 pg/ml; PMA/I, 15550 pg/ml; HMB-PP, 5188 pg/ml). Secondly, when $\gamma\delta^+$ T cells were cultured with the C.M Ag (1874 pg/ml) and OMP (2289 pg/ml) in the presence of PMA, an increase in IL-5 production was seen. All other Ags (WCE, Cyto Ags and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-5 beyond unstimulated controls. Finally, in the absence of PMA, all Ags (WCE, Cyto Ags, C.M Ag, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-5 beyond unstimulated controls.

IL-1 β : Firstly, all three positive controls induced IL-1 β production by $\gamma\delta^+$ T cells (PHA, 12.6 pg/ml; PMA/I, 4.8 pg/ml; HMB-PP, 8.8 pg/ml). Secondly, in the presence of PMA, all Ags (WCE, Cyto Ags, C.M Ag, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-1 β beyond unstimulated controls. Finally, in the

absence of PMA, when $\gamma\delta^+$ T cells were cultured with the WCE (2 pg/ml), Cyto Ags (18.6 pg/ml) and IMP (5.9 pg/ml) an increase in IL-1 β production was seen. All other Ags (C.M Ag and OMP) failed to stimulate $\gamma\delta^+$ T cells to produce IL-5 beyond unstimulated controls.

TNF- α : Firstly, all three positive controls induced TNF- α production by $\gamma\delta^+$ T cells (PHA, 3566 pg/ml; PMA/I, 8095 pg/ml; HMB-PP, 63 pg/ml). Secondly, in the presence of PMA, all Ags (WCE, Cyto Ags, C.M Ag, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- α beyond unstimulated controls. Finally, when $\gamma\delta^+$ T cells were cultured with the Cyto Ags (31 pg/ml) in the absence of PMA, an increase in TNF- α production was observed. All other Ags (WCE, C.M Ag OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- α beyond unstimulated controls.

TNF- β : Two of three positive controls (PHA, 222 pg/ml; PMA/I, 49.2 pg/ml) induced TNF- α production by $\gamma\delta^+$ T cells. This was in contrast to HMB-PP which failed to induce TNF- β by $\gamma\delta^+$ T cells. Secondly, when $\gamma\delta^+$ T cells were cultured with the C.M Ag (9.6 pg/ml) in the presence of PMA, an increase in TNF- β was found. All other Ags (WCE, Cyto Ags, OMP and IMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- β beyond unstimulated controls. Finally, in the absence of PMA, when $\gamma\delta^+$ T cells were cultured with the IMP (18 pg/ml), an increase in TNF- β production was seen. All other Ags (WCE, Cyto Ags, C.M Ag and OMP) failed to stimulate $\gamma\delta^+$ T cells to produce TNF- β beyond unstimulated controls. Table 4.5 summarises the cytokines produced by $\gamma\delta^+$ T cells upon stimulation with Ags derived from *H. pylori* J99. As shown, in the presence of PMA, the WCE did not induce any cytokine production; the Cyto Ags induced production of IL-12p70, IFN- γ and IL-4; the C.M Ag induced IFN- γ , IL-2, IL-4, IL-5, IL-8 and TNF- β production; OMP induced IFN- γ , IL-2, IL-4, IL-5 and IL-8 cytokine production while the IMP induced the production of IL-12p70, IL-1 β and

TNF- β by $\gamma\delta^+$ T cells. In the absence of PMA, the WCE, IL-12p70, IL-2, IL-4 and IL-1 β ; the Cyto Ags induced production of IFN- γ , IL-2 IL-1 β and TNF- α ; C.M Ag induced IFN- γ and IL-2 cytokine production; OMP induced IL-2 production only while IMP induced IL-12p70, IL-1 β and TNF- β production by $\gamma\delta^+$ T cells. It is worth noting that the addition of PMA to cultures did not enhance the production of any cytokine.

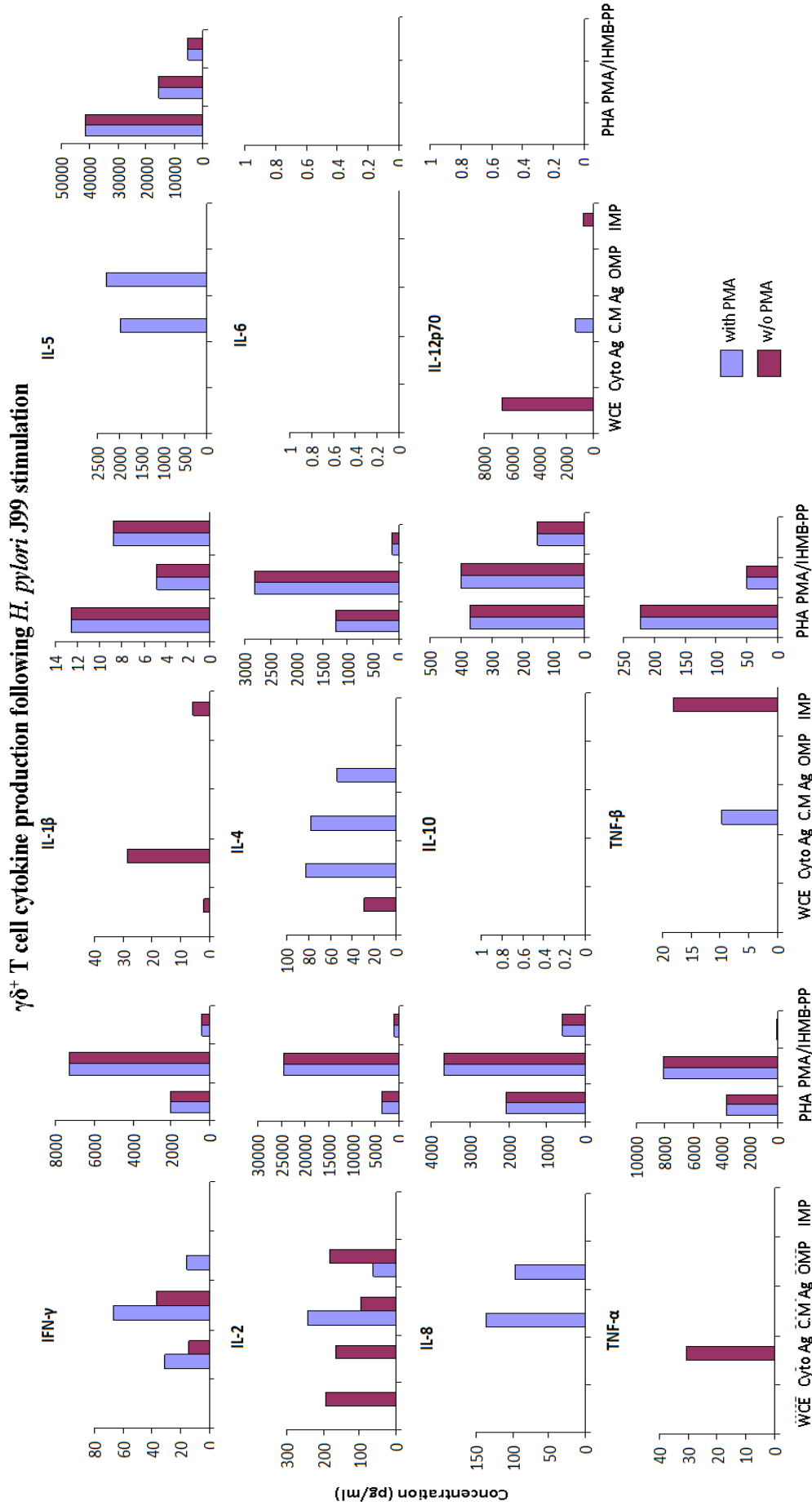


Fig. 4.15 Bar charts showing $\gamma\delta^+$ T cell cytokine production following stimulation with Ags derived from *H. pylori* J99. Data shows cytokine increases beyond levels in unstimulated control wells (n = 1).

Table 4.5 Summary of cytokine production by $\gamma\delta^+$ T cells following stimulation with Ags derived from *H. pylori* J99

	WCE wPMA	WCE w/o PMA	Cyto Ags wPMA	Cyto Ags w/o PMA	C.M Ag wPMA	C.M Ag w/o PMA	OMP wPMA	OMP w/o PMA	IMP wPMA	IMP w/o PMA
IL12p70	-	+	-	-	+	-	-	-	-	+
IFN-γ	-	-	+	+	+	+	+	-	-	-
IL-2	-	+	-	+	+	+	+	+	-	-
IL-10	-	-	-	-	-	-	-	-	-	-
IL-8	-	-	-	-	+	-	+	-	-	-
IL-6	-	-	-	-	-	-	-	-	-	-
IL-4	-	+	+	-	+	-	+	-	-	-
IL-5	-	-	-	-	+	-	+	-	-	-
IL-1β	-	+	-	+	-	-	-	-	-	+
TNF-α	-	-	-	+	-	-	-	-	-	-
TNF-β	-	-	-	-	+	-	-	-	-	+

WCE = whole cell extract, Cyto Ags = cytoplasmic antigens; C.M Ag = crude membrane antigens, OMP = outer membrane proteins; IMP = inner membrane proteins; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor; wPMA = with the addition of PMA to cultures; w/o PMA = without the addition of PMA in cultures. A + sign indicates cytokine production by NKT cells beyond that produced in control wells. A – sign indicates no significant cytokine production.

4.2.2.5 Serum analysis of cytokines in *H. pylori* positive and *H. pylori* negative patients.

Since *H. pylori* is known to induce both localised and systemic immune responses, a cytokine profile of the range of cytokines secreted into serum of both healthy (n =11) and *H. pylori* infected (n =11) patients was undertaken and differences in cytokine pattern in disease and non-disease states was compared using T_h1/T_h2 11plex FlowCytomix kit. As shown in Fig. 4.16, increases, no significant differences were detected in IL-12p70, IFN- γ , IL-10, IL-8, IL-6, IL-4, IL-5 and IL-1 β cytokine levels in serum from *H. pylori* infected individuals when compared with that of uninfected individuals. While 4 cytokines were decreased during *H. pylori* infection (**IL-2**: median = 44.26 pg/ml, mean = 43.26 \pm 11.01 pg/ml vs. 50.47 pg/ml, mean = 62.18 \pm 41.08 pg/ml ;*p* = 0.21; **TNF- α** : median = 0, mean = 10.62 \pm 13.41 pg/ml vs. median = 0, mean = 19.72 \pm 41.52 pg/ml ;*p* = 0.23; **TNF- β** : median = 0, mean = 18.78 \pm 32.6 pg/ml vs. median = 0, mean = 56.71 \pm 114.7 pg/ml ;*p* = 0.1; **IL-13**: median = 0, mean = 15 \pm

35.56 pg/ml vs. median = 0, mean = 50.66 ± 76.18 pg/ml ; $p = 0.3$) the difference compared with healthy controls was not significant. IL-6 did not yield any cytokine production (**IL-6**: median = 0, mean = 0). Large patient to patient variability was seen in levels of cytokine production in both *H. pylori* positive and *H. pylori* negative individuals. To summarise, no significant differences were seen amongst cytokines in the serum of *H. pylori* infected compared with uninfected individuals.

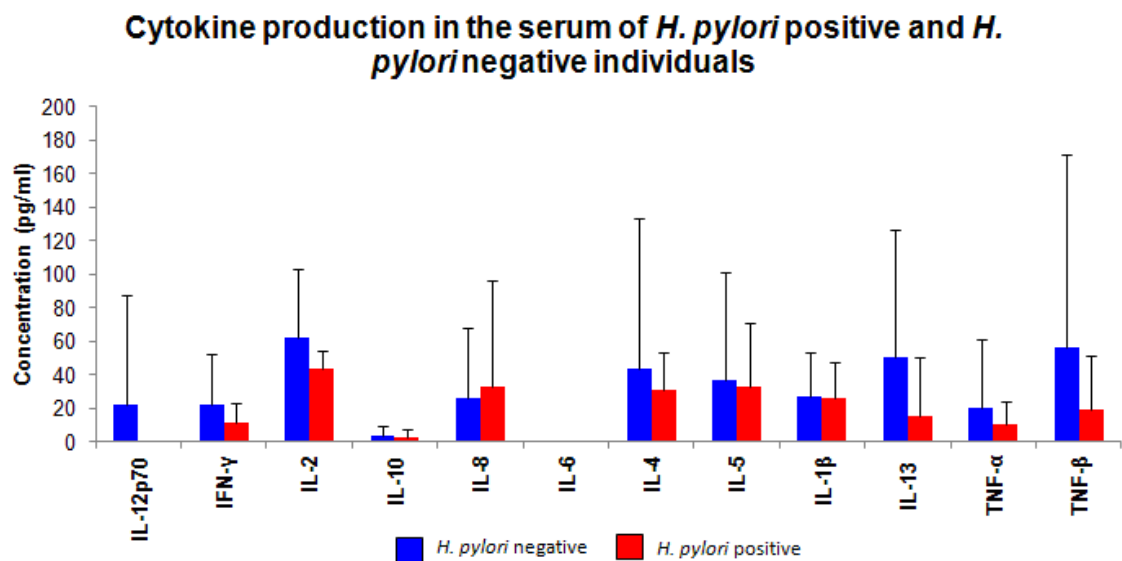


Fig. 4.16 Differences seen systemic immune responses in sera from *H. pylori* positive and *H. pylori* negative patients. Bar chart showing differences in cytokine production observed in serum of *H. pylori* positive (n = 11) and negative (n = 11) patients. Data shows mean values and the Y error bars indicate SD.

4.2.3 Cytotoxic immune responses using LDH assay

4.2.3.1 Analysing cell lysis in human NKT cell clones following stimulation with *H. pylori* derived Ags

NKT cells have been shown to respond quickly to foreign substances by carrying out immunosurveillance and killing of pathogenic invaders, possess potent anti-tumor properties and are now known to be activated by a small number of bacterial glycolipids (Matsuda *et al.*, 2008). To determine the cytotoxic activity of effector NKT cells to *H. pylori*, NKT cell clones (effector cells) were stimulated with a range of *H. pylori* Ags in

the presence of target Caco2 cells for 24 h and cell lysis of Caco2 cells was measured using LDH assay (n = 3). This was carried out in triplicate. As shown in Fig. 4.17, results showed a lack of LDH activity following *H. pylori* Ag stimulation as no changes were observed [control (no NKT cells) = $43 \pm 3.75\%$; α -GalCer = $38 \pm 3.85\%$; *H. pylori* 11637 = $38 \pm 3.12\%$; *H. pylori* 17874 = $39 \pm 6.74\%$; WCE = $42 \pm 2.1\%$; Cyto Ags = $38 \pm 4.72\%$; C.M. Ags = $37 \pm 3.75\%$; OMP = $36 \pm 5.1\%$; IMP = $40 \pm 5.58\%$].

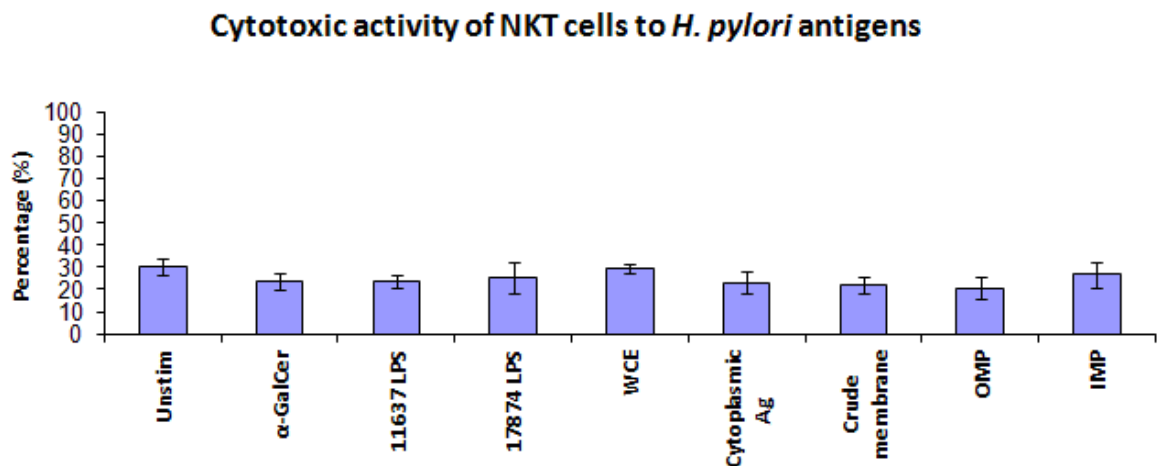


Fig. 4.17 Bar chart displaying lack of LDH activity by NKT cell clones following 24 h stimulation with *H. pylori* Ags (n = 3). In most cases, cells cultured with media alone had higher LDH levels than when co-cultured with glycolipid fractions. Bars represent mean values for 3 experiment. Y error bars indicate SD and each experiment was carried out in triplicate.

4.2.4 CD1 expression in normal gastric mucosa

CD1 molecules are a family of glycoproteins expressed on white blood cells that present lipid Ags to T cells (Dascher, 2007). Humans possess five isoforms (CD1a-e) four of which present Ags to T cells (CD1a-d) (Calabi *et al.*, 1989). CD1c has been shown to present Ags to $\gamma\delta^+$ T cells (Spada *et al.*, 2000) while CD1d presents glycolipids to NKT cells (see section 1.4.1.3). CD1 expression has already been characterised in normal and tumor bearing liver (Kenna *et al.*, 2007). Moreover, changes in CD1 mRNA expression were observed in disease state. This is significant as the liver is an organ where NKT cells are present in abundance (1%) and exert protective effects (Kenna *et al.*, 2007).

Regarding the GIT, to date, CD1 expression has been investigated in murine GIT (Bleicher *et al.*, 1990) and in human intestinal epithelium (Blumberg *et al.*, 1991). CD1 expression has not previously been determined in the human gastric mucosa. Here, RT-PCR was carried out on RNA from normal *H. pylori* negative mucosa with mild gastritis to explore CD1a, CD1b, CD1c and CD1d expression in the gastric mucosa of *H. pylori* negative individuals. The CD1a, CD1b, CD1c and CD1d primers are known to produce products of 530, 528, 529 and 535 bp, respectively (MWG). As a control, PCR was also carried out using primers specific for the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH). A molecular weight HyperLadder I (200 – 10037 bp) (Bioline, MyBio Ltd, Republic of Ireland) was loaded into lane 1. As shown in Fig. 4.18, the presence of GAPDH was observed in both epithelial and lamina propria layers of normal gastric mucosa. The presence of CD1c was recorded in the gastric lamina propria layer only and CD1d mRNA was seen the gastric epithelium while a faint indication of CD1d also noted in the lamina propria layer of one patient analysed. CD1a and CD1b were completely absent from gastric tissues (n = 1).

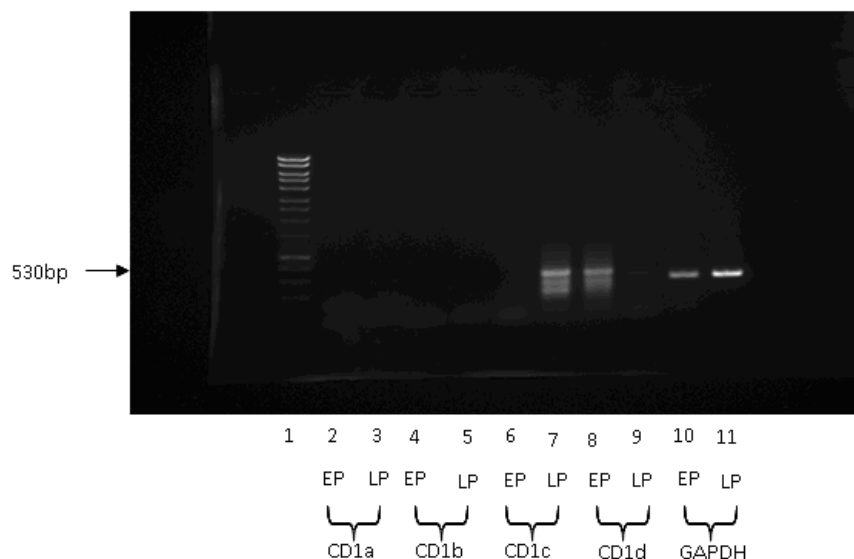


Fig. 4.18 Agarose gel showing RT-PCR of CD1 expression in the epithelial (EP) and lamina propria (LP) layer of gastric mucosa. Lane 1: molecular weight marker (hyperladder 1, bioline), lanes 2&3: CD1a in EP and LP, lanes 4&5: CD1b in EP and LP, lanes 6&7: CD1c in EP and LP, lanes 8&9 in EP and LP: CD1d, lanes 10&11: GAPDH in EP and LP.

4.3 DISCUSSION

H. pylori infection induces both local and systemic responses, firstly with an acute inflammatory response involving interaction of immune cells such as epithelial cells, DC's and macrophages with PAMPs (Robinson *et al.*, 2007; Sobala *et al.*, 1991). This interaction triggers the adaptive humoral and cellular immune responses, the cellular immune response is predominately T_h1 driven leading to the production of a range of cytokines and chemokines to regulate other immune cells (Pinto-Santini & Salama, 2005). However, this action rarely successfully eliminates infection and can actually contribute to *H. pylori* pathogenesis (D'Elis *et al.*, 1997b). A systemic response also occurs which elicits the production of anti-*H. pylori* Abs in the periphery. IgG and IgA Abs are not protective but instead are a good indicator of chronic *H. pylori* infection in gastric tissues (Andersen *et al.*, 1996; Rathbone *et al.*, 1986). Analysing the host's immune responses to *H. pylori* infection is important to control and eliminate infection. Therefore, key immunological responses such as proliferation, cytotoxicity and cytokine profiles from host immune cells are of interest here to provide an understanding of what is happening during *H. pylori* infection.

Proliferative responses of host immune cells were firstly tested as a marker of T cell activation following stimulation with a range of *H. pylori* derived Ags including WCE, Cyto Ags, C.M. Ags, OMP, IMP and LPS preparations. Results showed a lack of functional response of PBMCs from HS towards *H. pylori* LPS strains tested, while PBMCs did proliferate in response to the positive control PHA. Therefore, maybe these particular *H. pylori* LPS strains tested (NCTC11637 and CCUG17874) do not activate lymphocytes.

With the gastric lymphocyte populations derived epithelial and lamina propria layers, again no proliferative responses were seen following *H. pylori* LPS stimulation

for 7, 5, 4, 3, or 2 days albeit in the epithelium or the lamina propria, while control stimuli induced proliferation of lamina propria cells after 4 and 3 days only. Clinically, of the 12 gastric samples tested, 11 were deemed *H. pylori* negative possessing minimal inflammatory conditions and 1 sample was *H. pylori* positive as determined by the CLO test. The gastric epithelial layer lymphocytes may have died before culturing with bacterial Ags took place or alternatively could be refractory *in vitro*. Or indeed it is very possible that the *H. pylori* fractions tested on PBMC and gastric cultures possess low immunogenicity as *H. pylori* LPS is known for its weak endotoxic activity (Muotiala *et al.*, 1992)? Miller *et al.* (1978) found that *E. coli* and *S. typhimurium* LPS induced immune cell activation but only non-T cells were stimulated by LPS, while for optimum stimulation, helper T cells were needed. Other studies demonstrated the need for monocytes for T cell activation with LPS to occur and this action is MHC-unrestricted (Mattern *et al.*, 1994; Mattern *et al.*, 1998). Human peripheral blood T cells secreted IFN- γ after *E. coli* LPS stimulation when primed with IL-12 (Motegi *et al.*, 2006). Moreover, Rudnicka *et al.*, (2009) showed *H. pylori* LPS was a poor activator of mononuclear cells as proliferation was induced in only 3/10 and 2/11 of *H. pylori* positive and *H. pylori* negatively patients respectively after stimulation with *H. pylori* LPS. In fact, natural proliferation was hindered in cultures where *H. pylori* LPS was present which correlates with our data.

It is also worth noting that B7 molecules are involved in immune signalling by controlling stimulating and inhibiting T cell activation (Coyle & Gutierrez-Ramos, 2001; McAdam *et al.*, 1998). B7-H1, a member of this B7 family, down-regulates T cell activation (Dong *et al.*, 1999). Moreover, Das *et al.*, (2006) discovered gastric IELs expressed low levels of B7-H1, which were augmented following stimulation with *H. pylori*. B7-H1 inhibited proliferation of IELs during *H. pylori* infection and this action

was cancelled by blocking B7-H1. This could also explain the lack of gastric proliferative responses observed.

When proliferative responses of NKT cells were tested after *H. pylori* Ag stimulation, once again, a lack of proliferative responses of this unconventional T cell population to all Ags (WCE, Cyto Ags, C.M Ag, OMP and IMP) was observed after 24 h and 48 h. Taken together, our results indicate a lack of T cell proliferation by peripheral blood lymphocytes, gastric epithelial and lamina propria layer lymphocytes and NKT cells to *H. pylori*. *H. pylori* could be inducing immunosuppressive responses for persistence in gastric tissues. To support this, Hybenova *et al.*, (2010) analysed the proliferative response of lymphocytes from patients with *H. pylori*, patients without *H. pylori* and patients with *H. pylori* who also suffered from autoimmune thyroiditis (AT) as an association of this disease with *H. pylori* infection has been shown. Proliferation responses were compared both before and after *H. pylori* eradication treatment and concluded *H. pylori* infection resulted in a reduction in proliferative responses regardless of whether patients additionally suffered from AT, and once *H. pylori* bacteria was eradicated this decrease in proliferation was no longer seen (Hybenova *et al.*, 2010). In another paper, Grebowska *et al.*, (2010) analysed the proliferative responses of total peripheral blood mononuclear leukocytes (tPBML) following stimulation with *E. coli* and *H. pylori* LPS. Proliferative responses were undertaken firstly in a single stage culture where cells and LPS were co-cultured for 5 days before proliferation was measured and secondly in a two stage culture where macrophages were first isolated and matured for 5 days from peripheral blood of donors taking part in the study. Next mature macrophages were pulsed with bacterial LPS for 24 h. Finally, stimulated macrophages were co-cultured along with tPBML from the same donors and LPS for 5 days and proliferation was subsequently measured. Results showed that 5 of

21 healthy subjects in the single stage culture proliferated in response to LPS stimulation while the remaining individuals displayed a significant reduction in cell proliferation thought to be as a result of a decrease in the tPBMLs metabolic rate. Even in the two stage culture, the individuals that did not respond to initial LPS stimulation also showed a reduction in proliferative responses which is consistent with our results.

After proliferative responses of immune cells were tested, cytokine responses of host cells to *H. pylori* Ags were investigated next. Firstly, in PBMCs, increases in cytokine production beyond levels in control wells were observed after 24, 48 and 72 h stimulation of HS with *H. pylori* NCTC11637 LPS at a range of concentrations (1 ng-10 μ g). While NCTC11637 LPS induced IL-4, IL-5, IL-10, IL-1 β , TNF- α and TNF- β by PBMCs after 72 h stimulation, there were no distinct patterns of cytokine production observed at any time point. In gastric tissues, increases in epithelial layer cytokines IL-1 β , IL-2, IL-8, IL-18, TNF- α and IFN- γ have already been observed in *H. pylori* infection (Shimada *et al.*, 2008). When localised cytokine responses were examined after gastric epithelial and lamina propria layer stimulation with *H. pylori* LPS in culture, IL-2 production only was observed by epithelial and lamina propria layer cells after 72 h with *H. pylori* LPS from strains CCUG17874 and NCTC11637 respectively, are we observing immunosuppressive responses here too as no other distinct pattern of cytokine production was observed? Since *H. pylori* LPS demonstrates low biological activity (Muotiala *et al.*, 1992), it is also possible these particular strains do not induce host epithelial layer cytokine production or indeed that it is in fact a different component of *H. pylori* bacteria other than LPS triggering gastric epithelial and lamina propria layer immune responses. *H. pylori* NCTC11637 LPS is a rough LPS (see section 4.1.2.4), therefore containing a truncated O-specific chain. Could the structure of this particular LPS strain be a factor contributing to the lack of responses seen? To support

this, D'Elcios *et al.*, (2003) tested the antigenic specificity of gastric T cell clones by analysing immune responses to *H. pylori* derived Ags including a crude preparation of NCTC11637, CagA, VacA, urease and heat shock proteins. The authors reported a percentage (2-36) of CD4⁺ T cells and not CD8⁺ T cells proliferated in response to NCTC11637 lysate and concluded that while different *H. pylori* Ags are capable of inducing an immune response, CagA seems to be the most potent Ag for *H. pylori* mediated T cell responses in peptic ulcer cases (D'Elcios *et al.*, 2003).

Upon NKT cell stimulation with a variety of *H. pylori* derived bacterial Ags (WCE, Cyto Ags, C.M Ag, OMP and IMP from *H. pylori* 26695 and J99 in addition to LPS derived from *H. pylori* strains NCTC11637 and CCUG17874) small levels of cytokine production was observed beyond that in control wells. Ags from all *H. pylori* strains tested (26695, J99, NCTC11637 and CCUG17874) did induce small levels of IFN- γ and IL-8 production by NKT cells. We also observed small quantities of, IL-2, IL-4 and IL-5 after NKT cells were stimulated with several Ags derived from *H. pylori* strains 26695 in the presence of CD1d transfectants while CCUG17874 additionally induced small levels of IL-5 beyond those seen in control wells. However, none of these findings seem particularly significant as the levels of cytokine production were far lower than those recorded following NKT cell stimulation with α -GalCer; therefore while we show that Ags derived from strains 26695, J99, NCTC11637 and CCUG17874 are all capable of inducing an immune response, we can assume these particular *H. pylori* strains tested must not be natural ligands for NKT cell activation. NKT cells have however been shown to respond/ recognise a small number of other bacterial glycolipids such as *B. burgdorferi* and *Sphingomonas spp.* *B. burgdorferi*, the causative agent of Lyme disease possesses glycosylated diacylglycerols with one α -linked galactose sugar similar to α -GalCer that stimulates both mice and human NKT

cells (Kinjo & Ueno, 2011). *Sphingomonas* are gram negative bacteria that harbour glycosphingolipids with α -linked sugars which is similar to the structure of α -GalCer and therefore these sugars are capable of activating both mice and human NKT cells (Kinjo *et al.*, 2005). Kinjo *et al.*, (2011) believed that due to the high degree of conservation of Ag recognition by NKT cells, that invariant NKT cells must be involved in the immune response against severely pathogenic microbes. The authors discovered NKT cells recognise glycolipids from *Streptococcus pneumoniae* and group B *Streptococcus* (Kinjo *et al.*, 2011). Chang *et al.*, (2011) recently uncovered NKT cells recognise and are activated by a glycolipid derived from *H. pylori* called P157. P157 is a cholesterol derived lipid was discovered to trigger activation of NKT cells when presented by CD1d to produce IFN- γ when co-cultured with DCs. Both human and mouse NKT cells from NKT cell lines were activated by P157. This breakthrough was discovered when the authors were investigating the significance of viral respiratory infections in children in the onset of asthma. To do this, the authors infected suckling mice with H3N1, the influenza A virus and later analysed their susceptibility to allergen-induced airway hyperreactivity (AHR) in adulthood which was measured by the activation and expansion of NKT cells and found that H3N1 induces NKT cell expansion resulting in protection against AHR in adulthood. After infecting suckling mice with a number of other glycolipids, they discovered P157 Ag derived from *H. pylori* also activated NKT cells (Chang *et al.*, 2011). Therefore, *H. pylori* is capable of potently activating NKT cells and therefore it would be interesting to continue testing the stimulatory capacity of a variety of virulence factors from a range of *H. pylori* strains to further analyse the role of NKT cells in *H. pylori* infection.

As stated earlier, one of the best-documented glycolipids shown to stimulate and activate NKT cells is a marine sponge-derived glycolipid α -GalCer (see section 1.4.1.3).

α -GalCer has a unique structure with the sugar head attachment to the ceramide backbone by an α -glycosidic link. The α -glycosidic link is notable as higher organisms far more frequently have β -glycosidic links (Savage *et al.*, 2006). For NKT cell activation to occur, the C26 acyl chain of α -GalCer binds to A' while the sphingosine base is embedded in F' of CD1d binding groove with high affinity (Koch *et al.*, 2005). Since, α -GalCer, unlike the majority of other glycosphingolipids possesses α -anomeric linkage of sugar to lipid, determining the structure of natural ligand for NKT cell activation will prove to be a major step in NKT cell biology.

Next, when $\gamma\delta^+$ T cell clones were stimulated with *H. pylori* fractions, our results showed IFN- γ , IL-2, IL-4, IL-5, IL-8, IL-10, IL-1 β and TNF- β production after stimulation of this unconventional T cell population with Ags derived from *H. pylori* 26695. Notably, the WCE and Cyto Ags each induced activation of 6 cytokines by $\gamma\delta^+$ T cells. The C.M Ag induced IL-10 only while OMPs induced the production of IL-2 and TNF- β . Finally, IMP Ags induced IL-2, IL-4 and IL-1 β production. In addition, when $\gamma\delta^+$ T cells were stimulated with J99, IFN- γ , IL-2, IL-4, IL-5, IL-8, IL-12p70, IL-1 β TNF- α and TNF- β production was observed. Interestingly, the C.M Ag induced the production of 7 different cytokines by $\gamma\delta^+$ T cells while the OMP and Cyto Ags both activated $\gamma\delta^+$ T cells to secrete 5 cytokines. The WCE induced the production of IL-2, IL-4, IL-1 β and IL-12p70 while the IMP induced IL-12p70, IL-1 β and TNF- β secretion by $\gamma\delta^+$ T cells. It is important to note that the levels of cytokine production by $\gamma\delta^+$ T cells beyond unstimulated controls following stimulation with *H. pylori* derived Ags were far higher than those seen when NKT cells were stimulated with the same Ags. Are $\gamma\delta^+$ T cells more important than NKT cells in immune response to *H. pylori*? It would seem fitting since $\gamma\delta^+$ T cells primarily reside in epithelial tissues and play key roles in both innate and adaptive immunity. This importance $\gamma\delta^+$ T cells has already

been shown in mucosal immune responses (Inagaki-Ohara *et al.*, 2011; Komori *et al.*, 2006; Nilssen & Brandtzaeg, 2012) and in mice models of bacterial infections (Carding & Egan, 2002) and therefore following observation of cytokine responses to *H. pylori* antigenic stimulation, it is possible that $\gamma\delta^+$ T cells have an important role to play in *H. pylori* infection. Recently, live *H. pylori* bacteria from strain G27 activates purified CD3⁺ T cells and in particular $\gamma\delta^+$ T cells *in vitro* to induce cytokine and chemokine production in addition to up-regulating CD69 expression thus promoting an inflammatory response which may favour the bacteria to persist and cause a chronic disease state (Romi *et al.*, 2011). Therefore, it is likely that $\gamma\delta^+$ T cells have a role to play in *H. pylori* infection. Further experimental investigation will reveal more.

Finally, we stimulated NKT cell clones with *H. pylori* derived Ags and compared cytotoxicity levels with unstimulated controls. We observed no increases in LDH activity beyond control wells following stimulation of NKT cells with *H. pylori* derived Ags. Even though NKT cells are unconventional T cells and possess NK receptors, known for their MHC-unrestricted killing of bacterial or viral infected cells (Bluestone *et al.*, 2009; Lanier, 1998) and tumour cells, there is no increase in LDH activity following NKT cell stimulation with *H. pylori* derived Ags. This lack of cytotoxic activity could be explained by the fact that *H. pylori* has been shown to suppress lymphocyte activity. To support this theory, Rudnicka *et al.*, (2009) analysed functional responses of lymphocytes stimulated with *H. pylori* LPS and discovered that lymphocytes isolated from *H. pylori* positive (50%) patients, after stimulation with *H. pylori* LPS, exerted lower cytotoxicity levels than *H. pylori* negative (79%) patients. The authors recorded lower degree of cytotoxicity in lymphocytes from *H. pylori* positive patients than from *H. pylori* negative patients suggesting this may be as a result of fewer NK cells present in *H. pylori* positive patients. The same group also discovered

that *H. pylori* LPS actually hindered PBMCs cytotoxic effects, as decreases in IFN- γ , IL-2 and IL-10 were recorded after stimulation with *H. pylori* LPS (Rudnicka *et al.*, 2012). This action could be a mechanism of bacterial survival and persistence in the gastric mucosa. Taken together, other studies support that of our findings as the *H. pylori* derived Ags tested induced a negative response from host immune cells known for their potent killing effects.

Since, *H. pylori* is known to stimulate both local and systemic immune responses, serum was obtained from *H. pylori* positive and *H. pylori* negative individuals and compared the patterns of cytokine production in both, thus giving an insight into responses in the periphery. Decreases in IL-13, IL-2, IL-4, TNF- α and TNF- β were seen during infection. Therefore, both pro- and anti-inflammatory cytokines are differing between *H. pylori* disease and non-disease states, although these values did not reach significance as large patient to patient variability was seen. It could be that while T_h1 lymphocytes are recruited to gastric mucosa, the T_h2 producing cells remain at circulatory level or do the circulating blood lymphocytes recognises the stress that gastric lymphocytes are under and attempt to control inflammation. Or is the clinical pathology a major factor here that should not be overlooked in that only severe cases of *H. pylori* infection will document changes in cytokine production in serum? Of the 10 *H. pylori* infected patients studied, 7 possessed some form of gastritis, while no patients were implicated with peptic or duodenal ulcers, hence this clinical information may be relevant in explaining the lack of cytokine production in the periphery. To support these findings, in other studies, Bayraktaroğlu *et al.*, (2004) believe that IL-8, IL-6 and TNF- α levels in serum are not related to or affecting cytokine production in the gastric mucosa but is this also the case for all cytokines? Jafarzadeh & Sajjedi, (2006) have revealed changes in IL-18 levels in serum have been seen in *H. pylori* individuals suffering from

peptic ulcers compared with *H. pylori* patients who are asymptomatic. Moreover, the same group reports that this is due to the presence of *cagA* gene (Jafarzadeh & Sajjadi, 2006). To support this, a recent study by Ortiz-Princz *et al.*, (2011) analysed the cytokine profile from serum of patients infected with CagA⁺ *H. pylori* strains and compared cytokine levels in patients infected with CagA⁻ *H. pylori* strains. The outcome of this study showed significantly higher levels of IFN- γ , TNF and IL-6 in individuals infected with CagA⁺ *H. pylori* strains. The authors hypothesise *H. pylori* is cleverly striking a balance between inducing an immune response but employing mechanisms in order to persist in gastric tissues.

Finally, CD1 expression was explored in the gastric mucosa of *H. pylori* negative individuals. The presence of CD1c was recorded in the gastric lamina propria layer only and CD1d mRNA was seen the gastric epithelium while a faint indication of CD1d also noted in the lamina propria layer. CD1c has been shown to present Ags to $\gamma\delta^+$ T cells (Spada *et al.*, 2000) while CD1d is known to present glycolipids to NKT cells (Brigl & Brenner, 2004), therefore, their presence here in gastric tissues in this preliminary study suggests a role for unconventional T cell populations. For future projections, it would be interesting to obtain RNA from *H. pylori* positive individuals and do a comparative analysis of mRNA expression in *H. pylori* disease and non-disease states from a greater number of patients.

Overall, a lack of responses of PBMCs, gastric epithelial and lamina propria layer cells along with NKT cells dominated when proliferative, cytokine and cytotoxic responses of these immune cell populations to a variety of *H. pylori* Ags were tested. On the other hand, $\gamma\delta^+$ T cells are potentially important in mounting an immune response to *H. pylori*. Given their abundance in mucosal epithelial tissues, their increase in epithelial tissues during *H. pylori* infection as well as their marked cytokine

production shown here in response to stimulation with several *H. pylori* Ags, it would be interesting to test proliferation, specific cytotoxicity markers and other immunological responses of $\gamma\delta^+$ T cells to *H. pylori* and *H. pylori* derived products to reveal the exact role of this unconventional T cell population during *H. pylori* infection.

Chapter 5

Gene Expression Profiling of Human Gastric Mucosal

Derived CD2⁺ Cells in *H. pylori* Infection

5.1 INTRODUCTION

5.1.1 Introduction to microarrays

Microarrays are 2D arrays of microscopic spots of biological material fixed onto a solid support substrate to analyse the presence of target molecules in a biological sample of interest (Cheung *et al.*, 1999). Several types of microarrays are available including protein arrays, carbohydrate arrays, oligonucleotide arrays and DNA microarrays incorporating cDNA and single nucleotide polymorphism arrays. Simultaneous analysis of gene expression levels of thousands of genes can be achieved through the use of DNA microarrays. Gene expression describes the transcription of genetic information in DNA that leads to each cell possessing unique properties therefore displaying different functions. There are several other methods in place for the quantification of gene expression levels. Examples include differential display (De Felice *et al.*, 2011), northern blots (Santos *et al.*, 2011), cDNA library sequencing (Jiang *et al.*, 2011) and serial analysis of gene expression which incorporates cDNA (Song *et al.*, 2011) and oligonucleotide arrays (Meugnier *et al.*, 2011).

A microarray quantifies gene expression by measuring the amount of sample mRNA that hybridizes to a DNA probe. Expression levels are analysed with the aid of high throughput technology thereby constructing a gene expression profile (GEP). GEP enables one to study a large number of genes simultaneously utilizing a small starting sample size ultimately providing us with an insight into what is happening in the cell.

5.1.1.1 Principle of DNA microarrays

To design the array, DNA probes of interest are selected and amplified using PCR. Next a purification step occurs *via* gel filtration or precipitation to ensure removal of detergents, PCR primers and salts among others which may hinder the performance of

the array. Once purified, aliquots of the probes are printed onto a coated glass microscopic slide robotically. This fixation step is usually carried out using ultraviolet radiation. Next, labelling of the target sample occurs. The sample may be DNA, cDNA or mRNA. To analyse gene expression, pure RNA is vital for the hybridization process to occur efficiently. RNA isolated from test samples along with reference samples is reverse transcribed into cDNA with concurrent fluorescent labelling and fragmented for hybridization. Prior to hybridization, the adjusted cRNA yield is calculated for total RNA carryover in the IVT reaction. Hybridization of fluorescently labelled targets to the probes on the array occurs under stringent conditions. A confocal laser microscope is used to measure the degree of hybridization. Excitation occurs when the laser comes into contact with the array and fluorescence of a particular wavelength characteristic of a given fluorescent label is emitted. Information relating to each probe including gene name, intensity values etc is recorded. Finally, data processing and bioinformatics occurs (Duggan *et al.*, 1999; Khan *et al.*, 1999). The genes that are up and down regulated can then be analysed.

5.1.1.2 Applications of DNA microarray technology

There are several useful applications of DNA microarray technology including comparative genome evaluation (Kimmel *et al.*, 2006), identification of alternative splicing whereby arrays are designed to analyse splice sites of potential gene exons and genomic identification; for instance, the identification of oncogenes (McCannel *et al.*, 2010). This application is also useful for detecting the presence of mycoplasma in cell culture or organism spoiled foods. For example, Severgnini *et al.*, (2011) reviewed the recent progress in the field of DNA microarrays regarding the detection of pathogens that spoil food. To date, microarray technology has enabled the foodborne industry to

detect microbial pathogen levels as low as 1 bacterium/g of food. Hence, DNA microarray technology has provided both a sensitive and reliable method of efficiently monitoring the incidence of foodborne related illnesses worldwide. Microarray technology is also a useful tool for identification of single nucleotide polymorphisms, useful in forensic analysis (Amorim, 2011) and identification of specific mutations such as germline mutations which could be passed onto a developing foetus, or somatic mutations in cancer cases (Hacia *et al.*, 1999; Lisovich *et al.*, 2011). Moreover, microarray technology can be applied to the field of drug therapy. For example, if it is known that a gene is up-regulated in response to a particular infection or in cancer; new drugs can be tested to see if expression of this particular gene is decreased following drug treatment. Sinicrope *et al.*, (2011) applied DNA microarray technology for drug treatment evaluation. Here the efficiency of diflouromethlyornithine (DFMO) to prevent or delay the onset of oesophageal cancer in patients suffering from Barretts Oesophagus was tested. Gene expression levels of 25 genes were altered, notably genes encoding a transcription factor promoting cell proliferation and a protein which is directly involved with Proliferating Cell Nuclear Ag being down-regulated in the presence of DFMO. This study reiterates just how useful array technology can be in the development of new pharmaceutical drugs (Sinicrope *et al.*, 2011). Overall, DNA microarrays have facilitated a greater understanding of the biology of cells, expanded our knowledge of existing gene families, uncovered new genome information and revealed new patterns of gene expression

The application of microarray technology applicable to our study involves gene expression profiling whereby expression levels of thousands of genes are analysed to detect changes in gene expression in pathogen infected cells/tissues when compared with uninfected cells/tissues (Manger & Relman, 2000). This application was first

carried out in to investigate gene expression in mice with dimethylhydrazine induced colon carcinoma to investigate gene expression levels of tumour infected cells compared with uninfected cells (Augenlicht & Kobrin, 1982).

5.1.1.3 Utilising microarrays for investigating disease states

Microarrays have revolutionised the study of disease states by allowing comprehensive gene expression, protein modelling and genome mapping studies. A wide range of new advancements have been made in recent years due to transcriptomic microarray technology. Chung *et al.*, (2011) utilised microarrays to analyse disease states as their study involved generating a sexually transmitted disease DNA chip as a method of diagnosing genitourinary conditions. Also manipulating DNA microarrays for the study of disease states was Sutherland *et al.*, (2011) who concentrated on developing gene expression profiling for sepsis detection. Using earlier equine sepsis studies as a basis of biomarker gene expression, blood samples were obtained from patients suffering from sepsis, post-operative patients along with healthy controls, RNA was isolated and microarray GEP analysis was undertaken uncovering 42 markers for sepsis, including genes involved in innate and adaptive immunity (Sutherland *et al.*, 2011).

Microarrays have contributed to advancements in immunology by revealing both functions and functional disorders of biological systems. In recent years, GEP analysis has enabled significant breakthroughs in bacterial pathogenesis in plants (Mazarei *et al.*, 2011), animals (Buitenhuis *et al.*, 2011; De Zoysa *et al.*, 2011) and humans (Sumegi *et al.*, 2011; Toedter *et al.*, 2011). For instance, Sumegi *et al.*, (2011) utilised GEP to investigate familial hemophagocytic lymphohistiocytosis (FHL) in children. By comparing the GEP of PBMCs from children with and without FHL, the authors uncovered 550 genes with increased expression and 915 genes with decreased

expression involved in innate and adaptive immune responses including apoptosis, B and T cell differentiation and cell-cell interactions.

5.1.2 Bioinformatics

Bioinformatics is a computational method of analysing biological processes utilising both information technology and computer science to determine pattern recognition, data mining, algorithms and visualisation of biological samples (Benton, 1996). Bioinformatics was essential in our study for analysis of levels of gene expression in *H. pylori* infected and uninfected gastric tissues.

