An Investigation of the Role of the Hypersialylated Myeloma Cell Surface in Facilitating Evasion of Natural Killer Cell-Mediated Immunosurveillance

A thesis submitted to the

National University of Ireland, Galway

as fulfilment of the requirement for the degree of

Doctor of Philosophy

By

John Daly M.Sc.

Apoptosis Research Centre (ARC),

College of Science,

National University of Ireland, Galway

Supervisor:

Professor Michael O'Dwyer

Date submitted:

30/12/2021

Table of Contents

Declarations:

I declare that the work submitted presented herein describes work that was performed personally for the purpose of this thesis. Where other individuals have aided has been stated appropriately. This work was performed between 2016 and 2021 at the Apoptosis Research Centre, National University of Ireland, Galway. This work was supervised and mentored by Professor Michael O'Dwyer. Work was also performed at the Centre for Haematology and Regenerative Medicine at the Karolinska Institute, Stockholm. This work was supervised by Dr. Mattias Carlsten.

The work described within this thesis has not been submitted for degree, diploma or other qualification at any other University. I have no conflict of interest pertaining to the subject matter of this work.

JOHN DALY

12544467

Funding:

This work was funded by a Biomedical Research Scholarship (CRSDAL17) from the Irish Cancer Society. The Irish Cancer Society is a registered charity (Charity Regulatory Authority No. 20009502) and a company limited by guarantee.

Acknowledgements:

I would like to firstly thank my supervisor Prof. Michael O'Dwyer for his incredible support over the course of this project, and during my entire stay in your lab. I couldn't have picked a better supervisor and your constant availability for any issues I had throughout this project ensured that this study was a pleasure to be a part of from start to finish. Your patience while I was learning to become a competent researcher was very generous and the support you gave me for conferences allowed me to visit some outstanding places and make some valuable connections. Thank you!

I would like to secondly thank my co-supervisor Dr. Mattias Carlsten. I thoroughly enjoyed my stay with your group, and the secondment was one of the highlights of my PhD! Your knowledge was invaluable and critical to the advances made in this project. Your support for this project was above and beyond what was necessary, but you helped nonetheless and for that I will always be grateful!

I would like to take the time to thank Dr. Subhashis Sarkar and Dr. Alessandro Natoni for their support and input into this project. Thank you also for your close friendship and constant encouragement, and for being there to listen when I wanted to complain about my cells or experiments! I hope to see you in person in the near future!

I would especially like to thank Mom and Dad for their unwavering support over the last nine years at college and their constant encouragement. You're going to need to google a lot of definitions when you read this, but you'd never complain about it! I cannot wait for the day I graduate and I hope you can be there in person to see it!

Finally, thanks to all my friends for your company over the last few years. God himself only knows how much time I've spent playing Age of Empires with you while watching the Champions League, but it was well worth it! Finally, a special mention to Marcelo Bielsa who single-handily rescued Leeds United and gave me a club to be proud of supporting.

"Marching on Together"

Abstract

Abnormal glycosylation is a hallmark of cancer, and hypersialylation in particular has been shown to facilitate abnormal cell trafficking and drug resistance in many cancers, including Multiple Myeloma (MM) - a currently incurable plasma cell malignancy. Hypersialylation has also been implicated in facilitating evasion of natural killer (NK) cell-mediated immunosurveillance by tumour cells. NK cells are cytotoxic lymphocytes with an innate ability to recognize and destroy virally infected, genetically damaged and malignantly transformed cells and are vital effectors of the innate immune response. Further studies have implicated sialic acid-binding inhibitory NK cell receptors, termed Siglecs, in regulating NK cell functions against Siglec ligand-expressing tumour cells. While advancements in MM therapy have ensured patient outlook has improved considerably in recent decades, the majority of MM patients entering remission will inevitably relapse with more significant health maladies. Therefore, it is imperative to investigate potential mechanisms of resistance utilised by MM cells to evade immunosurveillance by the patient's immune system. In this study the role of hypersialylation in facilitating immune-escape from NK cells in MM was investigated, with a focus on generating novel NK cell-based therapies to enhance anti-MM functions.