5.1.3 Generation of RNA for microarray analysis

In this study, magnetic beads from Miltenyi Biotec were used to positively select CD2⁺ cells from epithelial and lamina propria layer suspensions derived from gastric antral biopsies. Following this, GEP analysis using an Affymetrix system in Karolinska institute was performed. CD2 is a glycoprotein present on CD4⁺ T cells, CD8⁺ T cells, unconventional T cells and NK cells. CD2 is an important co-stimulatory molecule which when paired with its ligand CD58, enhances the Ag-T cell interaction. CD2 is also involved in signalling in T cells. For instance, CD2 has been implicated in the positive selection of CD8⁺ T cells (Teh *et al.*, 1997) and in the activation of DCs (Crawford *et al.*, 2003). Therefore, the CD2 molecule is an important factor in immune responses against bacterial infection. Undertaking GEP on CD2⁺ epithelial and lamina propria cells would provide understanding of the responses of these immune cells in severely inflamed gastric mucosa during *H. pylori* infection compared with normal mucosa. This technique would allow simultaneous analysis of changes in gene expression of thousands of genes thereby investigating the function of these immune

cells in the disease versus non-disease state. Having identified that T cells and NK cells are present in the gastric mucosa (chapter 3), a study was undertaken in this chapter to isolate these populations and to analyse gene expression in *H. pylori* infection.

To generate and analyse genes differentially expressed by microarray analysis, there are three steps involved. These include preparation of sample, generation of the array and analysis followed by interpretation of array data (Bowtell, 1999) (Fig. 5.1). Preparation of RNA samples and analysis of array data were carried out by our group, the array was generated in the Karolinska Institute, Sweden while analysis was once again carried out by our group.

To generate the RNA samples, fresh gastric biopsies were obtained from the gastric antrum of *H. pylori* positive patients who presented with severe gastritis and *H. pylori* negative patients who displayed minimal gastritis. Gastric biopsies were firstly separated into epithelial and lamina propria tissues, cells were counted and viability was tested, next, CD2⁺ cells were isolated from each of these tissues using immuno-affinity magnetic bead technology. The purity of the CD2⁺ cells was analysed using flow cytometry and results revealed on average a purity of approximately 96%. Finally, total RNA was prepared from the CD2⁺ cells and pooled prior to microarray.

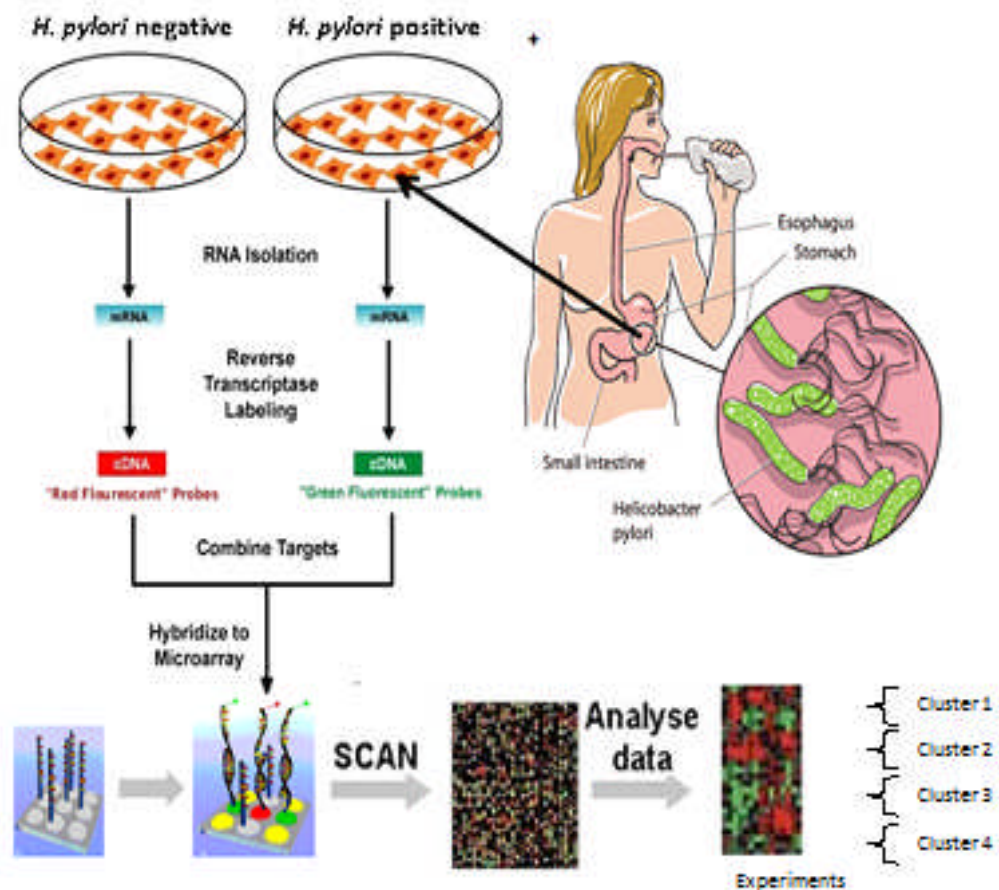


Fig. 5.1 Illustration showing the generation of RNA for Microarray analysis. Gastric biopsies obtained from *H. pylori* positive (HP⁺) and negative (HP⁻) patients were separated into epithelial and lamina propria layers before RNA was extracted from the CD2⁺ cells in each tissue. RNA was converted into cDNA which was hybridized to the microarray that was then scanned to reveal genes up-regulated and down-regulated in *H. pylori* disease and non-disease states. Image supplied by Dr. Ananya Gupta, University College Hospital, Galway. Permission to reproduce image granted by Ananya Gupta.

Each sample consisted of two biopsies, from these, approximately 600,000 epithelial layer cells/ml were isolated while the yield of lamina propria cells was approximately 1×10^6 cells/ml. This was the yield prior to CD2 positive selection. Poor quality RNA can lead to falsely identifying genes as being differentially expressed (Hinton *et al.*, 2004). Therefore, it was important to determine the quality of RNA. The quality of RNA from epithelial layer cells was sometimes poor due to epithelial layer cells losing their viability rapidly following isolation from the body while lamina propria cells were protected by the basement membrane. For each sample, RNA quality

was tested using nanodrop technology which measured an absorbance ratio of 260/280 and only good quality RNA with a ratio of >2 were included in the study. This procedure was carried out on samples from 36 different patients, the RNA was pooled into groups according to the patient's their clinical status and tissue type from which the CD2⁺ cells were isolated

- Group 1: epithelial layer of *H. pylori* negative patients,
- Group 2: epithelial layer of *H. pylori* positive patients,
- Group 3: lamina propria layer of *H. pylori* negative patients,
- Group 4: lamina propria layer of *H. pylori* positive patients

A quantity (2 μ g) of RNA from each group was used for generation of labelled cDNA for hybridization on the array. Therefore, 4 experimental replicates were included in this study (the four groups mentioned above) while there were three technical replicates of the HG-U133 Plus 2.0 array. Gene profiling was carried out with the RNA from CD2⁺ cells to investigate up-regulation and down-regulation of genes during *H. pylori* infection. Microarray analysis was carried out following bioinformatics on $>54,000$ oligonucleotides and involved analysis of $>33,000$ genes. Differentially expressed genes in this study provide an insight into CD2⁺ cell innate and adaptive immune responses to the gastric pathogen *H. pylori*.

5.1.4 Aims

To evaluate further the localised immune response to *H. pylori* in the gastric epithelial and lamina propria layers, a study investigating gene expression profiling was undertaken. The aims of this study were to:

- isolate RNA from purified CD2⁺ cells derived from the epithelial and lamina propria layers of *H. pylori* positive and *H. pylori* negative subjects;
- characterise using this RNA and Affymetrix technology, changes in gene expression amongst CD2⁺ cells of each gastric layer during *H. pylori* infection;
- investigate further the changes in gene expression in CD2⁺ cells with respect to surface marker expression, receptor gene usage, cytokine production, proliferation, signal transduction, cytotoxic function and cancer progression.

5.2 RESULTS

5.2.1 Optimisation experiments

Gastric antral biopsies were firstly separated into epithelial and lamina propria tissues as described in chapter 2. Each sample consisted of two to three biopsies, from these, approximately 6×10^5 epithelial cells/ml and 1×10^6 lamina propria cells/ml were isolated. This was the yield prior to CD2 positive selection. Cell viability was determined using trypan blue. Viability of lamina propria layer cells (on average 90%) was greater than that of epithelial cells (on average 60%). BerEP4 has previously been utilised in our lab and shown to verify that no cross-contamination of epithelial and lamina propria layer cells takes place when separating cells into their constituent layers and therefore we were presented with pure epithelial and lamina propria layer cell populations (see section 2.2). CD2⁺ cells were isolated from each of these tissues using immuno-affinity magnetic bead technology (Miltenyi Biotec, Gladbach, Germany). Pre-separation filters (Miltenyi Biotec, Gladbach, Germany) were also used to enhance yield and remove any excess tissue remaining after isolation of biopsies into their respective layers. The purity of the CD2⁺ cells was analysed using flow cytometry and results revealed a purity of approximately 98%.

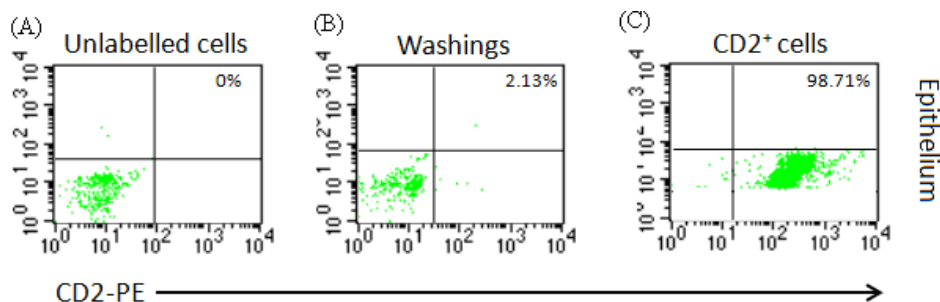


Fig. 5.2 Representative flow cytometric dot plots showing gastric epithelium layer cells (A) before CD2 positive selection, (B) percentage of CD2⁺ cells lost through the positive selection process through washings and (C) purified CD2⁺ cells eluted from beads after positive selection. Quadrants were gated on the basis of isotype matched controls.

Total RNA was prepared from the purified CD2⁺ cells and the quantity was determined using nanodrop technology. Obtaining sufficient quantities of cells for RNA isolation was challenging especially from epithelial cells. Poor quality RNA can lead to falsely identifying genes as being differentially expressed (Hinton *et al.*, 2004). Therefore, it was important to determine the quality of RNA. The quality of RNA from epithelial layer cells was sometimes poor, probably due to epithelial layer cells losing their viability rapidly following isolation from the body while lamina propria cells were protected by the basement membrane. For each sample, RNA quality was tested using nanodrop technology which measured an absorbance ratio of 260/280 and only good quality RNA with a ratio of >2 were included in the study. This procedure was carried out on samples from 36 different patients before RNA was pooled into groups according to the patient's clinical status and gastric layer from which the CD2⁺ cells were isolated.

5.2.2 Identification of differentially expressed genes in *H. pylori* infected and uninfected patients

Results in the following Figs. 5.3 and 5.4 show GEP of RNA isolated from *H. pylori*-infected and uninfected gastric mucosa. Both the epithelial and lamina propria layer of *H. pylori*-infected mucosa with moderate to severe gastritis were analysed and gene expression was compared and contrasted with that of uninfected mucosa (asymptomatic or mild gastritis) leading to analysis of four groups which included:

- Group 1. CD2⁺ cells in epithelial layer of *H. pylori* infected gastric mucosa compared with the epithelial layer of healthy mucosa designated (EP-HP⁺/HP⁻),

- Group 2. CD2⁺ cells in the lamina propria layer of *H. pylori* infected gastric mucosa compared with the lamina propria layer of healthy mucosa designated (LP- HP⁺/HP⁻),
- Group 3. CD2⁺ cells in epithelial layer of *H. pylori* infected gastric mucosa compared with *H. pylori* infected lamina propria designated (HP⁺- EP/LP) and
- Group 4. CD2⁺ cells in epithelial layer of normal mucosa compared with the lamina propria layer of normal mucosa designated (HP⁻- EP/LP).

The transcription profile revealed a large number of genes differentially expressed in CD2⁺ cells in the *H. pylori*-infected and uninfected groups in both the epithelial and lamina propria layer. The microarray contained a total of 54,675 oligos. The graphical representation of data generated by the microarray was illustrated using a heat map (Fig. 5.2) which here shows 737 of the genes with significantly increased (red) and reduced (blue) expression in the epithelium and lamina propria of *H. pylori* positive and negative patients. A heat map is a method of visualising data using colours (Perez-Llomas & Lopez-Bigas, 2011). In this case a microarray heat map was constructed to analyse and compare behaviour patterns of genes differentially expressed in the epithelial and lamina propria layers of *H. pylori* infected and uninfected mucosa.

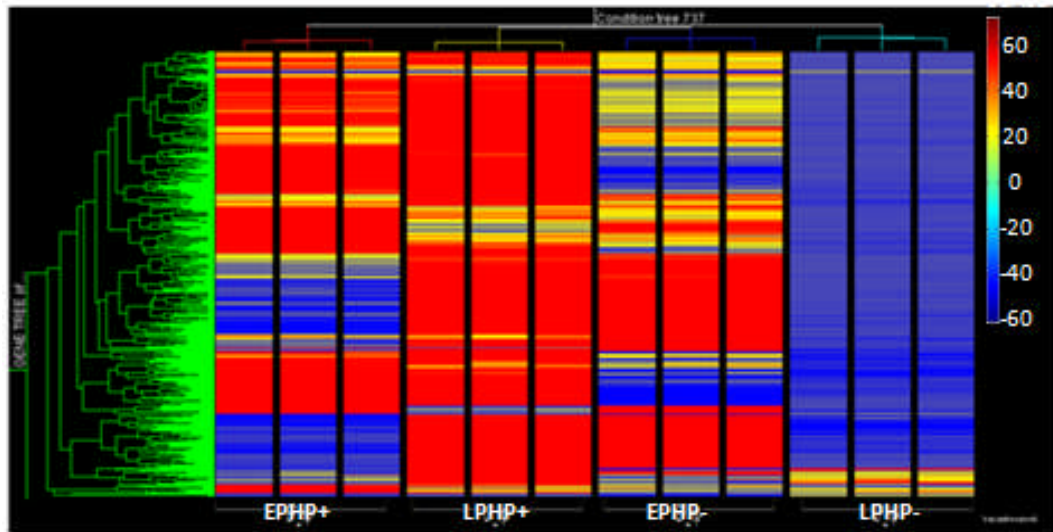


Fig. 5.3 Representative heat map Condition Tree view of 737 genes statistically different among four groups based on values of EP and LP layer of *H. pylori* positive and negative individuals. Each row represents a gene while each column represents a particular sample. The four groups include: the epithelium (EHPH⁺) and LP (LPHP⁺) of *H. pylori* positive subjects, the EP (EHPH⁻) and LP (LPHP⁻) of *H. pylori* negative subjects. Data for each group is represented in triplicate. Red colour represents genes significantly increased and those highlighted in blue represent genes significantly decreased. Linked to the heat map is a tree diagram which groups genes together according to hierarchy.

To characterise the overall patterns of gene expression hence analysing the degree of differences in GEP in normal and *H. pylori* infected antrum gastric mucosa; matrices comparing fold change in gene expression (Fig. 5.4) were next constructed for comparative analysis of the four different groups. Notably, 12, 311 genes and 1, 079 genes were significantly up-regulated in *H. pylori* infected epithelial and lamina propria layers respectively when compared with uninfected counterparts while an inter-layer comparison revealed 2, 156 genes up-regulated significantly in infected epithelial tissues compared with infected lamina propria layer tissues.

		UP REGULATED			
		EP Hp+	EP Hp-	LP Hp+	LP Hp-
DOWN REGULATED	EP Hp+	0	4 863	3 604	2 128
	EP Hp-	12 311	0	13 431	11 759
	LP Hp+	2 156	5 593	0	1 079
	LP Hp-	2 666	4 153	3 536	0

Fig. 5.4 Fold change comparison matrix of differentially expressed genes among the four groups based on genes significantly expressed two-fold and greater in CD2⁺ cells when the EP and LP layer of *H. pylori* positive and negative mucosa were compared. Reading the matrix from a vertical perspective indicates up-regulated genes and reading the matrix from a horizontal perspective indicated down-regulated genes in a group to group comparison.

To analyse the results further, differentially expressed genes found to be related to proinflammatory and immunological reactions, signal transduction, cytotoxicity, proliferation and other biological processes were examined. Genes were categorised in several functional groups involved in inflammatory responses and cancer progression for further analysis to gain understanding of the gastric mucosa genes, biology processes and molecular interactions associated with *H. pylori* infection.

5.2.3 Top 20 differentially expressed genes in *H. pylori* positive and *H. pylori* negative mucosa

Due to the large amount of data generated by this gene expression profiling study, the top 20 most significantly up-regulated and down-regulated genes in the RNA isolated from CD2⁺ cells in the epithelium and lamina propria layer of *H. pylori* infected compared with uninfected tissues were firstly analysed. The top 20 up-regulated and

down-regulated genes will be discussed in the forthcoming sections and are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelial layer of *H. pylori* infected compared with uninfected patients (EP-HP⁺/HP⁻)

Since approximately 85% of *H. pylori* bacteria reside on or above the gastric epithelial layer, the GEP of CD2⁺ cells from the epithelial layer in *H. pylori* infected and uninfected states were compared. As shown in Fig. 5.5, in the first group (EP-HP⁺/HP⁻), most notably, *CTLA4*, *GZMA*, *CD69* and *KLRC1*; *KLRC2* were among the significantly up-regulated genes in the CD2⁺ cells in the epithelial layer of *H. pylori* positive mucosa compared with *H. pylori* negative mucosa. Two of these genes have previously been associated with *H. pylori* infection including *CTLA4*, which is a cytotoxic T lymphocyte associated protein was the most highly up-regulated gene in this entire group (>344- fold). Cheng *et al.*, (2006) found an association of certain *CTLA4* gene polymorphisms with the onset of *H. pylori* related MALT lymphoma (Cheng *et al.*, 2006). *CD69*, expressed by NK cells among others, is a human transmembrane C-type lectin involved in T cell activation and proliferation (Ziegler *et al.*, 1994). Regarding *H. pylori* infection, *CD69* has been shown to be induced by *cagPAI*⁺ strains (Mori *et al.*, 2011). There have been no studies investigating the role of the other 18 genes in *H. pylori* infection. *GZMA* is a protein secreted by T_c cells and NK cells involved in cell lysis (reviewed in Henkart, 1994). *KLRC1*; *KLRC2*, also named *NKG2*, belongs to the killer cell lectin-like receptor subfamily, is expressed by NK cells and is involved in lysis of tumour infected cells. *RGS1*, a regulator of G protein signalling is involved in gut T cell trafficking (Gibbons *et al.*, 2011). *PTPRC*, also named as *CD45* is involved in cell signalling and cell growth. *RAB8B* is a transport regulator and is a member of the

ras oncogene family. *LCP2* is a lymphocyte cytosolic protein. Interestingly, a functional role for this protein in TCR signalling in response to precancerous lesions in oral buccal mucosa has been shown (Chen *et al.*, 2011). *PSCDBP* is a cytohesin binder which has been shown to regulate integrin mediated adhesion (Geiger *et al.*, 2000). *ZNFN1A1* encodes a zinc finger protein and is also known as Ikaros. This gene has been implicated in stress and inflammation (Chrousos & Kino, 2005). *DDX3Y* protein has been implicated in males with testicular tumours (Gueler *et al.*, 2012). Other over-expressed genes included *SFRS7*, *PTPN22*, *TRA*; *TRD*, *PTGER4*, *MALAT1*, *DDX3Y*, *TRGC2* and *ERBB2IP* along with an unknown gene. Overall, 3 markers of cytotoxicity were found to be amongst the top 20 most up-regulated genes in CD2⁺ cells when epithelium disease and non-disease states were compared, while many other immunologically relevant genes were also differentially expressed.

As shown in Fig. 5.5, T-complex 11, cancer/ testis Ag and *REG4* were significantly down-regulated in the CD2⁺ cells in the epithelial layer of *H. pylori* infected mucosa when compared with negative mucosa. REG is believed to be involved in controlling inflammation in the gastrointestinal epithelium through initiation of healing process (Zhang *et al.*, 2003). *EGFR* has already been shown to have an involvement in gastric cancer progression; hence its down-regulation may be of immense importance here (Pryczynicz *et al.*, 2009). *LRRC25*, encodes a leucine rich reactive protein may have a role to play in innate and adaptive immune responses as a recent study demonstrates an involvement of LRRC25 in Crohn's disease (Sim *et al.*, 2012). *CHES1* (checkpoint suppressor 1) has been shown to inhibit genes involved in tumorigenesis (Scott & Plon, 2005) and therefore is potentially important in *H. pylori* infection. TP73L, also referred to as p63 is a tumour protein known to be implicated in epithelial integrity (Ching *et al.*, 2010; Marcel *et al.*, 2011). It could be involved in

morphological epithelium changes due to *H. pylori* infection. BAGE4; BAGE2 forms part of the B melanoma Ag family which have been shown to encode Ags on the surface of melanomas that are recognised by T_c cells (Boel *et al.*, 1995). Their role in *H. pylori* infection has not yet been identified. Other genes with reduced expression included *FLJ22184*, *VGLL1*, *ADH4*, *PRSS7*, *MGC40368*, *SLC26A3*, *C19orf26*, *HSU79275* and *SRGAP2* along with several unknown genes whose functions in *H. pylori* remain elucidated. Once again, immunologically relevant genes not previously associated with *H. pylori* infection were amongst the down-regulated genes. When up- and down-regulated genes were compared, it is evident from Fig. 5.5, that there was a far greater fold-change amongst up-regulated genes in *H. pylori* infected epithelial layer.

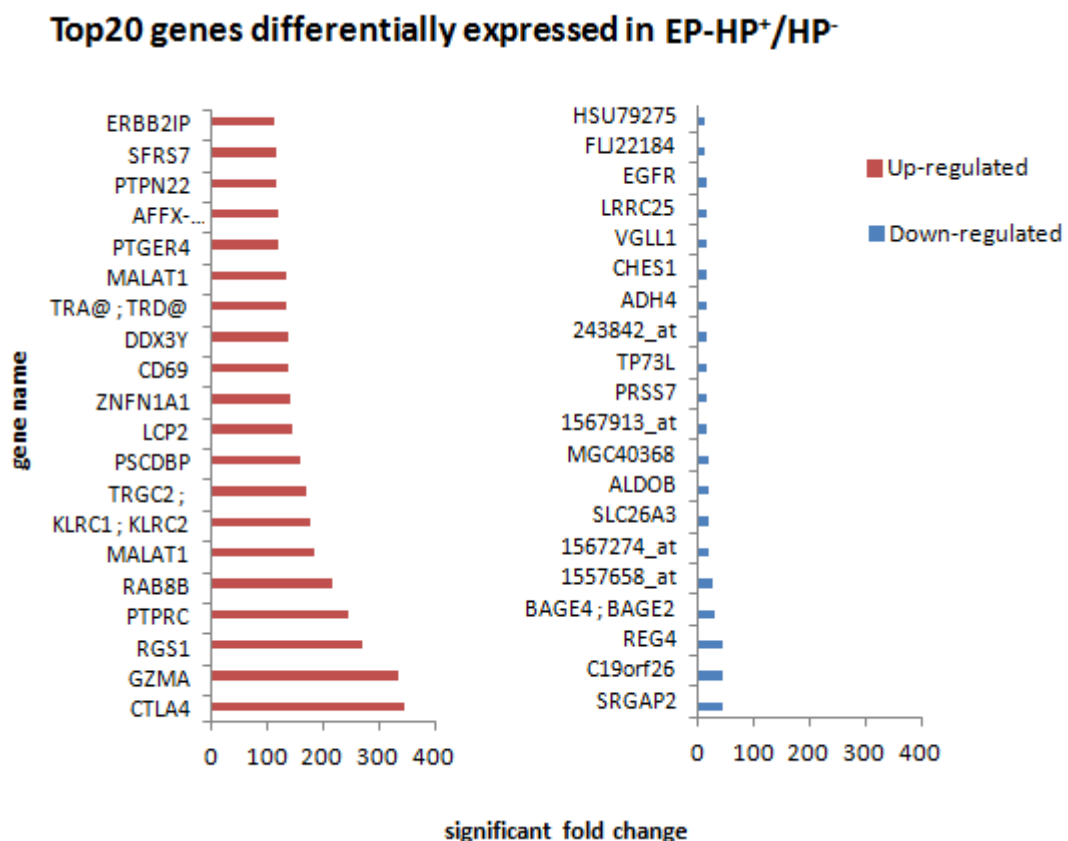


Fig. 5.5 Bar charts showing the top 20 up-regulated and down-regulated genes when the epithelium of *H. pylori* infected and uninfected patients were compared.

Group 2: Lamina propria layer of *H. pylori* infected compared with uninfected patients (LP-HP⁺/HP⁻)

H. pylori bacteria has been shown to extend into the underlying lamina propria tissues, hence the GEP of CD2⁺ lamina propria layer cells from *H. pylori* infected and uninfected mucosa were compared (Necchi *et al.*, 2007). Regarding the second group (LP-HP⁺/HP⁻), as shown in Fig. 5.6, of notable interest, *REG3A* and *OLFM4* expression was significantly up-regulated in the CD2⁺ cells in the lamina propria layer of *H. pylori* positive individuals when compared with healthy subjects. *PNLIP* was the most up-regulated gene in the microarray study (> 625-fold increase). PNLIP, a pancreatic lipase is an enzyme involved in the digestion of lipids. Interestingly, elevated levels of PNLIP have been found in peptic ulcer patients (Raffensperger, 1951). *REG3A* is implicated in both cell proliferation and differentiation; hence this gene may be implicated in activated T cell responses and T-dependant humoral responses. Moreover studies have shown that *REG3A* expression is down-regulated in gastric cancer cases (Choi *et al.*, 2007). Olfactomedin (*OLFM4*) is known to be expressed in the epithelial layer of patients suffering from inflamed colon (Seko *et al.*, 2010) and it is possible that it may be playing a role in the lamina propria layer of inflamed gastric tissue also. Moreover, recent data has hypothesised its role as a biomarker in gastric cancer (Oue *et al.*, 2009). *AMY1A*; *AMY1B* encode amylases which are digestive enzymes whose expression is increased in peptic ulcer cases (Rogers, 1960). No association has yet been made between these enzymes and the onset of peptic ulcers related to *H. pylori* infection so this is an interesting find. *ELA2A*, *ELA3A*, *ELA3B* are elastases which are a group of serine proteases. While their role in *H. pylori* infection has not yet been determined, elastases have a functional defensive role in the skin barrier (Nehmé-Pelluard *et al.*, 2011). *CPB1* is a tissue specific protein that has been implicated in pancreatitis (Müller

et al., 2002) but to date has no link with *H. pylori* infection. Other genes with increased expression in this group which have not yet been associated with *H. pylori* infection include *C20orf114*, *PRSS1*, *CEL*, *PLA2G1B*, *PNLIPRP2*, *CPA1*, *PRSS2*, *CTRB1*; *CTRB2* and *ZFP36L2*.

GKN1 and *ITGA6* were among the genes whose expression was significantly down-regulated in the CD2⁺ cells in lamina propria layer of *H. pylori* positive individuals when compared with healthy patients. Gastrosyne 1 (*GKN1*) is believed to be important for maintaining gastric mucosa integrity; it has also been linked to proliferation and differentiation of cells involved in the immune response (Oien *et al.*, 2004). *ITGA6* is a member of the integrin family. Integrins are cell surface receptors that form linkages with ECM and subsequently are involved in cell adhesion, integrity, proliferation and survival (Kwok *et al.*, 2007; Martin *et al.*, 2002). *CBX5*, also known as chromobox homolog 5 has already been implicated in gastric cancer as it is expressed at high levels in gastric tumour tissue (Claerhout *et al.*, 2011). Other genes with reduced expression in this group whose function in *H. pylori* infection remains unclear include *TMEM27*, *BCMP11*, *SCIN*, *bioB*, *bioC*, *FLJ10154*, *SFRS11*, *FOLR1*, *RPS26*, *IL6ST*, *SKI*, *GAST*, *LOC126295* and *DAZ1*; *DAZ3*.

Interestingly, there is no overlap in genes with increased or decreased expression in the epithelium and the lamina propria, thus highlighting just how differently CD2⁺ cells in each layer are responding during *H. pylori* infection. It is also worth noting, that out of the 80 genes discussed above, only 2 genes have previously been linked with *H. pylori* disease, 69 genes have no previous association with *H. pylori* infection and 9 genes are novel genes. Many of these 69 genes have however been implicated in immunological roles in other diseases such as the onset of cancer and therefore are potentially important in *H. pylori* infection. Once again, in the lamina propria layer, the

fold-change of up-regulated genes is far greater than down-regulated genes when infected and uninfected tissues were compared.

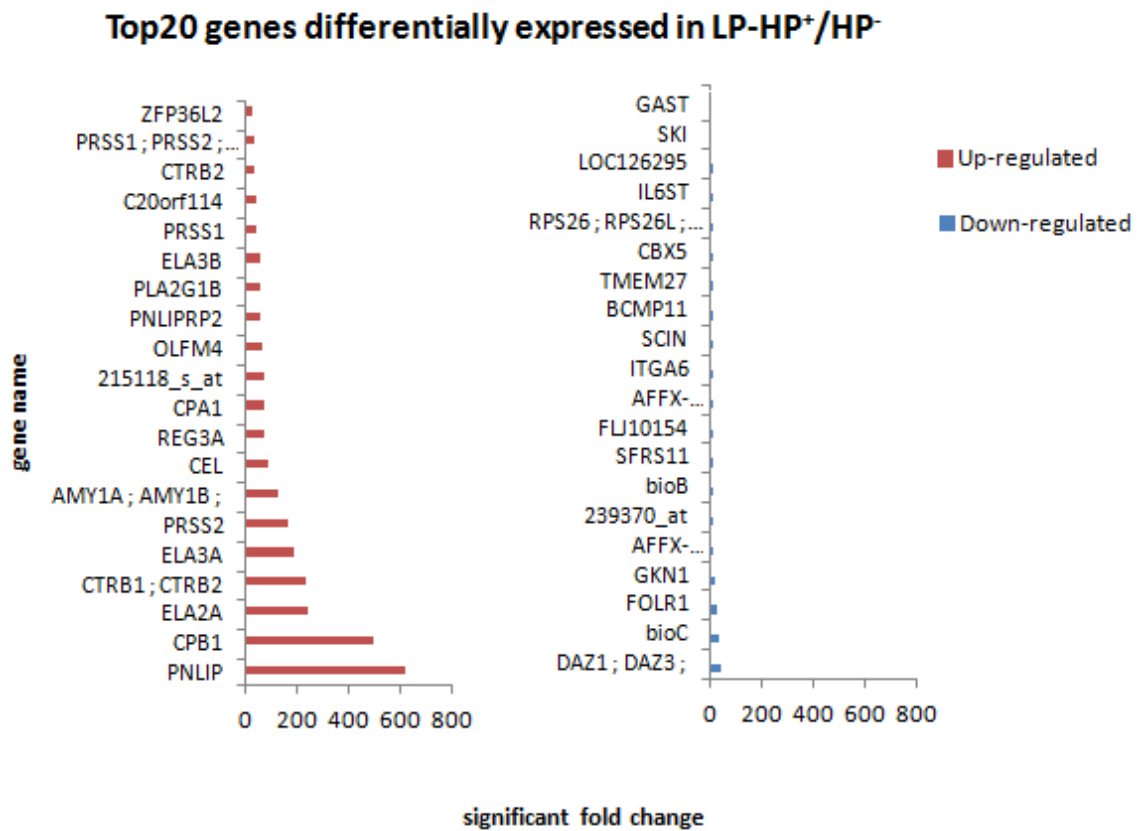


Fig. 5.6 Bar charts showing the top 20 up-regulated and down-regulated genes when the LP layer of *H. pylori* infected and uninfected patients were compared.

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺ EP/LP)

Since differences were observed in both epithelial and lamina propria layers of infected gastric mucosa when compared to healthy mucosa, the next question to be addressed was whether there were significant interlayer differences when the two gastric layers in *H. pylori* infection. In this third group (the interlayer comparison in *H. pylori* infection HP⁺-EP/LP), as shown in Fig. 5.7, significant up-regulation of various genes of interest was observed amongst the top 20 genes in CD2⁺ cells in the epithelial layer of *H. pylori*

infected mucosa when compared with infected lamina propria tissues. Notably, the TCR, *CD160*, *GKN1*, *GZMA*, killer cell Ig-like receptor two domains (*KIR2DL4*) and killer cell lectin like receptor subfamily C member 3 (*KLRC3*) and *TRAF3IP3* gene expression were significantly increased. *CD160* is expressed by both NK cells and $CD8^+$ cells (Anumanthan *et al.*, 1998). It has been linked proliferation and differentiation of cells involved in the immune response (Kaye, 2008). Granzyme is secreted by T_c cells and NK cells to lyse virus infected or tumour cells (Anthony *et al.*, 2010). *KIR2DL4* is a glycoprotein expressed on NK cells and certain subsets of T lymphocytes (Selvakumar *et al.*, 1996). *KLRC3* is an NK receptor and so is involved in cell apoptosis (Hidalgo *et al.*, 2008). *TRAF3IP3*, also named TRAF, is involved in cell proliferation (Ma *et al.*, 2007). *PYHIN1* forms part of the HIN domain family which function in regulation of cell differentiation and apoptosis. Moreover, a recent study has emerged highlighting the role of *PYHIN1* in host innate defence by binding microbial DNA (Schattgen & Fitzgerald, 2011). *SH2D1B*, also named EAT2, plays a role in signal transduction by negatively regulating NK cells (Veillette, 2006). It is currently unknown whether *SH2D1B* is involved in signal transduction in *H. pylori* mediated infection. A role for *FCRH3* has been hypothesised in autoimmune diseases such as rheumatoid arthritis (Chistiakov & Chistiakov, 2007), while its function in *H. pylori* infection remains unclear. Other genes over-expressed whose function in *H. pylori* infection remains elusive include *STS-1*, *FLJ10652*, *DAZ1*; *DAZ3*, *bioC*, *FOLR1*, *FLJ43663*, *SUPT16H*, *PELO* along with an unknown gene.

Next, *ELA2A* and *ELA3A* (encoding enzymes such as elastases) and *AMY1A*; *AMY1B* (encoding amylases) featured among the genes significantly down-regulated in $CD2^+$ cells in the infected gastric epithelium in comparison to infected lamina propria tissues (Fig. 5.7). Expression of *CXCL14* (encoding a chemokine) was also significantly

decreased in *H. pylori* infected epithelium. CXCL14 is involved in the activation of monocytes and DCs and is involved in migration of activated NK cells. Interestingly, the literature states that *CXCL14* expression is decreased in *in vitro* cancer studies when compared with normal cells and tissues (Frederick *et al.*, 2000). *EDIL3* encodes an integrin ligand and its differential expression has been shown in hepatocellular carcinoma (Sun *et al.*, 2010), but to date its differential expression has not been implicated in *H. pylori* infection or *H. pylori* mediated carcinoma. *COL3A1* encodes a collagen which functions to support tissues in the body such as the intestine (Vuorio & De Crombrughe, 1990). No correlation between *COL3A1* and *H. pylori* infection has been shown to date. *PRSS2* encodes a serine protease which encodes a trypsinogen. Studies have demonstrated a pro-inflammatory role for protease activated receptor 2 in *H. pylori* infected human gastric mucosa (Kandulski *et al.*, 2011). Other genes with reduced expression include *CEL*, *LOC387763*, *GJA1*, *ATP4A*, *PNLIPRP2*, *CTRB1*; *CTRB2*, *CPB1,211645_x_at*, *COL4A1*, *215118_s_at*, *PDGFRA*, *IGHM* and *PNLIP*. While there are a small number of studies reporting the differences in GEP in gastric biopsies from *H. pylori* positive and negative subjects, there are currently no studies to date where the gastric epithelial and lamina propria layer were separated and analysed separately. Therefore, these findings whereby the two gastric layers in *H. pylori* infected state are compared are of immense immunological importance.

Top20 genes differentially expressed in HP⁺EP/LP

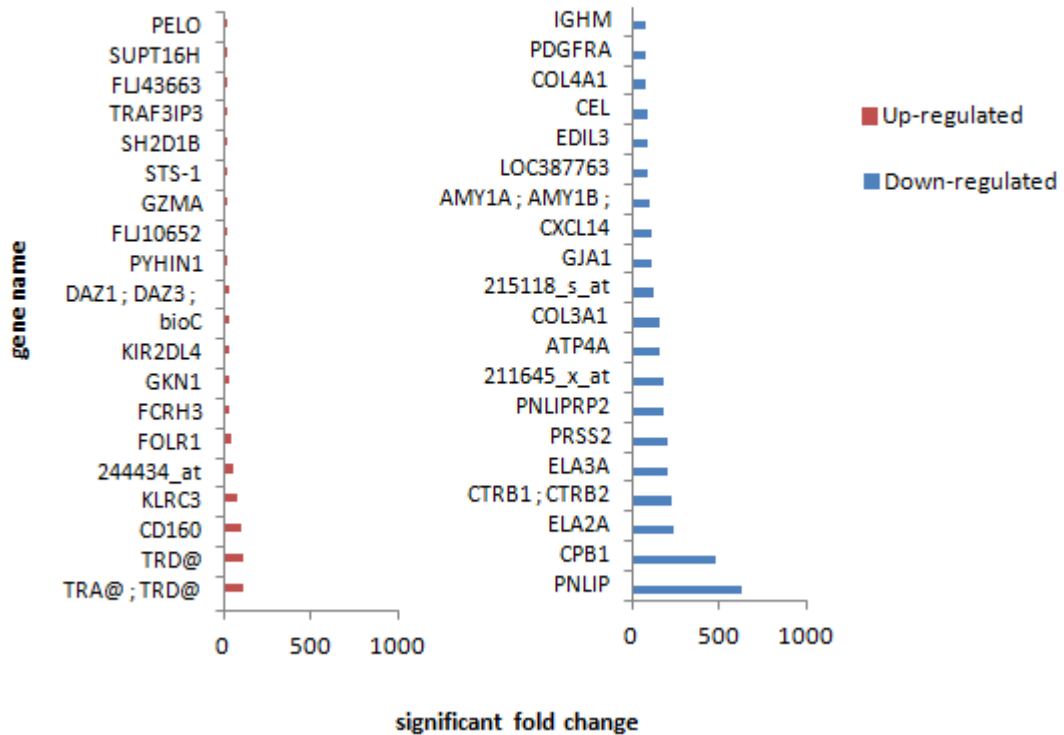


Fig. 5.7 Bar charts showing the top 20 genes up-regulated and down-regulated when the epithelium and LP layer of *H. pylori* infected patients were compared.

Group 4: Epithelium compared with the lamina propria of *H. pylori* uninfected patients (HP⁻EP/LP)

While gastrointestinal epithelium and lamina propria are known to participate in different functions due to their location and different environmental stresses they encounter, to our knowledge, GEP of the two individual gastric layers has not yet been explored in normal healthy mucosa. Therefore, in the final group (the interlayer comparison in *H. pylori* negative subjects HP⁻EP/LP), notably, significant differences in expression in the epithelium of the two genes *REG4* and *TP73L* (tumour protein p73-like) was observed (Fig. 5.8). *PCDH7* encodes is a member of the protocadherin gene family, is believed to be involved in cell signalling and cell adhesion (Yagi, 2008). *FUT6* forms part of the family of fucosyltransferases. Recently, *FUT* has been implicated in tumourigenesis (Guo *et al.*, 2012). Other genes with increased expression

in this group include *VGLL1*, *HSU79275*, *LRRC25*, *SI*, *FABP1*, *ADH4*, *PRSS7*, *BAGE4*; *BAGE2*, *SLC26A3*, *ALDOB*, *C19orf26*, *238103_at*, *C9orf10*, *MGC40368* and *SRGAP2*.

Next, *IL-8*, *IL-7*, *RNF138*, *BIRC3* and *LUM* are among the genes of interest significantly down-regulated in CD2⁺ cells in the epithelial layer of *H. pylori* negative mucosa when compared with the lamina propria layer counterpart (Fig. 5.8). *IL-8* is a chemokine involved in the initiation of the innate response to *H. pylori* infection. *IL-7* is a cytokine involved in T cell homeostasis (Fry & Mackall, 2002). *RNF138* is a ring finger protein. Regarding clinical pathologies, *RNF138* expression has recently been shown to be significantly increased in tumour tissue in the brain (Zhou *et al.*, 2012). *BIRC3*, or baculoviral IAP repeat containing 3, is involved in apoptosis. A role for a member of the IAP family has been hypothesised in *H. pylori* infection (Li *et al.*, 2011), although we are observing its differential expression in *H. pylori* negative mucosa. *LUM* forms part of the leucine rich proteoglycan family which function in epithelial cell migration and tissue repair (Saika *et al.*, 2000). Other genes with decreased expression in the epithelium whose role in gastric tissues are unknown include include *ZBTB10*, *ERBB2IP*, *IGH*; *IGHG1*, *ZNF165*, *CALD1*, *PBEF1*, *IGL*, *IGJ*, *COL1A1*, *IGLC2*, *IGHA1*, *IGFBP7*, *MMP1*, *PRNP* and *COL3A1* (Fig. 5.8). It is important to note what we are seeing here in this fourth group (HP⁻-EP/LP) is a normal pattern in gene expression and the differences that naturally exists between the two gastric layers. The composition of T and NK cells differs in the two gastric layers, for example CD8⁺ T cells predominate in the epithelium while CD4⁺ T cells are predominantly found in lamina propria tissues and these layers are exposed to different environmental stresses and Ags. The position of the epithelium at the interface between the lumen and the body's core and the lamina propria positioned underneath separated by the basement membrane implies that these two compartments are unique from each other. To the best of our

knowledge, this is the first time GEP has been undertaken on epithelium and lamina propria layers of *H. pylori* negative gastric mucosa.

Top20 genes differentially expressed in HP-EP/LP

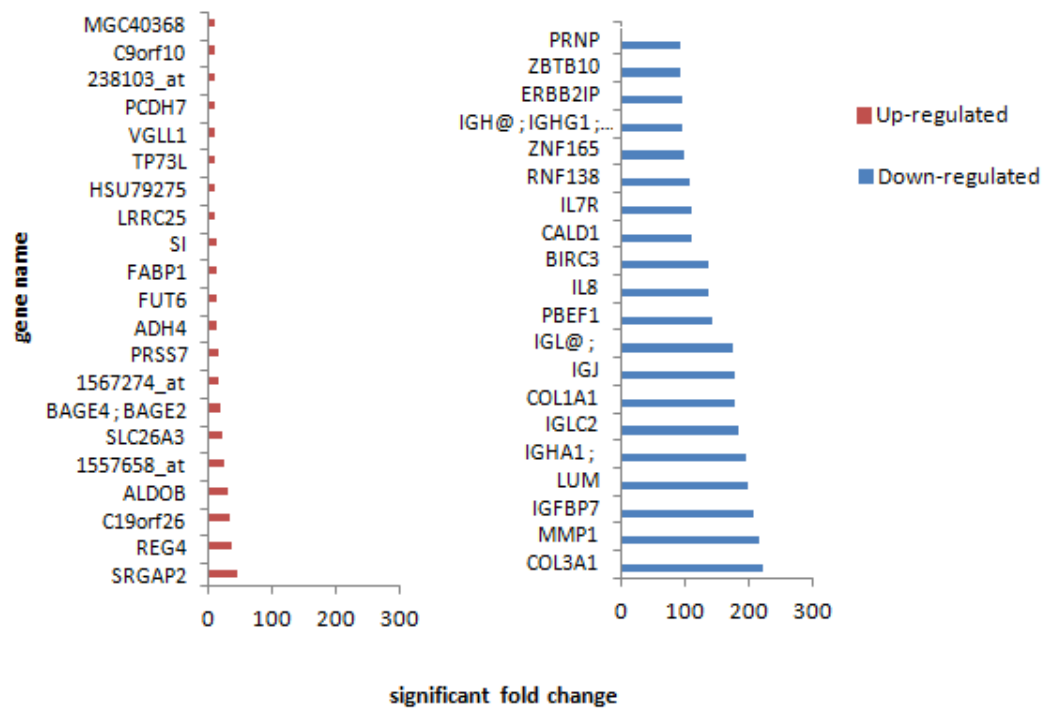


Fig. 5.8 Bar charts showing the top 20 genes up-regulated and down-regulated when the epithelium and LP layer of *H. pylori* uninfected patients were compared

5.2.4 Functional category breakdown of genes whose expression were significantly altered in *H. pylori* infection

Next, to identify additional genes of immunological relevance that could be acting as a signature for *H. pylori* infection, the microarray data was analysed further to observe which genes were up-regulated and down-regulated in *H. pylori* disease and non-disease states. Seeing as CD2⁺ cells incorporates both T and NK cells, here we are observing both innate and adaptive immune responses. The literature was reviewed to identify genes known to have an important role to play in infection; next immunologically

relevant genes involved in innate and adaptive responses were subsequently grouped under the following subheadings:

- Proliferation
- Cytotoxicity
- Cytokines
- Signal Transduction
- Receptors and corresponding ligands

One of the consequences of *H. pylori* infection is an inflammatory response resulting in the production of cytokines and signalling molecules. Hence, we looked at the cytokine GEP changes in order to investigate highly inflamed *H. pylori* infected versus non-inflamed normal gastric mucosa. Since *H. pylori* infection can induce cellular stress and apoptosis, cytotoxic genes were analysed to observe modifications in gene expression patterns in apoptotic genes. epithelial cells have already been shown to proliferate in response to *H. pylori* infection, therefore, up-regulation and down-regulation of markers of proliferation were compared in the gastric epithelium and lamina propria following *H. pylori* infection to analyse whether cells were proliferating in response to Ag stimulation. Next, cell surface receptors and signal transducers were scrutinized to see if there were changes implicated with *H. pylori* infection. Taken together, a comprehensive insight into the CD2⁺ immune responses during *H. pylori* infection were obtained through analysis of changes in the transcriptome in infected gastric mucosa when compared with uninfected mucosa.

Based on these five functional categories, a large number of significant differences were identified in both the epithelium and lamina propria in *H. pylori* positive subjects compared with controls. Values were obtained by comparing the numbers of differentially expressed genes with a fold change of >2 in each functional category. These results are shown in Fig. 5.9.

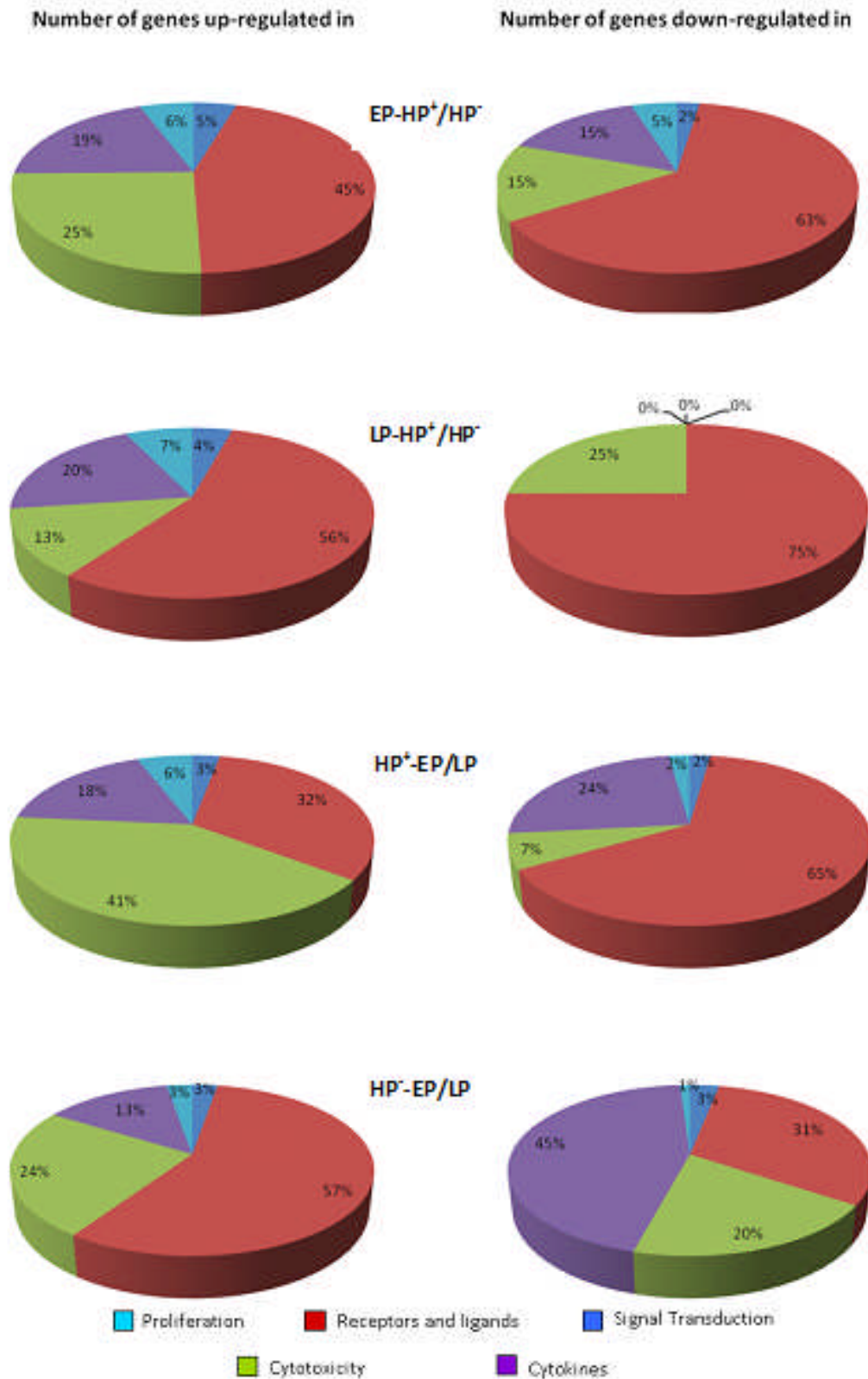


Fig. 5.9 Piecharts illustrating the broad functional category breakdown of immunologically relevant genes in the immune response to *H. pylori* infection. Included in these genes significantly altered in response to *H. pylori* are signal transducing genes, receptors and ligands involved in immunity, cytotoxic genes, cytokines and genes involved in proliferation.

5.2.4.1 Proliferation

Regarding mucosal cells and proliferation in *H. pylori* infection, an increase in proliferation in epithelial cells is one of the indicators for the development of *H. pylori* induced gastric adenocarcinoma (Murakami *et al.*, 1997) while a lack of proliferation of T cells in *H. pylori* infection has been reported (Chmiela *et al.*, 1996). Following cDNA array analysis, several genes were identified that serve as markers of proliferation in the CD2⁺ cells in the epithelial layer of *H. pylori* positive individuals compared with *H. pylori* negative individuals. The genes involved in proliferation are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelium of *H. pylori* infected compared with uninfected patients (EP-HP⁺ HP⁻)

In the first group (EP- HP⁺/HP⁻), as shown in Fig. 5.10, 7 genes involved in proliferation were found. The 5 up-regulated genes during infection were *ANXA1*, *ANXA4*, *IFI30*, *IFI16* and *IFNG* and the 2 down-regulated genes were *IL411* and *NUP62*. This finding correlates with a study carried out by Li-Ling Lin *et al.*, (2010) who hypothesise that *H. pylori* infection induces annexin A1 and A4 production to repair damaged tissue and that prolonged production could possibly be linked with carcinogenesis. Moreover, this study (Li-Ling Lin *et al.*, 2010) also reports a link between over-expression of *ANXA4* and human adenocarcinoma AGS cell proliferation. IFN- γ , a T_h1 pro-inflammatory and immunoregulatory cytokine with anti-proliferative activities was significantly up-regulated in the epithelial layer of *H. pylori* infected mucosa compared with healthy mucosa. This is in agreement with previous studies which reported a polarised T_h1 immune response to *H. pylori* (Karttunen *et al.*, 1995). Both pro- and anti-proliferation activity could be explained since there is a conflict occurring in the *H. pylori* gastric mucosa as this polarised T_h1 response leads to gastric

injury. Moreover, *H. pylori* employs immune evasion tactics all whilst evoking minimal host immune responses. A T_H1 pro-inflammatory response is signature for *H. pylori* infection while T_{reg} cells also have important roles to play in regulating immune response (Kandulski *et al.*, 2010; Kusters *et al.*, 2006). *H. pylori* is reported to suppress host cell proliferative responses (Chmiela *et al.*, 1996).

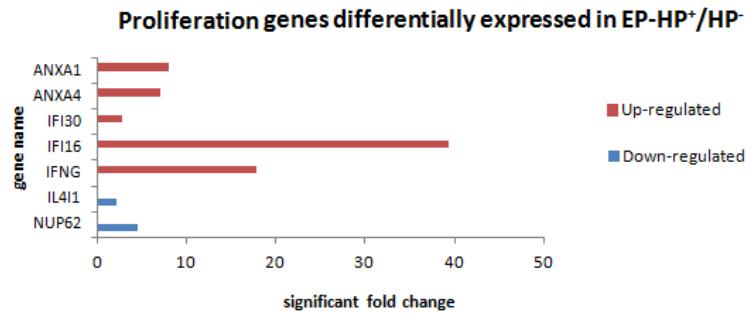


Fig. 5.10 Bar charts showing comparative analysis the number of proliferative genes up-regulated and down-regulated in the $CD2^+$ cells in the epithelium of *H. pylori* infected and uninfected subjects.

Group 2: Lamina propria layer of *H. pylori* infected patients compared with uninfected patients (LP-HP⁺/HP⁻)

In the second group (LP-HP⁺/HP⁻), as shown in Fig. 5.11, 5 genes involved in proliferation were found. Moreover, each of these 5 genes was up-regulated in disease-state. Included in this group were *ANXA1*, *IL4I1*, *IFI30*, *IFI16* and *IFNG*. Notably, the fold change in these proliferative genes in the lamina propria was not as marked as in the epithelium.

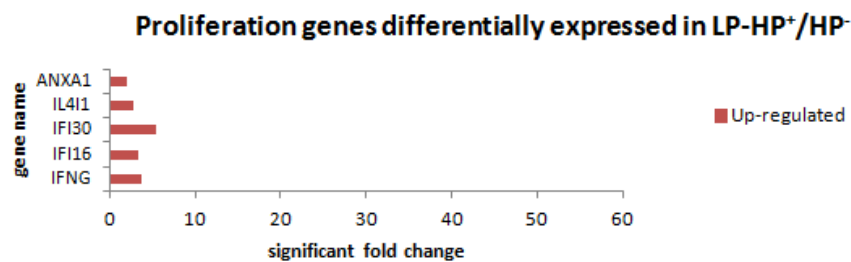


Fig. 5.11 Bar charts showing comparative analysis the number of proliferative genes up-regulated in the $CD2^+$ cells in the LP layer of *H. pylori* infected and uninfected subjects

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺ EP/LP)

In the third group, in the interlayer comparison in *H. pylori* infected patients (HP⁺ EP/LP), as shown in Fig. 5.12, 3 genes involved in proliferation were found. In the epithelium, the 2 up-regulated genes were *IL4I1* and *IFI30* and the 1 down-regulated gene was *IFNG*.

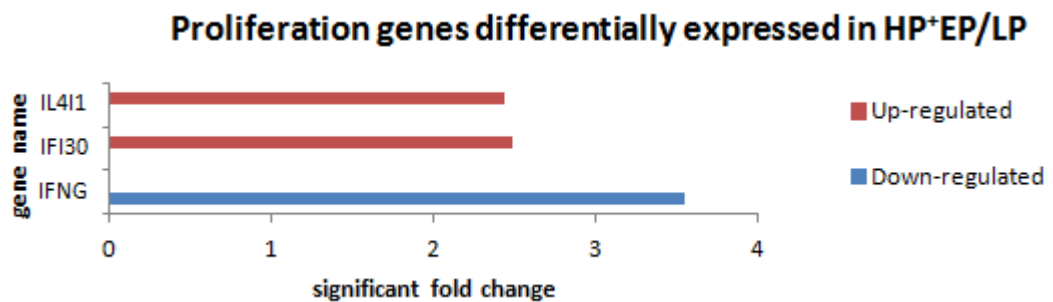


Fig. 5.12 Bar charts showing comparative analysis the number of proliferative genes up-regulated and down-regulated in the CD2⁺ cells in the epithelium and LP layer of *H. pylori* infected subjects

Group 4: Epithelium compared with lamina propria of *H. pylori* uninfected patients (HP⁻ EP/LP)

In the final group, the interlayer comparison in *H. pylori* negative subjects (HP⁻ EP/LP), as shown in Fig. 5.13, 2 genes involved in proliferation were found. The up-regulated gene in the epithelium was *NUP62* while the down-regulated gene was *IFI16*. The fold change here was low.

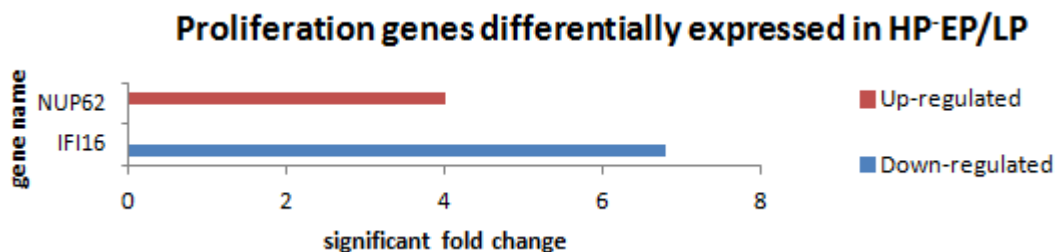


Fig. 5.13 Bar charts showing comparative analysis the number of proliferative genes up-regulated and down-regulated in the CD2⁺ cells in the epithelium and LP layer of normal healthy mucosa

5.2.4.2 Cytotoxicity

CD8⁺ T cells along with innate cells such as NK cells, innate like lymphocytes such as NKR⁺ T cells, unconventional T cells such as NKT cells and cell mediated T_c cells, all of which form part of the CD2⁺ cell population, are known for their cytotoxic activity. Moreover, in chapter 3 of this thesis we found an abundance of CD8⁺ T cells in the epithelial layer of both *H. pylori*- infected and uninfected gastric mucosa. All the genes involved in cytotoxicity are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelial layer of *H. pylori* infected patients compared with uninfected patients (EP- HP⁺/HP⁻)

When *H. pylori* -infected and uninfected epithelium were compared (EP- HP⁺/HP⁻), as shown in Fig. 5.14, 28 genes associated with this cytotoxic group were found. Among them, 22 genes were up-regulated during infection including *FAS*, *NKG7*, *PRF1*, *GZMK*, *GZMH*, *GZMB*, *GZMA*, *FASLG*, *FAIM*, *FADD*, *DAPK1*, *DAP*, *DEDD2*, *DAP3*, *CASP8*, *CASP7*, *CASP4*, *CASP3*, *CASP1*, *APAF1*, *ACIN1* and *API5* while *CASP10*, *NCR2*, *NCRI*, *CIDEC*, *DAPK3* AND *FLJ39616* were down-regulated during infection. Since *H pylori* bacteria mainly resides in epithelial tissues, either in the gastric mucus or on the surface of epithelial cells, this may explain the strong killing response occurring here in the epithelial layer of *H. pylori* positive subjects as the body attempts to eliminate this gastric pathogen. The most marked fold change was found with the *GZMA* gene (>300-fold). Other changes observed were less apparent.

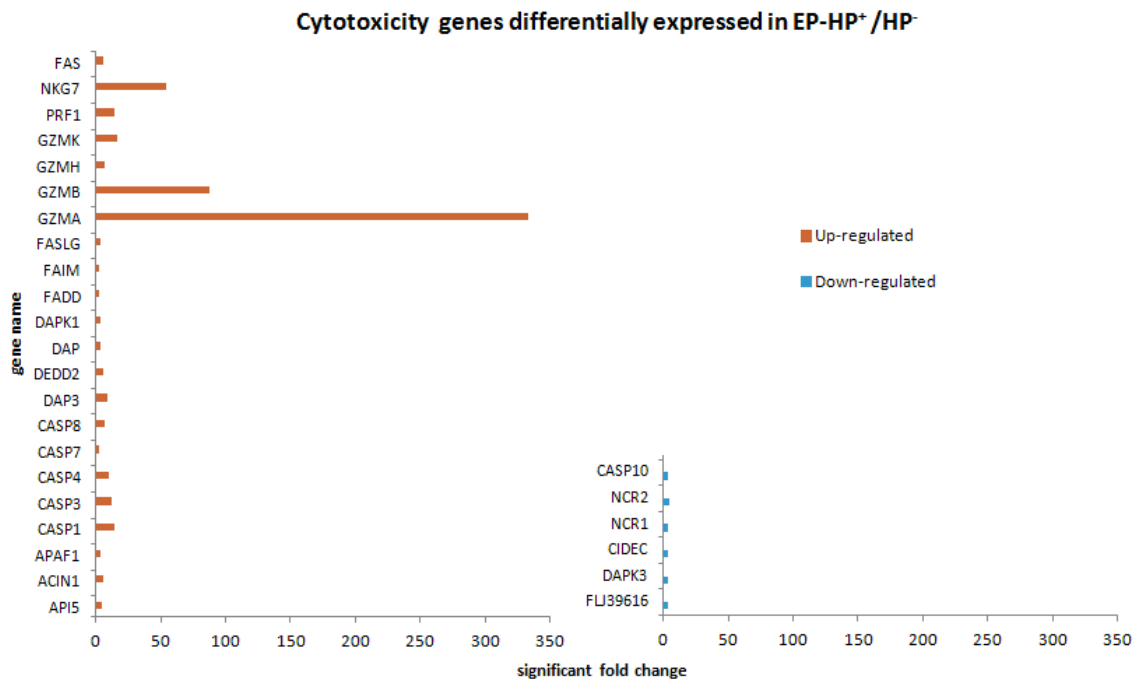


Fig. 5.14 Bar charts showing comparative analysis of cytotoxic genes differentially expressed in EP layer of *H. pylori* -infected and uninfected patients

Group 2: Lamina propria layer of *H. pylori* infected patients compared with uninfected patients (LP- HP⁺/HP⁻)

When *H. pylori* -infected and uninfected lamina propria were compared (LP- HP⁺/HP⁻), as shown in Fig. 5.15, 10 genes associated with cytotoxicity were found. Among them, 9 genes were up-regulated during infection including *PRF1*, *GZMK*, *GZMH*, *GZMB*, *GZMA*, *FAIM3*, *DEDD*, *AVEN* and *API5* while only *CASP6* was down-regulated during infection. The fold changes here were small (<10-fold difference). Interestingly, the fold change of *GZMA* and *GZMB* were markedly lower compared with the epithelium.

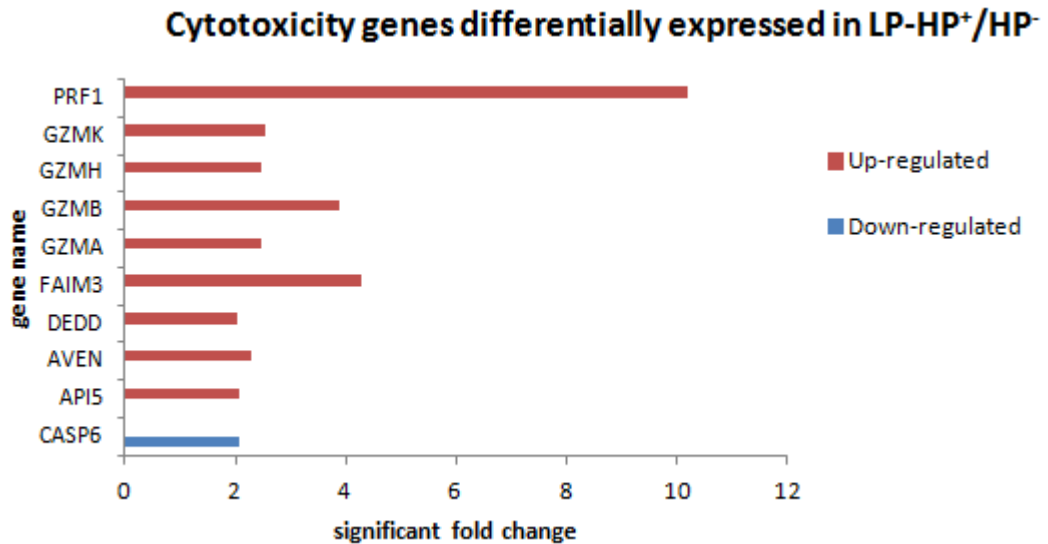


Fig. 5.15 Bar charts showing comparative analysis of cytotoxic genes differentially expressed in LP layer of *H. pylori* -infected and uninfected patients

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺ - EP/LP)

In the third group, in the interlayer comparison between epithelium and lamina propria of *H. pylori* -infected patients (HP⁺ -EP/LP), as shown in Fig. 5.16, 17 genes associated with cytotoxicity were found. Among them, 14 genes were up-regulated in the epithelium including *NKTR*, *NKG7*, *GZMK*, *GZMH*, *GZMB*, *GZMA*, *FASLG*, *DAPK2*, *CASP8*, *CASP4*, *CASP2*, *CASP1*; *COPI*, *CASP1* and *ACIN1* while *DAPK1*, *DAP* and *CASP10* were down-regulated in the epithelium. In infection, greater numbers of cytotoxic genes were up-regulated in the epithelium compared with the lamina propria and this correlates with where *H. pylori* normally reside within the gastric mucosa.

Cytotoxicity genes differentially expressed in HP⁺EP/LP

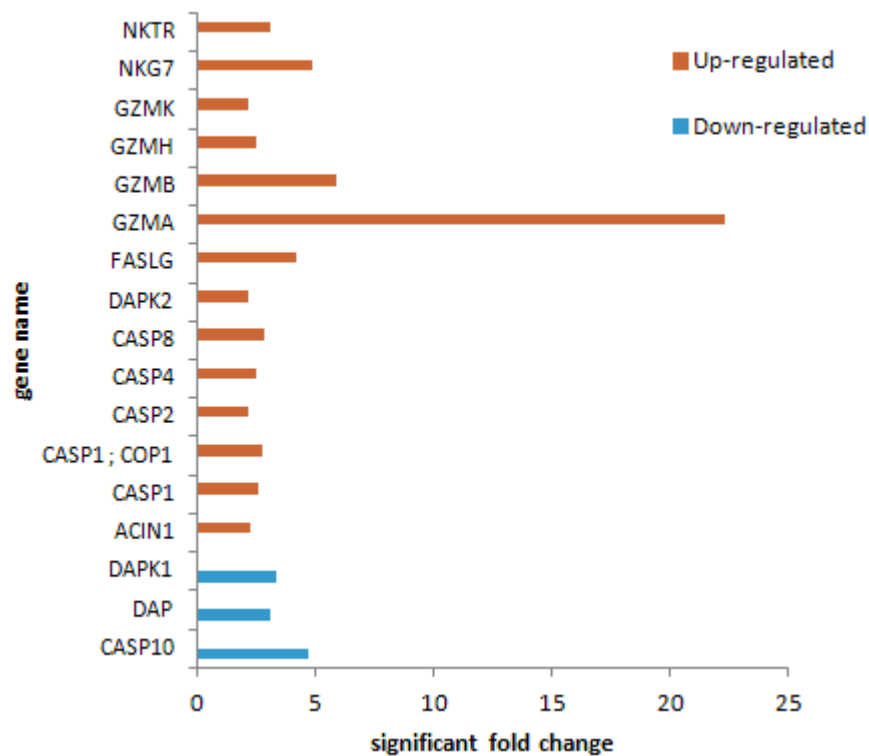


Fig. 5.16 Bar charts showing comparative analysis of cytotoxic genes differentially expressed in EP and LP layer of *H. pylori* infected patients

Group 4: Epithelium compared with lamina propria of *H. pylori* uninfected patients (HP⁻-EP/LP)

In the final group, in the interlayer comparison between the epithelial and lamina propria layer of *H. pylori* uninfected patients (HP⁻-EP/LP), as shown in Fig. 5.17, 28 genes associated with cytotoxicity were found. Among them, 9 genes were up-regulated in the epithelium including *FAF1*, *FAS*, *NKTR*, *FASTK*, *GZMK*, *GZMB*, *GZMA*, *DIP* and *DAPK1* while *DAP*, *DEDD2*, *DAP3*, *CASP8*, *CASP6*, *CASP4*, *CASP3*, *CASP2*, *CASP1; COP1*, *CASP1*, *ACIN1*, *FBF1*, *NCR2*, *NCRI*, *CIDECD*, *DAPK3*, *CASP10*, *FLJ39616* and *AATF* were the 19 down-regulated genes in the epithelium. Therefore, it is evident that there are greater numbers of down-regulated genes related to cytotoxicity in the epithelium than in the lamina propria layer in normal healthy gastric mucosa.

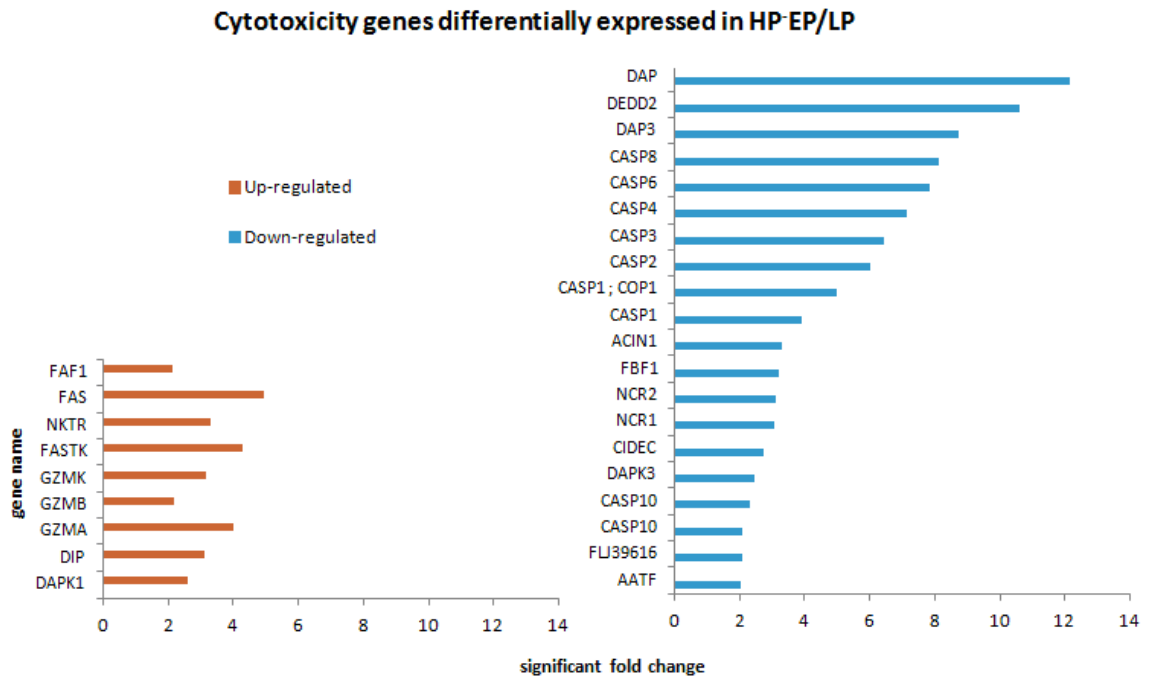


Fig. 5.17 Bar charts showing comparative analysis of cytotoxic genes differentially expressed in EP and lamina propria layer of *H. pylori* uninfected patients

5.2.4.3 Cytokines

Cytokines are proteins released by immune cells in response to various stimuli which play key roles in regulating inflammation, and innate and adaptive immunity (Janeway., 2008). In this study, the immune cells were T cells and NK cells and the stimulus was *H. pylori*. *H. pylori* is already known to induce IFN- γ , TNF- α , IL-1, IL-6, IL-8, IL-17 and IL-18 production (Karttunen *et al.*, 1995; Moss *et al.*, 1994; Noach *et al.*, 1994; Tomita *et al.*, 2001). A large number of genes related to cytokine production were found to be differentially expressed upon comparison of *H. pylori* -infected and uninfected patients. The genes involved in cytokine release are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelial layer of *H. pylori* infected patients compared with uninfected patients (EP- HP⁺/HP⁻)

In the first group analysed, when comparing the epithelial layer of *H. pylori* infected and uninfected subjects (EP- HP⁺/HP⁻), as shown in Fig. 5.18, 23 genes involved in cytokine production were found. The 17 up-regulated genes during infection were *LITAF*, *TBRG1*, *TNFSF5IP1*, *TNFSF8*, *TNFSF4*, *TNFSF14*, *TNFSF13B*, *TNFSF10*, *LTB*, *ILF3*, *ILF2*, *IL8*, *IL17*, *IL1B*, *IFI30*, *IFI16* and *IFNG* while the 6 down-regulated genes during infection were *LBP*, *TNFSF11*, *NUP62*, *IL28A*, *IL16* and *IL1F8*. Therefore, expression of genes encoding a number of pro-inflammatory cytokines including IFN- γ , IL-1- β and TNF was increased significantly. *IL8* was the most significantly up-regulated gene with a >50-fold increase observed. In addition, a significant increase in *IL17* expression was observed. IL-17 is produced mainly by a subset of CD4⁺ cells called T_h17 cells (Dong, 2006). The main function of IL-17 secreting cells is to mediate inflammation through stimulation of inflammatory cytokines and chemokines which in turn induce recruitment of neutrophils and macrophages to the infection site (Kabir, 2011). Recently, a role for IL-17 in *H. pylori* infection has been elucidated (see section 4.1).

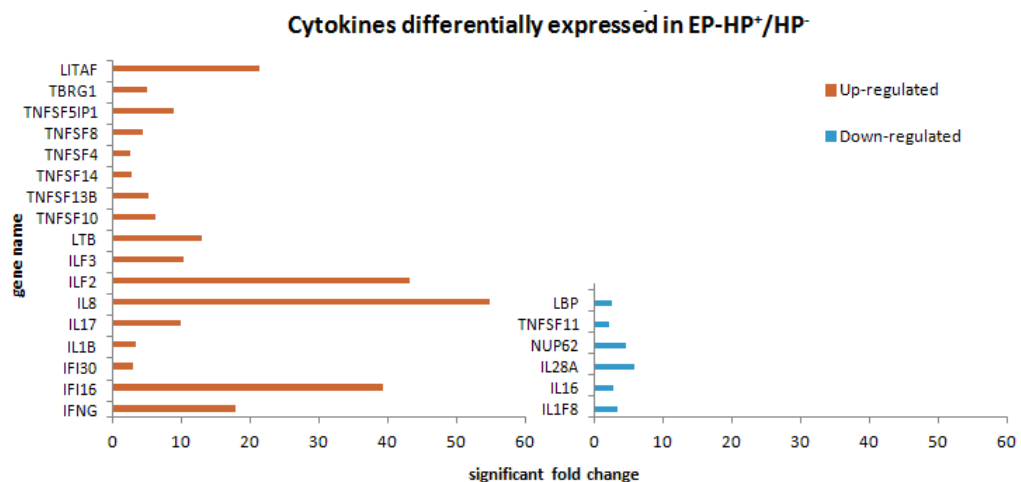


Fig. 5.18 Bar charts showing comparative analysis of genes involved in cytokine production differentially expressed in EP layer when *H. pylori* infected and uninfected patients were compared

Group 2: Lamina propria layer of *H. pylori* infected patients compared with uninfected patients (LP-HP⁺/HP⁻)

In the second group, when comparing the lamina propria layer of *H. pylori* infected and uninfected samples (LP- HP⁺/HP⁻), the expression of 14 genes involved in cytokine secretion were found to be altered (Fig. 5.19). All genes were up-regulated during infection and these included *TGFB1*, *TBRG1*, *TGFB111*, *TNF*, *TNFSF8*, *LITAF*, *ILF3*, *IL6*, *IL411*, *IL32*, *ISGF3G*, *IFI30*, *IFI16* and *IFNG*. TNF is important in the induction of apoptosis and inflammatory responses (Szlosarek & Balkwill, 2003) and a role in *H. pylori* infection for TNF has already been shown (Zhao *et al.*, 2010). While IL-8 was markedly increased in the *H. pylori* infected epithelium, it was not increased beyond that expressed in normal mucosal lamina propria. IL-6 is produced by T cells and is known to stimulate the immune response during infection (Odenbreit *et al.*, 2006). Fold changes were small reaching a maximum of 6-fold.

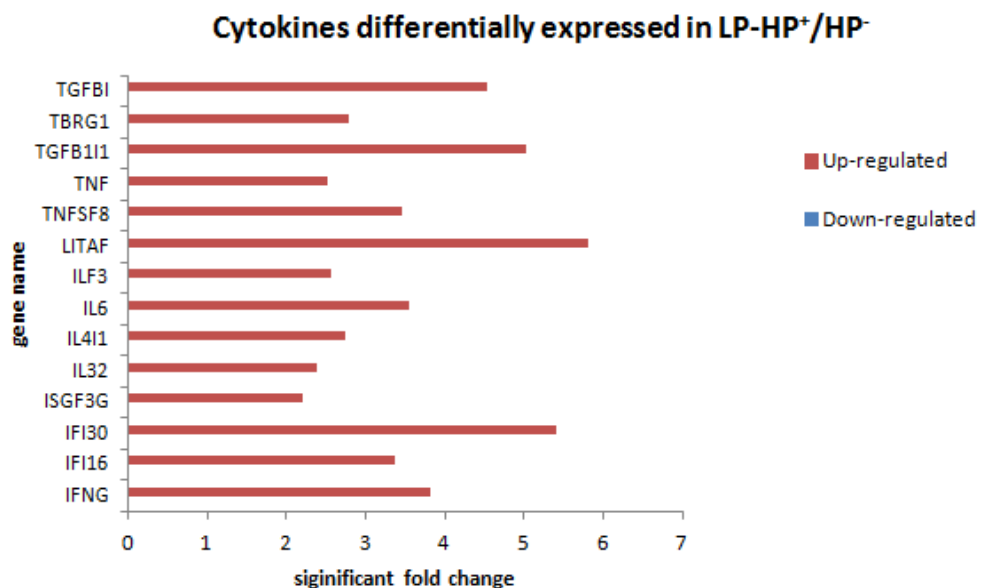


Fig. 5.19 Bar charts showing comparative analysis of genes involved in cytokine release differentially expressed in LP layer of *H. pylori* infected versus uninfected patients

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺ - EP/LP)

In the third group the interlayer comparison between the epithelial and lamina propria layer of *H. pylori* infected subjects (HP⁺ - EP/LP), 17 genes involved in cytokine secretion were found (Fig. 5.20). The 6 up-regulated genes in the epithelium were *TBRG1*, *TNFSF4*, *IL26*, *IL17*, *IFI16* and *IFNG* while the 11 down-regulated genes in the epithelium included *TGFBI*, *IL1B*, *TGFB111*, *IL1A*, *IL1B*, *IL6*, *TGFB1*, *IL411*, *IFI30*, *IL23A* and *IL16*. Therefore, modifications in gene expression patterns could be seen mainly among genes encoding the pro-inflammatory cytokines which included IFN- γ , IL-1, IL-16, IL-17, IL-23, IL-26 and TGF- β .

Overall, in the lamina propria layer, all genes involved in cytokine production were up-regulated, while in the epithelium, there was gene both up- and down-regulated in *H. pylori* infected tissues compared with controls. Moreover, there were a greater number of genes differentially expressed in the epithelium and the fold-change was far greater when compared with the lamina propria tissues.

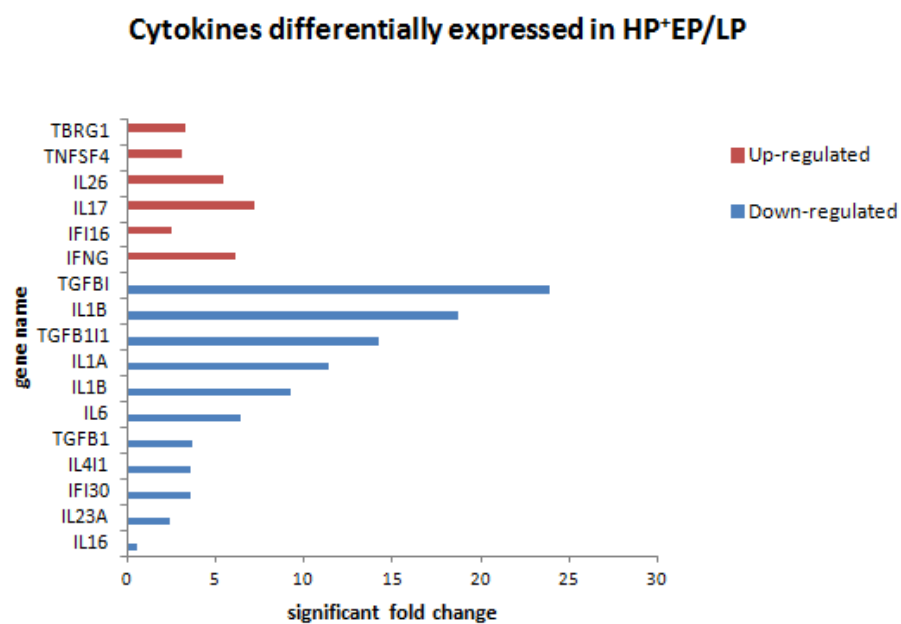


Fig. 5.20 Bar charts showing comparative analysis of genes involved in cytokine release differentially expressed in EP and LP layer of *H. pylori* infected patients

Group 4: Epithelium compared with lamina propria of *H. pylori* uninfected patients (HP⁻-EP/LP)

In the final group in the interlayer comparison between the epithelium and lamina propria layer of *H. pylori* -negative patients, (HP⁻- EP/LP), 48 genes involved in cytokine production were identified (Fig. 5.21). The 5 up-regulated genes in the epithelium were *TGFB2*, *NUP62*, *IL28A*, *IL17* and *IL16* while the 43 down-regulated genes in the epithelium included *IL6ST*, *IFRD1*, *IRF2BP2*, *TNFAIP8*, *IRF1*, *ILF2*, *IFRD1*, *IL1B*, *TACC1*, *TNFAIP3*, *IRF2BP2*, *IL1B*, *IL8*, *IL6ST*, *ISG20L2*, *TACC2*, *IFIH1*, *TNFSF10*, *IFIT3*, *TNFSF5IP1*, *ILF3*, *IFI16*, *IFITM3*, *ISG20*, *TNFAIP1*, *TNFSF13B*, *IFI44*, *IFITM2*, *IL6ST*, *IRF8*, *IRF3*, *IFI27*, *IRF6*, *ISG20L1*, *IL23A*, *TNFAIP2*, *TGFB11I*, *IFI35*, *IFIH1*, *TGFB1*, *IFI30*, *IFRD2* and *TNFSF4*. A change in gene expression of genes encoding both pro- and anti-inflammatory cytokines were evident in normal mucosa as the natural differences that exists between the two gastric layers was seen once again. Cytokines have many different functions in cell signalling, and since the two gastric layers differ in their composition of CD2⁺ cells, their function, their antigenic encounters and environmental stresses, it is understandable that the differentially expressed genes involved in cytokine production in normal gastric mucosa are revealed. The most marked change was *IL8* gene expression (>70 fold). Other notable increases were in the range of 2-60 fold. The findings highlight that in normal uninfected mucosa, many cytokine genes were expressed in the epithelium at significantly higher levels compared with the lamina propria.

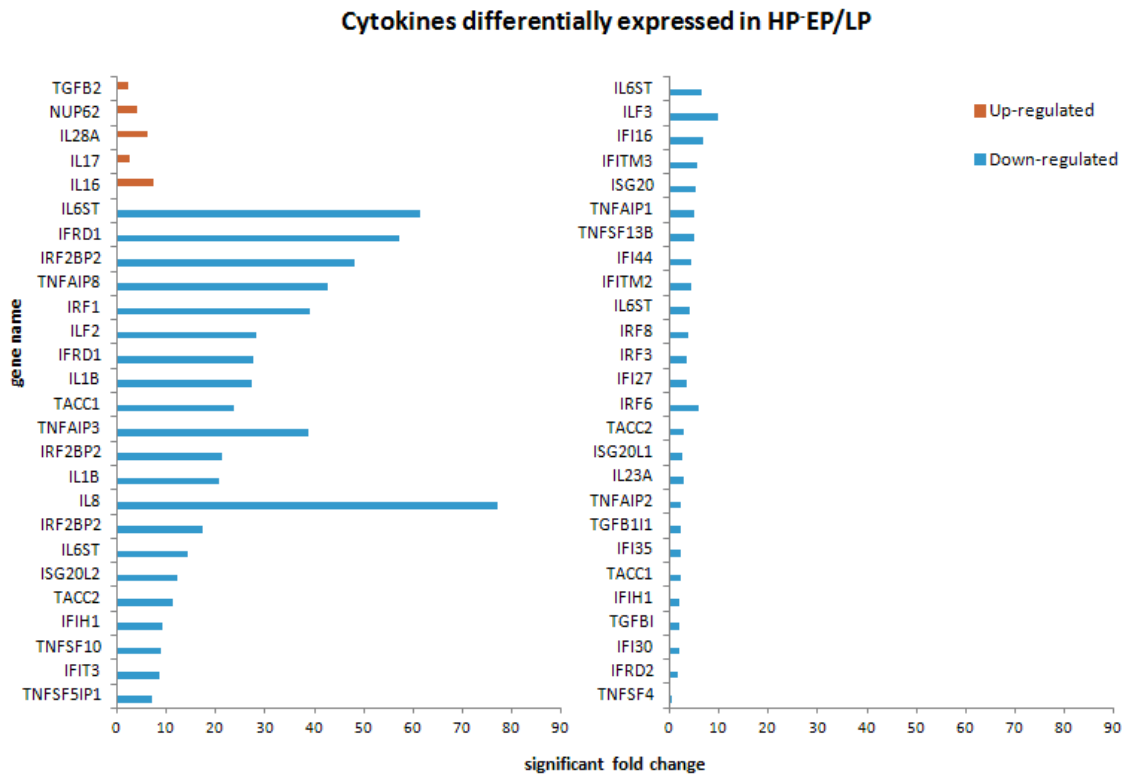


Fig. 5.21 Bar charts showing comparative analysis of genes involved in cytokine release differentially expressed in EP and LP layer of *H. pylori* uninfected patients

5.2.4.4 Signal Transduction

Modifications in expression of key genes involved in signal transduction or cell-cell interactions were observed in each of the four groups analysed. Signalling involves the interaction of various ligands or components with cell surface markers which in turn triggers an immune cascade of events leading to a physiological response. *H. pylori* triggers intracellular pathways in immune cells, such as NF- κ B (Peek Jr *et al.*, 2010). The genes involved in signal transduction are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelial layer of *H. pylori* infected patients compared with uninfected patients (EP- HP⁺/HP⁻)

In the first group when the epithelium of *H. pylori* infected and uninfected subjects were compared (EP- HP⁺/HP⁻), as shown in Fig. 5.22, there was altered expression of 5 genes associated with signal transduction. Among them, *PHPT1*, *CD8A*, *CD2BP* and *CD2BP2* were up-regulated, while *TCR* was the sole down-regulated gene during infection. When the TCR interacts with Ag and MHC molecules, T cells are subsequently activated so down-regulation of the TCR here implies lower levels of T cell activation in *H. pylori* infected epithelium. CD8 is expressed on T_c cells; T cells interact with MHC molecules upon Ag stimulation to initiate a cascade of immune events. Fold changes ranged from 4-19 fold.

Differentially expressed genes involved in signal transduction in EP-HP⁺/HP⁻

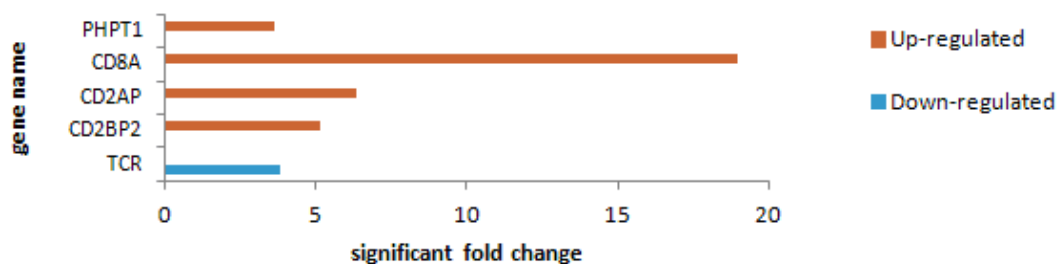


Fig. 5.22 Bar charts showing comparative analysis of genes involved in signal transduction differentially expressed in EP layer of *H. pylori* infected versus uninfected patients

Group 2: Lamina propria layer of *H. pylori* infected patients compared with uninfected patients (LP- HP⁺/HP⁻)

As shown in Fig. 5.23, when the lamina propria of *H. pylori* infected and uninfected subjects (LP- HP⁺/HP⁻) were compared, 3 genes associated with signal transduction were found, all of which were up-regulated during infection. These included *CD28*, *CD8B1* and *CD8A*. CD28 is a co-stimulatory molecule expressed on T cells involved in signalling (Riha & Rudd, 2010).

Differentially expressed genes involved in signal transduction in LP-HP⁺/HP⁻

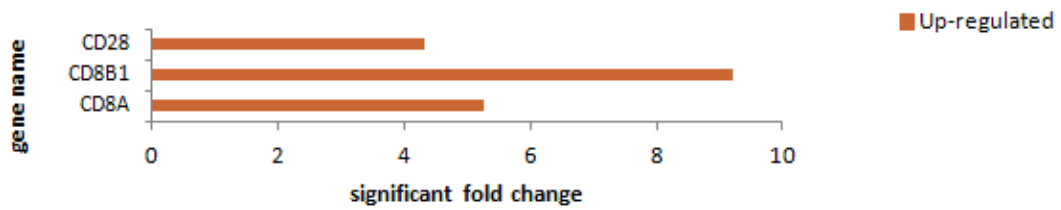


Fig. 5.23 Bar charts showing comparative analysis of genes involved in signal transduction up-regulated in LP layer of *H. pylori* infected versus uninfected patients

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺-EP/LP)

In the third group, when an interlayer comparison of the epithelium and lamina propria of *H. pylori* infected mucosa (HP⁺- EP/LP) was undertaken, 2 genes associated with signal transduction were found. Among these *CD8B1* was up-regulated while *CD28* was down-regulated in the epithelium (Fig. 5.24). The fold changes here however were only minimal.

Differentially expressed genes involved in signal transduction in HP⁺EP/LP

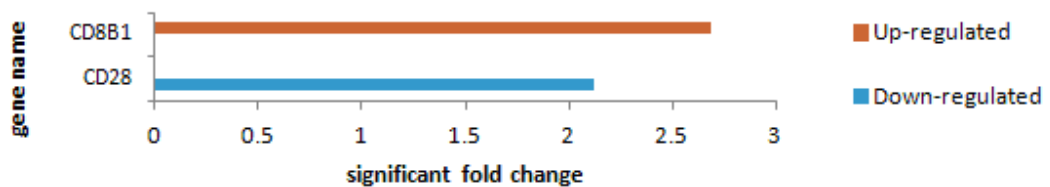


Fig. 5.24 Bar charts showing comparative analysis of genes involved in signal transduction differentially expressed in EP and LP layer of *H. pylori* infected patients.

Group 4: Epithelium compared with lamina propria of *H. pylori* uninfected patients (HP⁻-EP/LP)

In the fourth group, when an interlayer comparison of the epithelium and lamina propria layer of normal *H. pylori* negative mucosa (HP⁻-EP/LP) was carried out, 4 genes

associated with signal transduction were found. Among these, *CD8B1* was up-regulated, while *CD2AP*, *CD2BP2* and *CD8A* were down-regulated in the epithelium(Fig. 5.25).

Differentially expressed genes involved in signal transduction in HP-EP/LP

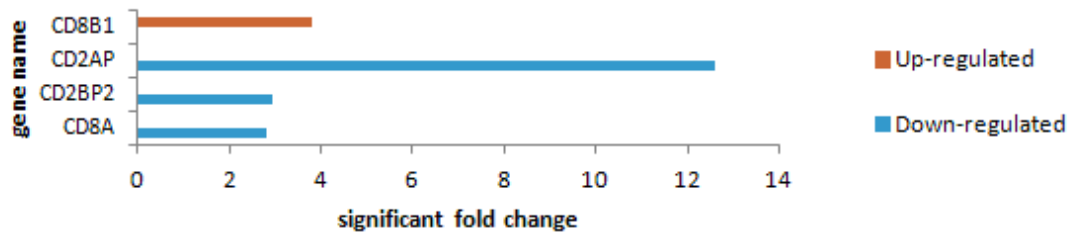


Fig. 5.25 Bar charts showing comparative analysis of genes involved in signal transduction differentially expressed in EP and LP layer of *H. pylori* uninfected patients

5.2.4.5 Receptors and their corresponding ligands

Modifications in receptor expression may be an important step in the body's ability to mount an immune response to *H. pylori*. For example, TLRs are PRRs of the innate immune system implicated in pathogen recognition (Harris *et al.*, 2006). The genes involved in receptor and ligand expression are listed by group, together with their full title, fold value and *p*-value in Appendix III.