While hypersialylation has been implicated in facilitating evasion of NK cell-mediated immunosurveillance by several cancers, prior to this project there had been no investigations into this axis within MM. Therefore, the first phase of this project was to complement existing research in other cancer types by determining whether desialylation of the MM cell surface could enhance NK cell cytotoxicity. Further initial exploratory assays were used to determine the expression of Siglec ligands, and their cognate receptors, on MM cells and NK cells respectively, to confirm whether the Siglec-Siglec ligand axis could potentially be hijacked by MM cells to dampen NK cell responses. Upon MM cell surface desialylation strongly enhanced

NK cell-mediated cytotoxicity was observed against several classical MM cell lines, confirming that cell surface hypersialylation protects MM cells from NK cell-mediated immunosurveillance. Screening of primary MM cells, isolated from patient bone marrow aspirates, and a panel of MM cell lines revealed strong expression of ligands for both Siglec-7 and Siglec-9. Furthermore, analysis of primary NK cells; either resting, activated or expanded, sourced from both healthy donors and NK cells from BMAs of MM patients revealed high expression of Siglec-7. Siglec-7 was expressed at higher levels in NK cells from MM patient BMAs than in NK cells from the peripheral blood of healthy donors, confirming the potential of Siglec-7/Siglec-7 ligand interactions within the MM tumor microenvironment to be exaggerated and therefore contribute to suppression of NK cell anti-cancer functions. Siglec-9 expression was also observed on subsets of naïve and IL-2 activated primary NK cells, but was absent on expanded NK cells and NK cell lines, presenting Siglec-7 as a promising candidate for further investigations. Finally, mass spectrometry of Siglec-7 Fc chimera-binding proteins after incubation with MM cell line lysates revealed PSGL-1 and CD43 as ligands for Siglec-7, with PSGL-1 more widely expressed than CD43. Elucidating the identity of these ligands presents PSGL-1 and CD43 as novel target antigens which can be targeted to abolish Siglec-7-mediated NK cell regulation against MM cells.

Hypersialylation of the tumour cell surface has been hypothesized to physically mask activating ligands which could otherwise promote NK cell anti-cancer functions. Desialylation of MM cells has previously been shown to increase detection levels of BCMA, an antigen targeted by monoclonal antibody therapy to stimulate NK cell-mediated ADCC. Upon desialylation of MM cell lines and screening for several different target antigens, increased detection of the glycoprotein CD38 was observed. CD38 is a target of anti-CD38 monoclonal antibodies used in the clinic to treat MM, such as Daratumumab (Dara). Following desialylation MM cells exposed to Dara were sensitized to NK cell-mediated ADCC compared

to Dara treatment alone. To address MM cells with low CD38 expression, which can be observed following Dara treatment in MM patients, MM cells were treated with ATRA to upregulate CD38 expression. Combining ATRA treatment with SIA-mediated desialylation and Dara elicited a potent NK cell-mediated cytotoxic response, which was significantly stronger than individual treatments of the same MM cells. Finally, CD38 KO in NK cells using CRISPR/Cas9 was proposed as a means of potentially preventing fratricide, a limiting factor of therapeutical response observed in moAb-based immunotherapies heavily dependent on NK cell-mediated responses, such as Dara.

The final phase of this project was to investigate the potential of targeting promising NK cell immune checkpoints such as Siglec-7 and CD96 to enhance NK cell anti-MM cytotoxicity. Having identified Siglec-7 as a promising novel checkpoint inhibitor in regulating NK cell cytotoxicity against MM and, using CRISPR/Cas9 to genetically delete Siglec-7 expression, Siglec-7^{KO} NK cells displayed enhanced cytotoxicity against Siglec-$7L^{+}$ MM cell lines, confirming that MM cells can evade NK cell-mediated immunosurveillance in a Siglec-7-dependent manner. However, it is not yet apparent whether enhanced NK cell-mediated cytotoxicity observed upon MM cell surface desialylation is entirely as a result of abolished Siglec-7-Siglec-7 ligand interactions, and may potentially be caused, at least in part, by exposing ligands for activating NK cell receptors previously masked by sialic acids. Further CRISPR/Cas9-based studies in this chapter targeted CD96 - an NK cell expressed receptor with an unclear role in human NK cells with contradictory data suggesting CD96 to be both an inhibitory and activating NK cell receptor. Cytotoxicity and cytokine release assays revealed conclusively that CD96 is an inhibitory NK cell receptor, with $CD96^{KO}$ NK cells mediating higher cytotoxicity and cytokine release against $CD155^{+}$ MM cell lines, compared to $CD96^{+}$ NK cells from the same donor. While blocking antibodies have been used to target NK cell immune checkpoints, highly efficient targeting of immune checkpoints using CRISPR/Cas9

MM has not previously demonstrated. Thus, this phase of the project highlights Siglec-7 and CD96 as important NK cell immune checkpoints, as well as proposing CRISPR/Cas9 as a promising tool to engineer potent NK cells lacking inhibitory pathways that can be hijacked by cancer.