Group 1: Epithelial layer of *H. pylori* infected patients compared with uninfected patients (EP- HP⁺/HP⁻)

In the first group when the epithelium of *H. pylori* infected and uninfected subjects were compared (EP- HP⁺/HP⁻), 65 genes involved in receptor and ligand expression were found to have altered expression. The 39 up-regulated genes during infection were *FASLG*, *CD8B1*, *CD8A*, *CD44*, *SELPLG*, *SLIC1*, *ILKAP*, *ITGB7*, *ITGB6*, *ITGB2*, *ITGB1*, *ITGAV*, *ITGAL*, *ITGAE*, *ITGA6*, *ITGA4*, *ITGB4BP*, *ITGB3BP*, *ITGB1BP1*, *ITM2C*, *CKLFSF6*, *CKLFSF4*, *CKLFSF3*, *CKLF*, *CXCR6*, *CXCR4*, *CXCL5*, *CXCL3*,

CXCL2, CXCL13, CXCL1, CCRL2, CCR6, CCL5, CCL4, CCL3; CCL3L1; CCL3L3, CCL20, XCL2 and *XCL1; XCL2* (Fig. 5.26). Regarding the function of some of these receptors and ligands, SELPLG, a selectin, is involved in recruitment to site of infection as well as cell adhesion along with CD44 whose functions also include cell migration, and lymphocyte activation were also modified in infected tissues (Nácher *et al.*; Washington *et al.*, 1994). FASLG, is involved in apoptosis. The most significant fold change was seen in integrin alpha E, also known as CD103, (*ITGAE* >100-fold increase in expression) Integrins receptor expression which is important in attachment of cells with its surrounding tissues and cell signalling and recruitment of neutrophils etc was altered following infection. The 26 down-regulated genes included *LIFR, CD8B1, IBSP, ITGB4, ITGAD, ITGA8, ITGA2B, ITGB1BP2, ITGB1BP3, CKLFSF7, CKLFSF1, CMKOR1, CCBP2, CXCL14, CXCL12, CX3CL1, CCL26, CCL25, CCL22, CCL2, CCL19, CCL14; CCL15, CCL13, CCL11* and *CCL1*(Fig. 5.26). Regarding the function of some of the proteins encoded by the above genes, LIFR, a leukemia inhibitory factor receptor is believed to play a role in cell growth and development (Kasukabe *et al.*, 1994). A large number of chemokine ligands were up-regulated in CD2⁺ cells in the infected gastric epithelium, these include, *XCL1;XCL2, CCL20, CCL25, CCL3, CCL4, CCL5, CXCL1, CXCL13, CXCL2, CXCL3, CXCL5, CXCL9, CD44, Selectin P ligand, CD8, CKLF, CKLFSF4, CKLFSF6, ITM2C, ITGB1BP1, ITGB3BP, ITGA4, ITGA6, ITGAE, ITGB1, ITGB7, ILKAP, ITGAL* and *ITGAV*. Up-regulation of chemokine receptors *CCR5, CCR6, CCRL2, CXCR4* and *CXCR6* was also evident in infected epithelial tissue. Chemokines are widely known to function through chemotaxis and have a wide range of functions from immune surveillance to inflammatory effects and immune response to infection. Among the chemokine ligands down-regulated in CD2⁺ cells in the infected epithelium include *CCL14, CCL15, CCL2, CCL25, CXCL12,*

CCBP2, CKLFSF1, CKLFSF7, ITGA8, ITGB4, IBSP, ITGBL1, CD8B1 and LIFR. CMKOR1 was the sole chemokine receptor whose gene expression was down-regulated in infected gastric epithelium.

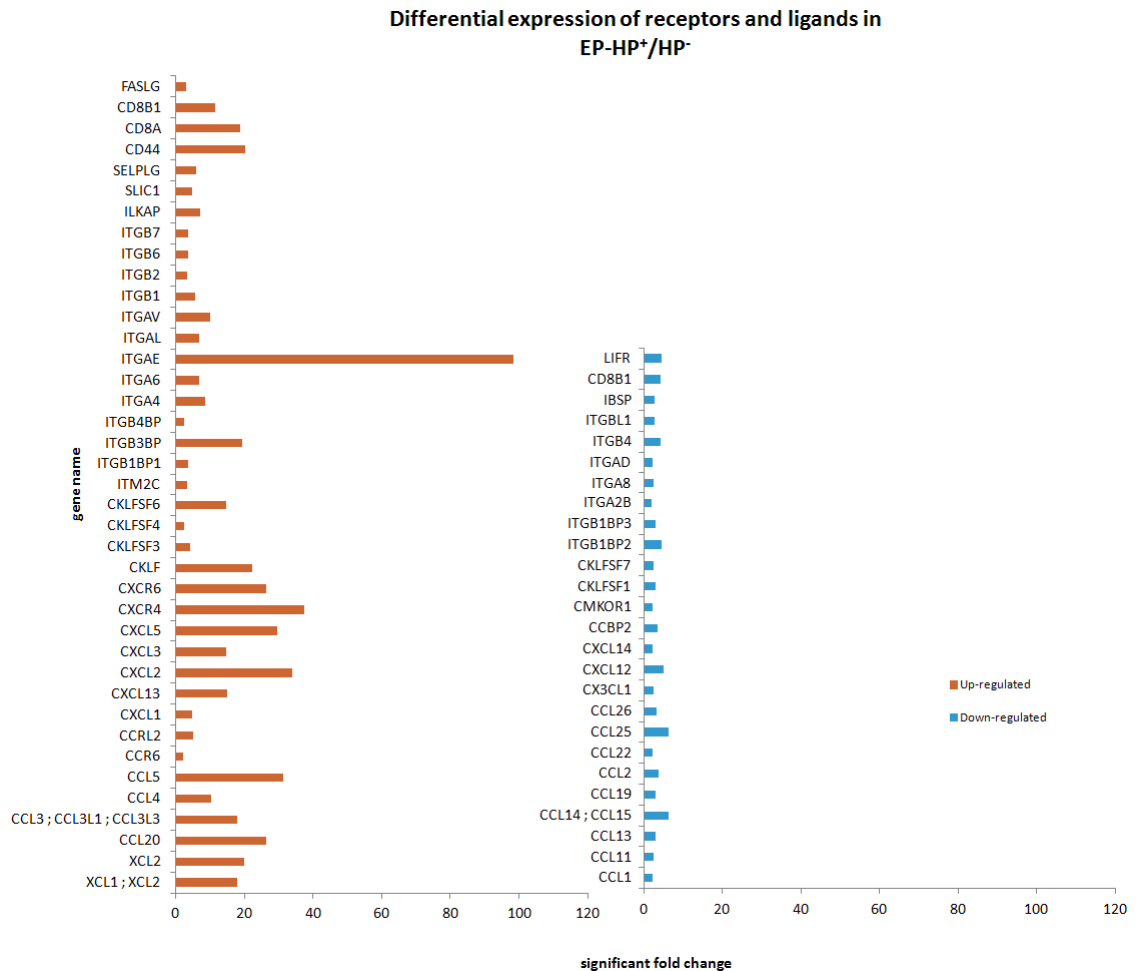


Fig. 5.26 Bar charts showing analysis of genes involved in receptor and ligand expression up-regulated and downregulated when the EP layer of *H. pylori* infected and uninfected patients were compared

Group 2: Lamina propria layer of *H. pylori* infected patients compared with uninfected patients (LP- HP⁺/HP⁻)

As shown in Fig. 5.27, when the lamina propria of *H. pylori* infected and uninfected subjects were compared (LP- HP⁺/HP⁻), 42 genes involved in receptor and ligand expression were found. The 39 up-regulated genes during infection were *LIF*, *CD8B1*, *CD8A*, *CD44*, *SELPLG*, *SLIC1*, *SELL*, *SELE*, *ITGB7*, *ITGB2*, *ITGB1*, *ITGAL*, *ITGAE*,

ITGA9, ITGA8, ITGA4, ITGB1BP1, CKLFSF6, CKLF, CMKOR1, CXCR6, CXCR4, CXCL5, CXCL14, CXCL13, CXCL12, CXCL10, CCR7, CCR6, CCR5, CCR10, CCL5, CCL4, CCL3; CCL3L1; CCL3L3, CCL20, CCL2, CCL11, XCL2 and XCL1; XCL2, while the 3 down-regulated genes were *ITGB6, ITGA6* and *ITGB5*. In comparison to group one, there are similarities in the families of receptors and ligands being differentially expressed in *H. pylori* infected gastric mucosa. For instance, integrins, selectins, chemokines were differentially expressed in both *H. pylori* epithelium and lamina propria when compared with uninfected controls.

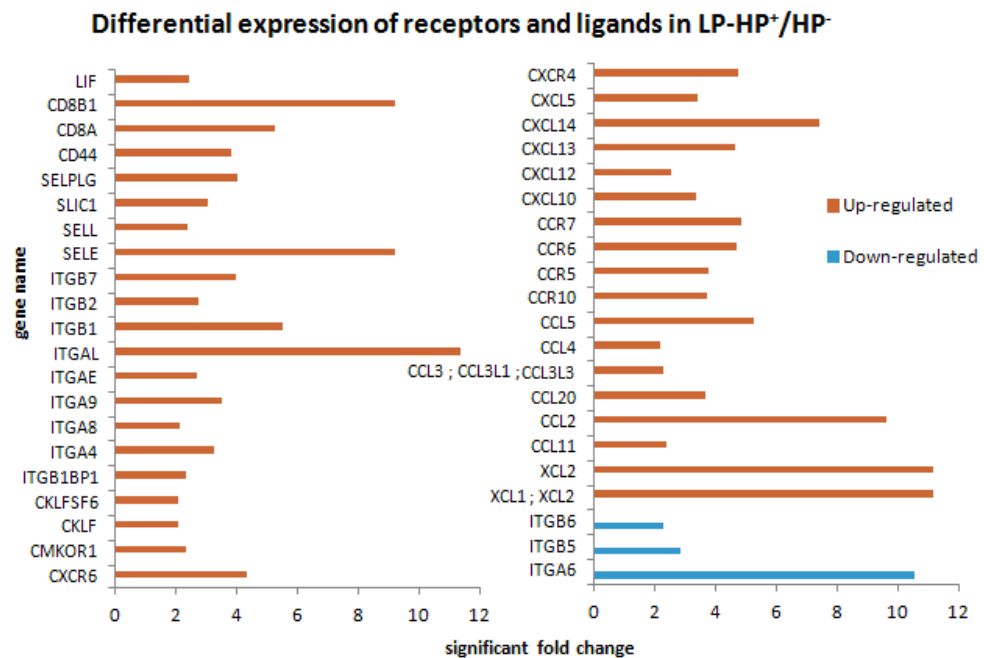


Fig. 5.27 Bar charts showing analysis of genes involved in receptor and ligand expression up-regulated and down-regulated when the LP layers of *H. pylori* infected and uninfected patients were compared

Group 3: Epithelium compared with lamina propria of *H. pylori* infected patients (HP⁺ - EP/LP)

In the third group following an interlayer comparison of *H. pylori* infected epithelial and lamina propria layer tissues (HP⁺ -EP/LP), 40 genes associated with receptor and ligand expression were found. The 11 up-regulated genes in the epithelium were *FASLG, CD8B1, SLIC1, ITGAE, ITGA6, CKLF, CXCR6, CXCL13, CCRL2, CCR5* and

CCL5 (Fig. 5.28) while the 29 down-regulated genes were *LIFR*, *LIF*, *OSMR*, *CD44*, *SELE*, *ITGB1*, *ITGAM*, *ITGAL*, *ITGA9*, *ITGA8*, *ITGA6*, *ITGA5*, *CMKOR1*, *CXCL9*, *CXCL3*, *CXCL2*, *CXCL16*, *CXCL14*, *CXCL12*, *CXCL10*, *CXCL1*, *CX3CL1*, *CCR7*, *CCR6*, *CCR10*, *CCL4*, *CCL3*; *CCL3L1*; *CCL3L3*, *CCL2* and *CCL11* (Fig. 5.28). *CXCL14* was the most significantly down-regulated gene (>100-fold decrease). Regarding function, literature suggests *OSMR* may be involved in inflammation (Pradeep *et al.*, 2010). Moreover, this correlates with a study by Zeaiter *et al.*, (2011) showed that oncostatin M and oncostatin M receptor expression was increased in *H. pylori* infection (Zeaiter *et al.*, 2011). Up-regulation of genes encoding chemokine receptors *CCR5*, *CXCR4*, *CXCR6*, *CMKOR1* and *CKLFSF6* was detected. Up-regulation of members of the integrin family involved in cell adhesion and cell signalling were also detected along with *SELE*, involved in inflammation and tumour cell response (Läubli & Borsig, 2010; Svensson *et al.*, 2009), *SELPLG*, *CD44*, *CD8B*, *SL1C1* and *CD8B1*. The fold change of up-regulated genes was low (<6-fold increase) in comparison with down-regulated genes (>100-fold decrease)

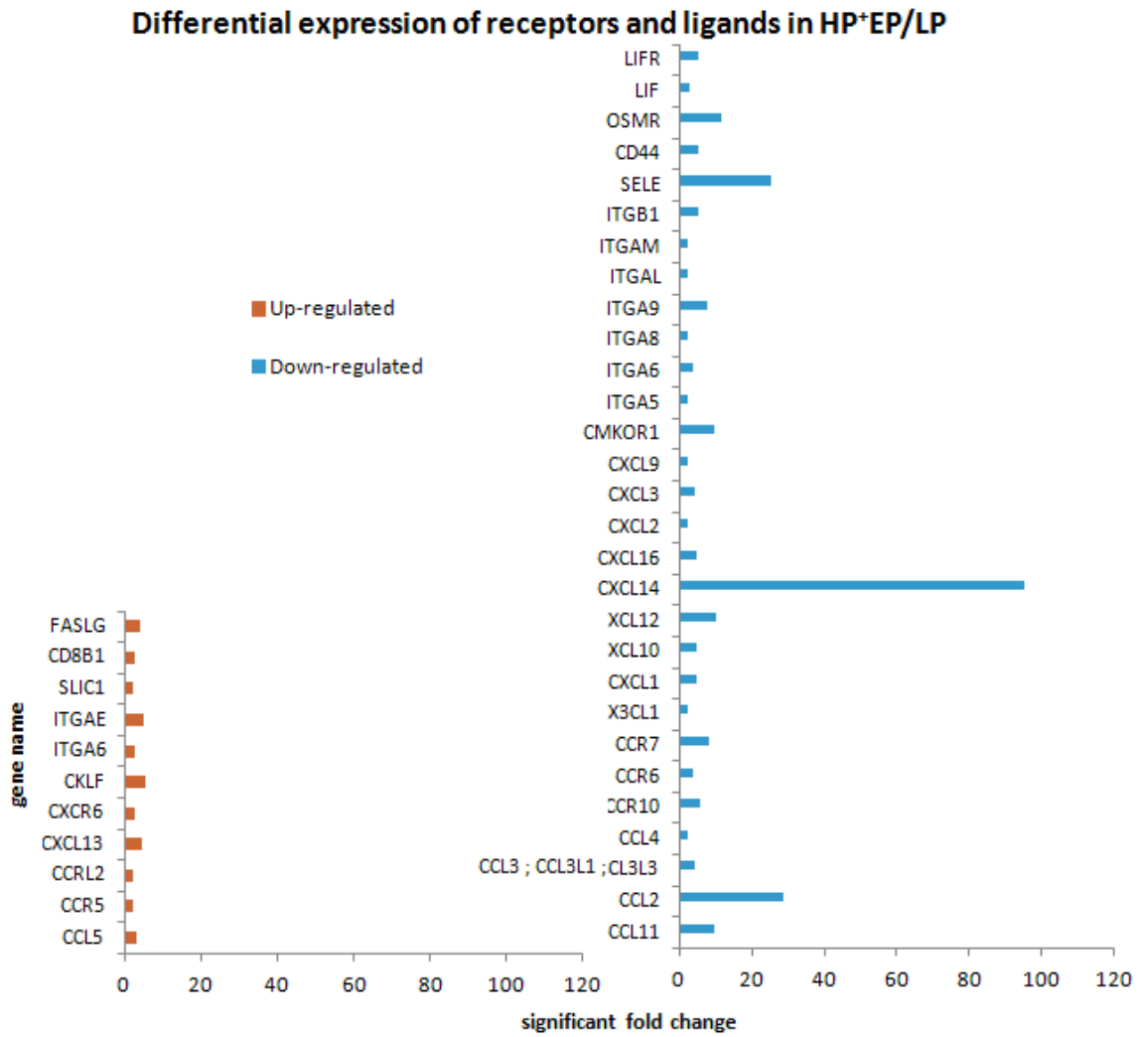


Fig. 5.28 Bar charts showing analysis of genes involved in receptor and ligand expression up-regulated and down-regulated when the EP and LP layers of *H. pylori* infected patients were compared

Group 4: Epithelium compared with lamina propria of *H. pylori* uninfected patients (HP⁻-EP/LP)

In the fourth group, following an interlayer comparison between the epithelium and lamina propria of *H. pylori* negative subjects (HP⁻-EP/LP), 50 genes involved in receptor and ligand expression were found. Among these, 21 genes were up-regulated in the epithelium including *CD8B1*, *IBSP*, *ITGBL1*, *ITGB4*, *ITGB3*, *ITGAD*, *ITGA8*, *ITGA2B*, *ITGB1BP3*, *ITGB1BP2*, *CKLFSF1*, *CMKOR1*, *CCBP2*, *CXCL12*, *CCL5*, *CCL26*, *CCL25*, *CCL14*; *CCL15*, *CCL13* and *CCL1* (Fig. 5.29), while the 30 remaining down-regulated genes in the epithelium included *OSMR*, *CD44*, *CD8A*, *CD2BP2*, *ILKAP*, *ITGB6*, *ITGB5*, *ITGB1*, *ITGAV*, *ITGAE*, *ITGA6*, *ITGB3BP*, *CKLFSF8*, *CKLFSF6*, *CKLFSF4*, *CMKOR1*, *CXCR6*, *CXCR4*, *CXCL9*, *CXCL5*, *CXCL3*, *CXCL2*, *CXCL16*, *CXCL14*, *CXCL12*, *CXCL1*, *CCL5*, *CCL4*, *CCL3*; *CCL3L1*; *CCL3L3* and *CCL20* (Fig. 5.29).

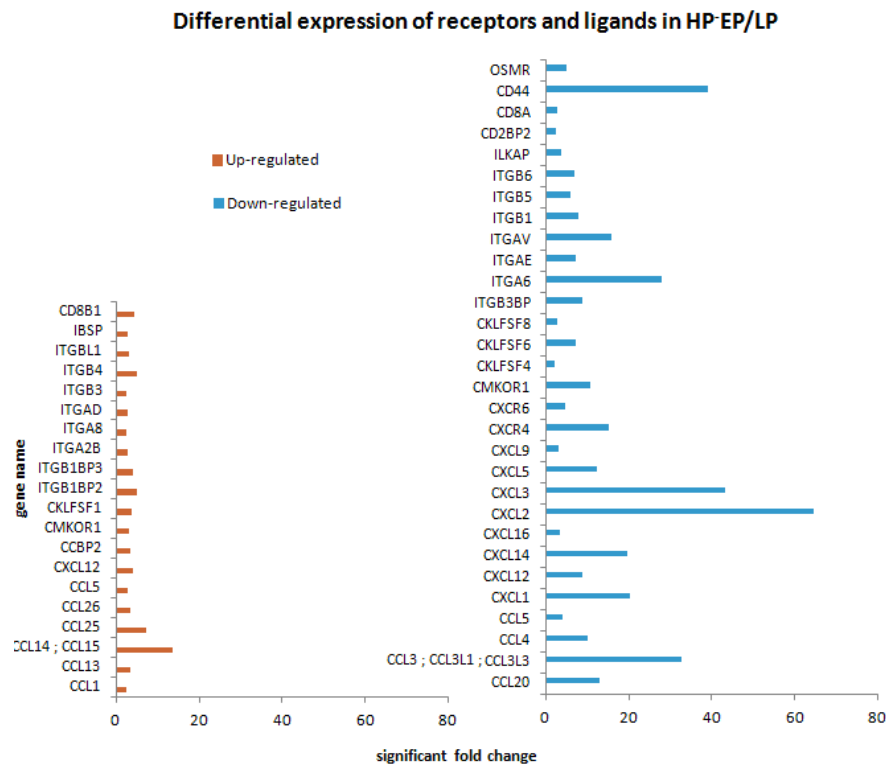


Fig. 5.29 Bar charts showing analysis of genes involved in receptor and ligand expression up-regulated and downregulated in the EP layer compared with the LP layer of normal healthy mucosa

5.2.4.6. Other genes of interest

Several additional genes were differentially expressed in *H. pylori* infected and uninfected patients. For instance, other immunologically relevant genes that do not fall into the above functional categories or genes potentially involved in lipid binding membrane integrity or cancer progression, in innate or inflammatory responses listed by group, together with their full title, fold value and *p*-value in Appendix III.

Since *H. pylori* is classed as a Class I Carcinogen by IARC (IARC, 1994), it is important to analyse the microarray data in further detail, for potential markers of cancer differentially expressed in *H. pylori* disease and non disease states. When genes involved in cancer progression were examined, as shown in Table 5.1, when CD2⁺ cells in the epithelium of *H. pylori* infected and uninfected subjects were compared, our study revealed and differential expression of 10 genes. The 8 up-regulated genes included *ECT2*, *GDDR*, *ALOX5*, *ALOX5AP*, *COTL1*, *LEPR*, *LEPROTL1* and *PTGS2* while the two down-regulated genes were *PGA5* and *ST5*. Next, when CD2⁺ cells in the lamina propria layer of *H. pylori* infected and uninfected subjects were compared, 5 genes were differentially expressed, all of which were up-regulated. These included *GIF*, *ALOX5AP*, *LEPROTL1*, *MMP9* and *PTGS1*. GIF has recently been proposed as a potential marker for cancer diagnosis (Wu *et al.*, 2012), hence its up-regulation here in *H. pylori* infected lamina propria tissues compared with controls is an interesting finding. *LEPR* (leptin receptor) and *LEPROTL1* (leptin receptor overlapping transcript-like 1) were up-regulated in *H. pylori* infected epithelium when compared with controls while *LEPROTL1* alone was up-regulated in infected lamina propria layer tissues. Leptin is believed to have an involvement in the link between obesity and cancer. Carroll *et al.*, (2011) examined and compared mRNA expression of leptin and others in mammary adipose tissue from cancer patients and cancer patients with known breast

cancer risk factors such as obesity and metabolic syndrome and found significant increases in leptin expression in cancer patients with metabolic syndrome (Carroll *et al.*, 2011). In keeping with the link between obesity and cancer progression, Allott *et al.*, (2011) examined the relationship between visceral obesity and oesophageal adenocarcinoma (OAC) and observed increases in *MMP2* and *MMP9* expression in OAC cell lines cultured with visceral adipose tissues. Moreover, upon examination of biopsies from patients with OAC, the authors recorded elevated levels of *MMP9* gene expression linked to visceral obesity and poor tumour differentiation (Allott *et al.*). Interestingly, our study revealed increased *MMP9* (matrix metalloproteinase 9) expression in lamina propria tissues of *H. pylori* positive patients when compared with control tissues. *ALOX5* (arachidonate 5-lipoxygenase) and *ALOX5AP* (arachidonate 5-lipoxygenase activating protein) were up-regulated in the epithelium when *H. pylori* infected and uninfected tissues were compared. Moreover, *COTL1* expression was also increased. This is another interesting finding since *COTL1* interacts with lipoxygenase 5. Lipoxygenase is a catalyst involved in the oxidation of polyunsaturation of fatty acids resulting in alterations of cell structure and metabolism as well as the formation of molecules involved in cell signalling (Brash, 1999). Lipoxygenase expression changes at different stages of cancer (Pidgeon *et al.*, 2007) and therefore lipoxygenase up-regulation in *H. pylori* infected epithelial and lamina propria layer tissues here may be relevant since *H. pylori* is one of the leading causes of gastric cancer (Wroblewski *et al.*, 2010). Other genes encoding eicosanoids differentially expressed in disease and non-disease states include *PTGS1* (prostaglandin endoperoxide synthase 1) and *PTGS2* (prostaglandin endoperoxide synthase 2). In summary, gene expression profiling has uncovered differential expression of several markers involved in cancer progression when CD2⁺ cells from *H. pylori* infected and uninfected were compared. Since *H. pylori*

is classed as a Class I carcinogen (IARC, 1994), this in an interesting find in understanding the progression of *H. pylori* infection in some patients from gastritis to more severe forms such as gastric adenocarcinoma and MALT lymphoma.

Table 5.1 Other differentially expressed genes of interest in *H. pylori* infected and uninfected patients grouped by function in cancer.

Genes related to cancer				
	Gene name	Gene symbol	Fold change	p-value
EP- HP ⁺ /HP ⁻	ECT2	EP cell transforming sequence 2 oncogene	11.26	0.001
	GDDR	down-regulated in gastric cancer GDDR	10.92	0.001
	ALOX5	arachidonate 5-lipoxygenase	7.15	0.007
	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	11.22	0.0005
	COTL1	coactosin-like 1	60.24	0.016
	LEPR	leptin receptor	4.13	0.0021
	LEPROTL1	leptin receptor overlapping transcript-like 1	14.27	0.013
	PTGS2	prostaglandin-endoperoxide synthase 2	4.45	0.006
	PGA5	pepsinogen 5, group I (pepsinogen A)	-7.85	6.63E-05
	ST5	suppression of tumorigenicity 5	-3.83	0.004
LP- HP ⁺ /HP ⁻	GIF	gastric intrinsic factor (vitamin B synthesis)	3.94	3.65E-05
	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	2.74	0.023
	LEPROTL1	leptin receptor overlapping transcript-like 1	7.04	0.007
	MMP9	matrix metalloproteinase 9	2.4	0.004
	PTGS1	prostaglandin-endoperoxide synthase 1	2.6	0.013

EP = epithelium; LP = lamina propria; HP = *H. pylori*; p indicates significance; minus numbers represent genes under-expressed

5.2.5 Quantitative real-time PCR

Quantitative Real-Time PCR was used to confirm expression data obtained for 5 genes (albeit with increased expression, decreased expression and with no difference in fold-change) detected by microarray analysis. When stored RNA was analysed prior to quantitative Real-Time PCR, epithelial layer RNA had degraded and so only lamina propria layer RNA was used for this portion of the study (n = 2) (Fig. 5.30).

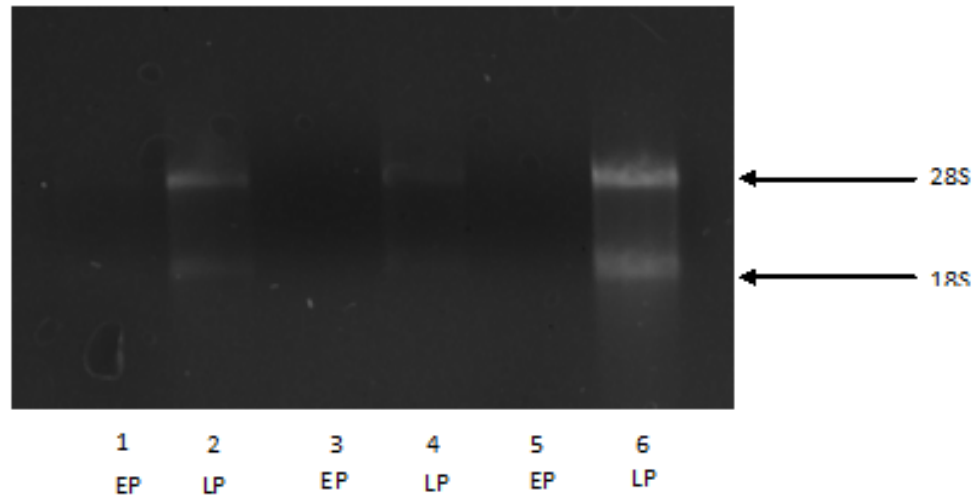


Fig. 5.30 Agarose gel image showing RNA from *H. pylori* negative patients with minimal gastritis and gastric inflammation (lanes 1 & 2), *H. pylori* negative patients with severe gastritis (lanes 3 & 4) and *H. pylori* positive patients (lanes 5 & 6). EP layer RNA has degraded in all gastric samples.

Real time RT-PCR results were normalised by using a reference gene (GAPDH and B2M) whose expression remains constant under the conditions being investigated. Although the fold changes recorded by the microarray and real time RT-PCR differed for certain genes, the general profile of genes differentially expressed albeit up-regulated or down-regulated was seen (Table 5.2).

Table 5.2 Fold change comparison of five genes of LP CD2⁺ RNA by real-time RT-PCR and microarray analysis

Gene	Fold change	
	Microarray	Real-Time RT-PCR
CXCL14	11.2	3.99
ITGA6	10.5	3.27
CD160	-0.6	0
CTLA4	-1	0
IL-8	-1.9	-1

For example, CXCL14 displayed increased expression (11.2-fold increase) by microarray analysis and when expression was recorded by real time RT-PCR (3.99-fold), albeit to a slightly lesser extent (Fig 5.31). Overall, these similar results are in concordance with the changes in gene expression seen in the microarray data despite the

slight quantitative variations in the values obtained using the two different methodologies.

Amplification curves of CXCL14 in lamina propria layer of *H. pylori* positive and *H. pylori* negative mucosa

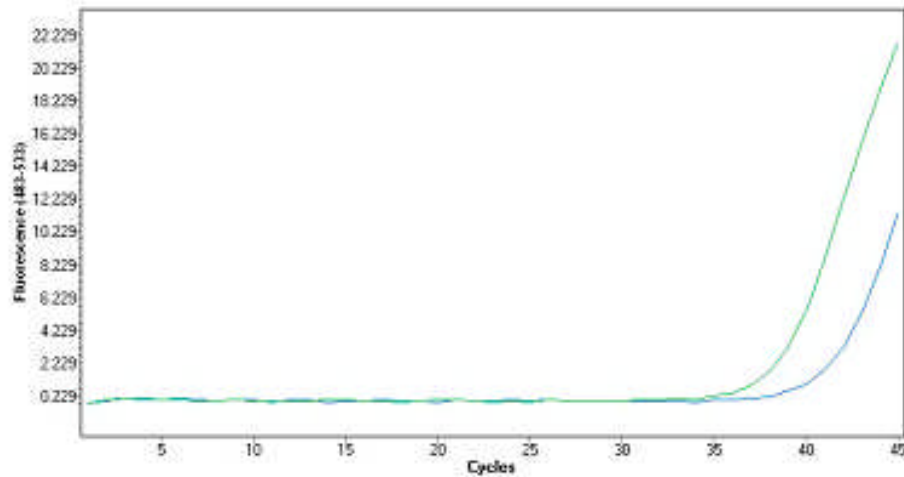


Fig. 5.31 represents the amplification of CXCL14 in *H. pylori* negative LP while shows the amplification of CXCL14 in *H. pylori* positive lamina propria. Green curve represents LP from *H. pylori* positive patients and blue curve represents the LP from *H. pylori* negative mucosa

5.3 DISCUSSION

Here, we reported microarray findings following gene expression profiling in gastric antral mucosa (epithelium and lamina propria layers) from *H. pylori* infected and uninfected subjects. Our results demonstrated that gene expression in the epithelial and lamina propria layer of *H. pylori* positive patients differed in relation to inflammatory and immunological reactions as well as in the functional categories of proliferation, signal transduction, cytokine production and receptor expression among others. In this chapter, the GEP focused on the CD2⁺ cell population which incorporates both innate and adaptive components as well as being comprised of unconventional T cells thus providing a new and comprehensive way of comparing inflammatory and immune responses in the gastric mucosa with and without *H. pylori* infection. Utilising gene expression profiling as a method of analysing thousands of genes simultaneously in *H. pylori* infection as a useful tool for the identification of genes differentially expressed in disease versus non disease state.

To our knowledge, this is the first time gene expression profiling of human epithelial and lamina propria layer tissues of fresh gastric biopsies have been investigated. Recent literature analysing GEP during *H. pylori* infection mainly focuses on cDNA microarray cell line (e.g. AGS, Caco2) or *in vitro* studies (Bach *et al.*, 2002; Chiou *et al.*, 2001; Cox *et al.*, 2001; Maeda *et al.*, 2001). However, such methodologies may not accurately reflect what actually happens *in vivo* in *H. pylori* infected gastric mucosa. Some of these cell lines do not mimic the gastric tissues physiologically and do not support productive infection. There have been similar GEP studies carried out on animal models such as rhesus macaques (Huff *et al.*, 2004) and it is debatable to what extent immunological responses of animals infected with *H. pylori* in a laboratory situation can be compared to what is actually happening in the GIT when it comes in

contact with this gastric pathogen. Rhesus macaques when in captive environments may become infected with *H. pylori* were inoculated with a human strain *H. pylori* J166, gastric antral biopsies were extracted and following analysis of DNA microarray genes involved in cytokine and chemokine production, innate immunity, apoptosis and signal transduction were among the genes differentially expressed when disease and non-disease states were compared. The publications that concentrated on human tissues worked with whole gastric biopsies (Hofman *et al.*, 2007; Mannick *et al.*, 2004; Wen *et al.*, 2004; Yang *et al.*, 2012) where differentially expressed genes involved in cancer progression as well as signal transduction and inflammatory responses were investigated. Hence, our study is novel since we separated the whole gastric biopsies into its constituent layers therefore providing an in depth insight into responses of both the epithelial and lamina propria layers to *H. pylori* infection individually. Moreover, we concentrated on a population of cells called the CD2⁺ cell population which includes both T cells and NK cells thereby focusing on the response of this specialised group of lymphocytes. Therefore, we are obtaining an insight into both innate and adaptive immune responses to *H. pylori* which is important as *H. pylori* is known to trigger both innate and adaptive immune systems (see chapter 1). Our approach led to the identification of thousands of genes whose expression was modified in disease state and through data processing, transcriptional changes of key immunological/inflammatory genes were observed whose expression has changed in *H. pylori* infected state.

Top 20 most differentially expressed genes

Upon analysis of the top 20 genes with the highest fold change, many novel immunologically relevant genes not previously associated with *H. pylori* infection were found.

When the epithelium of *H. pylori* positive patients was compared with the epithelium from *H. pylori* negative patients, amongst the top 20 differentially expressed genes, many genes implicated in key immunological responses such as stress and inflammation, T cell activation and gut T cell trafficking were found. Moreover, only two of the top 20 differentially expressed genes identified have been previously associated with *H. pylori* infection (CTLA4 and CD69). In addition, 3 genes encoding markers of cytotoxicity were found [*CTLA4*, *GZMA* and *KLRC1;KLRC2* (Cheng *et al.*, 2006; Gunturi *et al.*, 2004; Henkart, 1994)] implying a strong killing response by epithelial lymphocytes. Taken together, it appears there is a complex cascade of events occurring in the *H. pylori* infected epithelium whereby the body is attempting to control infection through initiation of such immune responses. It would be interesting to analyse these results further by investigating whether these genes are translated into proteins as this would provide us with more comprehensive understanding of the host immunologic response to this gastric pathogen. The lamina propria layer appears to play a very different role than that of the epithelium as none of the genes differentially expressed in *H. pylori* infected lamina propria layer overlapped with the top 20 genes differentially expressed in the epithelium. When the lamina propria of *H. pylori* infected and uninfected subjects were compared, an increase in *PNLIP* which is implicated in peptic ulcers (Raffensperger, 1951) was seen. *PNLIP* was the most up-regulated gene in this microarray study (>625-fold). Increased expression of other genes linked to an involvement with peptic ulcers were also seen here [*AMYL1A*; *AMLY1B* (Rogers, 1960)]. Hence, could these genes now be linked to peptic ulcers as a result of *H. pylori* infection? Genes encoding markers involved in membrane integrity [*GKN1* and *ITGA6* (Kwok *et al.*, 2007; Martin *et al.*, 2002; Oien *et al.*, 2004)] were amongst the top 20 most down-regulated genes when the lamina propria of *H. pylori* infected subjects were

compared with controls. Therefore, severe gastric injury must also be taking place even in the lamina propria during *H. pylori* infection. This also poses the question whether CD2⁺ cells are attempting to control and suppress inflammatory responses in the lamina propria layer. Interestingly, upon analysis of the top 20 differentially expressed genes, we also uncovered genes involved in tumourigenesis and gastric cancer, all interesting finds since approximately 20% of *H. pylori* infected individuals progress on to develop more severe forms of disease. Therefore, uncovering the possible genes responsible for the disease progression in a certain percentage of the population would undoubtedly impact on future therapy possibilities. Of the 80 most differentially expressed genes, many possess key immunological roles such as T cell activation, cell activation and differentiation, apoptosis, cell adhesion and membrane integrity and cytokine production in other infection and disease states but few have been implicated in *H. pylori* infection to date.

Genes implicated in innate immunity

CD2⁺ cells (T and NK cells) are implicated in both innate and adaptive immunity and since *H. pylori* is a known potent stimulator of the innate immune system (Lee & Josenhans, 2005) we firstly analysed innate immune response genes differentially expressed due to *H. pylori* infection. Transcriptional changes of genes involved in innate immunity induced by *H. pylori* infection have been documented in the literature. Hofman *et al.*, (2007) carried out GEP on both whole gastric antral and fundus biopsies and reported up-regulation of *TLR2* and *LY96*, both of which are associated with signal transduction and bacterial recognition in the gastric antrum following infection. In addition, we found up-regulation of the several other genes involved in innate immunity. Among them, *SELE* which plays a role in cell adhesion and inflammation;

STAT1, important for cell viability following interaction with pathogens; *TNFSF13B*, *PLSCR1* and *NKG7* involved in immunosurveillance and apoptosis and members of the KIR gene family, including KIR2DL4, KIR3DL2 and KIR3DL1, involved in regulation of the immune response. It is possible that unconventional T cells (which are known to bridge innate and adaptive immunity), NK cells (which form part of the CD2⁺ cell population), and/or indeed NKR⁺ T cells (which bear both T and NK receptors) may be the orchestrators in innate immune responses to *H. pylori* infection. We have already shown significant differences in NKR⁺ T cell populations in the gastric epithelium and lamina propria during *H. pylori* infection. As stated previously, a study by our group revealed significant increases in CD161⁺ T cells in the epithelium and decreases in CD56⁺ T cells in the lamina propria of *H. pylori* infected subjects compared to controls (O'Keeffe *et al.*, 2008), therefore it is possible NKR⁺ T cells are playing a role in the immune response to this gastric pathogen.

Inflammatory genes

Both acute and chronic *H. pylori* infection is known to be a causative agent of gastric inflammation. *H. pylori* infection triggers the recruitment of immune cells to the infection site following recognition of bacterial PAMPs by host cell PRRs. This leads to a cascade of innate events involving the activation of transcription factors such as AP-1 and NF- κ B which leads to differentiation and activation of immune cells as well as chemokine and cytokine production. A polarised T_h1 response (IFN- γ and IL-12) is a signature of *H. pylori* infection; this complex list of cytokines attempting to rid the body of this pathogen is rarely effective in clearing infection and in fact can contribute to pathogenesis by causing severe gastric injury (reviewed in O'Keeffe *et al.*, 2008).

Hence, analysing genes involved in inflammatory responses may play a role in determining the clinical outcome of disease.

Wen *et al.*, (2004) investigated the GEP of inflammatory genes in *H. pylori* infected gastric mucosa and reported elevation in *IL7R*, *CXCR4*, *CXCL2*, *CXCL13*, *CCL18*, *VCAM1* and Ag presenting genes in *H. pylori* infection. In agreement with these findings, our data revealed an increase in *IL7R*, *CXCL2*, *CXCR4*, *CXCL13*, *VCAM1* expression in the epithelium of *H. pylori* infected mucosa when compared with healthy mucosa and significant up-regulation in *IL7R*, *CXCR4* and *CXCL13* in *H. pylori* infected lamina propria layer tissue when compared with healthy tissues. Furthermore, we found significant up-regulation of many other inflammatory genes in infected epithelium when compared with uninfected epithelium. Among these, genes encoding inflammatory markers such as *CEBPB*, *BCL6* and *ABCF1* which may be induced by TNF and possibly has a role to play in inflammation (Klein *et al.*, 1999; Richard *et al.*, 1998), *SCYE1* a small inducible cytokine induced by apoptosis, *IL2RB* and *FOSL2* are involved in cell proliferation and differentiation (Hein *et al.*, 2009; Matsui *et al.*, 1990); while *ELMO2*, *CCL20* (Ito *et al.*, 2011). Interestingly *CASP1*, which is known to be implicated in inflammation (Mariathasan *et al.*, 2004; Sewell *et al.*, 1986) was up-regulated in epithelial tissues.

Next, when *H. pylori* infected lamina propria tissue was compared with uninfected tissue the data revealed that *CXCL14*, a known tumour suppressing chemokine (Komori *et al.*, 2010), *MIF*, *ABCF1*, *C4A*; *C4B*, *IL-1R1* and *IL-1RN* were all up-regulated. Finally when an interlayer comparison was made in *H. pylori* infected subjects, the expression of *IFNGR1*, *IL1R2*, *IL6* and *IL17* which stated earlier is important for neutrophil recruitment were all increased. In addition, a notable finding was an increase in expression of *IL26*. This gene encodes a cytokine expressed by T_H1

and T_h17 cells that has been implicated with intestinal inflammation and the recent literature has shown that its expression is up-regulated in Crohn's disease (Dambacher *et al.*, 2009). Here we report *IL26* is up-regulated in *H. pylori* infected epithelium when compared with infected lamina propria.

The above findings indicate just how differently the cells in the epithelium and the lamina propria layer tissues respond following *H. pylori* infection. While both induce an inflammatory response, the markers of inflammation are quite different in the epithelial and lamina propria layer. Results showed significant increases in the epithelium of a large set of genes encoding cytokines known to be vital in the body's immune response to infection. While certain cytokines such as IFN- γ and IL-17 have already been shown to play important roles in *H. pylori* infection, our study has revealed differential expression of many other genes involved in cytokine and chemokine production that have not been previously linked with *H. pylori* infection. Moreover, considerable decreases in a number of chemokines and cytokines were observed in the epithelium when compared with the lamina propria. Upon analysis of the number of genes differentially expressed and the degree of fold change, it can be hypothesised that the epithelium plays a more significant role in the inflammatory response to *H. pylori*. Knowing that 85% of *H. pylori* bacteria are known to reside in epithelial tissues while 10% live in mucous linings and the remaining 5% at intracellular matrices, it is not surprising that there are so many changes in gene expression of inflammatory genes in the epithelium. Since host responses, and in particular the polarised T_h1 response to *H. pylori* infection is known to be important to the overall clinical outcome of infection (Lindholm *et al.*, 1998), the manner which CD2⁺ cells of the epithelium and lamina propria respond are quite important.

Cytokines and chemokines

H. pylori is known to induce cytokine production and it is widely known that *H. pylori* infection causes a polarised T_H1 response resulting in the production of pro-inflammatory cytokines such as IFN- γ (Kusters *et al.*, 2006). The microarray data determined alterations in expression of cytokines and chemokines, along with their receptors and ligands during *H. pylori* infection. While chemokines such as IL-8 and RANTES have already been implicated in *H. pylori* infection (Shimada & Terano, 1998), this study has uncovered many chemokines not previously associated with *H. pylori* infection. IFN- γ , TNF- α , LTB (which is also a member of the TNF family), IL-10RA and IL-17R (which is implicated in neutrophil recruitment to *H. pylori* infected tissues (Kabir, 2011)) cytokines were increased upon infection. The main function of IL-17 secreting cells is to mediate inflammation through secretion of inflammatory cytokines and chemokines which in turn promote recruitment of neutrophils and macrophages to the infection site (Kabir, 2011). Several members of the TNF family were up-regulated in *H. pylori* infected epithelial and lamina propria layers when compared with controls, relevant since TNF is an important factor in inflammatory and apoptotic responses (Szlosarek & Balkwill, 2003). In addition, *IL8* expression was increased >54 fold in *H. pylori* infected epithelium which correlates with previous findings (Goll *et al.*, 2007). IL-8 is a pro-inflammatory cytokine that triggers the onset of inflammation. IL-8 is secreted by various cells including T cells and epithelial cells known to be involved in innate immunity through neutrophil recruitment which has a part to play in *H. pylori* induced gastritis (Ibraghimov & Pappo, 2000). Overall, both pro- and anti-inflammatory cytokines are differentially expressed in the epithelium when *H. pylori* disease and control states were compared. This could be explained by the fact that *H. pylori* infection triggers a polarised T_H1 response involving IFN- γ , IL-1,

IL-8, IL-12, IL-18, IL-23 and more recently IL-17 (Karttunen *et al.*, 1995; Moss *et al.*, 1994; Noach *et al.*, 1994; Tomita *et al.*, 2001). This polarised T_h1 inflammatory immune response can ultimately contribute to the onset of more severe pathologies such as peptic ulcers and gastric cancer. T_{reg} cells have been shown to play a part in *H. pylori* mediated infection by producing cytokines such as IL-10 to minimise gastric injury (Kandulski *et al.*, 2010). Therefore, the body's attempt to elicit an immune response all whilst minimising host injury ultimately leads to the complicated cytokine profile.

In the lamina propria layer, following comparative analysis of genes encoding cytokines and their corresponding receptors, expression of *IL-1*, *IL-2*, *IL-4R*, *IL-6*, *IL-7R*, *IL-10RA*, *IL-18RAP*, *IL-21R*, *IL-32*, *IFNG*, *IFI16*, *IFI30*, *IFNGR1*, *TGFB* and members of the *TNF* family were all increased upon *H. pylori* infection. No down-regulated genes involved in cytokine production were found following a comparative analysis of the lamina propria layer of *H. pylori* infected and uninfected mucosa. Once again, both pro- and anti-inflammatory genes were up-regulated indicating not unlike the epithelium, the host response in the lamina propria is attempting to invoke an immune response while minimising gastric injury or indeed it could be the case that *H. pylori* may be inducing suppressive immune responses from host cells in order to persist in gastric tissues.

Either way, our data strongly suggests CD2⁺ cells in both the epithelium and lamina propria contribute significantly in the body's immune response to *H. pylori* infection through cytokine production. Since cytokines and chemokines form an extracellular signalling network functioning in various aspects of both innate and adaptive immune responses including T and B cell proliferation, inflammation and defence against viruses, changes in their gene expression observed here indicate immense biological relevance.

Cytotoxicity

In this study, several markers of cytotoxicity were among the genes up-regulated the most in the epithelial layer of *H. pylori* infected tissue when compared with healthy tissue. This suggests that there is a very strong cytotoxic killing response taking place in the gastric epithelium upon *H. pylori* infection. A greater number of genes with a higher fold change were recorded in *H. pylori* infected epithelium than in *H. pylori* infected lamina propria following comparison with uninfected control subjects. For instance GZMA was up-regulated >300 fold in *H. pylori* infected epithelium where as this gene was only up-regulated >3 fold in *H. pylori* infected lamina propria layer tissues when each layer was compared with their control counterparts. This may explain the gastric atrophy associated with *H. pylori* infection. Similarly in lamina propria layer tissues, CFLAR which encodes an apoptosis regulator expression was up-regulated in *H. pylori* infected tissue when compared with controls. In fact, 4 anti-apoptotic genes were up-regulated when the lamina propria layer of infected tissues was compared with uninfected tissues (CFLAR, AVEN, API5 and FAIM3). One could hypothesise *H. pylori* is initiating an immune evasion mechanism in order to minimise apoptosis to survive in the gastric epithelium. A study by Müller *et al.*, 2011 also suggests this.

Signal transduction

Signalling involves the interaction of various ligands or components with cell surface markers which in turn triggers an immune cascade of events leading to a physiological response. *H. pylori* triggers intracellular pathways in immune cells, such as NF- κ B, MAPK, beta-catenin as well as signal transducers (Franco *et al.*, 2005; Hisatsune *et al.*, 2008). Beta-catenin (CTNNB1), an important modulator of the Wnt signal transducing pathway during *H. pylori* infection which is involved in tumourigenesis and here we

report its up-regulation in *H. pylori* infected epithelium (10.15-fold; $p = 0.0037$) and lamina propria (2.61, 0.06), albeit to a lesser extent when compared with healthy tissues. This correlates with previous findings which report CTNNB1 has a role to play in cell-cell interactions during *H. pylori* infection (Yang *et al.*, 2012). *TANK*, a TRAF family member associated with NF- κ B activation was up-regulated in *H. pylori* infected epithelium (51.54-fold, $p = 0.0002$) along with *LOC497661*, another NF- κ B activating protein (13.47-fold, $p = 0.002$). Yang *et al.*, (2012) recently carried out a similar study analysing the GEP of whole biopsies in *H. pylori* infected and uninfected subjects and focused on genes involved in signal transduction. The authors recorded differential expression of three genes, notably, over-expression of *PHPT1* while *SCGP* and *MST4* were under-expressed. Our data also revealed under-expression of *SCGP*, encoding a calcium binding protein (Rogstam *et al.*, 2007), occurring specifically in lamina propria layer tissues. Taken together, findings suggest *H. pylori* infection induces up-regulation of several factors involved in signal transduction, important in cell-cell communication when mounting an effective immune response to *H. pylori*.

Membrane integrity/adhesion molecules

During *H. pylori* infection, chronic gastritis and inflammation leads to compromised epithelial layer barrier. This gastric tissue injury is caused by the polarised Th1 immune response and as mentioned earlier can lead to more severe forms of disease. Therefore, analysing changes in gene expression of genes involved in membrane integrity is immunologically important. The transcriptional response of genes involved in maintenance of membrane integrity and intracellular adhesion in *H. pylori* infection was analysed and subsequent altered expressions were identified. *PNN* and *ITGAE*, *ECT2*, *ICAM3* known to bind integrin were up-regulated in infected epithelium. *H. pylori*

infection in the lamina propria elicited transcriptional changes of Reg gene family members with up-regulation of *REG1A*, *REG3A* along with members of the integrin family, genes encoding the intracellular adhesion molecules which are of great interest and relevance.

Gastric cancer

Genes potentially involved in gastric cancer were also examined since *REG4* which encodes a gastrointestinal secretory protein that is a member of the superfamily of C-type lectins is believed to play a part in gastric cancers (Zhang *et al.*, 2003) was among the genes most down-regulated in *H. pylori* infected epithelium when compared with its uninfected counterpart. Zheng *et al.*, (2010) reported up-regulation of *REG4* gene expression in cases of gastric intestinal adenoma and down-regulation occurred following malignant changes in gastric epithelial layer cells. The question arises whether *H. pylori* bacteria have a role to play in the link between RegIV and gastric cancer or whether changes may be an outcome of disease state and progression.

Other down-regulated markers noted in the epithelial layer as a result of *H. pylori* infection included LPS binding protein, BACE1, involved in pepsin A activity. More notable genes with altered GEP in infected lamina propria include *LAT* which is a linker for activation of T cells, *SOCS3* which is a suppressor of cytokine signalling and genes encoding integral membrane proteins, *SLAMF1* which encodes a signalling lymphocytic activation molecule. In contrast, *TP53* (which encodes a tumor protein), *PORIMIN* (pro-oncosis receptor inducing membrane injury) gene along with *ECT2* which encodes an epithelial cell transforming sequence 2 oncogene and *LYZ* (lysozyme) were all up-regulated in *H. pylori* infected epithelium.

Next, transcriptional changes in the lamina propria such as *FYN* oncogene, *CASC3* (cancer susceptibility candidate), *TPT1* (tumour protein), *GIF* (gastric intrinsic factor) and *PIMI* oncogene caught our attention as their role may indeed be important to the CD2⁺ gastric immune response to *H. pylori* infection.

Genes of potential importance were also found to be differentially expressed in the two layers of *H. pylori* infected gastric mucosa. *GDDR* which is a gene down-regulated in gastric cancer, *GIF* which has recently been proposed as a potential marker for cancer diagnosis (Wu *et al.*, 2012), *PCNA* which encodes a proliferating cell nuclear Ag, , *LAT* which encodes a linker for activation of T cells, *ST5* (a suppressor of tumorigenicity), and *OSMR* which has recently been linked with a potential role as a biomarker in gastric cancer (Oue *et al.*, 2009) were all down-regulated.

The above genes of interest are all associated with cancer and hence may be of relevance here as approximately 20% of individuals who are infected with *H. pylori* develop more severe forms of disease such as peptic ulcers and gastric cancer. According to recent statistics by the WHO, gastric cancer is one of the most common cancers worldwide with 736,000 deaths recorded in 2008. *H. pylori* infection is a major risk factor in the onset of gastric cancer; therefore it is important to understand what is happening during infection with this gastric pathogen. Further analysis would need to be undertaken to determine whether some of these genes may be used in the future as possible biomarkers for *H. pylori* associated cancer.

Other genes of immunological relevance

Other genes of potential importance whose expression was modified in *H. pylori* infected were also identified. This is particularly the case amongst the top 20 differentially expressed genes in infected and uninfected subjects. For instance,

ZNFN1A1 was up-regulated in *H. pylori* infected epithelium when compared with uninfected epithelium and may be of importance as it has previously been shown to play a role in stress and inflammation by maintaining homeostasis (Chrousos & Kino, 2005). *EGFR* which was under-expressed in *H. pylori* infected epithelium when compared with uninfected epithelium has been associated with the progression of gastric cancer (Pryczynicz *et al.*, 2009). Other genes of interest not in the top 20 but significantly altered in infection include *MICA*, a gene expressed by NK and T cells and in particular $\gamma\delta^+$ T cells which was significantly up-regulated in *H. pylori* infected epithelium when compared with that of healthy epithelium (4.05-fold increase). Since *MICA* is known to be induced by stresses such as oxidative stress, heat shock and bacterial infection (Groh *et al.*, 1996), its increased expression here may be showing us a responsive role of $\gamma\delta^+$ T cells residing in the epithelium to *H. pylori* infection. Also of interest is the increased expression of *SLAM* genes, important factor for the development of $\gamma\delta^+$ T cells. Data revealed up-regulation of *SLAMF1* (2.90-fold increase), *SLAMF7* (12.19-fold increase) and *SLAMF8* (2.56-fold increase) epithelial tissues during disease state, this finding once again points to a role for $\gamma\delta^+$ T cells in *H. pylori* infection. Significant increases in *SLAMF6* (3.19-fold increase) and *SLAMF8* (2.80-fold increase) were also observed in *H. pylori* infected epithelium when compared with infected lamina propria tissues.

In conclusion, undertaking cDNA microarrays using gastric on CD2⁺ cells isolated from gastric epithelial and lamina propria layers is a useful procedure for investigating changes in the GIT following *H. pylori* infection. Our data adds to a limited group of studies undertaken mainly on cell line cultures, animal models and a small number of human whole gastric biopsies. We have identified a large number of genes differentially expressed in *H. pylori* disease and control healthy mucosa that have not previously been shown. Of particular interest are novel genes identified amongst the

top 20 up-regulated and down-regulated genes that have not previously been linked with *H. pylori* infection. Moreover, we have expanded our analysis to incorporate both epithelial and lamina propria interlayer analysis separately and we have found differences in gene expression patterns with *H. pylori* infection here also. Therefore, our study sheds light to the differences in the immune response obtained from the two layers. Since care was taken from the beginning to ensure only true CLO[®] test negative (asymptomatic) with and CLO[®] test positive (moderate to severe gastritis) fresh gastric biopsies this approach was to purify CD2⁺ cells from epithelial and lamina propria layer of gastric biopsies isolated from severely inflamed *H. pylori* positive and *H. pylori* negative patients devoid of gastric inflammation. This means the purity and composition of the CD2⁺ population differed between the four groups analysed (for example, results from chapter 3 revealed differences in numbers in T and NK cell populations in epithelium and lamina propria layers of normal healthy mucosa and in *H. pylori* infection) and some differences seen in gene expression may be as a result of differential cell purity in the samples in different states so are some genes responsive because of gastritis or is *H. pylori* inducing all of the changes we are seeing? One way to address this would be to carry out a microarray including RNA isolated from *H. pylori* positive patients with severe gastritis, *H. pylori* negative patients with severe gastritis and *H. pylori* negative patients with minimal gastritis. Future studies would involve using this approach to analyse GEP in purified NKT cells or $\gamma\delta^+$ T cells from human gastric biopsies. Presently, the difficulty in obtaining sufficient numbers of these cells from 2-3 biopsies and without generating a cell line limits this approach. In doing this, one would overcome any differences seen in gene expression due to differences in cell composition of the CD2⁺ cell population. There was great difficulty involved in obtaining a sufficient yield of high quality RNA from subjects and in particular from *H.*

pylori positive subjects even though care was taken to ensure that these human samples were handled properly and utilised to their full potential. Since 4 microarrays were carried out, we can hypothesise what is happening *H. pylori* infection. This study gives the opportunity for one to delve into a form of data biomining as this study has generated a huge data resource from which, through data manipulation, further analyses could open doors for subsequent more focused studies, for instance further experimental analysis on genes differentially expressed here that are affiliated with cancer. Hence, this type of study forms an important basis for uncovering new bio-markers of disease and new modes of intervention which in turn offers a deeper insight into *H. pylori* pathogenesis.

Overall, *H. pylori* is eliciting a complex immune response involving both innate and adaptive components. Novel genes involved in tumour progression, ulcers and cancer were amongst the top 20 differentially expressed genes in *H. pylori* infected epithelial and lamina propria tissues when compared with controls. It is evident there is a stronger killing response occurring in epithelial layer tissues as 3 markers of cytotoxicity were found amongst the top 20 most up-regulated genes in CD2⁺ cells when the epithelium of *H. pylori* infected and uninfected subjects were compared. Also, the epithelium seems to play more significant role in *H. pylori* infection as there are higher numbers genes involved in proliferation, cytotoxic, signal transducing, receptors and cytokine production with a greater fold-change occurring in the epithelium than in the lamina propria of *H. pylori* infected mucosa when compared with healthy mucosa.

Chapter 6

General Discussion

6.1 DISCUSSION

H. pylori is a gastric pathogen affecting approximately 50% of the world's population and is now classed as a Class I Carcinogen by the international agency the research of cancer (IARC, 1994). Infection causes gastritis in all patients with the majority of these being asymptomatic (Kusters *et al.*, 2006). However, approximately 10-15% of infected patients develop more severe forms of disease such as severe gastritis, peptic ulcers and a small proportion (1-2%) are implicated with gastric cancers such as gastric adenocarcinoma and MALT lymphoma (Ernst & Gold, 2000). The symptoms, severity and associated conditions vary depending on factors such as bacterial strain, host immune responses, host genetics and environmental factors such as diet (Blaser & Atherton, 2004; Montecucco & Rappuoli, 2001). Infection elicits both systemic and localised immune responses (Ferrero, 2005; Suarez *et al.*, 2006; Velin & Michetti, 2006).

Acute infection is characterised by a localised inflammatory response involving the recruitment of B and T lymphocytes, neutrophils, eosinophils and mast cells to infected tissues, occurring following activation of the transcription factor NF κ B and this process ultimately leads to chemokine (such as RANTES, IL-8 and GRO-1) and cytokine (such as IFN- γ) production (Baeuerle & Henkel, 1994; Bodger & Crabtree, 1998). There is a prerequisite for interaction of immune cells such as epithelial cells, DCs and macrophages with PAMPs such as TLRs and Nod proteins for this acute inflammatory response to occur (Takeda & Akira, 2003; Takeuchi & Akira, 2010). Systemic responses are also induced involving the production of anti-*H. pylori* Abs such as IgA and IgG. However, these Abs are not protective but merely reflect chronicity of infection (Andersen *et al.*, 1996; Rathbone *et al.*, 1986). While both humoral and cellular responses occur, infection with *H. pylori* is predominantly T_h1 driven (Bamford

et al., 1998). This adaptive polarised T_h1 cytokine immune response is ineffective in clearing infection and can actually contribute to pathogenesis leading to chronic infection (D'Elia *et al.*, 1997). The T_h1 response leads to the production of pro-inflammatory cytokines such as IFN- γ which can induce severe inflammation and gastric epithelial injury. The involvement of T_{reg} cells as well as the fact *H. pylori* has developed evasion mechanisms for survival and persistence in the gastric mucosa all contribute to chronicity of infection (Suarez *et al.*, 2006). As stated above, host factors such as the immune response and genetic polymorphisms, bacterial factors such as virulence genes and environmental factors such as smoking and diet all influence severity of infection. Immune cells possess PRRs which interact with bacterial PAMPs such as LPS, peptidoglycan, flagellins lipoproteins etc. TLR4 is a PRR which interacts with bacterial LPS (Miller *et al.*, 2005), however, *H. pylori* bioactivity is 500-1000 times weaker than that of LPS derived from other bacteria (Muotiala *et al.*, 1992) and therefore is a poor activator of host PRRs. Moreover, it seems to be TLR2 and not TLR4 that interacts with *H. pylori* LPS (Smith Jr *et al.*, 2003). The altered structure of *H. pylori* LPS is likely responsible for its weak immunogenicity. *H. pylori* possesses modification of its lipid A structure by removal of phosphate groups from the lipid A disaccharide backbone. *H. pylori* also alters its chemical LPS structure through possession of longer acyl chains or complete removal of acyl chains thereby partaking in immune evasion (Moran, 1998; Moran & Aspinall, 1998; Moran, 2007). Another method of immune evasion is through Lewis^x and Lewis^y blood group Ags that are present in healthy gastric mucosa, the O chains of repeating units on smooth LPS strains are involved in molecular mimicry as the LPS can mimic the structure of host and through molecular mimicry of self-epitopes, and the bacterium may go undetected and persist in gastric tissues. Therefore, the above mechanisms employed by *H. pylori* in

addition to host and environmental factors all play a role in *H. pylori* pathogenesis (Appelmeik *et al.*, 2000; Aspinall *et al.*, 1996; Moran, 2007).

While classical T cells are recognised as the primary orchestrators of adaptive immune responses, the importance of unconventional T cells in infection and immunity is emerging (O’Keeffe Moran 2008; Bendelac *et al.*, 2007; Brigl & Brenner, 2010; Hayday, 2000; Kabelitz, 2011; Kronenberg & Gapin, 2002). Unconventional T cells are unique by displaying both innate and adaptive attributes. NKT cells are defined by a conserved invariant V α 24J α 18 paired with V β 11 TCR canonical arrangement (Porcelli *et al.*, 1993). NKT cells differ from their classical counterparts by recognising glycolipid Ags presented by CD1d present on the surface of immune cells (Sköld & Behar, 2003). This pathway enables NKT cells to react to numerous infectious agents. NKT cell activation can result in either stimulation or suppression of the hosts’ immune responses as NKT cells are capable of inducing different responses depending on particular circumstances (Bendelac *et al.*, 2007; Godfrey *et al.*, 2000; Matsuda *et al.*, 2008). NKT cells mainly populate the liver, spleen and bone marrow; both variant and invariant NKT cell populations also reside in intestinal tissues constituting a portion of IEL and LPL populations (Wingender & Kronenberg, 2008; O’Keeffe *et al.*, 2004). Their location in mucosal tissues along with their innate ability to rapidly produce cytokines, display cytotoxicity and trigger adaptive immune responses makes them an ideal candidate in localised immune responses (Meresse & Cerf-Bensussan, 2009; Wingender & Kronenberg, 2008). $\gamma\delta^+$ T cells are another unconventional T cell population comprising approximately 1-10% of peripheral blood lymphocytes and have a higher incidence in epithelial and intestinal tissues (Bonneville *et al.*, 2010; Carding & Egan, 2002; Kabelitz, 2011). There are several $\gamma\delta^+$ T cell subsets residing in different tissues and possessing varying functions. $\gamma\delta^+$ T cells partake in regulation of

inflammation, pro-inflammatory cytokine production, potent killing effects following encounters with phosphorylated metabolite Ags or mycobacterial ligands. Other known functions of $\gamma\delta^+$ T cells include maintenance of epithelial homeostasis, wound repair, response to infectious pathogens and tumours as well as triggering adaptive immune responses through Ag processing and presentation to classical T cells (Bonneville *et al.*, 2010; Carding & Egan, 2002; Kabelitz, 2011). Their importance in other mucosal tissues and in mucosal infections have already been highlighted therefore $\gamma\delta^+$ T cells may be important in *H. pylori* infection also.

In this thesis we discuss our findings of gastric epithelial and lamina propria layer lymphocyte responses paying particular attention to unconventional T cells in *H. pylori* infection. In chapter 3, we phenotypically characterise a number of T cell subsets including NKT cells and $\gamma\delta^+$ T cells in gastric epithelial and lamina propria tissues and compare the frequency of these T cell populations in *H. pylori* disease and non-disease states. In chapter 4, functional characterisation of the immune response to *H. pylori* was studied using proliferation and cytokine analysis. Here in initial experiments, PBMCs and cell suspensions derived from the epithelium or lamina propria layers were used to analyse the functional response of immune cells to an array of *H. pylori* derived Ags. Following this, purified NKT cells or $\gamma\delta^+$ T cells were used to address the fine specificity of the response to *H. pylori*. In chapter 5, a large study investigating the gene expression profile was undertaken. Here, $CD2^+$ cells were isolated from gastric epithelial and lamina propria layers. RNA was then prepared from the purified population and gene expression profiling was performed using the Affymetrix technology in association with the Karolinska Institute in Sweden where we uncovered large numbers of genes differentially up and down regulated in *H. pylori* infection.

The gastric mucosa is a site where distinct lymphocyte populations differentially distribute in health and in *H. pylori* associated disease state. Many cell populations have been implicated as immune cells involved in the pathophysiology of *H. pylori* infection, however, the present study, is the first to focus on phenotyping on populations of T cells, $\gamma\delta^+$ T cells, NKT cells and NK cells in the gastric epithelium and lamina propria of *H. pylori* infected and uninfected patients. Flow cytometry revealed here that $CD4^+$ and $CD8^+$ classical T cells were significantly higher and lower respectively in the lamina propria of *H. pylori* infected individuals when compared with uninfected individuals. As regards the epithelial layer, there were far greater numbers of $CD8^+$ T cells present than their $CD4^+$ counterparts although there were no significant differences found in $CD8^+$ T cell numbers during *H. pylori* infection. These findings are consistent with other studies showing that $CD8^+$ T cells that are primarily found in the gastric epithelium while $CD4^+$ T cell and $CD8^+$ T cells are found in the lamina propria (reviewed in Kunisawa *et al.*, 2007).

Double positive T cells (DP T cells) bearing both the CD4 and CD8 co-receptors were also examined here using flow cytometry. While the numbers of these cells were low in the normal, healthy gastric epithelium and lamina propria, in the two *H. pylori* positive subjects studied, the numbers of DP T cells were markedly higher in the epithelium and in the lamina propria when compared with controls. Expression of the CD4 marker on helper T cells and CD8 marker on cytotoxic T cells was once considered to be mutually exclusive. DP T cells are unique in that they display both CD4 and CD8 cell surface markers and therefore possess properties attributed to both. Also in chapter 3, our findings revealed a significant reduction in the numbers of NK cells ($CD3^+CD56^+$) in *H. pylori* infection. This was accompanied by a reduction in T cells bearing the CD56 molecule ($CD3^+CD56^+$). These findings extend our previous studies (O’Keeffe *et al.*,

2008) where we reported alterations in NKR⁺ T cells (T cells expressing CD56 or CD161 or CD94) in *H. pylori* infection. The reductions in the numbers of both NK cells and T cells bearing NK markers like CD56⁺ T cells might contribute to chronicity of infection and ineffective immuno-surveillance impacting on gastric cancer development. Although it could be proposed that a reduction in the numbers of NK or NKR⁺ T cells displaying cytotoxic properties during *H. pylori* infection may contribute to a lack of control of pathogenic T_h1 cells during gastritis development, whereas expansion of distinct cell populations reflect chronic local antigenic stimulation or dysregulated cytokine production, further functional studies are needed in order to establish the immune contribution of these lymphocyte populations in *H. pylori* infection.

Chapter 3 findings also demonstrated novel findings of NKT cells in the normal healthy gastric mucosa and in *H. pylori* disease state. The results showed only a small population of NKT cells as identified by expression of the V α 24V β 11 TCR and when NKT cell numbers were compared between *H. pylori*-positive and -negative individuals, we found an increase in numbers, although not significant, in the *H. pylori* infected epithelium. There were no changes found in the *H. pylori* infected lamina propria layer compared with controls. When NKT cell subsets were further examined by triple staining based upon co-expression of either CD4 or CD8, there were significantly more CD8⁺V α 24J α 28⁺ T cells in epithelial tissues than in lamina propria layer tissues in normal gastric mucosa. Due to the nature and location of this cell subset, it is possible CD8⁺V α 24J α 28⁺ T cells are involved in immunosurveillance of epithelial tissues and display cytotoxic properties to Ags entering the lumen. The function of NKT cells in the human gastric mucosa is presently unknown and the changes in the functional response including their cytokine profiles in *H. pylori* infection remains to be elucidated. Of

interest, lower NKT cell numbers have been reported in patients implicated with autoimmune diseases such as coeliac disease and ulcerative colitis (Grose *et al.*, 2007). The authors hypothesise the overall decrease may have an impact on the activation of T cells that become sensitised as a result of coeliac disease. The studies here showed an increase in the numbers of $\gamma\delta^+$ T cells in the *H. pylori* infected gastric epithelium when compared with controls. The increased numbers of these potent cytotoxic cells is interesting given the functional roles for these cells in regulation of immunity, control of infection and oral tolerance. As in previous studies (Futagami *et al.*, 1996), the numbers of $\gamma\delta^+$ T cells were higher in normal, gastric epithelium when compared with the lamina propria layer compartments. Since approximately 85% of *H. pylori* reside in gastric epithelium (Lee *et al.*, 1993; Moran, 1996) and since vast numbers of Ags including food derived Ags and indigenous flora interact with the epithelium of the gastrointestinal tract high numbers of $\gamma\delta^+$ T cells in epithelial layer tissues would suggest a protective role whereby tissue homeostasis is maintained, regulation of inflammation is occurring and potent killing effects are being exerted.

It is still difficult to postulate whether the changes in the numbers $\gamma\delta^+$ T cells are more important than changes in the numbers of NKT cells in infections with *H. pylori* and further studies are required to address this question. One unconventional T cell population not studied here that may be of immense importance in *H. pylori* infection is MAIT cells. Due to their location in mucosal tissues and the fact that these cells are absent in germ-free mice (Treiner *et al.*, 2003), it would be interesting to compare MAIT cell numbers in *H. pylori* infected and uninfected states and once phenotypically characterised, it would be fascinating to investigate possible functions of this unconventional T cell subset in *H. pylori* infection through proliferative responses, cytokine responses and cytotoxic responses of these cells to *H. pylori* derived Ags.

After lymphocyte populations were defined and phenotyped in the gastric mucosa of *H. pylori* infected and uninfected patients, the reactivity of these immune cells to bacterial stimulation were examined. With this in mind, key immunological responses such as proliferation, cytokine production and cytotoxicity of host immune cells (PBMCs, gastric epithelial and lamina propria layer cells, NKT cells and $\gamma\delta^+$ T cells) to *H. pylori* were investigated in chapter 4. In addition, a variety of *H. pylori* derived fractions were used in these functional studies in order to investigate further the specificity of the immune response to *H. pylori*. The range of *H. pylori* derived Ags studied included the WCE, Cyto Ag, C.M Ag, OMP and IMP from *H. pylori* strains 26695 and J99 as well as LPS derived from *H. pylori* strains NCTC11637 and CCUG17874. To measure proliferation, A BrdU ELISA based assay was used to examine the responses of PBMCs, gastric epithelial and lamina propria cells and NKT cells to *H. pylori* derived antigenic stimulation. While PHA, the positive mitogenic control induced proliferation in PBMCs after 7 day stimulation, a lack of proliferative responses by PBMC and gastric cells to the *H. pylori* bacterial (NCTC11637 LPS and CCUG17874 LPS) stimulation was observed. Given the variability observed here in the proliferative responses and the failure to detect any significant proliferative or cytokine responses to many of the *H. pylori* fractions tested when using cell suspensions derived from the peripheral blood or the gastric epithelium or lamina propria, the study was extended to include NKT and $\gamma\delta^+$ T cell lines. For these experiments, the following *H. pylori* derived Ags were used: WCE, Cyto Ag, C.M Ag, OMP and IMP from *H. pylori* 26695 and J99. In this chapter, the results showed that when NKT cells were stimulated *in vitro* in the presence of CD1d transfected cells, a lack of proliferation was seen in response to the *H. pylori* Ags. In order to address these results and to confirm whether or not a proliferative response can be detected, additional experiments are required

including employing a different methodology for detection of cell proliferation. A lack of proliferation to *H. pylori* derived Ags is consistent with other findings which reported attenuated responses of immune cells after *H. pylori* stimulation (Chmiela *et al.*, 1996; Hybenova *et al.*, 2010) but given the failure of NKT cells to respond to α -GalCer by this method, it is difficult for us to draw similar conclusions.

Next, the immune responses of PBMCs, gastric cells, NKT cells and $\gamma\delta^+$ T cells to *H. pylori* bacterial stimulation were examined through analysis of cytokine production using T_h1/T_h2 11plex FlowCytomix kit. When NKT cell were examined in the presence of stimuli and CD1d transfectants small increases in the levels (<50 pg/ml in most cases) of IFN- γ , IL-2, IL-4, IL-5, IL-8 and TNF- β cytokine production were recorded following *H. pylori* Ag stimulation. The production of IL-8 by NKT cells was particularly interesting. Here, the WCE, Cyto Ag, C.M Ag and OMP from *H. pylori* 26695 all induced small levels of IL-8 production beyond levels in unstimulated cultures. In addition all Ags derived from *H. pylori* J99 (WCE, Cyto Ag, C.M Ag, OMP and IMP) in addition to the LPS preparations tested from the two *H. pylori* strains, NCTC11637, CCUG17874 and from the *E. coli* clinical isolate induced IL-8 production. In these experiments, the positive control stimulus α -GalCer significantly induced secretion from NKT cells of a broad range of cytokines, confirming that the cells themselves, the α -GalCer and the assay system were all working optimally. Notably, the levels of cytokines induced by α -GalCer stimulation were higher in all cases than the levels induced by the *H. pylori* derived Ags.

While *H. pylori* or products derived from *H. pylori* may be a source of antigenic stimulation for NKT cells, it is possible that the bacterial Ags we tested are not natural glycolipids for NKT cell activation. The nature of the glycolipid response to NKT cells has been explored in several studies (Kawano *et al.*, 1997; Kronenberg & Gapin, 2002;

Sköld & Behar, 2003). Numerous glycolipids, including mammalian phospholipids (Fischer *et al.*, 2004), mammalian glycosphingolipids, iGb3 (Godfrey *et al.*, 2004; NingYin *et al.*, 2009; Zhou *et al.*, 2004), ganglioside GD3 (Wu *et al.*, 2003) and bacterial glycolipids (Bendelac *et al.*, 2007; Brigl & Brenner, 2010; Fischer *et al.*, 2004; Kinjo *et al.*, 2005; Kinjo *et al.*, 2006) have been identified as ligands for NKT cells. In this study, tested a range of *H. pylori* Ags (WCE, Cyto Ags, C.M Ags, OMP, IMP) derived from *H. pylori* strains strains 26695, J99 as well as LPS derived from *H. pylori* NCTC11637 and from *H. pylori* CCUG17874. Of interest to this thesis, more recently P157, a *H. pylori* derived glycolipid has been discovered and reported as a ligand for NKT cell activation (Chang *et al.*, 2011). In order for NKT cell activation to occur, the C26 acyl chain of α -GalCer binds to A' hydrophobic pocket of CD1d while the sphingosine base is embedded in F' hydrophobic pocket of CD1d binding groove with high affinity (Koch *et al.*, 2005). Since, α -GalCer, unlike the majority of other glycosphingolipids possesses α -anomeric linkage of sugar to lipid, determining the structure of natural ligand for NKT cell activation will prove to be a major step in NKT cell biology. Certainly the structure of *H. pylori* LPS and its low immunogenicity properties are factors contributing to its ability to evade immune recognition. Thus NKT cells may not be interacting with the *H. pylori* Ags tested here. In addition to quantifying NKT cell numbers in the gastric mucosa and to examining reactivity to *H. pylori* derived Ags in the presence of CD1d transfectants, we also examined mRNA expression of CD1d in gastric epithelium and lamina propria. Using RT-PCR we revealed that CD1d is present in the gastric epithelium and not in the lamina propria. To investigate further, upon analysis of microarray data in chapter 5, it was also evident that CD1d was present in the gastric epithelium of normal healthy mucosa but was not altered in expression levels during *H. pylori* infection. These findings indicate that not

only are NKT cells present in normal gastric epithelium but also their preferred antigen presenting molecule, CD1d is also expressed. The findings implicate that NKT cells are important local immunoregulatory cells within the normal gastric mucosa. As a future projection it would be fascinating to test the stimulatory capacity of various other *H. pylori* Ags such as *H. pylori* 7.13. *H. pylori* 7.13 has been shown to cause reproducible mucosal damage and gastric cancer in 17%, 59% and 59% of *H. pylori* infected gerbils by 4, 8 and 16 wks (Sugimoto *et al.*, 2011). Thus it would be interesting to test immune responses of *H. pylori* 7.13 with human cells to see if this particular Ag exerts similar effects outside of animal models.

With regards to $\gamma\delta^+$ T cells, the results in chapter 4 showed that stimulation with Ags from *H. pylori* strains 26695 and J99 produced IFN- γ , IL-2, IL-4, IL-5, IL-8, IL-12p70, IL-1 β , TNF- α and TNF- β production (up to 7163 pg/ml) in response to WCE, Cyto Ag, C.M Ag, OMP or IMP stimulation. The cytokine levels produced by $\gamma\delta^+$ T cells were more pronounced than levels produced by NKT cells, suggesting therefore that this cell population could potentially be involved in *H. pylori* mediated immunity. The importance of $\gamma\delta^+$ T cells has already been shown in other mucosal infections. For instance, human $\gamma\delta^+$ T cells expressing V γ 9V δ 2 chains secrete T_H1 cytokines IFN- γ and TNF- α following stimulation with viable a bacterial component called *iso*-butylamine (IBA) which is produced by pathogenic bacteria such as *S. typhimurium* and *L. monocytogenes* (Hara *et al.*, 1992; Jouen-Beades *et al.*, 1997). In addition, live *H. pylori* bacteria from strain G27 activates purified CD3⁺ T cells and in particular $\gamma\delta^+$ T cells *in vitro* to induce cytokine and chemokine production in addition to up-regulating CD69 expression thus promoting an inflammatory response which may favour the bacteria to persist and cause chronic disease state (Romi *et al.*, 2011). Now, we are reporting Ags derived from *H. pylori* strains 26695 and J99 (WCE, Cyto Ag, C.M Ag, OMP and IMP)

are also capable of inducing cytokine responses by $\gamma\delta^+$ T cells *in vitro* following stimulation. These studies along with our findings suggest a role for $\gamma\delta^+$ T cells in *H. pylori* infection. Roark *et al.*, (2008) demonstrate $\gamma\delta^+$ T cells secrete effector cytokines such as IFN- γ and IL-17 which have a part to play in infiltration of specialised immune cells, polarisation of immune responses and elimination of infectious agents. Therefore, this poses the question for future projections whether gastric $\gamma\delta^+$ T cells produce IL-17 in response to *H. pylori* infection? (Roark *et al.*, 2008). Their location in mucosal tissues and enrichment in epithelial layer would suggest a function for these cells in mucosal infections. $\gamma\delta^+$ T cells recognise unconventional Ags such as phosphorylated microbial metabolites and lipid Ags rather than processed Ags through APC (Hayday, 2000). It is presently unknown if *H. pylori* Ags could be classed in this category. Romi *et al.*, (2011) has shown that *H. pylori* can induce activation of CD3⁺ T cells and in particular $\gamma\delta^+$ T cells *in vitro* to secrete cytokines and chemokines as well as up-regulation of CD69, thus promoting an inflammatory response which may favour persistence and cause chronic disease state. We also observed mainly pro-inflammatory cytokine production by $\gamma\delta^+$ T cells (IFN- γ , IL-2, IL-8 and IL-12p70, TNF- α , IL-1 β), therefore leading to a polarised T_h1 immune response. Upon analysis of our results in addition to findings shown in similar studies (D'Elios *et al.*, 1997), *H. pylori* seems to have evolved to strike a balance between inducing inflammatory responses but at the same time controlling the severity of host immune responses to persist and cause chronicity of infection. Another area of functional analysis regarding human immune cell responses to *H. pylori* infection was the studies performed examining cytotoxicity. Here, we tested the cytotoxic responses of NKT cells after stimulation with *H. pylori* derived bacterial Ags. In these results, we observed a lack of cytotoxicity as measured by LDH activity. Rudnicka *et al.*, (2012) recorded a lower degree of cytotoxicity in

lymphocytes from *H. pylori* positive patients than from *H. pylori* negative patients. The authors suggest this may be as a result of fewer NK cells present in *H. pylori* positive patients. The same group also discovered that *H. pylori* LPS actually hindered PBMC cytotoxic effects as decreases in IFN- γ , IL-2 and IL-10 were recorded after stimulation with *H. pylori* LPS (Rudnicka *et al.*, 2012). Therefore, is *H. pylori* hindering NKT cell cytotoxic effects here too as a mechanism of survival and persistence? Future work would entail carrying out more specialised cytotoxicity assays. Analysis of more specific markers of cytotoxicity such as FasL, granzyme or perforin might better reflect the cytotoxic activity of NKT cells. Also it would be interesting to determine cytotoxicity levels of $\gamma\delta^+$ T cells stimulated with *H. pylori* Ags and compare these results to unstimulated controls to observe whether there is also a lack of responsiveness with this unconventional T cell population also following *H. pylori* stimulation.

In addition to analysis of functional responses to *H. pylori* derived Ags, the cytokine profiles of serum from *H. pylori* infected and uninfected patients were compared. The results showed that there was no significant difference in the serum levels of IFN- γ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-1 β , TNF- α or TNF- β in the *H. pylori* positive subjects compared with controls. The *H. pylori* positive patients studied here were all positive for CLO test and while 7 out of 10 patients possessed some form of mild to moderate gastritis, no patients were implicated with peptic or duodenal ulcers. It is possible that if our study was extended to include with patients with more severe inflammatory conditions, changes in serum cytokines would be more apparent.

The final part of this thesis in chapter 5 utilised cDNA arrays and Affymetrix technology to analyse CD2⁺ cells from human gastric epithelial layer and lamina propria layer tissues and to identify changes in gene expression of key immunological and

inflammatory genes in the gastric mucosa following infection with *H. pylori*. Gene expression profiling (GEP) was carried out in epithelial and lamina propria layers of *H. pylori* infected subjects compared with controls (groups 1 and 2 respectively). We also performed interlayer comparisons of epithelial and lamina propria tissues in both *H. pylori* disease and non-disease states (groups 3 and 4 respectively). Upon analysis of the top 20 genes with the highest fold change, many novel immunological genes not previously associated with *H. pylori* infection were found. Novel genes involved in membrane integrity, T cell activation, tumour progression, ulcers and cancer were amongst the top 20 differentially expressed genes in *H. pylori* infected epithelial and lamina propria tissues when compared with controls. Moreover, 3 markers of cytotoxicity were found amongst the top 20 most up-regulated genes in CD2⁺ cells when the epithelium of *H. pylori* infected and uninfected subjects were compared suggesting a strong killing response is occurring in epithelial tissues upon *H. pylori* infection. Next, we examined genes differentially expressed in *H. pylori* disease and non-disease states in functional areas important in host innate and adaptive immune responses such as proliferation, cytokine production, cytotoxicity responses, signal transduction and receptor/ligand expression. In addition, we included other unattributed genes deemed important in host immune responses to *H. pylori* infection such as genes involved in cancer progression, lipid binding, membrane integrity and adhesion among others. Upon microarray analysis, we discovered many novel genes differentially expressed in infection that had not previously been associated with *H. pylori* infection. Cytotoxic genes were amongst the most significantly up-regulated genes in *H. pylori* infected epithelium when compared with controls. The microarray data suggests that the epithelium plays more significant role in *H. pylori* infection as there are higher numbers genes involved in key functional areas such as proliferation, cytotoxic, signal

transducing, receptors and cytokine production with a greater fold-change occurring in the epithelium than in the lamina propria of *H. pylori* infected mucosa when compared with healthy mucosa. Moreover, a large number of differentially expressed genes were seen when an interlayer comparison was undertaken between the epithelium and lamina propria layer tissues of normal healthy mucosa, highlighting just how different these mucosal compartments are. To the best of our knowledge, such interlayer comparisons at the protein level have not been undertaken. This study provided us with a large data resource for further host pathogen interaction analysis. Future studies would involve using this approach to analyse GEP in purified NKT cells or $\gamma\delta^+$ T cells from human gastric biopsies. Therefore, it would be possible to assign specificity of immune responses observed to one particular cell type since CD2⁺ cells are a mixture of CD4⁺ T cells, CD8⁺ T cells, NK cells and unconventional T cells. Presently, the difficulty in obtaining sufficient numbers of these cells from 2-3 biopsies and without generating a cell line limits this approach. Since the composition of the CD2 enriched cells vary naturally between *H. pylori* positive patients suffering from a high degree of inflammation and *H. pylori* negative patients devoid of any gastric inflammation, future projections would also include cells isolated from inflamed gastric biopsies from *H. pylori* negative individuals. The present study provides an excellent basis for subsequent, more focused studies. For instance, it would be interesting to select genes among the top 20 most up-regulated and down-regulated genes in infected epithelial and lamina propria layer tissues such as biomarkers for cancer and focus on uncovering their roles in *H. pylori* infection.

In conclusion, the results and findings from this thesis have demonstrated novel findings of immune populations which may have central roles in immunity to *H. pylori*. We have found significant differences in the numbers of lymphocyte populations in

gastric epithelium and lamina propria layers when *H. pylori* infected and uninfected tissues were compared. We confirmed the presence of small numbers of NKT cells and $\gamma\delta^+$ T cells in the two gastric compartments of normal gastric mucosa and observed differences in their frequency in *H. pylori* disease state. NKT cells are well recognised cells of the immune system through their capacity to rapidly kill targets and by potent cytokine secretion. $\gamma\delta^+$ T cells are another important lymphocyte population and are enriched in epithelial and mucosal tissues and previously have been shown to play key roles in other mucosal diseases. While further experimental analysis would reveal more, it is clear from our findings so far that our *H. pylori* derived antigenic preparations from strains 26695 and J99 are capable of inducing immune responses in key immune cells. *H. pylori* immune evasion mechanisms in addition to polarised T_H1 immune response leading to suppression of immune responses and persistence in gastric tissues may be contributing factors of great importance regarding our overall findings. Finally, following gene expression profiling of RNA isolated from gastric epithelial and lamina propria derived $CD2^+$ cells from *H. pylori* infected and uninfected gastric tissues, a large resource of data was generated containing information relating to genes differentially expressed in *H. pylori* disease and non-disease states as well as epithelial and lamina propria interlayer comparisons. GEP uncovered novel genes possibly related to innate and inflammatory responses, apoptosis, cytokine production and onset of cancer among others differentially expressed during *H. pylori* infection. Therefore, this microarray is extremely useful as stepping stone for more specialised insights into host pathogen interactions during *H. pylori* infection thus contributing to understanding mechanisms of *H. pylori* immunopathogenesis. A clear understanding of the mechanisms that *H. pylori* may use to control lymphocyte activity would give valuable

insights into how this bacterium counteracts the host immune response to perpetuate chronic infection and could reveal new strategies for future therapeutic exploitation.

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Appendix I

**Proliferation of gastric epithelial and lamina propria
layer cells following stimulation with *H. pylori* derived**

Ags

Appendix ITable (i) Proliferation of gastric epithelial and lamina propria layer cells following 7 day stimulation with *H. pylori* LPS (n = 3)

7 day proliferation		
Stimuli	EP	LP
	Mean ± SD	Mean ± SD
Unstim	0.25 ± 0.12	0.37 ± 0.16
PHA	0.23 ± 0.12	0.39 ± 0.22
α-GalCer (25µg/ml)	0.24 ± 0.11	0.38 ± 0.12
α-GalCer (10µg/ml)	0.22 ± 0.1	0.37 ± 0.11
α-GalCer (5µg/ml)	0.19 ± 0.08	0.3 ± 0.08
<i>E. coli</i> clinical isolate LPS (100 ng)	0.44 ± 0.23	0.35 ± 0.15
<i>H. pylori</i> NCTC11637 LPS (100 ng)	0.4 ± 0.18	0.42 ± 0.17
<i>H. pylori</i> CCUG17874 LPS (100 ng)	0.4 ± 0.15	0.49 ± 0.15
<i>E. coli</i> clinical isolate LPS (10 ng)	0.33 ± 0.17	0.42 ± 0.12
<i>H. pylori</i> NCTC11637 LPS (10 ng)	0.31 ± 0.15	0.42 ± 0.09
<i>H. pylori</i> CCUG17874 LPS (10 ng)	0.3 ± 0.12	0.34 ± 0.09

EP = epithelium; LP = lamina propria; Unstim = unstimulated; PHA = phytohaemagglutinin; α-GalCer = α-galactosylceramide; *E. coli* = *Escherichia coli*; *H. pylori* = *Helicobacter pylori*; NCTC = national collection of type cultures; CCUG = culture collection, University of Göteborg

Table (ii) Proliferation of gastric epithelial and lamina propria layer cells following 5 day stimulation with *H. pylori* LPS (n = 3)

5 day proliferation		
Stimuli	EP	LP
	Mean ± SD	Mean ± SD
Unstim	0.28 ± 0.04	0.88 ± 0.63
PHA	0.28 ± 0.06	0.46 ± 0.07
α-GalCer (25µg/ml)	0.29 ± 0.04	0.42 ± 0.09
α-GalCer (10µg/ml)	0.29 ± 0.03	0.4 ± 0.12
α-GalCer (5µg/ml)	0.27 ± 0.04	0.35 ± 0.09
<i>E. coli</i> clinical isolate LPS (100 ng)	0.32 ± 0.001	0.49 ± 0.09
<i>H. pylori</i> NCTC11637 LPS (100 ng)	0.3 ± 0.002	0.43 ± 0.08
<i>H. pylori</i> CCUG17874 LPS (100 ng)	0.33 ± 0.06	0.51 ± 0.1
<i>E. coli</i> clinical isolate LPS (10 ng)	0.33 ± 0.008	0.44 ± 0.1
<i>H. pylori</i> NCTC11637 LPS (10 ng)	0.28 ± 0.01	0.41 ± 0.1
<i>H. pylori</i> CCUG17874 LPS (10 ng)	0.29 ± 0.02	0.37 ± 0.1

EP = epithelium; LP = lamina propria; Unstim = unstimulated; PHA = phytohaemagglutinin; α-GalCer = α-galactosylceramide; *E. coli* = *Escherichia coli*; *H. pylori* = *Helicobacter pylori*; NCTC = national collection of type cultures; CCUG = culture collection, University of Göteborg

Table (iii) Proliferation of gastric epithelial and lamina propria layer cells following 4 day stimulation with *H. pylori* LPS (n = 3)

4 day proliferation		
Stimuli	EP	LP
	Mean ± SD	Mean ± SD
Unstim	0.28 ± 0.01	0.81 ± 0.16
PHA	0.25 ± 0.04	1.54 ± 0.01
α-GalCer (25µg/ml)	0.22 ± 0.03	0.33 ± 0.01
α-GalCer (10µg/ml)	0.24 ± 0.01	0.74 ± 0.1
α-GalCer (5µg/ml)	0.26 ± 0.008	0.67 ± 0.04
<i>E. coli</i> clinical isolate LPS (100 ng)	0.26 ± 0.06	0.68 ± 0.15
<i>H. pylori</i> NCTC11637 LPS (100 ng)	0.25 ± 0.06	0.62 ± 0.08
<i>H. pylori</i> CCUG17874 LPS (100 ng)	0.22 ± 0.03	0.66 ± 0.04
<i>E. coli</i> clinical isolate LPS (10 ng)	0.24 ± 0.05	0.7 ± 0.11
<i>H. pylori</i> NCTC11637 LPS (10 ng)	0.24 ± 0.02	0.77 ± 0.12
<i>H. pylori</i> CCUG17874 LPS (10 ng)	0.29 ± 0.05	0.6 ± 0.02

EP = epithelium; LP = lamina propria; Unstim = unstimulated; PHA = phytohaemagglutinin; α-GalCer = α-galactosylceramide; *E. coli* = *Escherichia coli*; *H. pylori* = *Helicobacter pylori*; NCTC = national collection of type cultures; CCUG = culture collection, University of Göteborg

Table (iv) Proliferation of gastric epithelial and lamina propria layer cells following 3 day stimulation with *H. pylori* LPS (n = 2)

3 day proliferation		
Stimuli	EP	LP
	Mean ± SD	Mean ± SD
Unstim	0.17 ± 0.03	0.56 ± 0.1
PHA	0.17 ± 0.03	0.9 ± 0.2
α-GalCer (25µg/ml)	0.15 ± 0.02	0.48 ± 0.09
<i>E. coli</i> clinical isolate LPS (100 ng)	0.14 ± 0.02	0.46 ± 0.08
<i>H. pylori</i> NCTC11637 LPS (100 ng)	0.15 ± 0.02	0.42 ± 0.08
<i>H. pylori</i> CCUG17874 LPS (100 ng)	0.19 ± 0.03	0.48 ± 0.08
<i>E. coli</i> clinical isolate LPS (10 ng)	0.18 ± 0.03	0.4 ± 0.07
<i>H. pylori</i> NCTC11637 LPS (10 ng)	0.14 ± 0.03	0.41 ± 0.08
<i>H. pylori</i> CCUG17874 LPS (10 ng)	0.17 ± 0.03	0.47 ± 0.09

EP = epithelium; LP = lamina propria; Unstim = unstimulated; PHA = phytohaemagglutinin; α-GalCer = α-galactosylceramide; *E. coli* = *Escherichia coli*; *H. pylori* = *Helicobacter pylori*; NCTC = national collection of type cultures; CCUG = culture collection, University of Göteborg

Appendix II

Cytokine production by PBMCs, gastric epithelial and lamina propria layer cells, NKT cells and $\gamma\delta^+$ T cell clones following stimulation with *H. pylori* derived Ags

Appendix IITable (i) Cytokine production by PBMCs after 24 h stimulation with *H. pylori* NCTC11637 LPS

Cytokine	Unstim (pg/ml)	<i>H. pylori</i> NCTC11637 (pg/ml)					
		(10 µg)	(1 µg)	(0.5 µg)	(0.1 µg)	(10 ng)	(1 ng)
IFN-γ	234.46	0	0	0	0	0	0
IL-1β	0	44.65	25.2	4.5	308	0	0
IL-2	0	0	0	0	0	0	0
IL-4	2751.7	1432.13	4091.2	2455.83	1439.36	0	0
IL-5	0	28.66	33.29	0	0	31.38	0
IL-6	0	0	0	0	0	0	0
IL-8	0	0	0	0	18.9	0	0
IL-10	16	20.47	26.44	5.25	133.22	7.38	0
IL-12p70	0	0	0	0	0	0	0
IL-13	0	3.7	0	0	0	0	0
TNF-α	5417.3	7496.93	23231.88	7701.6	18784.25	276.94	0
TNF-β	7.1	217.8	191.9	20.3	1671.5	0	0

Unstim = unstimulated; *H. pylori* = *Helicobacter pylori*; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor

Table (ii) Cytokine production by PBMCs after 48 h stimulation with *H. pylori* NCTC11637 LPS

Cytokine	Unstim (pg/ml)	<i>H. pylori</i> NCTC11637 (pg/ml)					
		(10 µg)	(1 µg)	(0.5 µg)	(0.1 µg)	(10 ng)	(1 ng)
IFN-γ	277.5	156.5	282.3	0	0	0	0
IL-1β	1.5	23.43	21.72	23.43	215.08	0	0
IL-2	0	0	0	0	0	0	0
IL-4	2665.9	3894.1	0	5239.2	7030.4	5797	0
IL-5	0	0	29.4	0	0	0	0
IL-6	0	0	0	0	0	0	0
IL-8	0	0	0	0	0	0	0
IL-10	3.9	52.57	40.33	23.05	121.21	2.17	0
IL-12p70	0	0	0	0	0	0	0
IL-13	12.4	0	0	0	3.5	0	0
TNF-α	1019.9	13866.27	13386.5	16682.4	8486.58	279.74	231.59
TNF-β	0	447.64	315.66	158.15	1796.54	0	0

Unstim = unstimulated; *H. pylori* = *Helicobacter pylori*; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor

Table (iii) Cytokine production by PBMCs after 72 h stimulation with *H. pylori* NCTC11637 LPS

Cytokine	Unstim (pg/ml)	<i>H. pylori</i> NCTC11637 (pg/ml)					
		(10 µg)	(1 µg)	(0.5 µg)	(0.1 µg)	(10 ng)	(1 ng)
IFN-γ	0	0	0	0	0	0	0
IL-1β	0	44.65	31.37	1.76	263.23	0	0
IL-2	0	0	0	0	0	0	0
IL-4	1097.9	4789.19	23407.08	5316.81	19538.25	0	0
IL-5	0	28.66	33.29	0	0	0	0
IL-6	0	0	0	0	0	0	0
IL-8	0	0	0	0	0	0	0
IL-10	9.5	94.6	80.43	36.27	211.51	9.51	2.88
IL-12p70	0	0	0	0	0	0	0
IL-13	0	0	0	0	0	0	0
TNF-α	532.39	9120.6	7958.49	7496.43	4456	882.47	609.62
TNF-β	0	763.08	492.36	49.23	3174.55	0	0

Unstim = unstimulated; *H. pylori* = *Helicobacter pylori*; IFN = interferon; IL = interleukin; TNF = tumour necrosis factor

Table (iv) Cytokine production by gastric epithelial cells after 72 h stimulation with *H. pylori* NCTC11637 and CCUG17874 LPS

Cytokine	<i>H. pylori</i> NCTC11637 (pg/ml)			<i>H. pylori</i> CCUG17874 (pg/ml)		
	Unstim	PHA	LPS	Unstim	PHA	LPS
IFN-γ	0	0	0	0	0	0
IL-1β	0	0	0	0	0	0
IL-2	0	0	0	0	0	28.21
IL-4	0	0	0	0	0	0
IL-5	0	0	0	0	0	0
IL-6	0	0	0	0	0	0
IL-8	23.34	21.05	21.94	593.6	436.79	286.96
IL-10	0	0	0	9.24	11.58	3.72
IL-12p70	0	0	0	0	0	0
IL-13	6.05	12.53	7.32	0	14.13	0
TNF-α	0	0	0	0	0	0
TNF-β	0	0	0	0	0	0

H. pylori = *Helicobacter pylori*; Unstim = unstimulated; PHA = phytohaemagglutinin; LPS = lipopolysaccharide; IL = Interleukin; IFN = interferon; TNF = tumour necrosis factor

Table (v) Cytokine production by gastric lamina propria layer cells after 72 h stimulation with *H. pylori* NCTC11637 and CCUG17874 LPS

Cytokine	<i>H. pylori</i> NCTC11637 (pg/ml)			<i>H. pylori</i> CCUG17874 (pg/ml)		
	Unstim	PHA	LPS	Unstim	PHA	LPS
IFN-γ	0	0	0	0	0	0
IL-1β	0	0	0	0	0	0
IL-2	0	0	3.73	0	0	0
IL-4	0	0	0	0	0	0
IL-5	0	0	0	0	0	0
IL-6	603.33	392.8	471.9	162.26	184.15	196.41
IL-8	1078.32	1512.09	1421.77	5456.21	5456.21	5456.21
IL-10	24.29	21.48	14.23	5.97	0	0
IL-12p70	0	0	0	0	0	0
IL-13	8.74	6.04	6.48	13.61	0	0
TNF-α	0	0	0	0	0	0
TNF-β	0	0	0	0	0	0

H. pylori = *Helicobacter pylori*; Unstim = unstimulated; PHA = phytohaemagglutinin; LPS = lipopolysaccharide; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

Table (vi) NKT cell cytokine production following stimulation with α -GalCer

Cytokine	NKT cells + α -GalCer	
	with mock cell line	with CD1d cell line
IFN-γ	0	3492.64
IL-1β	0	1.32
IL-2	53.3	3084.5
IL-4	23.9	4020.3
IL-5	81.68	6165.3
IL-6	251.4	2020.6
IL-8	114.7	4620.1
IL-10	0	0.33
IL-12p70	0	0
TNF-α	0	802.2
TNF-β	0	81.4

H. pylori = *Helicobacter pylori*; Unstim = unstimulated; PHA = phytohaemagglutinin; LPS = lipopolysaccharide; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

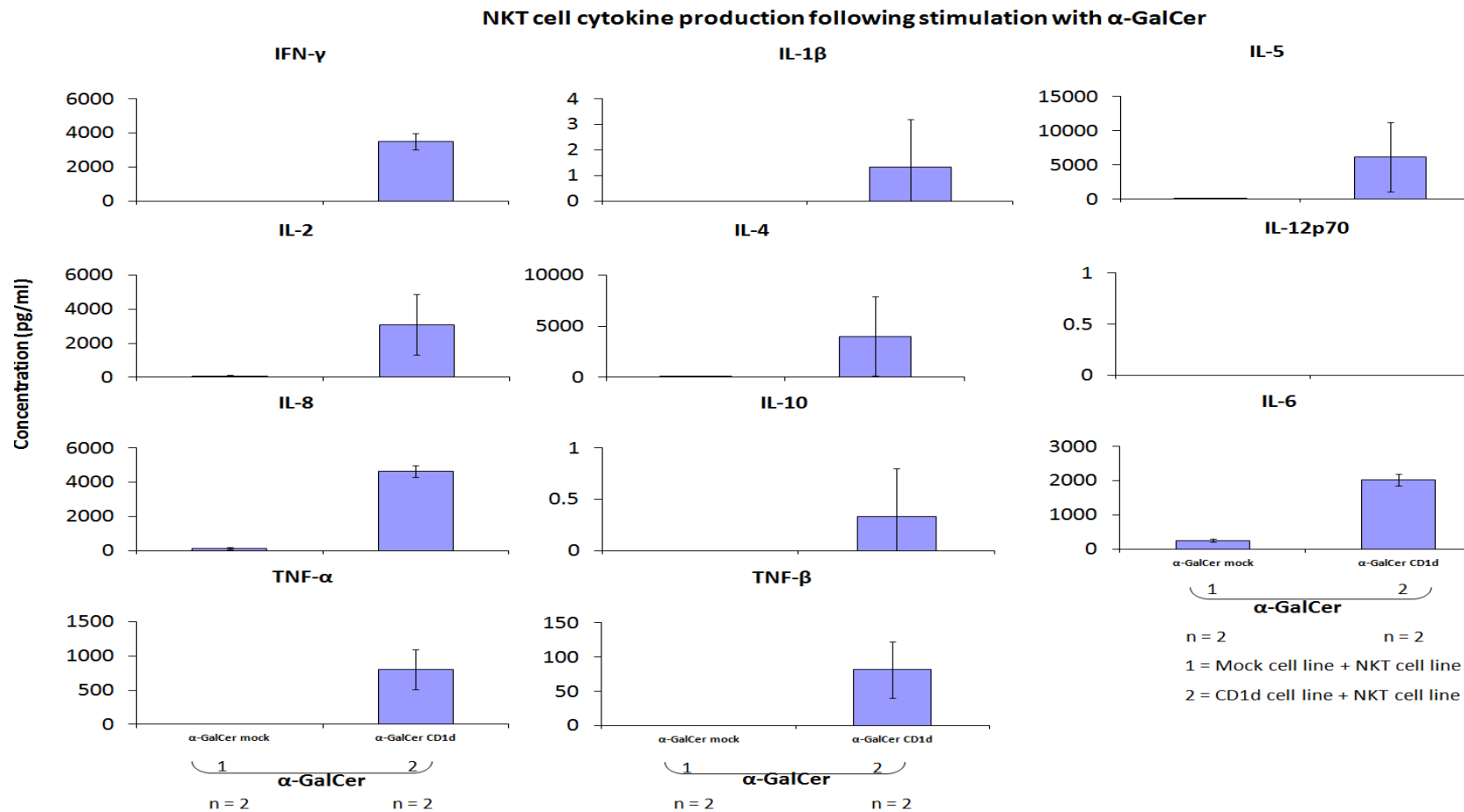


Fig. (i) NKT cell cytokine production following stimulation with the positive control α -GalCer

Table (vii) Cytokine production by NKT cells after *H. pylori* 26695 antigenic stimulation (n = 2)

Cytokine		Controls			<i>H. pylori</i> 26695									
		Vehicle		Unstim	WCE		Cyto Ag		C.M Ag		OMP		IMP	
		Mock	CD1d		Mock	CD1d	Mock	CD1d	Mock	CD1d	Mock	CD1d	Mock	CD1d
IFN-γ	Mean \pm SD	0	0	0	0	10.03 \pm 7.09	0	6.45 \pm 4.56	6.45 \pm 4.56	0	0	0	13.74 \pm 9.71	0
IL-1β	Mean \pm SD	0	0	0	0	0	0	0	0	0	1.27 \pm 0.28	0	0	0
IL-2	Mean \pm SD	0	119.01 \pm 84.15	0	0	102.55 \pm 72.51	102.55 \pm 72.51	125.18 \pm 88.51	0	108.73 \pm 76.88	104.61 \pm 73.97	124.27 \pm 87.87	0	0
IL-4	Mean \pm SD	61.41 \pm 4 3.4	50.38 \pm 22.6	27.67 \pm 9.52	8.08 \pm 5.71	52.63 \pm 1.28	20.94 \pm 14.8	39.22 \pm 13.7	32.03 \pm 22.64	39.23 \pm 21.48	27.03 \pm 22.67	55.24 \pm 6.28	36.69 \pm 25.94	71.22 \pm 50.36
IL-5	Mean \pm SD	183.44 \pm 129	259.28 \pm 183.3	128.1 \pm 9 0.58	160.34 \pm 133.7	322.25 \pm 227.8	196.18 \pm 138.7	277.64 \pm 196.3	169.46 \pm 119.8	224.27 \pm 158.6	204.29 \pm 144.4	262.92 \pm 185.9	151.36 \pm 107	161.85 \pm 114.4
IL-6	Mean \pm SD	164.83 \pm 23.8	47.29 \pm 1.89	0	151.89 \pm 1.11	65.03 \pm 35.01	157.89 \pm 0.57	51.03 \pm 23.86	184.44 \pm 3.94	65.72 \pm 31.15	168.88 \pm 20.66	72.82 \pm 8.73	152.66 \pm 23.37	45.58 \pm 6.21
IL-8	Mean \pm SD	114.35 \pm 44.1	150.71 \pm 15.33	0	97.5 \pm 44.6	173.18 \pm 34.2	114.01 \pm 56.9	161.61 \pm 29.97	109.63 \pm 50.72	163.26 \pm 17.75	104.56 \pm 48.34	214.02 \pm 42.71	102.66 \pm 44.1	137.5 \pm 1.74
IL-10	Mean \pm SD	0	0	0	0	0	0	0	0	0	0	0	0	0
IL12p70	Mean \pm SD	15.48 \pm 1 0.9	18.89 \pm 13.35	0	18.515 \pm 1.08	19.27 \pm 13.63	17.73 \pm 12.54	16.23 \pm 11.5	0	0	0	15.48 \pm 10.94	0	0
TNF-α	Mean \pm SD	0	0	0	0	0	15.58 \pm 11.01	0	0	0	0	0	0	0
TNF-β	Mean \pm SD	10.36 \pm 7 .32	0	17.36 \pm 12.27	16.49 \pm 11.66	0	6.72 \pm 5.14	0	8.14 \pm 1.9	8.58 \pm 6.06	0	17.36 \pm 12.27	0	3.08 \pm 2.2

H. pylori = *Helicobacter pylori*; NKT = Natural killer T cells; WCE = whole cell extract; Cyto Ag = cytoplasmic antigens; C.M Ag = crude membrane antigens; OMP = outer membrane proteins; IMP = inner membrane proteins; SD = standard deviation; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

NKT cell cytokine production after *H. pylori* 26695 stimulation

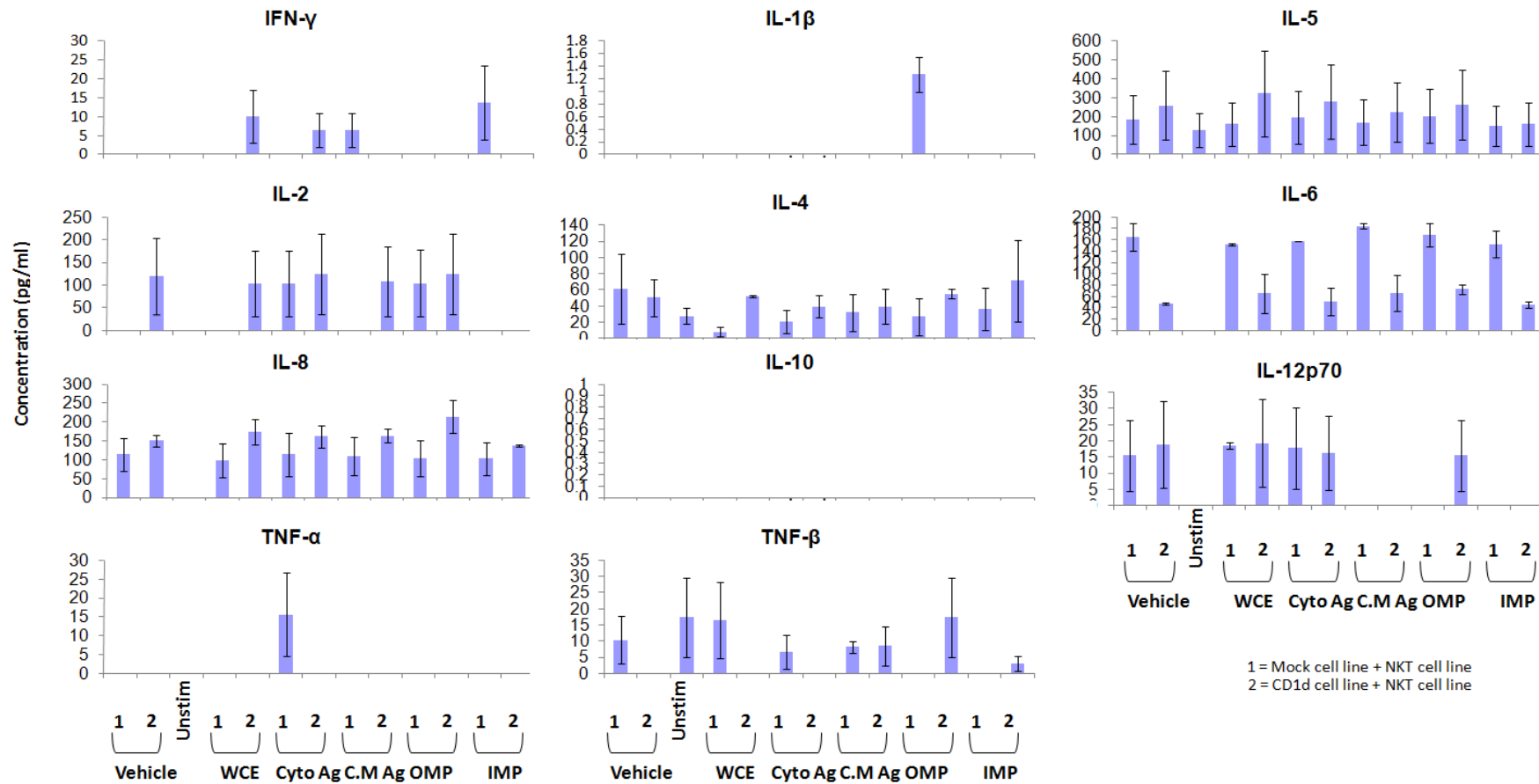


Fig.(ii) NKT cell cytokine production following stimulation with antigens derived from *H. pylori* 26695

Table (viii) Cytokine production by NKT cells after *H. pylori* J99 antigenic stimulation (n = 2)

Cytokine	Controls			<i>H. pylori</i> J99										
	Vehicle		NKT	WCE		Cyto Ag		C.M Ag		OMP		IMP		
	Mock	CD1d		Mock	CD1d	Mock	CD1d	Mock	CD1d	Mock	CD1d	Mock	CD1d	
IFN-γ	Mean \pm SD	0	0	0	0	7.35 \pm 5.19	0	0	2.8 \pm 1.98	6.45 \pm 4.56	0	0	0	0
IL-1β	Mean \pm SD	0	0	0	0	0	0	0	0	1.07 \pm 0.75	0	0	0	0
IL-2	Mean \pm SD	0	119.01 \pm 84.15	0	94.28 \pm 66.66	0	106.67 \pm 75.42	116.46 \pm 6.55	104.61 \pm 73.97	0	0	103.59 \pm 7.31	0	0
IL-4	Mean \pm SD	61.41 \pm 4 3.4	50.38 \pm 22.6	27.67 \pm 9.52	26.89 \pm 19.01	35.99 \pm 5.6	24.04 \pm 0.01	30.81 \pm 6.5	51.61 \pm 2.00	25.46 \pm 6.5	51.61 \pm 3.44	31.96 \pm 3.84	38.28 \pm 3.84	38.88 \pm 27.49
IL-5	Mean \pm SD	183.44 \pm 129	259.28 \pm 183.3	128.1 \pm 90.6	157.33 \pm 111.2	290.8 \pm 205.6	155.84 \pm 110.2	217.53 \pm 153.8	178.74 \pm 126.4	207.57 \pm 146.7	148.4 \pm 104.9	224.27 \pm 158.6	146.9 \pm 103.9	245.01 \pm 173.2
IL-6	Mean \pm SD	164.83 \pm 23.8	47.28 \pm 1.88	0	145.37 \pm 4.82	61.72 \pm 31.19	174.51 \pm 7.51	55.01 \pm 16.45	177.16 \pm 6.35	56.3 \pm 23.1	160.67 \pm 15.73	58.58 \pm 17.4	137.68 \pm 27.99	48.47 \pm 16.61
IL-8	Mean \pm SD	114.35 \pm 44.1	139.87 \pm 15.3	0	91.75 \pm 17.2	163.37 \pm 20.38	109.08 \pm 46.68	162.06 \pm 8.5	109.7 \pm 43.42	165.16 \pm 27.81	102.92 \pm 46.01	158.04 \pm 12.84	108.2 \pm 50.31	154.74 \pm 5.69
IL-10	Mean \pm SD	0	0	0	0	0	0	0	0	0	0	0	0	0
IL-12p70	Mean \pm SD	15.48 \pm 1 0.9	18.89 \pm 13.35	0	17.73 \pm 12.57	0	0	0	0	0	15.9 \pm 11.24	17.73 \pm 12.57	21.61 \pm 15.28	0
TNF-α	Mean \pm SD	0	0	0	0	0	0	0	19.91 \pm 14.07	0	0	0	0	0
TNF-β	Mean \pm SD	10.36 \pm 7 .32	0	17.36 \pm 12.27	0	0	4.49 \pm 0.65	4.96 \pm 3.5	0	12.12 \pm 8.57	0	0	6.8 \pm 4.8	0

H. pylori = *Helicobacter pylori*; NKT = Natural killer T cells; WCE = whole cell extract; Cyto Ag = cytoplasmic antigenic; C.M Ag = crude membrane antigens; OMP = outer membrane proteins; IMP = inner membrane proteins; SD = standard deviation; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

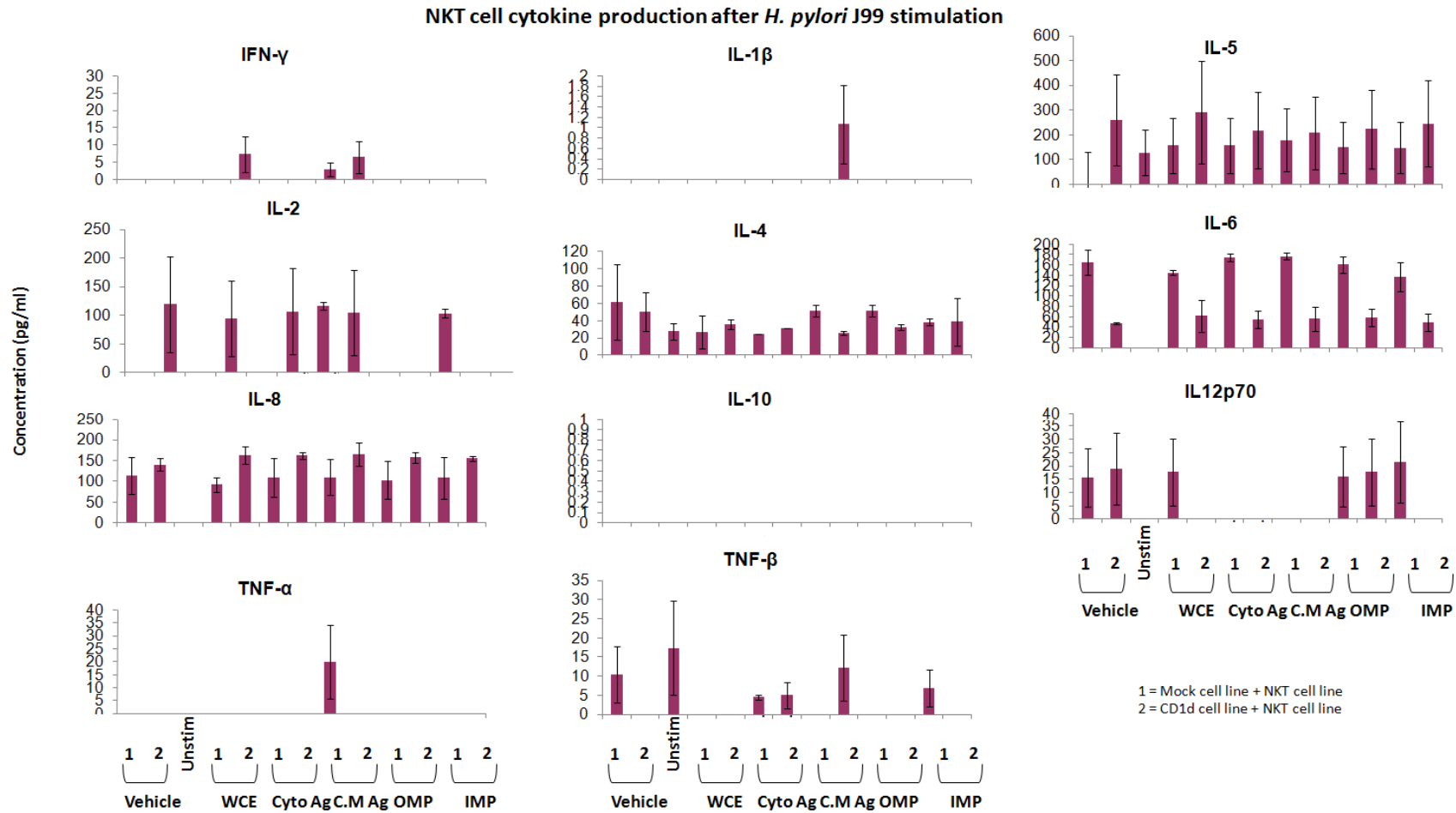


Fig. (iii) NKT cell cytokine production following 24 h stimulation with antigens derived from *H. pylori* J99

Table (ix) Cytokine production by NKT cells after *H. pylori* LPS stimulation (n = 2)

Cytokine		Controls			<i>E. coli</i> LPS		<i>H. pylori</i> LPS			
		Vehicle		NKT	Clinical Isolate		NCTC11637		CCUG17874	
		Mock	CD1d		Mock	CD1d	Mock	CD1d	Mock	CD1d
IFN-γ	Mean \pm SD	0	0	0	2.8	0	0	6.22	14.21	1.46
					\pm 1.97			\pm 4.12	\pm 10.04	\pm 1.03
IL-1β	Mean \pm SD	0	0	0	0	0.26	0	0	0	0
						\pm 0.18				
IL-2	Mean \pm SD	0	119.01	0	0	98.42	106.67	169.2	119.01	101.54
			\pm 84.15			\pm 69.59	\pm 75.42	\pm 119.6	\pm 84.15	\pm 71.79
IL-4	Mean \pm SD	61.41	50.38	27.67	44.12	43.06	14.51	76.23	39.94	29.81
		\pm 43.42	\pm 22.6	\pm 9.51	\pm 17.09	\pm 30.44	\pm 9.09	\pm 9.36	\pm 1.49	\pm 8.16
IL-5	Mean \pm SD	183.44	259.28	128.1	175.63	234.54	139.63	217.53	166.4	279.52
		\pm 129.7	\pm 183.33	\pm 90.58	\pm 124.18	\pm 165.84	\pm 98.73	\pm 153.81	\pm 117.66	\pm 197.65
IL-6	Mean \pm SD	164.83	47.28	0	162.85	58.26	136.89	65.58	164.45	56.3
		\pm 23.78	\pm 1.89		\pm 4.13	\pm 22.83	\pm 5.09	\pm 15.04	\pm 16.64	\pm 23.09
IL-8	Mean \pm SD	114.35	139.87	0	104.71	171.24	94.2	179.08	104.33	178.25
		\pm 44.05	\pm 15.33		\pm 35.51	\pm 33.96	\pm 50.37	\pm 16.89	\pm 31.6	\pm 21.05
IL-10	Mean \pm SD	0	0	0	0	0	0	0	0	0
IL-12p70	Mean \pm SD	15.48	18.89	0	0	0	560.34	78.11	15.11	0
		\pm 10.94	\pm 13.35				\pm 396.2	\pm 55.23	\pm 10.68	
TNF-α	Mean \pm SD	0	0	0	0	0	0	0	0	0
TNF-β	Mean \pm SD	10.36	0	17.36	0	10.36	13.87	23.46	0	6.79
		\pm 7.32		\pm 12.27		\pm 7.32	\pm 9.81	\pm 16.58		\pm 4.8

H. pylori = *Helicobacter pylori*; NKT = Natural killer T cells; WCE = whole cell extract; Cyto Ag = cytoplasmic antigens; C.M Ag = crude membrane antigens; OMP = outer membrane proteins; IMP = inner membrane proteins; SD = standard deviation; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

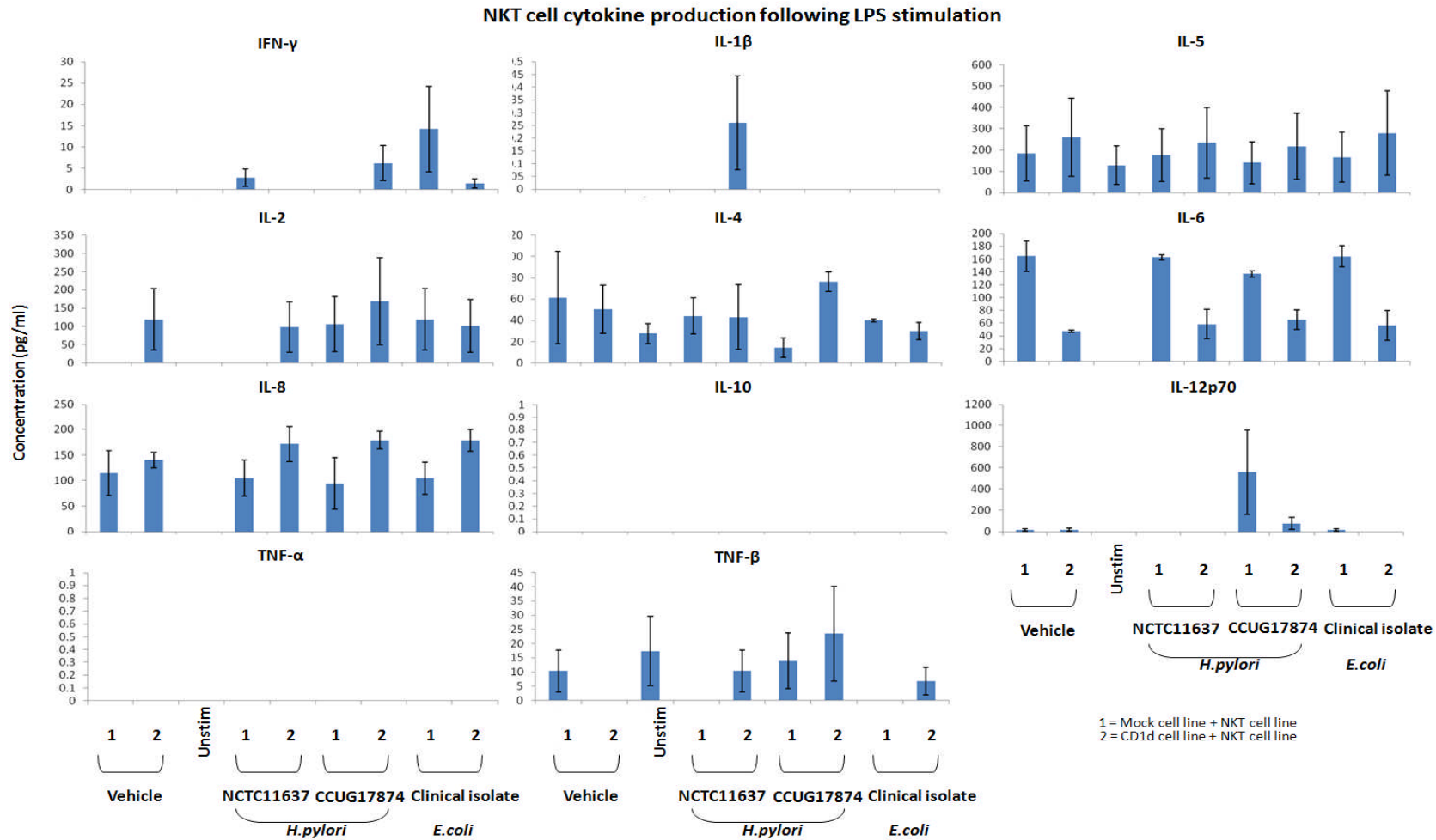


Fig. (iv) NKT cell cytokine production following 24 h stimulation with LPS derived from *H. pylori* and *E. coli*

Table (x) Cytokine production by $\gamma\delta^+$ T cells after *H. pylori* 26695 antigenic stimulation in the presence of PMA (n = 1)

Cytokine		PMA								
		Controls				<i>H. pylori</i> 26695				
		Unstim	PHA	PMA/I	HMB-PP	WCE	Cyto Ag	C.M Ag	OMP	IMP
IFN-γ	pg/ml	183.51	2009.67	7254.63	431.21	201.96	232.71	173.34	137.33	16.66
IL-1β	pg/ml	4.81	12.56	4.81	8.78	0	0	0	0	0
IL-2	pg/ml	1168.13	3572.9	24414.45	1147.6	1145.8	1042	1119.38	1312.04	1312
IL-4	pg/ml	170.6	1233.8	2814.17	139.3	288.84	178.23	93.49	101.13	183.76
IL-5	pg/ml	2631.3	41591.19	15550	5188.22	5655.49	9795.15	2649.03	2117.95	2176.85
IL-6	pg/ml	0	0	0	0	0	0	0	0	0
IL-8	pg/ml	460.6	2052.27	3657.98	597.2	591.81	589.12	433.58	342.32	354.2
IL-10	pg/ml	182.49	372.56	400.74	152.13	144.16	158.9	146.77	12.49	0
IL-12p70	pg/ml	0	0	0	0	0	0	95.74	0	0
TNF-α	pg/ml	18.87	3566.2	8095.14	62.68	0	0	0	0	0
TNF-β	pg/ml	0	222.05	49.21	0	34.5	0	0	9.61	0

Table (xi) Cytokine production by $\gamma\delta^+$ T cells after *H. pylori* 26695 antigenic stimulation in the absence of PMA (n = 1)

Cytokine		w/o PMA								
		Controls				<i>H. pylori</i> 26695				
		Unstim	PHA	PMA/I	HMB-PP	WCE	Cyto An	C.M An	OMP	IMP
IFN-γ	pg/ml	183.51	2009.67	7254.63	431.21	203.34	166.05	172.08	141.94	0
IL-1β	pg/ml	4.81	12.56	4.81	8.78	0	31.49	0	0	0
IL-2	pg/ml	1168.13	3572.9	24414.45	1147.6	1093.3	1008.5	1159.18	1471.97	1760.89
IL-4	pg/ml	170.6	1233.8	2814.17	139.3	76.83	245.1	0	0	93.49
IL-5	pg/ml	2631.3	41591.19	15550	5188.22	2443.51	2443.44	2578.78	2443.44	2018.12
IL-6	pg/ml	0	0	0	0	0	0	0	0	0
IL-8	pg/ml	460.6	2052.27	3657.98	597.2	387.64	416.56	428.71	392.44	354.2
IL-10	pg/ml	182.49	372.56	400.74	152.13	136.18	168.5	185.33	68	0
IL-12p70	pg/ml	0	0	0	0	0	0	0	0	0
TNF-α	pg/ml	18.87	3566.2	8095.14	62.68	0	0	0	0	0
TNF-β	pg/ml	0	222.05	49.21	0	0	0	0	0	0

H. pylori = *Helicobacter pylori*; *E. coli* = *Escherichia coli*; Unstim = unstimulated; PHA = phytohaemagglutinin; PMA = Phorbol myristate acetate; I = Ionomycin; HMB-PP = (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; WCE = whole cell extract; Cyto Ag = cytoplasmic antigens; C.M Ag = crude membrane antigens; OMP = outer membrane proteins; IMP = inner membrane proteins; n = sample size; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

Table (xii) Cytokine production by $\gamma\delta^+$ T cells after *H. pylori* J99 antigenic stimulation in the presence of PMA (n = 1)

Cytokine		with PMA								
		Controls				<i>H. pylori</i> J99				
		Unstim	PHA	PMA/I	HMB-PP	WCE	Cyto Ag	C.M Ag	OMP	IMP
IFN-γ	pg/ml	183.51	2009.67	7254.63	431.21	183.51	214.53	250.46	199.28	0
IL-1β	pg/ml	4.81	12.56	4.81	8.78	0	0	0	4.81	0.18
IL-2	pg/ml	1168.13	3572.9	24414.45	1147.6	983.71	1163.68	1410.54	1231.88	258.78
IL-4	pg/ml	170.6	1233.8	2814.17	139.3	147.44	253.57	248.49	224.55	151.44
IL-5	pg/ml	2631.3	41591.19	15550	5188.22	2476.65	2298.91	4605.37	4920.69	2176.85
IL-6	pg/ml	0	0	0	0	0	0	0	0	0
IL-8	pg/ml	460.6	2052.27	3657.98	597.2	445.82	406.91	597.2	557.27	373.28
IL-10	pg/ml	182.49	372.56	400.74	152.13	137.5	146.77	181.08	138.82	0
IL-12p70	pg/ml	0	0	0	0	0	0	1289.6	0	0
TNF-α	pg/ml	18.87	3566.2	8095.14	62.68	1.45	0	0	0	0
TNF-β	pg/ml	0	222.05	49.21	0	0	0	9.61	0	0

Table (xiii) Cytokine production by $\gamma\delta^+$ T cells after *H. pylori* J99 antigenic stimulation in the absence of PMA (n = 1)

Cytokine		w/o PMA								
		Controls				<i>H. pylori</i> J99				
		Unstim	PHA	PMA/I	HMB-PP	WCE	Cyto Ag	C.M Ag	OMP	IMP
IFN-γ	pg/ml	183.51	2009.67	7254.63	431.21	172.08	197.97	219.87	178.39	0
IL-1β	pg/ml	4.81	12.56	4.81	8.78	6.83	23.45	4.81	0	10.69
IL-2	pg/ml	1168.13	3572.9	24414.45	1147.6	1360.71	1331.37	1264.55	1350.92	287.08
IL-4	pg/ml	170.6	1233.8	2814.17	139.3	200.01	126.57	163.15	143.41	0
IL-5	pg/ml	2631.3	41591.19	15550	5188.22	2476.65	2631.3	2314.58	2338.28	1716.68
IL-6	pg/ml	0	0	0	0	0	0	0	0	0
IL-8	pg/ml	460.6	2052.27	3657.98	597.2	387.64	411.72	421.41	394.85	282.8
IL-10	pg/ml	182.49	372.56	400.74	152.13	107.87	182.49	141.46	116.79	0
IL-12p70	pg/ml	0	0	0	0	6723.74	0	0	0	747.49
TNF-α	pg/ml	18.87	3566.2	8095.14	62.68	0	49.73	0	14.31	0
TNF-β	pg/ml	0	222.05	49.21	0	0	0	0	0	18.02

H. pylori = *Helicobacter pylori*; *E. coli* = *Escherichia coli*; Unstim = unstimulated; PHA = phytohaemagglutinin; PMA = phorbol myristate acetate; I = ionomycin; HMB-PP = (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; WCE = whole cell extract; Cyto Ag = cytoplasmic antigens; C.M Ag = crude membrane antigens; OMP = outer membrane proteins; IMP = inner membrane proteins; n = sample size; IL = interleukin; IFN = interferon; TNF = tumour necrosis factor

Appendix III

List of genes grouped by function differentially expressed in CD2⁺ cells in the epithelial and lamina propria layer of *H. pylori* positive and negative patients.

Table (i) top 20 genes up-regulated when the epithelial layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
CTLA4	cytotoxic T-lymphocyte-associated protein 4	344.82	0.0061
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) ; granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	333.33	0.000002
RGS1	regulator of G-protein signalling 1	270.27	0.0000003
PTPRC	protein tyrosine phosphatase, receptor type, C	243.9	0.0003
RAB8B	RAB8B, member RAS oncogene family	217.39	0.00003
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	185.18	0.0043
KLRC1 ; KLRC2	killer cell lectin-like receptor subfamily C, member 1 ; killer cell lectin-like receptor subfamily C, member 2	178.57	0.0016
TRGC2 ; TRGV9 ; LOC442532 ; LOC442670 ; TARP	T cell receptor gamma constant 2 ; T cell receptor gamma variable 9 ; similar to T-cell receptor gamma chain C region PT-gamma-1/2 ; similar to T-cell receptor gamma chain V region PT-gamma-1/2 precursor ; TCR gamma alternate reading frame protein ; TCR gamma alternate reading frame protein	169.49	0.00002
PSCDBP	pleckstrin homology, Sec7 and coiled-coil domains, binding protein ; pleckstrin homology, Sec7 and coiled-coil domains, binding protein	158.73	0.00004
LCP2	lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	144.93	0.0007
ZNFN1A1	Zinc finger protein, subfamily 1A, 1 (Ikaros)	140.85	0.0029
CD69	CD69 antigen (p60, early T-cell activation antigen)	138.88	0.00004
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	136.99	0.0113
TRA@ ; TRD@	T cell receptor alpha locus ; T cell receptor delta locus	135.14	0.0005
MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	135.14	0.0023
PTGER4	prostaglandin E receptor 4 (subtype EP4)	120.48	0.0049
AFFX-HUMRGE/M10098_5_at		119.04	0.0036
PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	116.28	0.0073
SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	114.94	0.0025
ERBB2IP	erbb2 interacting protein	113.63	0.00002

Table (ii) top 20 genes down-regulated when the epithelial layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	43.75	0.00032
C19orf26	chromosome 19 open reading frame 26	42.78	0.0085
REG4	regenerating islet-derived family, member 4	42.4	0.0022
BAGE4 ; BAGE2	B melanoma antigen family, member 4 ; B melanoma antigen family, member 2	28.69	0.0143
1557658_at	CDNA FLJ33670 fis, clone BRAMY2028783	27.96	0.0005
1567274_at		19.97	0.0004
SLC26A3	solute carrier family 26, member 3	18.09	0.0009
ALDOB	aldolase B, fructose-bisphosphate	17.49	0.0017
MGC40368	T-complex 11 (mouse) like 2	17.45	0.0114
1567913_at	Cancer/testis antigen CT45-1	16.94	0.0013
PRSS7	protease, serine, 7 (enterokinase)	15.77	0.0004
TP73L	tumor protein p73-like	15.43	0.0030
243842_at	Hypothetical LOC400236	15.3	0.0008
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	14.74	0.0054
CHES1	checkpoint suppressor 1	14.53	0.0028
VGLL1	vestigial like 1 (Drosophila)	14.27	0.0027
LRRRC25	leucine rich repeat containing 25	14.19	0.0023
EGFR	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	14.26	0.0001
FLJ22184	hypothetical protein FLJ22184	13.37	0.0003
HSU79275	hypothetical protein HSU79275	13.36	0.0134

Table (iii) top 20 genes up-regulated when the lamina propria layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
PNLIP	pancreatic lipase	625	0.00006
CPB1	carboxypeptidase B1 (tissue)	500	0.0028
ELA2A	elastase 2A	243.9	0.00001
CTRB1 ; CTRB2	chymotrypsinogen B1 ; chymotrypsinogen B2	238.1	0.00004
ELA3A	elastase 3A, pancreatic ; elastase 3A, pancreatic	188.68	0.0028
PRSS2	protease, serine, 2 (trypsin 2)	169.49	0.0065
AMY1A ; AMY1B ; AMY1C ; AMY2A ; AMY2B	amylase, alpha 1A; salivary ; amylase, alpha 1B; salivary ; amylase, alpha 1C; salivary ; amylase, alpha 2A; pancreatic ; amylase, alpha 2B; pancreatic	126.58	0.00002
CEL	carboxyl ester lipase (bile salt-stimulated lipase)	93.46	0.00008
REG3A	regenerating islet-derived 3 alpha	75.19	0.0043
CPA1	carboxypeptidase A1 (pancreatic)	72.46	0.0008
215118_s_at	Translocation associated fusion protein IRTA1/IGA1 (IRTA1/IGHA1)	71.43	0.0112
OLFM4	olfactomedin 4	66.22	0.0005
PNLIPRP2	pancreatic lipase-related protein 2 ; pancreatic lipase-related protein 2	60.97	0.0082
PLA2G1B	phospholipase A2, group IB (pancreas)	60.6	0.0004
ELA3B	elastase 3B, pancreatic	57.14	0.0003
PRSS1	protease, serine, 1 (trypsin 1)	42.01	0.0001
C20orf114	chromosome 20 open reading frame 114	42.01	0.0044
CTRB2	chymotrypsinogen B2	36.49	0.0141
PRSS1 ; PRSS2 ; PRSS3 ; TRY6	protease, serine, 1 (trypsin 1) ; protease, serine, 2 (trypsin 2) ; protease, serine, 3 (mesotrypsin) ; trypsinogen C	32.57	0.004
ZFP36L2	zinc finger protein 36, C3H type-like 2	28.01	0.0004

Table (iv) top 20 genes down-regulated when the lamina propria layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
DAZ1 ; DAZ3 ; DAZ2 ; DAZ4	deleted in azoospermia 1 ; deleted in azoospermia 3 ; deleted in azoospermia 2 ; deleted in azoospermia 4	42.59	0.0063
bioC	biotin biosynthesis; reaction prior to pimeloyl CoA	34.21	0.0098
FOLR1	folate receptor 1 (adult) ; folate receptor 1 (adult)	28.51	0.0058
GKN1	gastrokine 1	20.18	0.0137
AFFX-HUMRGE/M10098_M_at 239370_at		15.28	0.0151
bioB	biotin synthesis, sulfur insertion?	14.54	0.0004
SFRS11	splicing factor, arginine/serine-rich 11	14.01	0.0041
FLJ10154	Hypothetical protein FLJ10154	12.6	0.0002
AFFX-HUMRGE/M10098_5_at		11.52	0.0003
ITGA6	integrin, alpha 6	11.24	0.0072
SCIN	scinderin	10.53	0.0064
BCMP11	breast cancer membrane protein 11	10.06	0.0191
TMEM27	transmembrane protein 27	9.78	0.0177
CBX5	Chromobox homolog 5 (HP1 alpha homolog, Drosophila)	9.72	0.0002
RPS26 ; RPS26L ; LOC440440	ribosomal protein S26 ; 40S ribosomal protein S26-like ; similar to 40S ribosomal protein S26	9.52	0.0125
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	9.34	0.0132
LOC126295	hypothetical protein LOC126295	9.33	0.0027
SKI	v-ski sarcoma viral oncogene homolog (avian)	9.04	0.0016
GAST	gastrin	8.61	0.0115
		8.27	0.0038

Table (v) top 20 genes up-regulated when an interlayer comparison of epithelial and lamina propria layers of *H. pylori* subjects was carried out

Gene symbol	Description	Fold-change	p-value
TRA@ ; TRD@	T cell receptor alpha locus ; T cell receptor delta locus	116.1	0.0029
TRD@	T cell receptor delta locus	105.44	0.009
CD160	CD160 antigen	96.36	0.00003
KLRC3	killer cell lectin-like receptor subfamily C, member 3	73.11	0.0002
244434_at	Transcribed locus, weakly similar to XP_521021.1 PREDICTED: similar to calcium/calmodulin-dependent serine protein kinase [Pan troglodytes]	56.89	0.0046
FOLR1	folate receptor 1 (adult) ; folate receptor 1 (adult)	40.95	0.0011
FCRH3	Fc receptor-like 3	35.51	0.0001
GKN1	gastrokine 1	30.67	0.0088
KIR2DL4	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	29.57	0.01
bioC	biotin biosynthesis; reaction prior to pimeloyl CoA	28.41	0.0041
DAZ1 ; DAZ3 ; DAZ2 ; DAZ4	deleted in azoospermia 1 ; deleted in azoospermia 3 ; deleted in azoospermia 2 ; deleted in azoospermia 4	25.73	0.0005
PYHIN1	pyrin and HIN domain family, member 1	24.34	0.0138
FLJ10652	hypothetical protein FLJ10652	23.35	0.0039
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) ; granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	22.33	0.0015
STS-1	Cbl-interacting protein Sts-1	20.67	0.00013
SH2D1B	SH2 domain containing 1B	20.63	0.0019
TRAF3IP3	TRAF3 interacting protein 3	19.19	0.0014
FLJ43663	Hypothetical protein FLJ43663	18.39	0.0072
SUPT16H	suppressor of Ty 16 homolog (<i>S. cerevisiae</i>)	18.04	0.0014
PELO	Pelota homolog (<i>Drosophila</i>)	17.77	0.0071

Table (vi) top 20 genes down-regulated when an interlayer comparison of epithelial and lamina propria layers of *H. pylori* subjects was carried out

Gene symbol	Description	Fold-change	p-value
PNLIP	pancreatic lipase	625	0.0023
CPB1	carboxypeptidase B1 (tissue)	476	0.0013
ELA2A	elastase 2A	232.56	0.00001
CTRB1 ; CTRB2	chymotrypsinogen B1 ; chymotrypsinogen B2	227.27	0.0009
ELA3A	elastase 3A, pancreatic ; elastase 3A, pancreatic	208.33	0.00043
PRSS2	protease, serine, 2 (trypsin 2)	200	0.0026
PNLIPRP2	pancreatic lipase-related protein 2 ; pancreatic lipase-related protein 2	181.81	0.0015
211645_x_at	Immunoglobulin kappa light chain (IGKV) mRNA variable region, joining region, and constant region ; Immunoglobulin kappa light chain (IGKV) mRNA variable region, joining region, and constant region	178.57	0.0002
ATP4A	ATPase, H+/K+ exchanging, alpha polypeptide	153.84	0.0045
COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	151.51	0.0066
215118_s_at	Translocation associated fusion protein IRTA1/IGA1 (IRTA1/IGHA1)	125	0.0083
ELA3A	elastase 3A, pancreatic	109.89	0.0087
GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	108.69	0.00002
CXCL14	chemokine (C-X-C motif) ligand 14	107.53	0.0059
AMY1A ; AMY1B ; AMY1C ; AMY2A ; AMY2B	amylase, alpha 1A; salivary ; amylase, alpha 1B; salivary ; amylase, alpha 1C; salivary ; amylase, alpha 2A; pancreatic ; amylase, alpha 2B; pancreatic	96.15	0.0008
LOC387763	hypothetical LOC387763	93.46	0.0128
EDIL3	EGF-like repeats and discoidin I-like domains 3	90.9	0.0054
CEL	carboxyl ester lipase (bile salt-stimulated lipase)	87.72	0.0008
COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	82.64	0.0048
COL4A1	collagen, type IV, alpha 1	81.97	0.0158
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	78.13	0.0001
IGHM	immunoglobulin heavy constant mu	78.13	0.0001

Table (vii) top 20 genes up-regulated when an interlayer comparison of epithelial and lamina propria layers of normal healthy gastric mucosa was carried out

Gene symbol	Description	Fold-change	p-value
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	46.98	0.0049
REG4	regenerating islet-derived family, member 4	37.7	0.0012
C19orf26	chromosome 19 open reading frame 26	33.43	0.0072
ALDOB	aldolase B, fructose-bisphosphate	32.19	0.0061
1557658_at	CDNA FLJ33670 fis, clone BRAMY2028783	26.69	0.0002
SLC26A3	solute carrier family 26, member 3	22.9	0.0063
BAGE4 ; BAGE2	B melanoma antigen family, member 4 ; B melanoma antigen family, member 2	19.54	0.0221
1567274_at		16.35	0.0004
PRSS7	protease, serine, 7 (enterokinase)	15.77	0.0005
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	14.75	0.0062
FUT6	Fucosyltransferase 6 (alpha (1,3) fucosyltransferase)	14.2	0.0008
FABP1	fatty acid binding protein 1, liver	13.77	0.0031
SI	sucrase-isomaltase (alpha-glucosidase)	13.23	0.0291
LRRC25	leucine rich repeat containing 25	12.72	0.0062
HSU79275	hypothetical protein HSU79275	12.52	0.0092
TP73L	tumor protein p73-like	12.29	0.0019
VGLL1	vestigial like 1 (Drosophila)	12.28	0.0059
PCDH7	BH-protocadherin (brain-heart)	12.25	0.002
238103_at	CDNA clone IMAGE:4806358	12.01	0.0007
C9orf10	chromosome 9 open reading frame 10	11.92	0.0004
MGC40368	T-complex 11 (mouse) like 2	11.9	0.0003

Table (viii) top 20 genes down-regulated when an interlayer comparison of epithelial and lamina propria layers of normal healthy gastric mucosa was carried out

Gene symbol	Description	Fold-change	p-value
COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	222.2	0.0003
MMP1	matrix metalloproteinase 1 (interstitial collagenase)	217.39	0.0017
IGFBP7	insulin-like growth factor binding protein 7	208.33	0.0008
LUM	lumican	200	0.0028
IGHA1 ; IGHA2 ; MGC27165	immunoglobulin heavy constant alpha 1 ; immunoglobulin heavy constant alpha 2 (A2m marker) ; hypothetical protein MGC27165	196.1	0.0001
IGLC2	Immunoglobulin lambda joining 3	185.18	0.0006
COL1A1	collagen, type I, alpha 1	178.57	0.0003
IGJ	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	178.57	8.03E-09
IGL@ ; IGLC1 ; IGLC2 ; IGLV3-25 ; IGLV2-14 ; IGLJ3	immunoglobulin lambda locus ; immunoglobulin lambda constant 1 (Mcg marker) ; immunoglobulin lambda constant 2 (Kern-Oz- marker) ; immunoglobulin lambda variable 3-25 ; immunoglobulin lambda variable 2-14 ; immunoglobulin lambda joining 3	175.44	0.0038
PBEF1	pre-B-cell colony enhancing factor 1	142.86	0.0043
IL8	interleukin 8	136.98	0.009
BIRC3	baculoviral IAP repeat-containing 3	136.98	0.0004
CALD1	caldesmon 1	111.11	0.0004
IL7R	Interleukin 7 receptor	111.11	0.00002
RNF138	ring finger protein 138	107.52	0.0003
ZNF165	zinc finger protein 165	98.03	0.0076
IGH@ ; IGHG1 ; IGHG2 ; IGHG3 ; IGHM	immunoglobulin heavy locus ; immunoglobulin heavy constant gamma 1 (G1m marker) ; immunoglobulin heavy constant gamma 2 (G2m marker) ; immunoglobulin heavy constant gamma 3 (G3m marker) ; immunoglobulin heavy constant mu	96.15	0.0012
ERBB2IP	erbb2 interacting protein	95.23	0.0019
ZBTB10	Zinc finger and BTB domain containing 10	93.45	0.0018
PRNP	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	93.45	0.0014

Table (ix) Genes involved in proliferation up-regulated when the epithelial layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
IFNG	interferon, gamma	17.79	0.0052
IFI16	interferon, gamma-inducible protein 16	39.27	0.00002
ANXA4	annexin A4	7.05	0.002
IFI30	interferon, gamma-inducible protein 30	2.84	0.017
ANXA1	annexin A1	8.04	0.0029

Table (x) Genes involved in proliferation down-regulated when the epithelial layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
NUP62	nucleoporin 62kDa	4.6055	0.036
IL4I1	interleukin 4 induced 1	2.1685	0.08

Table (xi) Genes involved in proliferation up-regulated when the lamina propria layer of *H. pylori* positive and negative subjects was compared

Gene symbol	Description	Fold-change	p-value
IFNG	interferon, gamma	3.813883	0.0107
IFI16	interferon, gamma-inducible protein 16	3.376	0.0019
IFI30	interferon, gamma-inducible protein 30	5.417118	0.0038
IL4I1	interleukin 4 induced 1	2.745744	0.0196
ANXA1	annexin A1	2.025111	0.0008

Table (xii) Genes involved in proliferation differentially expressed in the epithelial and lamina propria layer of *H. pylori* positive subjects were compared

Gene symbol	Description	Fold-change	p-value
IFNG	interferon, gamma	-3.54	0.0078
IFI30	interferon, gamma-inducible protein 30	2.48	0.0008
IL4I1	interleukin 4 induced 1	2.43	0.0131

Table (xiii) Genes involved in proliferation differentially expressed in the epithelial and lamina propria layer of *H. pylori* negative subjects were compared

Gene symbol	Description	Fold-change	p-value
NUP62	Interleukin 4 induced 1	4.05	0.064
IFI16	interferon, gamma-inducible protein 16	-6.78	0.0004

Table (xiv) Genes involved in cytotoxicity up-regulated when the epithelium of *H. pylori* positive and negative subjects were compared

Gene symbol	Description	Fold-change	p-value
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) ; granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	333.33	0.000002
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) ; granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	86.95	0.0038
GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX) ; granzyme H (cathepsin G-like 2, protein h-CCPX)	6.9	0.0234
GZMK	granzyme K (granzyme 3; tryptase II) ; granzyme K (granzyme 3; tryptase II)	16.75	0.0095
PRF1	perforin 1 (pore forming protein)	24.27	0.0076
NKG7	natural killer cell group 7 sequence	54.05	0.0018
NKTR	natural killer-tumor recognition sequence	13.17	0.0006
FASLG	Fas ligand (TNF superfamily, member 6)	3.73	0.0091
DAP3	death associated protein 3	9.19	0.0065
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	31.54	0.00011
API5	apoptosis inhibitor 5	7.12	0.028
FAS	Fas (TNF receptor superfamily, member 6)	6.07	0.0069
FAIM	Fas apoptotic inhibitory molecule	2.23	0.0139
FADD	Fas (TNFRSF6)-associated via death domain	2.61	0.0433
DAPK1	death-associated protein kinase 1	3.23	0.0045
DAP	death-associated protein	3.22	0.0052
DEDD2	death effector domain containing 2	5.15	0.0006
DAP3	death associated protein 3	9.19	0.0064
CASP8	caspase 8, apoptosis-related cysteine peptidase	6.53	0.006
CASP7	caspase 7, apoptosis-related cysteine peptidase	2.68	0.033
CASP4	caspase 4, apoptosis-related cysteine peptidase	16.55	0.0021
CASP3	caspase 3, apoptosis-related cysteine peptidase	12.06	0.0104
APAF1	apoptotic peptidase activating factor	3.24	0.0222
ACIN1	Apoptotic chromatin condensation inducer 1	5.33	0.0010

Table (xv) Genes involved in cytotoxicity down-regulated when the epithelium of *H. pylori* positive and negative subjects were compared

Gene symbol	Description	Fold-change	p-value
CASP10	caspase 10, apoptosis-related cysteine peptidase	3.64	0.0003
NCR2	natural cytotoxicity triggering receptor 2	4.47	0.0046
NCR1	natural cytotoxicity triggering receptor 1	3.32	0.0107
CIDEc	cell death-inducing DFFA-like effector c	3.6	0.0212
DAPK3	death-associated protein kinase 3	3.15	0.0301
FLJ39616	apoptosis-related protein PNAS-1	3.12	0.0156

Table (xvi) Genes involved in cytotoxicity differentially expressed when the lamina propria of *H. pylori* positive and negative subjects were compared

Gene symbol	Description	Fold-change	p-value
PRF1	perforin 1 (pore forming protein) ; perforin 1 (pore forming protein)	10.19	0.00089
GZMK	granzyme K (granzyme 3; tryptase II) ; granzyme K (granzyme 3; tryptase II)	2.52	0.0027
GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX) ; granzyme H (cathepsin G-like 2, protein h-CCPX)	2.46	0.0137
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) ; granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	3.89	0.0046
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) ; granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	2.46	0.0099
FAIM3	Fas apoptotic inhibitory molecule 3 ; Fas apoptotic inhibitory molecule 3	4.27	0.0069
DEDD	death effector domain containing	2.02	0.0045
AVEN	apoptosis, caspase activation inhibitor	2.3	0.0284
API5	apoptosis inhibitor 5	2.08	0.0061
CASP6	caspase 6, apoptosis-related cysteine peptidase	-2.08	0.0077

Table (xvii) Genes involved in cytotoxicity up-regulated when an interlayer comparison of epithelium and lamina propria of *H. pylori* positive subjects was carried out

Gene symbol	Description	Fold-change	p-value
NKTR	natural killer-tumor recognition sequence	3.71	0.0106
NKG7	natural killer cell group 7 sequence	4.85	0.0201
GZMH	granzyme H (cathepsin G-like 2, protein h-CCPX) ; granzyme H (cathepsin G-like 2, protein h-CCPX)	2.51	0.0098
GZMK	granzyme K (granzyme 3; tryptase II) ; granzyme K (granzyme 3; tryptase II)	2.15	0.0005
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	5.9	0.007
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	22.33	0.0015
FASLG	Fas ligand (TNF superfamily, member 6)	4.17	0.0345
DAPK2	death-associated protein kinase 2	2.14	0.013
CASP8	caspase 8, apoptosis-related cysteine peptidase	2.84	0.0029
CASP4	caspase 4, apoptosis-related cysteine peptidase	2.5	0.024
CASP2	caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally down-regulated 2)	2.13	0.0015
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	3.12	0.0222
CASP1 ; COP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) ; caspase-1 dominant-negative inhibitor pseudo-ICE	2.76	0.0225
ACIN1	Apoptotic chromatin condensation inducer 1	2.52	0.004

Table (xviii) Genes involved in cytotoxicity down-regulated when an interlayer comparison of the epithelium and lamina propria of *H. pylori* positive subjects was carried out

Gene symbol	Description	Fold-change	p-value
DAPK1	death-associated protein kinase 1	3.37	0.0057
DAP	death-associated protein	3.11	0.0059
CASP10	caspase 10, apoptosis-related cysteine peptidase	4.71	0.0136

Table (xix) Genes involved in cytotoxicity up-regulated when an interlayer comparison of the epithelium and lamina propria of normal healthy mucosa was carried out

Gene symbol	Description	Fold-change	p-value
FAF1	Fas (TNFRSF6) associated factor 1	2.12	0.0392
NKTR	Natural killer-tumor recognition sequence	3.32	0.0232
FASTK	Fas-activated serine/threonine kinase	2.36	0.0081
GZMK	granzyme K (granzyme 3; tryptase II) ; granzyme K (granzyme 3; tryptase II)	3.15	0.0031
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	2.17	0.0128
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	4.02	0.002
DIP	death-inducing-protein	3.12	0.0183
DAPK1	death-associated protein kinase 1	2.61	0.017
FAS	Fas (TNF receptor superfamily, member 6)	4.9	0.032

Table (xx) Genes involved in cytotoxicity down-regulated when an interlayer comparison of the epithelium and lamina propria of normal healthy mucosa was carried out

Gene symbol	Description	Fold-change	p-value
AATF	Apoptosis antagonizing transcription factor	2.04	0.0132
FLJ39616	apoptosis-related protein PNAS-1	2.06	0.02
CASP10	caspase 10, apoptosis-related cysteine peptidase	2.19	0.005
DAPK3	death-associated protein kinase 3	2.3	0.033
CIDEC	cell death-inducing DFFA-like effector c	2.73	0.0113
NCR1	natural cytotoxicity triggering receptor 1	3.06	0.007
NCR2	natural cytotoxicity triggering receptor 2	3.12	0.006
FBF1	Fas (TNFRSF6) binding factor 1	3.19	0.06
ACIN1	Apoptotic chromatin condensation inducer 1	3.3	0.0365
CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	3.9	0.00048
CASP1;COP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase) ; caspase-1 dominant-negative inhibitor pseudo-ICE	4.97	0.0121
CASP2	caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally down-regulated 2)	6.03	0.0515
CASP3	caspase 3, apoptosis-related cysteine peptidase	6.42	0.0163
CASP4	caspase 4, apoptosis-related cysteine peptidase	7.12	0.0002
CASP6	caspase 6, apoptosis-related cysteine peptidase	7.84	0.0262
CASP8	caspase 8, apoptosis-related cysteine peptidase	8.12	0.0143
DAP3	death associated protein 3	8.74	0.005
DEDD2	death effector domain containing 2	8.12	0.0064
DAP	death-associated protein kinase	12.65	0.03

Table (xxi) Genes involved in cytokine production up-regulated in the epithelium of *H. pylori* positive subjects compared with controls

Gene symbol	Description	Fold-change	p-value
IFNG	interferon, gamma	17.79	0.00519
IFI16	interferon, gamma-inducible protein 16	39.26	0.00002
IFI30	interferon, gamma-inducible protein 30	2.84	0.0168
IL1B	interleukin 1, beta	3.33	0.0271
IL17	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	9.97	0.0105
IL8	interleukin 8	54.6	0.007
ILF2	interleukin enhancer binding factor 2, 45kDa ; interleukin enhancer binding factor 2, 45kDa	43.1	0.005
ILF3	interleukin enhancer binding factor 3, 90kDa	10.17	0.00006
LTB	lymphotoxin beta (TNF superfamily, member 3)	12.98	0.0214
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	6.16	0.009
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	5.19	0.0183
TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	2.65	0.0012
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	2.5	0.0332
TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	4.26	0.0038
TNFSF5IP1	tumor necrosis factor superfamily, member 5-induced protein 1	8.88	0.0116
TBRG1	Transforming growth factor beta regulator 1	5.06	0.0226
LITAF	lipopolysaccharide-induced TNF factor	21.37	0.005

Table (xxii) Genes involved in cytokine production down-regulated in the epithelium of *H. pylori* positive subjects compared with controls

Gene symbol	Description	Fold-change	p-value
LBP	lipopolysaccharide binding protein ; lipopolysaccharide binding protein	2.45	0.018
TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	2.03	0.0105
NUP62	Interleukin 4 induced 1	4.6	0.0366
IL28A	interleukin 28A (interferon, lambda 2)	5.74	0.0014
IL16	interleukin 16 (lymphocyte chemoattractant factor)	2.78	0.0134
IL1F8	interleukin 1 family, member 8 (eta)	3.44	0.007

Table (xxiii) Genes involved in cytokine production up-regulated in the lamina propria of *H. pylori* positive subjects compared with controls

Gene symbol	Description	Fold-change	p-value
TGFB1	transforming growth factor, beta-induced, 68kDa	4.53	0.0005
TBRG1	transforming growth factor beta regulator 1	2.77	0.025
TGFB1I1	transforming growth factor beta 1 induced transcript 1	5.03	0.0025
TNF	tumor necrosis factor (TNF superfamily, member 2)	2.51	0.0058
TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	3.44	0.0172
LITAF	lipopolysaccharide-induced TNF factor	5.8	0.0005
ILF3	interleukin enhancer binding factor 3, 90kDa	2.57	0.004
IL6	interleukin 6 (interferon, beta 2)	3.53	0.004
IL4I1	interleukin 4 induced 1	2.75	0.0196
IL32	interleukin 32 ; interleukin 32	2.37	0.0428
ISGF3G	interferon-stimulated transcription factor 3, gamma 48kDa	2.2	0.0126
IFI30	interferon, gamma-inducible protein 30	5.41	0.0038
IFI16	interferon, gamma-inducible protein 16	4.37	0.0019
IFNG	interferon, gamma	3.81	0.0107

Table (xxiv) Genes involved in cytokine production up-regulated when the epithelium and the lamina propria of *H. pylori* positive subjects were compared

Gene symbol	Description	Fold-change	p-value
IFNG	interferon, gamma	6.10	0.0078
IFI16	interferon, gamma-inducible protein 16	2.46	0.0165
IL17	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	7.12	0.0194
IL26	interleukin 26	5.42	0.024
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	3.1	0.0228
TBRG1	transforming growth factor beta regulator 1	3.28	0.0032

Table (xxv) Genes involved in cytokine production down-regulated when the epithelium and the lamina propria of *H. pylori* positive subjects were compared

Gene symbol	Description	Fold-change	p-value
IL23A	interleukin 23, alpha subunit p19	2.37	0.0192
IFI30	interferon, gamma-inducible protein 30	3.53	0.0008
IL4I1	interleukin 4 induced 1	3.59	0.01
TGFB1	transforming growth factor, beta-induced, 68kDa	23.92	0.00256
IL6	interleukin 6 (interferon, beta 2)	6.4	0.0018
IL1B	interleukin 1, beta	9.19	0.00003
IL1A	interleukin 1, alpha	11.37	0.0154
TGFB1I1	transforming growth factor beta 1 induced transcript 1	14.18	0.0011
IL1B	interleukin 1, beta	18.69	0.006
TGFBI	transforming growth factor, beta 1 (Camurati-Engelmann disease)	3.63	0.008

Table (xxvi) Genes involved in cytokine production up-regulated when the epithelium and the lamina propria of normal healthy mucosa compared

Gene symbol	Description	Fold-change	p-value
TGFB2	transforming growth factor, beta 2	7.33	0.036
NUP62	Interleukin 4 induced 1	4	0.0364
IL28A	interleukin 28A (interferon, lambda 2)	6.01	0.0021
IL17	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	2.47	0.0286
IL16	interleukin 16 (lymphocyte chemoattractant factor)	7.33	0.0131

Table (xxvii) Genes involved in cytokine production down-regulated when the epithelium and the lamina propria of normal healthy mucosa compared

Gene name	Gene description	Fold change	p -value
IFITM2	interferon induced transmembrane protein 2 (1-8D)	4.34	0.014
IFITM3	interferon induced transmembrane protein 3 (1-8U)	5.7	0.0007
IFIH1	interferon induced with helicase C domain 1	9.23	0.0256
IFIH1	interferon induced with helicase C domain 1	2.01	0.0105
IRF1	interferon regulatory factor 1	39.06	0.0025
IRF2BP2	interferon regulatory factor 2 binding protein 2	48.08	0.0038
IRF3	interferon regulatory factor 3	3.44	0.0425
IRF6	interferon regulatory factor 6	8.79	0.0167
IRF6	interferon regulatory factor 6	2.9	0.0064
IRF8	interferon regulatory factor 8 ; interferon regulatory factor 8	3.7	0.0288
ISG20	interferon stimulated exonuclease gene 20kDa	5.22	0.0204
ISG20L1	interferon stimulated exonuclease gene 20kDa-like 1	2.52	0.0086
ISG20L2	interferon stimulated exonuclease gene 20kDa-like 2	12.33	0.0001
IFI27	interferon, alpha-inducible protein 27	3.34	0.0029
IFI16	interferon, gamma-inducible protein 16	7.67	0.0004
IFI30	interferon, gamma-inducible protein 30	1.86	0.031
IFI35	interferon-induced protein 35	2.18	0.0418

IFI44	interferon-induced protein 44	4.49	0.0036
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	8.45	0.0132
IFRD1	interferon-related developmental regulator 1	57.14	0.0073
IFRD2	interferon-related developmental regulator 2	1.58	0.0133
IL1B	interleukin 1, beta	27.4	0
IL23A	interleukin 23, alpha subunit p19	2.4	0.0216
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	61.35	0.0126
IL8	interleukin 8	136.99	0.009
ILF2	interleukin enhancer binding factor 2, 45kDa ; interleukin enhancer binding factor 2, 45kDa	28.25	0.0071
ILF3	interleukin enhancer binding factor 3, 90kDa	13.61	0.0004
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10 ; Tumor necrosis factor (ligand) superfamily, member 10	8.96	0.0007
TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	4.85	0.0084
TNFSF5IP1	tumor necrosis factor superfamily, member 5-induced protein 1	7.05	0.0149
TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)	4.91	0.002
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	2.38	0.0364
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	55.25	0.0088
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	45.66	0.0052
TGFB1I1	transforming growth factor beta 1 induced transcript 1	2.32	0.0056
TBRG1	transforming growth factor beta regulator 1	2.84	0.0216
TGFA	transforming growth factor, alpha	4.29	0.0054

Table (xxviii) Signal Transduction genes differentially expressed in EP-HP⁺/HP⁻

Gene name	Gene description	Fold change	p-value
CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	18.9	0.0073
CD2AP	CD2-associated protein	6.34	0.0046
CD2BP2	CD2 antigen (cytoplasmic tail) binding protein 2	5.13	0.0176
PHPT1		4.2	0.002
TCR	T-cell receptor alpha chain (TCRA) mRNA (HLA-A1, 24; B7, 8; DR 1, 3)	3.8	0.0499

Table (xxix) Signal Transduction gene differentially expressed in LP-HP⁺/HP⁻

Gene name	Gene description	Fold change	p-value
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	2.17	0.0072
CD28	CD28 antigen (Tp44)	-2.15	0.0135

Table (xxx) Signal Transduction genes differentially expressed in EP-HP⁺/HP⁻

Gene name	Gene description	Fold change	p-value
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	4.33	0.012
CD2AP	CD2-associated protein	-11.25	0.0046
CD2BP2	CD2 antigen (cytoplasmic tail) binding protein 2	-2.5	0.0176
CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	-2.45	0.0113

Table (xxxii) Signal Transduction genes differentially expressed in HP⁺-EP/LP

Gene name	Gene description	Fold change	p-value
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	3.99	0.0059
CD2AP	CD2-associated protein	-12.25	0.0046
CD2BP2	CD2 antigen (cytoplasmic tail) binding protein 2	3.01	0.0371
CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	2.89	0.0113

Table (xxxii) Genes associated with immune receptors/ligands up-regulated when epithelium of *H. pylori* positive and negative subjects were compared

Gene name	Gene description	Fold change	p-value
XCL1	chemokine (C motif) ligand 1	2	0.0091
XCL1 ; XCL2	chemokine (C motif) ligand 1 ; chemokine (C motif) ligand 2	18.02	0.0198
XCL2	chemokine (C motif) ligand 2	19.84	0.0229
CCL20	chemokine (C-C motif) ligand 20	26.53	0.0022
CCL3;CCL3L1;CCL3L3	chemokine (C-C motif) ligand 3 ; chemokine (C-C motif) ligand 3-like 1 ; chemokine (C-C motif) ligand 3-like 3	17.79	0
CCL4	chemokine (C-C motif) ligand 4	10.45	0.0004
CCL5	chemokine (C-C motif) ligand 5 ; chemokine (C-C motif) ligand 5	41.84	0.0113
CCR5	chemokine (C-C motif) receptor 5	7.7	0.0381
CCR6	chemokine (C-C motif) receptor 6	2.14	0.0235
CCRL2	chemokine (C-C motif) receptor-like 2	5.05	0.0012
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4.69	0.0038
CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	15.06	0.0038
CXCL2	chemokine (C-X-C motif) ligand 2	33.9	0.0114
CXCL3	chemokine (C-X-C motif) ligand 3	14.68	0.0055

CXCL5	chemokine (C-X-C motif) ligand 5	29.5	0.0002
CXCR4	chemokine (C-X-C motif) receptor 4	68.97	0.0068
CXCR6	chemokine (C-X-C motif) receptor 6	50	0.0031
CKLF	chemokine-like factor	26.74	0.0039
CKLFSF3	chemokine-like factor superfamily 3	4.28	0.0046
CKLFSF4	chemokine-like factor superfamily 4	2.6	0.0026
CKLFSF6	chemokine-like factor superfamily 6	16	0.0009
ITM2C	integral membrane protein 2C ; integral membrane protein 2C	3.23	0.0041
ITGB1BP1	integrin beta 1 binding protein 1	4.69	0.0009
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	19.34	0.0051
ITGB4BP	integrin beta 4 binding protein	2.59	0.009
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	15.11	0.0437
ITGA6	integrin, alpha 6	8.83	0.0022
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	99.01	0.0024
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	7.1	0.0086
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	12.53	0.0064
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	11.09	0.0012
ITGB2	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	3.44	0.0405
ITGB6	integrin, beta 6	3.54	0.0251
ITGB7	integrin, beta 7	3.76	0.0178
ILKAP	integrin-linked kinase-associated serine/threonine phosphatase 2C	7.25	0.0265
SLIC1	Selectin ligand interactor cytoplasmic-1	4.95	0.0011
SELPLG	selectin P ligand	6.02	0.0183
CD44	CD44 antigen (homing function and Indian blood group system)	55.87	0.0041

CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	18.9	0.0073
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	19.46	0.0102
FASLG	Fas ligand (TNF superfamily, member 6)	3.73	0.0091

Table (xxxiii) Genes associated with immune receptors/ligands down-regulated when epithelium of *H. pylori* positive and negative subjects were compared

Gene name	Gene description	Fold change	p-value
CCL1	chemokine (C-C motif) ligand 1	2.2	0.0302
CCL11	chemokine (C-C motif) ligand 11	2.44	0.0448
CCL13	chemokine (C-C motif) ligand 13	3.03	0.0056
CCL14;CCL15	chemokine (C-C motif) ligand 14 ; chemokine (C-C motif) ligand 15	10.6	0.0004
CCL19	chemokine (C-C motif) ligand 19	2.93	0.0499
CCL2	chemokine (C-C motif) ligand 2	3.75	0.0095
CCL22	chemokine (C-C motif) ligand 22	2.18	0.0177
CCL25	chemokine (C-C motif) ligand 25	6.21	0.0024
CCL26	chemokine (C-C motif) ligand 26	3.22	0.0105
CX3CL1	chemokine (C-X3-C motif) ligand 1	2.58	0.0237
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	5.03	0.0112
CXCL14	Chemokine (C-X-C motif) ligand 14	2.18	0.0333
CCBP2	Chemokine binding protein 2	3.49	0.0161
CMKOR1	Chemokine orphan receptor 1	2.13	0.0142
CKLFSF3	chemokine-like factor superfamily 3	2.96	0.0243
CKLFSF8	Chemokine-like factor superfamily 8	2.34	0.0022
ITGB1BP1	integrin beta 1 binding protein 1	4.63	0.0256
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	3.02	0.0029
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	2.02	0.0217
ITGA9	Integrin, alpha 9	2.49	0.0031

ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	2.28	0.0117
ITGB4	integrin, beta 6	4.33	0.0073

Table (xxxiv) Genes associated with immune receptors/ligands up-regulated when lamina propria layer of *H. pylori* positive and negative subjects were compared

Gene name	Gene description	Fold change	p-value
XCL1 ; XCL2	chemokine (C motif) ligand 1 ; chemokine (C motif) ligand 2	11.12	0.0032
XCL2	chemokine (C motif) ligand 2	11.12	0.0147
CCL11	chemokine (C-C motif) ligand 11	2.39	0.0102
CCL2	chemokine (C-C motif) ligand 2	9.62	0.0002
CCL20	chemokine (C-C motif) ligand 20	3.66	0.0028
CCL3;CCL3L1CCL3L3	chemokine (C-C motif) ligand 3 ; chemokine (C-C motif) ligand 3-like 1 ; chemokine (C-C motif) ligand 3-like 3	2.25	0.0055
CCL4	chemokine (C-C motif) ligand 4	2.17	0.0279
CCL5	chemokine (C-C motif) ligand 5	6.78	0.002
CCR10	chemokine (C-C motif) receptor 10	3.71	0.0303
CCR5	chemokine (C-C motif) receptor 5	3.75	0.0006
CCR6	chemokine (C-C motif) receptor 6	4.69	0.0157
CCR7	chemokine (C-C motif) receptor 7 ; chemokine (C-C motif) receptor 7	4.84	0.0006
CXCL10	chemokine (C-X-C motif) ligand 10	3.34	0.009
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.51	0.015
CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	4.62	0.0079
CXCL14	chemokine (C-X-C motif) ligand 14	11.82	0.0009
CXCL5	chemokine (C-X-C motif) ligand 5	3.38	0.0452
CXCR4	chemokine (C-X-C motif) receptor 4	6.49	0
CXCR6	chemokine (C-X-C motif) receptor 6	4.34	0.0013
CMKOR1	chemokine orphan receptor 1	2.48	0.009

CKLF	chemokine-like factor	2.05	0.0045
CKLFSF6	chemokine-like factor superfamily 6	2.08	0.0473
ITGB1BP1	integrin beta 1 binding protein 1	2.34	0.0115
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	0.56	0.0473
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	3.33	0.01
ITGA8	integrin, alpha 8	2.1	0.0429
ITGA9	Integrin, alpha 9	3.49	0.0193
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	2.67	0.0043
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	18.32	0.0002
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	5.49	0.0008
ITGB2	integrin, beta 2 (antigen CD18 (p95), lymphocyte function-associated antigen 1; macrophage antigen 1 (mac-1) beta subunit)	2.73	0.0364
ITGB7	integrin, beta 7	3.96	0.0283
SELE	selectin E (endothelial adhesion molecule 1)	9.22	0.001
SELL	selectin L (lymphocyte adhesion molecule 1)	2.39	0.0283
SLIC1	Selectin ligand interactor cytoplasmic-1	3.06	0.0224
SELPLG	selectin P ligand	4.01	0.0223
CD2	CD2 antigen (p50), sheep red blood cell receptor ; CD2 antigen (p50), sheep red blood cell receptor	5.98	0.0014
CD44	CD44 antigen (homing function and Indian blood group system)	5.69	0.0032
CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	5.26	0.0073
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	9.21	0.0072
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	2.4	0.0426

Table (xxxv) Genes associated with immune receptors/ligands down-regulated when lamina propria layer of *H. pylori* positive and negative subjects were compared

Gene name	Gene description	Fold change	p-value
ITGA6	integrin, alpha 6	10.53	0.0064
ITGB5	integrin, beta 5	2.82	0.0083
ITGB6	integrin, beta 6	2.26	0.02

Table (xxxvi) Genes associated with immune receptors/ligands up-regulated when the epithelium and lamina propria of *H. pylori* positive subjects were compared

Gene name	Gene description	Fold change	p-value
CCL5	chemokine (C-C motif) ligand 5	3.6	0.0043
CCR10	chemokine (C-C motif) receptor 10	2.81	0.0004
CCR6	chemokine (C-C motif) receptor 6	2.31	0.0017
CX3CL1	chemokine (C-X3-C motif) ligand 1	2.08	0.0127
CXCL14	chemokine (C-X-C motif) ligand 14	4.5	0.0014
CXCR6	chemokine (C-X-C motif) receptor 6	2.5	0.0068
CMKOR1	Chemokine orphan receptor 1	2.35	0.0113
CKLF	chemokine-like factor	7.58	0.0042
CKLFSF1	chemokine-like factor superfamily 1	2.33	0.0124
CKLFSF4	chemokine-like factor superfamily 4	1.65	0.015
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	3.92	0.0038
ITGA6	integrin, alpha 6	2.51	0.0093
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	5.02	0.0034
CD44	CD44 antigen (homing function and Indian blood group system)	2.04	0.0143
CD8A	Fas ligand (TNF superfamily, member 6)	2.24	0.0205
CD8B1	Oncostatin M receptor	2.68	0.0356
OSMR	leukemia inhibitory factor receptor	4.17	0.0345

Table (xxxvii) Genes associated with immune receptors/ligands down-regulated when the epithelium and lamina propria of *H. pylori* positive subjects were compared

Gene name	Gene description	Fold change	p-value
CCL11	chemokine (C-C motif) ligand 11	9.78	0.029
CCL2	chemokine (C-C motif) ligand 2	28.9	0.008
CCL3;CCL3L1CCL3L3	chemokine (C-C motif) ligand 3 ; chemokine (C-C motif) ligand 3-like 1 ; chemokine (C-C motif) ligand 3-like 3	4.14	0.003
CCL4	chemokine (C-C motif) ligand 4	2.07	0.013
CCR10	chemokine (C-C motif) receptor 10	5.63	0.014
CCR6	chemokine (C-C motif) receptor 6	3.85	0.007
CCR7	chemokine (C-C motif) receptor 7 ; chemokine (C-C motif) receptor 7	7.92	0.003
CX3CL1	chemokine (C-X3-C motif) ligand 1	2.44	0.015
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	4.9	0.002
CXCL10	chemokine (C-X-C motif) ligand 10	4.91	0
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	17.36	0.003
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	2.46	0.01
CXCL14	chemokine (C-X-C motif) ligand 14	107.53	0.006
CXCL16	chemokine (C-X-C motif) ligand 16	4.55	0.028
CXCL2	chemokine (C-X-C motif) ligand 2	2.36	0.008
CXCL3	chemokine (C-X-C motif) ligand 3	3.98	0.012
CXCL9	chemokine (C-X-C motif) ligand 9	2.32	0.015
CXCR4	chemokine (C-X-C motif) receptor 4	1.59	0.009
CMKOR1	chemokine orphan receptor 1	17.18	0
CMKOR1	Chemokine orphan receptor 1	2.42	0.016
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	2.42	0.046
ITGA6	integrin, alpha 6	3.62	0.001
ITGA8	integrin, alpha 8	2.47	0.003
ITGA9	Integrin, alpha 9	7.62	0.001
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	2.13	0.035

ITGAM	integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide) ; integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide)	2.36	0.001
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	8.61	0.002
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2.07	0
SELE	selectin E (endothelial adhesion molecule 1)	25.19	0.007
CD44	CD44 antigen (homing function and Indian blood group system)	6.47	0
CD44	CD44 antigen (homing function and Indian blood group system)	2.79	0.012
OSMR	Oncostatin M receptor	11.43	0.015
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	2.66	0.045
LIFR	leukemia inhibitory factor receptor	9.39	0.002

Table (xxxiii) Genes associated with immune receptors/ligands up-regulated when the epithelium and lamina propria of *H. pylori* negative subjects were compared

Gene name	Gene description	Fold change	p-value
CCL1	chemokine (C-C motif) ligand 1	2.09	0.037
CCL13	chemokine (C-C motif) ligand 13	2.82	0.0064
CCL14;CCL15	chemokine (C-C motif) ligand 14 ; chemokine (C-C motif) ligand 15	11.84	0.0034
CCL25	chemokine (C-C motif) ligand 25	6.21	0.0094
CCL26	chemokine (C-C motif) ligand 26	2.78	0.0359
CCL5	chemokine (C-C motif) ligand 5	2.28	0.0206
CX3CL1	chemokine (C-X3-C motif) ligand 1	2.05	0.0107
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3.55	0.03
CMKOR1	Chemokine orphan receptor 1	2.52	0.0138
CKLFSF1	chemokine-like factor superfamily 1	3.27	0.0097
ITGB1BP2	integrin beta 1 binding protein (melusin) 2	4.13	0.0168

ITGB1BP3	integrin beta 1 binding protein 3	3.32	0.004
ITGAD	integrin, alpha D	2.39	0.0116
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	2.01	0.0345
ITGB4	integrin, beta 4	4.33	0.0081
ITGBL1	Integrin, beta-like 1 (with EGF-like repeat domains)	2.5	0.0185
IBSP	integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	2.35	0.0032
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	3.81	0.0171
LIFR	Leukemia inhibitory factor receptor	5.6	0.0077

Table (xxxix) Genes associated with immune receptors/ligands down-regulated when the epithelium and lamina propria of *H. pylori* negative subjects were compared

Gene name	Gene description	Fold change	p-value
CCL20	chemokine (C-C motif) ligand 20	12.84	0.0009
CCL3;CCL3L1CCL3L3	chemokine (C-C motif) ligand 3 ; chemokine (C-C motif) ligand 3-like 1 ; chemokine (C-C motif) ligand 3-like 3	32.68	0
CCL4	chemokine (C-C motif) ligand 4	9.97	0.0031
CCL5	chemokine (C-C motif) ligand 5 ; chemokine (C-C motif) ligand 5	4.13	0.0348
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	20.16	0.0006
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	8.92	0.0055
CXCL14	chemokine (C-X-C motif) ligand 14	28.99	0
CXCL16	chemokine (C-X-C motif) ligand 16	3.39	0.0001
CXCL2	chemokine (C-X-C motif) ligand 2	64.52	0.0115
CXCL3	chemokine (C-X-C motif) ligand 3	43.1	0.0042
CXCL5	chemokine (C-X-C motif) ligand 5	12.36	0.0065
CXCL9	chemokine (C-X-C motif) ligand 9	2.92	0.0035

CXCR4	chemokine (C-X-C motif) receptor 4	33.9	0.0117
CXCR6	chemokine (C-X-C motif) receptor 6	4.61	0.0089
CMKOR1	chemokine orphan receptor 1	10.6	0.0063
CKLF	chemokine-like factor	1.61	0.0374
CKLFSF4	chemokine-like factor superfamily 4	2.22	0.0218
CKLFSF6	chemokine-like factor superfamily 6	8.12	0.0047
CKLFSF8	chemokine-like factor superfamily 8	2.64	0.0457
ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	8.79	0.019
ITGB4BP	integrin beta 4 binding protein	1.54	0.0345
ITGA6	integrin, alpha 6	37.04	0.0057
ITGA6	integrin, alpha 6	18.52	0.0009
ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen 1; alpha polypeptide)	7.36	0.0097
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	28.41	0
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	16.39	0.0026
ITGB5	integrin, beta 5	5.8	0.0088
ITGB6	integrin, beta 6	7.03	0.0113
ILKAP	integrin-linked kinase-associated serine/threonine phosphatase 2C	3.8	0.0381
CD2BP2	CD2 antigen (cytoplasmic tail) binding protein 2	2.94	0.0371
CD2	CD2 antigen (p50), sheep red blood cell receptor ; CD2 antigen (p50), sheep red blood cell receptor	3.53	0.0137
CD8A	CD8 antigen, alpha polypeptide (p32) ; CD8 antigen, alpha polypeptide (p32)	2.82	0.0246
CD44	CD44 antigen (homing function and Indian blood group system)	75.19	0.0032
OSMR	Oncostatin M receptor	4.95	0.0099
LIFR	leukemia inhibitory factor receptor	3.8	0.0114

Table (xI) Other innate and inflammatory genes

	Gene name	Gene description	Fold change	p-value	
EP-HP⁺/HP⁻	IRAK4	interleukin-1 receptor-associated kinase 4	2.95	0.047	
	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	9.09	0.001	
	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	40.48	0.010	
	ABCF1	ATP-binding cassette, sub-family F (GCN20), member 1	4.27	0.013	
	SCYE1	small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	5.48	0.022	
	ELMO2	engulfment and cell motility 2 (ced-12 homolog, <i>C. elegans</i>)	4.36	0.005	
	STAT1	signal transducer and activator of transcription 1, 91Da	46.73	0.007	
	PLSCR1	phospholipid scramblase 1	18.08	0.0002	
	KIR2DL4	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4	16.81	0.002	
	KIR3DL1	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	2.89	0.006	
	KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2	3.78	0.001	
	FOSL2	FOS-like antigen 2	12.12	0.019	
	LP-HP⁺/HP⁻	C4B	complement component 4A ; complement component 4B	6.72	0.011
		MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	3.80	0.002
HP⁺ - EP/LP	SOCS4	suppressor of cytokine signaling 4	4.12	0.002	

EP = epithelium; LP = lamina propria; HP = *H. pylori*; p indicates significance; minus numbers represent genes under-expressed

Table (xli) Lipid binding genes

	Gene name	Gene description	Fold change	p-value
EP-HP⁺/HP⁻	PICALM	phosphatidylinositol binding clathrin assembly protein	20	0.002
	PLEKHA2	Pleckstrin homology domain containing, family A member 2	33.89	8.04E-05
	CHMP4A	chromatin modifying protein 4A	28.57	0.004
	IGKC; IGKV1-5	immunoglobulin kappa constant ; immunoglobulin kappa variable 1-5	9.35	0.012
	FLJ11088	GGA binding partner	16.61	0.001
	PITPNA	phosphatidylinositol transfer protein, alpha	5.21	0.008
	ENTH	enthoprotin	5.26	0.003
	STARD4	START domain containing 4, sterol regulated	3.24	0.018
	SDPR	serum deprivation response (phosphatidylserine binding protein)	-3.04	0.004
	IGHG3	Immunoglobulin heavy constant mu	-3.04	0.005
	EPN3	epsin 3	-3.12	0.009

EP = epithelium; LP = lamina propria; HP = *H. pylori*; p indicates significance; minus numbers represent genes under-expressed

Table (xlii) Membrane integrity and adhesion

	Gene name	Gene description	Fold change	p-value
EP-HP⁺/HP⁻	PNN	pinin, desmosome associated protein	52.91	0.002
	ECT2	epithelial cell transforming sequence 2 oncogene	11.26	0.001
	UPK1B	uroplakin 1B	5.55	0.002
	SPON1	spondin 1, extracellular matrix protein	-2.71	0.003
LP-HP⁺/HP⁻	CAPN9	calpain 9	-4.67	0.016
	ADRM1	adhesion regulating molecule 1	2.43	0.017
	AMICA1	adhesion molecule, interacts with CXADR antigen 1	4.81	0.001
	CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	3.42	0.034

EP = epithelium; LP = lamina propria; HP = *H. pylori*; *p* indicates significance; minus numbers represent genes under-expressed

Table (xliii) Genes related to cancer

	Gene name	Gene description	Fold change	p-value
EP-HP⁺/HP⁻	FYN	FYN oncogene related to SRC, FGR, YES	85.47	0.001
	CASC3	cancer susceptibility candidate 3	5.27	0.0006
	TPT1	tumor protein, translationally-controlled 1	14.01	0.006
	PTGS1	prostaglandin-endoperoxide synthase 1	2.6	0.013
	PORIMIN	pro-oncosis receptor inducing membrane injury gene	47.61	6.68E-05
	ECT2	epithelial cell transforming sequence 2 oncogene	11.26	0.001
	GDDR	down-regulated in gastric cancer GDDR	10.92	0.001
	PCNA	proliferating cell nuclear antigen	47.84	0.006
	DEK	DEK oncogene (DNA binding)	47.39	0.003
	STMN1	stathmin 1/oncoprotein 18	7.28	0.019
	APRIN	androgen-induced proliferation inhibitor	10.64	0.002
	MTCP1	mature T-cell proliferation 1	2.52	0.014
	HCLS1	hematopoietic cell-specific Lyn substrate 1	50	3.47E-05
	ALOX5	arachidonate 5-lipoxygenase	7.15	0.007
	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	11.22	0.0005
	COTL1	coactosin-like 1	60.24	0.016
	LEPR	leptin receptor	4.13	0.0021
	LEPROTL1	leptin receptor overlapping transcript-like 1	14.27	0.013

	PTGS2	prostaglandin-endoperoxide synthase 2	4.45	0.006
LP-HP ⁺ /HP ⁻	MUC6	mucin 6, gastric	3.98	0.023
	GIF	gastric intrinsic factor (vitamin B synthesis)	3.94	3.65E-05
	PIM1	pim-1 oncogene	3.39	0.008
	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	2.74	0.023
	LEPROTL1	leptin receptor overlapping transcript-like 1	7.04	0.007
	MMP9	matrix metalloproteinase 9	2.4	0.004
HP ⁺ -EP/LP	LIPF	lipase, gastric	-6.21	0.0002
	EMCN	endomucin	-7.34	0.009
	PGA5	pepsinogen 5, group I (pepsinogen A)	-7.85	6.63E-05
	ST5	suppression of tumorigenicity 5	-3.83	0.004
	TRA1	tumor rejection antigen (gp96) 1	4.59	0.010
	PIM3	pim-3 oncogene	-4.37	0.003

EP = epithelium; LP = lamina propria; HP = *H. pylori*; *p* indicates significance; minus numbers represent genes under-expressed

Appendix VI

Publications arising from this thesis

Papers

1. O'KEEFFE, J., GATELY, C.M., O'DONOGHUE, Y., ZULQUERNAIN, S.A., STEVENS, F.M. & MORAN, A.P. 2008. Natural killer-cell receptor-positive T-lymphocytes in normal and *Helicobacter pylori*-infected human gastric mucosa. *Helicobacter* **13**: 500-505.

Papers in Preparation

2. Syed A. Zulquernain,^{*‡} Yvonne O'Donoghue,^{*} Laurence J. Egan,[‡] John M. Lee,[‡] Joan O'Keefe,^{†¶} Anthony P. Moran^{*¶} 2010. Raised levels of IL-17 producing T-cells in gastric mucosa of patients with *H. pylori* infection. *Helicobacter* (paper submitted).
3. O'DONOGHUE, Y., MASHAYEKHI, K., MOSHFEGH, A., ZULQUERNAIN, S.A., O'KEEFFE, J. S. & MORAN, A.P. 2010. Gene expression profiling in gastric epithelial and lamina propria derived CD2⁺ cells from *H. pylori* infected and uninfected patients (in preparation).

Meetings abstracts (poster presentations)

1. O'DONOGHUE, Y., ZULQUERNAIN, S.A., MORAN, A.P. & O'KEEFFE, J. 2008. Natural killer T-cells (NKT-cells) in normal and *Helicobacter pylori*-infected gastric mucosae. *In* Abstracts of the Society for General Microbiology, Irish Branch Symposium, Regulatory Mechanisms in Host-Pathogen Interactions, p. 42. National University of Ireland, Galway.
2. O'DONOGHUE, Y., ZULQUERNAIN, S.A., MORAN, A.P. & O'KEEFFE, J. 2008. Unconventional T-cell population in normal and *Helicobacter pylori*-infected gastric mucosae. *In* Abstracts of the 8th International Workshop on Pathogenesis and Host Response in *Helicobacter* Infections, HP-73. The European Study Group on Pathogenesis and Immunology in *Helicobacter* Infections and the European *Helicobacter* Study Group, Helsingør, Denmark.
3. O'DONOGHUE, Y., MORAN, A.P., ZULQUERNAIN, S.A. & O'KEEFFE, J. 2008. NKT-cell and $\gamma\delta^+$ T-cell populations in normal and *Helicobacter pylori*-infected gastric mucosa. *In* Abstracts of the Joint Meeting of the Irish Society for Immunology & Ulster Immunology Group 2008: From Immune Regulation to Immunotherapeutics, p. 25. Royal Dublin Society, Dublin.
4. ZULQUERNAIN, S.A., O'DONOGHUE, Y.M., RAHA ALI, R.A., LEE, J., EGAN, L.E., & O'KEEFFE, J. MORAN, A.P. 2009. Comparison of IL-17 producing CD3⁺ lymphocytes (predominantly Th17-cells) in epithelial and lamina-propria layers in *Helicobacter pylori*-infected gastric mucosae. *In* Abstracts of the Joint Meeting of the Irish Society of Gastroenterology and American College of Gastroenterology, p. 18. Irish Society for Gastroenterology, Tralee, Ireland.
5. O'DONOGHUE, Y., MASHAYEKHI, K., MOSHFEGH, A., ZULQUERNAIN, S.A., O'KEEFFE, J. S. & MORAN, A.P. 2010. Immune response to the gastric pathogen *Helicobacter pylori*: microarray analysis of gastric CD2⁺ cells. *In* Abstracts of College of Science Open Day, p. 64. National University of Ireland, Galway.

6. **O'DONOGHUE, Y., MASHAYEKHI, K., MOSHFEGH, A., ZULQUERNAIN, S.A., O'KEEFFE, J. S. & MORAN, A.P.** 2010. Microarray analysis of CD2⁺ cell gene expression in *H. pylori* infected and non-infected gastric mucosa. Abstracts of College of Science Open Day, p. 53. National University of Ireland, Galway.

Meeting abstracts (oral presentation)

7. **MORAN, A.P., O'DONOGHUE, Y., MASHAYEKHI, K., MOSHFEGH, A., ZULQUERNAIN, S.A., O'KEEFFE, J. S.** 2010. Immune response to the gastric pathogen *Helicobacter pylori*: microarray analysis of gastric CD2⁺ cells. *Helicobacter* **15**: 317. The European Study Group on Pathogenesis and Immunology in Helicobacter Infections and the European *Helicobacter* Study Group, Helsingør, Denmark.

Natural Killer Cell Receptor⁺ T-Lymphocytes in Normal and *Helicobacter pylori*-Infected Human Gastric Mucosa

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Abstract

Background: *Helicobacter pylori* infection is associated with development of chronic inflammation and infiltration of immune cells into the gastric mucosa. As unconventional T-lymphocytes expressing natural killer cell receptors are considered to play central roles in the immune response against infection, a study investigating their frequencies in normal and *H. pylori*-infected gastric mucosa was undertaken.

Materials and Methods: Flow cytometry was used to quantify T-cells expressing the natural killer cell markers CD161, CD56, and CD94 in freshly isolated lymphocytes from the epithelial and lamina propria layers of gastric mucosa. Thirteen *H. pylori*-positive and 24 *H. pylori*-negative individuals were studied.

Results: CD94⁺ T-cells were the most abundant (up to 40%) natural killer receptor-positive T-cell population in epithelial and lamina propria layers of *H. pylori*-negative gastric mucosa. CD161⁺ T-cells accounted for about one-third of all T-cells in both compartments, but the lowest proportion were of CD56⁺ T-cells. Compared with *H. pylori*-negative mucosa, in *H. pylori*-infected mucosa the numbers of CD161⁺ T-cells were significantly greater ($p = 0.04$) in the epithelium, whereas the numbers of CD56⁺ T-cells were lower ($p = 0.01$) in the lamina propria. A minor population (< 2%) of T-cells in both mucosal layers of *H. pylori*-negative subjects were natural killer T-cells, and whose proportions were not significantly different ($p > 0.05$) to those in *H. pylori*-infected individuals.

Conclusions: The predominance, heterogeneity, and distribution of natural killer cell receptor-positive T-cells at different locations within the gastric mucosa reflects a potential functional role during *H. pylori* infection and warrants further investigation.

Keywords

Helicobacter pylori, gastric mucosa, T-cells, natural killer receptor⁺ T-cells, natural killer T-cells

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Innate immune cells, which include T-cell populations activated by glycolipids rather than peptides, are central to the outcome of the ensuing immune response but have not been studied in *Helicobacter pylori* infection [1]. Included in this cell population are T-cells that bear natural killer (NK) receptors, termed natural killer receptor-positive (NKR⁺) T-cells. NKR⁺ T-cells become activated rapidly after stimulation, are capable of MHC-unrestricted cytotoxicity and can secrete large amounts of cytokines including interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), interleukin (IL)-2, and IL-4 [2–4]. Several NK receptors exist, including CD56, CD57, CD161, CD94, and the killer Ig-like receptors. Human NK receptors unlike T-cell receptors (TCRs) are not clonally distributed, antigen-specific receptors, but instead, they recognize HLA-A, -B, -C, or -E molecules and deliver either inhibitory or activatory signals (reviewed in [5]). Moreover, some NK receptors function primarily as co-receptors amplifying

the signal delivered through other receptors on the cell surface. Human CD56⁺ T-cells display both NK-like and T-cell-restricted cytotoxicity in vitro [6] and exhibit potent cytokine secretion [4,7]. The NK marker CD161 is a co-stimulatory molecule that regulates the activity of CD1d-dependent T-cells [8], whereas CD94 expression on T-cells is considered protective against viral infection and against the potential development of autoimmunity [9]. NKR⁺ T-cells bearing the invariant TCR alpha chain, V α 24J α 18, are termed NKT-cells [10] and are recognized for their roles in regulating autoimmunity [11], as well as in defense against certain microbial infections [12]. These cells are

activated by α -galactosylceramide (α -GalCer) presented by the CD1d molecule [13], by endogenous glycolipids such as isoglobotrihexosylceramide [14], and by microbial glycolipids derived from the membrane of some bacteria [15,16]. Altered numbers and functions of NKT-cells have been previously reported in autoimmune diseases [17], in cancer [18] and in human infections [12]. Like other innate lymphocyte populations such as $\gamma\delta$ T-cells [19], it is thought that NKR⁺ T-cells, including NKT-cells, act as frontline immune regulatory cells [4,20].

The human gastrointestinal tract contains several phenotypically and functionally distinct populations of T-cells that occupy different tract compartments [21,22]. T-cells isolated from the epithelial layer (so-called intraepithelial lymphocytes (IELs)) and the lamina propria of the human colon are cytotoxic and kill carcinoma cell lines in vitro [23]. While studies have indicated that NKR⁺ T-cells are present in the human small and large intestines [18], to date no study has addressed the potential roles of these cells in the human gastric mucosa and in infection with *H. pylori*. Therefore, the aims of this study were, firstly, to evaluate the expression of the NK receptors CD56, CD 161, and CD94 on freshly isolated gastric epithelial and lamina propria layer T-cells; secondly, to enumerate V α 24J α 18-bearing NKT-cells among gastric epithelial and lamina propria layer T-cells; and thirdly, to compare the numbers of NKR⁺ T-cells and NKT-cells in each of these compartments during *H. pylori* infection. Given the putative central role of NKR⁺ T-cells in the immune response to microbial infections, their numbers in *H. pylori*-positive and -negative gastric mucosa were compared.

Materials and Methods

Subjects

The study cohort consisted of 37 patients (15 males; 22 females) undergoing investigation for gastric symptoms at an open-access endoscopy unit at University College Hospital Galway. Average age of women was 38.5 years (range, 16–69 years), and for men, 50 years (range, 26–72 years). Antral biopsy samples (n = 4) were obtained after written informed consent and patient confidentiality was maintained. The study was approved by the Research and Ethics Committee of University College Hospital Galway and that of the National University of Ireland Galway. *H. pylori* status of each patient was established by urease testing of an antral and a fundal biopsy using the CLO[®] test, and *H. pylori*-positive or -negative status was further confirmed in histologic examination of additional antral and fundal biopsies with hematoxylin and eosin stain. The *H. pylori*-negative status of biopsies was further confirmed by the lack of *H. pylori* growth after conventional

culture of antral and fundal biopsies. On the basis of this testing, 13 of 37 patients were *H. pylori* positive. Grading of gastric mucosal inflammation showed that of the 13 *H. pylori*-positive patients, 10 had mild gastritis, and three had moderate to severe gastritis; whereas of the 24 *H. pylori*-negative patients, 14 had no gastritis, eight had a grade of nil to minimal gastritis, one had moderate and another had severe gastritis. For the latter two patients, the use of nonsteroidal anti-inflammatory drugs could not be excluded and hence they were not included for further analysis. The remaining biopsy samples were transported to the laboratory in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS; Gibco-BRL, Paisley, UK) for isolation of lymphocytes and flow cytometric analyses.

Preparation of Epithelial and Lamina Propria Layer Single-Cell Suspensions from Gastric Biopsies

Single-cell suspensions of the epithelial layer and lamina propria were prepared from gastric biopsy specimens as previously described [24]. Briefly, biopsy samples were rotated at 37 °C for 1 hour in calcium- and magnesium-free HBSS supplemented with 5% fetal calf serum, 1 mmol/L dithiothreitol (DTT), and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (all from Sigma Chemical Co., St. Louis, MO, USA). This resulted in the complete removal of the epithelial layer, leaving the lamina propria intact and attached to the basement membrane [25]. The resulting single-cell suspension was washed in RPMI-1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum and antibiotics and viable cells were enumerated by conventional Trypan blue (Sigma) exclusion staining. To obtain lamina propria cells, the remaining tissue was placed in 5 mL of supplemented RPMI-1640 medium containing 130 U/mL collagenase (Type 1A, Sigma) and rotated at 37°C for 3 hours. The resulting single-cell suspension was washed in RPMI-1640 medium and viability counts were performed as above. Confirming the purity of the epithelial and lamina propria preparations, immunohistochemistry and flow cytometry experiments following EDTA/DTT treatment showed that (a) the basement membrane remained intact and (b) that B-cells were absent from the epithelial layer single cell suspensions (data not shown), and (c) that the CD3⁺ T-cells were predominantly CD8⁺, consistent with an epithelial layer phenotype (Table 1).

Antibodies and Flow Cytometry

Fluorochrome -labeled monoclonal antibodies specific for human CD3, CD4, CD8, CD56, CD161, CD94, and isotype-matched controls were obtained from Serotec (Oxford, UK). Anti-V α 24J α 18-PE, specific for invariant NKT-cells

Table 1 Comparison of T-cell subsets and natural killer (NK)-cells in gastric epithelium and lamina propria of *H. pylori*-negative and -positive individuals

	% of cells	
	Epithelium	Lamina propria
<i>H. pylori</i> -negative subjects ^a		
CD4 ⁺ T-cells ^b	11.4(2.5–34) ^c	35.7(17.6–63.9)
CD8 ⁺ T-cells ^b	85.9(36.6–98.1)	55.3(32.2–72.5)
NK-cells ^b	4.6(0.3–10.8)	8.3(2–17.6)
<i>H. pylori</i> -positive subjects		
CD4 ⁺ T-cells	13.4(2.2–88.6)	53.4(8–94.9) ^d
CD8 ⁺ T-cells	79.6(51.1–90.1)	40.3(23.2–55) ^d
NK-cells	3.3(1.3–5.4)	4.3(1.3–7.9) ^d

^a*H. pylori*-negative individuals (n = 24) and *H. pylori*-positive patients (n = 13).

^bCD4⁺ T-cells were defined as CD3⁺CD4⁺; CD8⁺ T-cells as CD3⁺CD8⁺ and NK-cells as CD3[−]CD56⁺.

^cMedian percentage values (with range in parentheses) of cells for each subset are shown.

^dSignificant difference where $p < .05$ between *H. pylori*-positive and -negative subjects.

[26], was obtained from BD Pharmingen (Oxford, UK).

Single-cell suspensions (1×10^5) derived from the epithelium and lamina propria were stained using the above antibodies as previously described [4] and analyzed by flow cytometry using FACsCalibur[®] and CellQuest[®] lysis software (Becton Dickinson, Oxford, UK).

Statistical Analyses

Flow cytometric results are expressed as median values with ranges, and analyzed for statistical significance ($p < 0.05$) using the Mann–Whitney *U*-test.

Results

T- and NK-Cell Composition Among Gastric Mononuclear Cell Populations

Two- and three-color flow cytometry of single-cell suspensions prepared from human gastric antral mucosa was used to determine the T-cell composition in the epithelium and lamina propria layers in control subjects, classified as *H. pylori*-negative (Table 1). In the epithelial layer, the numbers of CD8⁺ T-cells were more abundant than CD4⁺ T-cells (medians, 85.9% vs 11.4%), whereas in the lamina propria, the numbers of each population were not significantly different. In *H. pylori*-positive subjects, however, the numbers of CD4⁺ T-cells and CD8⁺ T-cells were significantly ($p = 0.04$) higher (median, 53.4%) and lower (median, 40.3%), respectively, in the lamina propria compared with control subjects. Among epithelial layer

lymphocytes, there were no significant differences in the numbers of CD4⁺ or CD8⁺ T-cells when compared with control subjects. NK-cell numbers (CD3[−]CD56⁺) were significantly lower ($p = 0.03$) in the lamina propria of *H. pylori*-positive subjects (median, 4.3%) compared with controls (median, 8.3%), whereas there were no differences between the numbers of these cells in the epithelia of the two subject groups.

Compartmentalization of NKR⁺ T-cells in Gastric Epithelium and Lamina Propria with Altered Numbers in *H. pylori* Infection

NKR⁺ T-cells were quantified by dual staining on the basis of CD3 expression (T-cell marker) and either CD56, CD161, or CD94 (NK-cell markers), and therefore, were classified as CD56⁺ T-cells, CD161⁺ T-cells, or CD94⁺ T-cells. Flow cytometric analysis revealed differential distribution of NKR⁺ T-cell populations in the epithelium and lamina propria compartments of *H. pylori*-negative control subjects (Fig. 1). In the epithelial layer, noteworthy populations of NKR⁺ T-cells were found with almost 40% of T-cells expressing CD94 (median, 38.4%). In the lamina propria, CD161⁺ T-cells were the most abundant population (median, 33.9%). The proportion of CD56⁺ T-cells was lower than either CD94⁺ T-cells or CD161⁺ T-cells, in both the epithelial layer (15.1%) and the lamina propria (19.9%).

When NKR⁺ T-cell populations were examined in the gastric tissue samples of *H. pylori*-positive individuals, marked differences in the numbers of these T-cells were found when compared with *H. pylori*-negative control subjects (Fig. 1). In the epithelium, the numbers of CD161⁺ T-cells were significantly higher ($p = 0.04$) in *H. pylori*-positive subjects (median, 38.7%). Other differences in the epithelium of *H. pylori*-positive subjects included lower numbers of CD56⁺ T-cells (median, 12.1%) and CD94⁺ T-cells (median, 28.2%) than in controls, although the differences did not reach significance ($p = 0.8$ and $p = 0.7$, respectively). In contrast to the epithelium, the numbers of CD56⁺ T-cells (median, 6.7%) were markedly lower in the lamina propria of *H. pylori*-positive subjects ($p = 0.01$). Moreover, unlike the epithelial layer, there were no significant differences in the numbers of lamina propria CD161⁺ T-cells or CD94⁺ T-cells in *H. pylori*-positive subjects compared with controls.

Invariant NKT-Cells in Gastric Epithelium and Lamina Propria

The expression of the V α 24J α 18⁺ TCR defines the invariant CD 1d-restricted NKT-cell population in humans [10], and flow cytometric analysis was used to quantify these cells in

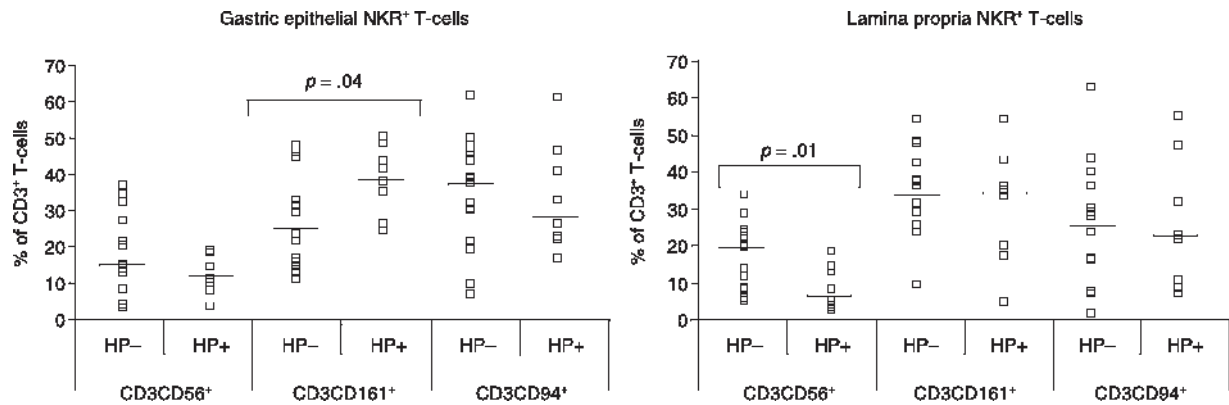


Figure 1 Natural killer receptor-positive (NKR⁺) T-cells in the gastric mucosa of *H. pylori*-negative and -positive individuals. Percentages of NKR⁺ T-cells (CD56⁺, CD161⁺, and CD94⁺ T-cell populations) among epithelial and lamina propria layer CD3⁺ T-cells in *H. pylori* infected (HP⁺) and uninfected controls (HP⁻) are shown. Percentages of each T-cell population are shown and horizontal bars indicate median values.

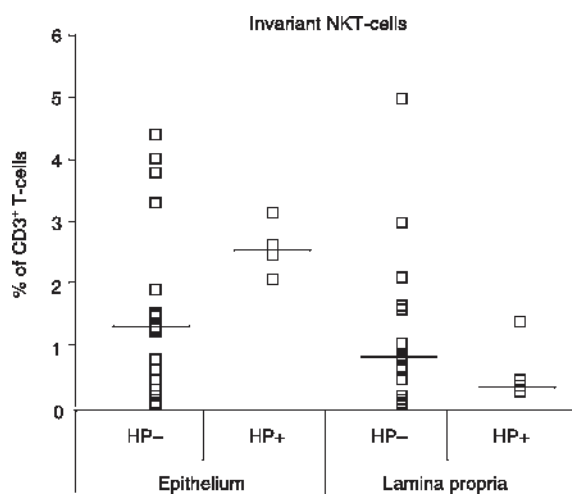


Figure 2 Percentages of invariant NKT-cells in the gastric epithelium and lamina propria of *H. pylori*-negative (HP⁻) and -positive (HP⁺) individuals. Horizontal bars indicate median values.

the epithelial and lamina propria layers. As shown in Fig. 2, a small minority of T-cells in both mucosal compartments of *H. pylori*-negative patients expressed the V α 24J α 18⁺ TCR (medians, 1.23% (epithelium) and 1.55% (lamina propria), respectively). In *H. pylori*-positive subjects, the numbers of V α 24J α 18⁺ T-cells were greater in the epithelium (median, 2.5%), but lower in the lamina propria (median, 0.38%), compared to *H. pylori*-negative individuals. However, these differences did not reach statistical significance ($p = 0.3$ and 0.09 , respectively) for the low numbers of subjects studied.

Discussion

The gastric mucosa is a site where distinct lymphocyte populations differentially distribute in healthy state and during *H. pylori* infection. The present study shows that in

the epithelial and lamina propria layers of normal gastric mucosa, there is an abundance of CD8⁺ and CD4⁺ T-cells as well as noteworthy populations of NKR⁺ T-cells and NK-cells, but low numbers of invariant NKT-cells. When the lymphocyte populations were compared between *H. pylori*-positive and -negative individuals, marked differences in the proportions of these cells in the two gastric mucosal layers were found. In the epithelium, lower numbers of CD56⁺ T-cells and CD94⁺ T-cells and higher numbers of CD161⁺ T-cells and NKT-cells were found in *H. pylori*-infected mucosa compared with controls; whereas in the lamina propria, lower numbers of CD8⁺ T-cells, NK-cells, and CD56⁺ T-cells but greater numbers of CD4⁺ T-cells were observed in *H. pylori*-positive individuals.

Infection with *H. pylori* is associated with infiltration of many immune cells into the gastric mucosa including T- and B-lymphocytes, mast cells, macrophages, and dendritic cells [27,28]. Our findings of predominantly CD8⁺ T-cells among epithelial layer lymphocytes are consistent with reports showing that this is the most abundant T-cell population found in intestinal epithelial layers (reviewed in [29]). These intraepithelial lymphocytes regulate mucosal homeostasis, induce tolerance to dietary antigens, and are actively involved in the inflammatory response during infections [29]. In this study, the most abundant NK receptor-bearing T-cell population in the human gastric epithelium were the CD94⁺ T-cells, representing up to 40% of all T-cells. CD94 binds HLA-E, and ligation can be stimulatory or inhibitory depending on its dimerization status with NKG2A [30]. While there were no significant differences between CD94⁺ T-cell numbers in *H. pylori*-infected and noninfected individuals, the numbers of CD161⁺ T-cells among epithelial layer T-cells were greater when compared with controls. CD161⁺ T-cells are predominantly memory T-cells [31] and human small intestinal CD161⁺ T-cells are capable of secreting IFN- γ upon stimulation [18]. Whether

the increased numbers of CD161⁺ T-cells reflect local expansion due to chronic antigen-driven stimulation during *H. pylori* infection requires further investigation.

The intestinal lamina propria contains the largest numbers of T-cells in the body which maintain oral tolerance to dietary antigens and participate in immune protection against infections [32]. The present data are consistent with previous findings showing predominance of CD4⁺ versus CD8⁺ T-cells in the lamina propria of *H. pylori*-infected tissues [24,33]. A polarization towards a Th1-dominated T-cell response in the antral lamina propria is associated with *H. pylori* infection [34,35] and the Th1-defining cytokine, IFN- γ , is expressed by a higher percentage of gastric T-cells during *H. pylori* infection [33–35]. In addition to total numbers of CD4⁺ and CD8⁺ T-cells, flow cytometric analysis showed that the proportions of lymphocytes that express CD56 were significantly lower in the lamina propria of *H. pylori*-infected individuals compared with uninfected controls; these cells included both CD56⁺ (NKR⁺) T-cells and NK-cells (which are CD3⁻ lymphocytes). The findings of reduced numbers of NK-cells in the *H. pylori*-infected mucosa are in agreement with previous investigations [36]. Additionally, gastric NK-cells are highly activated during *H. pylori* infection [37] and secrete high levels of IFN- γ upon stimulation with *H. pylori* antigens [36]. CD56 expression on immune cells correlates with a cytotoxic function [38]; and T-cells expressing CD56 display NK- and T-cell-restricted cytotoxicity and are capable of secreting cytotoxic cytokines IFN- γ and TNF- α [4,6]. Thus, a reduction in the proportions of NK-cells and of CD56⁺ T-cells during *H. pylori* infection could prove clinically relevant.

As in previous studies in human peripheral blood and the intestine [10,18], the results of the present investigation have shown that in freshly isolated gastric biopsy specimens, only a small minority of T-cells expressed the Va24Ja18⁺ TCR, which defines the NKT-cell population. Nevertheless, in the studies described herein, a greater number of these cells was found among epithelial layer T-cells in *H. pylori*-positive than *H. pylori*-negative individuals. Consistent with this observation, *H. pylori*-derived antigens, in particular lipopolysaccharide, induced expansion of gastric NKT-cells [O'Keeffe J, Gately CM, Moran AP, unpublished results]. The importance of NKT-cells in the immune response against microbial pathogens has been highlighted in a number of infections where these cells promote microbial clearance, and deficiencies in their numbers in animal models have been shown to result in disease exacerbation (for review see [12]). Nonetheless, while expanded numbers of NKT-cells might be intimately associated with *H. pylori* infection, especially at the gastric epithelium, analysis of a greater number of infected subjects is required, particularly since these cells represent a small proportion of the cellular population.

In conclusion, the results of this study have shown that adult human gastric mucosa is a site where populations of T-cells bearing NK receptors, including NKT-cells, accumulate among epithelial and lamina propria layer lymphocyte populations. Given their abundance in the mucosa, the observed differences in the proportions of these T-cell populations in *H. pylori*-positive and -negative individuals suggest that they may play an important role in the immune response against *H. pylori*. Moreover, as a T-cell population lying within the gastric niche of *H. pylori*, NKR⁺ T-cells could influence the outcome of bacterial colonization as seen in other infections at various body sites [39]. Although it can be inferred that a loss of distinct NKR⁺ T-cells with cytotoxic potential during infection may contribute to a lack of control of pathogenic Th1 -cells (secreting IFN- γ) during gastritis development, whereas expansion of distinct cell populations reflect chronic local antigenic stimulation or dysregulated cytokine production, further investigations are required to establish the immune contribution of these cells in *H. pylori* infection.

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SHORT COMMUNICATION

Raised Levels of Interleukin-17 Producing T-cells in the Gastric Mucosa of Patients with *Helicobacter Pylori* Infection

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Running title: Raised levels of IL- 17 producing T-cells in the gastric mucosa of patients with *H. pylori* infection

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Key words

Helicobacterpylori, gastric mucosa, Th1 7 cells, Interleukin- 17

Abstract

Background: *Helicobacter pylori* infection is associated with infiltration of immune cells in the gastric mucosa and the induction of multiple pro-inflammatory cytokines. As IL-17 has a central role in chronic inflammation, a study investigating serum levels of IL-17 and enumeration of IL-17 producing T-cells amongst freshly isolated epithelial and lamina propria layer lymphocytes in *H. pylori* infection was undertaken.

Materials and Methods: Serum samples, gastric (antral) biopsy samples and relevant clinical data were collected from 25 *H. pylori* -positive and 56 *H. pylori* negative subjects. Levels of IL-17 in serum and culture supernatants were detected by ELISA and results were correlated with gastric inflammation. Flow-cytometry was used to quantify Th17 cells amongst epithelial and lamina propria layer cells.

Results: ELISA determination of serum IL-17 showed that in the *H. pylori* -positive group, 47.3% patients had significantly elevated levels of serum IL-17 compared to 13.9% in the *H. pylori* -negative group (odds ratio 5.55). Flow cytometry showed that amongst epithelial layer cells the percentage of Th17 cells was significantly higher in *H. pylori* -infected group [mean (SD):13.7% (6.9)] when compared with uninfected controls [0.2% (0.20)]. Likewise amongst lamina propria cells, the percentage of Th17 cells was higher in *H. pylori* -infected subjects compared with *H. pylori* -negative controls.

Conclusion: IL-17 plays an important role in *H. pylori* infection. *H. pylori* infection is not only associated with high serum IL-17 levels but importantly there is an increased capacity for IL-17 production by cells occupying the epithelial and lamina propria layers of gastric mucosa.

Introduction

Helicobacter pylori (*H. pylori*), a gastro-duodenal pathogen, induces an inflammatory mucosal response characterized by infiltration of multiple cell types both within epithelial glands and the underlying lamina propria layers [1-3]. Most *H. pylori*-infected patients are asymptomatic, but *H. pylori*-driven gastritis can lead to the development of gastroduodenal ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma [4]. The severity of inflammation increases the risk of clinical and histological disease [5]. *H. pylori* produces various cytokines, such as interleukin (IL-) 1 β , interferon- (IFN-) γ , IL-6, and tumor necrosis factor- (TNF)- α resulting in a powerful immune response [6, 7]. Antigens released by *H. pylori* can stimulate endothelial cells, macrophages and epithelial cells to make significant amounts of chemokines, such as interleukin (IL)-8 and growth-regulated oncogene- α , that produce a chemotactic gradient for the migration of neutrophils into the gastric mucosa [8, 9].

IL-17 was originally named cytotoxic T lymphocyte-associated-8 (CTLA-8), subsequently IL-17, and more recently IL-17A, since it is one of six related members belonging to the IL-17 family (IL-17A-F) [10]. IL-17, a pro-inflammatory cytokine, induces a wide array of inflammatory effectors in target cells including certain cytokines, chemokines and other effectors that promote the recruitment of neutrophils and macrophages [11, 12]. IL-17 was detected at the mRNA level in human peripheral blood activated CD4 memory T-cells and subsequent studies have also shown that IL-17 can be also made by activated CD8 T-cells, TCR $\alpha\beta$ T-cells, and neutrophils [10]. Of note, IL-17 is produced by a specific subset of CD4 T-cells, termed T helper (Th) 17-cells, that are distinct from, and antagonized by the classical T helper type 1 (Th1) or T helper

type 2 (Th2) cells [13]. Th17 cells also produce, but to a lesser extent, TNF- α , IL-6, IL-17F, IL-22, and granulocyte macrophage-colony stimulating factor [14]. The molecular pathways governing the development of Th17 cells in humans have not yet been elucidated, but murine studies indicate that Th17 cell differentiation is driven by IL-6, transforming growth factor beta (TGF- β) [15] and IL-23 [16]. Studies have previously shown that IL-17 is produced in excess in the *H. pylori*-infected gastric mucosa [16, 17].

The human gastrointestinal tract contains several phenotypically and functionally distinct populations of T-cells including innate T-cells residing [18] in different tract compartments. T-cells isolated from epithelial (intraepithelial lymphocytes; IELs) and lamina propria layers are cytotoxic and kill cancer cells in vitro [19, 20]. While studies have shown IL-17 production in *H. pylori* infected gastric biopsies [21], to date no study has identified IL-17 producing T-cells in distinct epithelial and lamina propria layers in the uninfected human gastric mucosa and in infection with *H. pylori*. Therefore the aims of this study were, firstly, to enumerate Th17 cells amongst freshly isolated IELs and lamina propria lymphocytes; secondly to compare numbers of Th17 cells in each of these compartments during *H. pylori* infection; and thirdly to correlate serum IL-17 levels in *H. pylori* infection and inflammation.

Materials and Methods

Subjects

In this prospective study the enrolment of 81 patients was done from an open access endoscopy unit attending for oesophago-gastro-duodenoscopy (OGD). The study was started after obtaining specified ethical approvals from University Hospital Galway and the National University of Ireland, Galway. Voluntary written consents were obtained from the patients and confidentiality maintained throughout the project. Further information about the disease process and the study was given to the participants as information leaflets.

Exclusion criterion. A strict exclusion criterion was followed in all the phases of the study to limit the presence of other possible inflammatory processes in the participants. Patients, receiving antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin, on immunosuppressants, or with history of alcohol abuse, with previous history of *H. pylori* infection or gastric surgery, or with history of any chronic inflammatory diseases and co-morbidities were excluded from the study. Most patients included in the study had dyspeptic symptoms and were otherwise well.

Patient sampling and data. Clinical data including age, gender, background history, medication history, symptom record with alarm score, smoking history, brief physical examination and endoscopy results were recorded for each participant at the time of the endoscopy procedure. At the time of the procedure serum samples and multiple biopsy samples from gastric antrum were obtained from the participants for the study and confidentially transported to the laboratory. Each participant underwent CLO[®] testing and histological analysis (Sydney classification) to establish *H. pylori* status. The

remaining gastric samples from each patient were transported to the laboratory in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS; Gibco-BRL, Paisley, UK) for isolation of lymphocytes, culturing, ELISA and flow cytometric analysis.

Preparation of Epithelial Layer and Lamina Propria Mononuclear Cell (LPMC) Single Cell Suspensions

Single-cell suspensions of the epithelial layer and lamina propria were prepared from 4 to 5 gastric biopsy specimens as previously described [22]. Briefly, biopsy samples were rotated at 37°C for 1 hour in calcium- and magnesium-free HBSS supplemented with 5% foetal calf serum, 1 mM dithiothritol and 1 mM ethylenediamine tetraacetic acid (all from Sigma Chemical Co, St, Louis, MO). This resulted in the complete removal of the epithelial layer leaving the lamina propria intact and attached to the basement membrane [22]. The resulting single-cell suspension was washed in RPMI-1640 medium (Gibco-BRL) supplemented with 10% foetal calf serum and antibiotics and viable cells were enumerated by conventional trypan blue (Sigma) exclusion staining. To obtain LPMC's, the remaining tissue was placed in 5 ml of supplemented RPMI-1640 medium containing 130 U/ml collagenase (Type 1A, Sigma) and rotated at 37°C for 3 hours. The resulting single-cell suspension was washed in RPMI-1640 medium and viability counts were performed as above. Viable cells were used for further culturing and immunological techniques.

Culture

Different culturing conditions were optimized on (a) whole mucosal biopsy samples and on (b) viable separated epithelial layer and lamina propria layer cells after initial experiments on peripheral blood mononuclear cells (data not shown). For whole biopsy cultures, mucosal tissue specimens were cultured in a 50ml/L CO₂ incubator on a culture insert (Falcon, Oxnard, CA, USA) placed over polystyrene plates (Falcon) containing RPMI 1640 (Life Technologies, Inc., Rockville, MD, USA) medium with 5% heat-inactivated foetal calf serum and antibiotics (culture medium) and under the stimulated conditions of phorbol- 1 2-myristate- 13-acetate (50ng/ml) and ionomycin (1 µg/ml)(PMA/I) or in medium alone at 37°C for 16 hours. The supernatants were then removed and frozen at -80°C before analysis for IL- 17 levels using an IL- 17 specific ELISA (see below). Likewise in separate experiments, isolated epithelial layer lymphocytes or LPMC were stimulated with PMA/I or medium alone for 16 hours. Supernatants from these cultures were subsequently frozen at -80°C for measurement of IL- 17. For analysis of intracellular expression of IL- 17 in CD3⁺ T-cells among epithelial and lamina propria layer lymphocytes, brefeldin A (2µg/ml; Sigma) was included in the cultures for the final 4 hours.

Measurement of IL-17 by Enzyme-Linked Immunosorbent Assay

(ELISA)

Analysis of IL- 17 was carried out in (a) serum samples and (b) culture supernatants using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA). The procedure was carried out according to the manufacturer's

instructions and the lower detection level of IL-17 was 1.2 pg/ml. For serum analysis of IL-17, peripheral blood samples were collected from patients before the endoscopy procedure. Serum was prepared from each sample by centrifugation for 10 minutes and then storage at -80°C. For analysis of IL-17 in cultures, the whole biopsy or the isolated epithelial layer or LPMC samples were cultured (stimulated or unstimulated; as above), the supernatants removed and then stored at -80°C. IL-17 levels were then measured using the ELISA system.

Detection of Intracellular IL-17 in CD3⁺ Epithelial and Lamina Propria Layer Lymphocytes by Flow Cytometry

Fluorochrome-labelled monoclonal antibodies specific for human CD3 (anti-CD3-Cy-5), for IL-17 (anti-IL-17-PE) and isotype matched IgG control antibodies (PE and Cy5) were obtained from Serotec (Oxford, UK) and from R&D Systems (MN, USA). For Th17 cell detection, single-cell suspensions (1×10^5) derived from the epithelium and lamina propria layers were superficially stained with the anti-CD3-Cy-5 antibody. Cells were then fixed with 4% paraformaldehyde followed by permeabilization with 0.1% w/v saponin buffer. Non-specific binding sites were blocked by incubating the permeabilized cells with 10% heat incubated normal human serum/saponin. The cells were then incubated with the anti-IL-17-PE antibody or isotype IgG control followed by washing in phosphate buffer saline (PBS) before final fixation in 0.5% paraformaldehyde.

Intracellular fluorescence was measured by flow-cytometry using FACsCalibur[®] and CellQuest[®] lysis software (Becton Dickinson, Oxford, UK).

Statistical Analyses

SPSS software was used for data recording and statistical analysis. Pearson χ^2 ($p < 0.05$) with odds ratio were used to analyse the serum IL-17 results. Flow cytometric results were expressed as median values with ranges, and analysed for statistical significance ($p < 0.05$) using the Mann-Whitney U test.

Results

Patient Demographics

A total of 81 patients were enrolled in the study with 44 (54%) males and 37 (46%) females. The mean age was 43 years (range 18 to 75 years). Of the 81 patients enrolled in the study 25 (31%) were determined as *H. pylori* infected and 56 (69%) uninfected. In the *H. pylori* positive group 17 (68%) were males and 8 (32%) females. In the *H. pylori* negative group there were 27 (48%) males and 29 (52%) females. All of these patients attended the endoscopy unit for OGD for the first time and most of them were referrals from the family doctors.

Serum IL-17 levels

In the first part of the study serum IL-17 levels were compared between subjects with and without *H. pylori* infection. A control group of 10 healthy patients was established with normal endoscopies, normal histological biopsies and no other related symptoms. Further 62 participants were enrolled in this part of the study without any evidence of other inflammatory and infective processes. In this cohort of 62 patients, 19 (31%) were *H.*

pylori infected (positive) and 43 (69%) un-infected (negative). ELISA determination of serum IL-17 (Fig.1) showed that in the *H. pylori*-positive group, 9 (47.3%) patients had significantly ($p = 0.005$) elevated levels of serum IL- 17 (mean 31 pg/ml; range 12-48pg/ml) compared to 6 (13.9%) in the *H. pylori*-negative group (mean 16pg/ml; range 12 to 23pg/ml) with an odds ratio of 5.55. All patients with increased levels of serum IL-17, irrespective of *H. pylori* status ($n = 15$), had endoscopically and histologically proven inflammation.

IL-17 Levels in Supernatants from Gastric Cultures

IL-17 levels were then measured in organ culture supernatants from PMA/I stimulated whole mucosal biopsy tissue or cell suspensions from separated epithelium and lamina propria layers (Table 1). In the organ culture of mucosal biopsies, three of 4 patients who were *H. pylori*-negative and displayed no gastritis had low levels of IL- 17 detected in culture supernatants (<15 pg/ml). In contrast, high levels of IL- 17 were detected in the organ culture supernatants of one *H. pylori*-positive sample with endoscopically and histologically proven inflammation. In the next 4 biopsy samples IL- 17 was measured in supernatants from the separated epithelial layer or LPMC cell suspensions. As shown in Table 1, the highest levels of IL- 17 were detected in culture supernatants from the *H. pylori*-positive subjects with gastritis; with markedly higher levels found in LPMC cultures. In addition, significant levels of IL- 17 were also found in epithelial and LPMC cultures from *H. pylori*-negative samples with severe (endoscopic and histological; chemical) gastritis.

Intracellular IL-17 Expression in CD3 + T-Cells from Epithelium and Lamina Propria Layer Single Cell Suspensions

In the next experiments, intracellular expression of IL-17 was studied in freshly isolated CD3 T-cells (Th17 cells) from separated epithelium and lamina propria layers using flow cytometry (Fig. 2). In this cohort, 4 (33%) subjects were *H. pylori*-positive and 8 (66%) - negative. All 4 *H. pylori* infected subjects had endoscopically and histologically proven gastritis. As shown in the representative example in Fig. 2, both IL-17 and IFN- γ were detectable in CD3 T-cells from the epithelial layer and lamina propria layers with proportions of cells ranging from 1.5% to 27.3% for IL-17 and from 23% to 87% for IFN- γ . Summarised data from this cohort of *H. pylori*-positive and *H. pylori*-negative subjects (Fig. 3), showed that when the proportions of Th17 cells were quantified amongst epithelial layer lymphocytes, the frequencies were higher in *H. pylori*-positive subjects (mean, 13.7%; range, 12.2 - 23.4%] when compared with uninfected controls (0.2%; 0 - 0.9%) ($p = 0.03$). Likewise amongst LPMC, the percentage of Th17 cells was significantly higher in *H. pylori*-positive subjects (mean 11.2%; range 1 - 7%) compared with *H. pylori*- uninfected controls (3.5%; 3.5 - 18.2%) ($p = 0.07$).

Discussion

A number of cytokines like IL-8, TNF- α , MPO, IL-23 and IL-17 have been proposed to orchestrate the inflammatory response in *H. pylori*-associated gastro-duodenal disease [1, 18, 23, 24]. Emerging studies [16, 17] and our study have shown that IL-17 levels were significantly increased in the peripheral blood and in the gastric mucosa in *H. pylori* infection. Our study further provides evidence that in *H. pylori* infection, the proportions of Th17 cells were increased amongst IEL populations in the gastric epithelium and amongst lamina propria lymphocytes in the gastric lamina propria. Furthermore our data showed that serum IL-17 levels were increased in *H. pylori* infected patients with gastritis, which is consistent with previous observations [17, 25].

IL-17, predominantly released by Th17 cells, plays a key role in inflammatory and autoimmune conditions (asthma, rheumatoid arthritis, inflammatory bowel disease and psoriasis) as well as in chronic infections [11]. To investigate the increased IL-17 response in *H. pylori* infection, serum IL-17 levels were correlated with gastric *H. pylori* infection in the absence of other inflammatory and infective processes. The criterion was designed to exclude any cause of an ongoing inflammatory process other than *H. pylori* related gastric inflammation. Consistent with this, we noted that antral gastritis was related to IL-17 production where all the patients with a detectable serum IL-17 level had endoscopically and histologically proven inflammation. Furthermore, in the *H. pylori*-infected group, patients had varying degrees of gastric inflammation (Sydney classification) correlating with detectable serum IL-17 levels. Hence it was noted that *H. pylori* infection with endoscopic and histological evidence is significantly associated with elevated serum IL-17 levels and, thus, reiterates the pro-inflammatory role of IL-17 in

chronic *H. pylori* infection. Raised serum levels of IL- 17 have previously been reported with development of duodenal ulcers in *H. pylori* infected patients [25].

In the present study when whole mucosal biopsies were cultured under stimulated conditions, there was an elevated level of IL- 17 detected in culture supernatants in *H. pylori*-infected patients compared with controls, although the number of such samples was small for any significant results. Consistent with the increased secretion of IL-17 in organ cultures, when single cell suspensions from gastric lamina propria and epithelial layers were separated and stimulated, IL- 17 levels were again increased in *H. pylori* infection compared with controls. *H. pylori* infection increases in IL- 17 and IL- 17 RNA transcripts were previously reported in human gastric mucosa and amongst LPMC [26, 27] . Moreover the findings here are in agreement with previous studies on patients with gastric ulcerations [17, 25], where elevated IL- 17 levels are directly related to inflammation and *H. pylori* infection.

The gastric mucosa differentially separated into the epithelium and lamina propria layers contains the largest numbers of T-cells in the body which maintain tolerance to dietary antigens and participate in immune protection [28]. In addition to the measurement of IL-17 in serum and organ culture supernatants, flow cytometry was used to quantify Th 17 cells amongst freshly isolated T cells from the epithelium and lamina propria from *H. pylori*-infected subjects and uninfected controls. The present data shows markedly increased numbers of Th 17 cells amongst both the IEL and lamina propria T cell populations in *H. pylori* infection. Furthermore while Th17 cell numbers were higher in the epithelial layer compared with the lamina propria in the cohort of *H. pylori* infected subjects, the difference was not significant. The results suggest that the *H. pylori*

infection and inflammation is associated with an influx of IL-17 producing T-cells in both the epithelial and lamina propria layers, but this change is more pronounced in epithelial layer.

Results of this study provide new evidence showing that not only serum levels of IL-17 but also an increased capacity for IL-17 production by T-cells occupying the epithelial and lamina propria layers of gastric mucosa in *H. pylori* infection. While isolated changes in IL-17 are unlikely to explain the entire pathological features on the *H. pylori* infected gastric mucosa, an increase in the numbers of Th 17 cells amongst IEL and lamina propria T cells may be relevant, considering the biological function of this cytokine. During infection with *H. pylori*, IL-17 produced locally by tissue resident T-cells can act upon stromal, epithelial and endothelial cells producing pro-inflammatory cytokines and chemokines that rapidly recruit neutrophils to the site of infection. In conclusion, the present study indicates that IL-17 serum levels and Th17 cell numbers in the gastric epithelium and lamina propria were increased in *H. pylori* infected subjects. These IL-17 producing (Th17) cells may participate in *H. pylori* infection and inflammation and may be potential targets in novel and evolving therapies.

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