Taken together, this project demonstrates several novel mechanisms of enhancing NK cell activity against MM. Targeted desialylation of the hypersialylated MM cell surface is a promising strategy to enhance NK cell efficacy against MM, which can be combined with existing frontline therapies, such as anti-CD38 monoclonal antibodies, to elicit a potent anti-MM response by combing enhanced cytotoxicity through abolished Siglec-7 inhibitory signalling while simultaneously enhancing ADCC. Finally, CRISPR/Cas9 represents a novel mechanism for generating highly potent NK cells, potentially suitable as part of adoptive NK cell-based therapies which could deliver more enhanced anti-MM responses.

List of Figures

Chapter 1: Introduction

Chapter 3:

Chapter 4:

Chapter 5:

Appendix A:

Appendix B:

List of Tables

Chapter 2:

Chapter 5:

Abbreviations

ABCG2	APT-binding cassette transporter G2
ADAM17	a disintegrin and metalloprotease 17
ADC	antibody-drug conjugate
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cell-mediated phagocytosis
AKT	AKT serine/threonine kinase
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	antigen-presenting cell
ASCT	autologous stem cell transplants
ATRA	all-*trans* retinoic acid
BCMA	B-cell maturation antigen
bFGF	basic fibroblast growth factor
BiKE	bi-specific killer engager
BM	bone marrow
BMA	bone marrow aspirate
BME	bone marrow microenvironment
CAR	chimeric antigen receptor
CAR-NK	CAR-NK cell
CAR-T	CAR-T cell
CCND1	cyclin D1
CCND3	cyclin D3
CCR7	C-C chemokine receptor type 7
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
cDNA	complementary DNA
CLL	chronic lymphocytic leukaemia
CLP	common lymphoid progenitor
CMAH	CMP-Neu5Ac hydroxylase

CMP	cytidine-5'-monophosphate
CR	complete remission
CRBN	cereblon
CRISPR	clustered regularly spaced palindromic repeats
crRNA	CRISPR RNA
CSR	class switch recombination
CUL4A	Cullin 4A
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
DDB1	DNA-binding protein 1
DKK1	Dickopff-1
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
E:T	effector cell:target cell
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular-signal-related kinase pathway
FA	Fanconi anaemia
FBS	foetal bovine serum
FDA	Food and Drug Administration
FMO	fluorescence minus one
FSC	forward scatter
GalNAc	N-acetylgalactosamine
GDF15	growth differentiating factor 15
GlcNAc	N-acetylglucosamine
GM-CSF	granulocyte/macrophage colony stimulating factor
gRNA	guide RNA
haCD16	high affinity CD16

HER	Herceptin
HDR	homology directed repair
HIF1α	hypoxia inducible factor 1 subunit alpha
HLA	human leukocyte antigen
HSC	haematopoietic stem cell
iDC	immature dendritic cell
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor
IMiD	immunomodulatory drug
iNK	immature NK cell
ITAM	immunoreceptor tyrosine-based inhibitory motif
ITIM	immunoreceptor tyrosine-based activating motif
KIR	killer cell immunoglobulin-like receptors
KO	knockout
MAA	*Maackia amurensis agglutinin*
MAC	membrane attack complex
MAF	Musculoaponeurotic fibrosarcoma
MAFB	Musculoaponeurotic fibrosarcoma oncogene homolog B
mDC	mature dendritic cell
MDSC	myeloid-derived suppressor cells
MFI	mean fluorescence intensity or median fluorescence intensity
MGUS	monoclonal gammopathy of undetermined signifigance
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1-alpha
MM	multiple myeloma
MMSET	multiple myeloma SET domain
mNK	mature NK cell
moAb	monoclonal antibody
M-protein	monoclonal protein
MRD	minimal residual disease
mRNA	messenger RNA

MYC	MYC Proto-Oncogene, BHLH Transcription Factor
NCR	natural cytotoxicity receptor
ND-MM	newly diagnosed MM
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NEURA	neuraminidase
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non homologous end joining
NK	natural killer
NKG2A	natural killer group 2, member A
NKG2D	natural killer group 2, member D
NKp	NK progenitor
OS	overall survival
PAM	protospacer adjacent motif
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed death receptor
PDCD1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PFS	progression free survival
PI	proteosome inhibitor
PI3K	phosphatidylinositol-3 kinase
P. Iodide	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PSMB5	proteasome 20S subunit beta 5
RAF	rapidly accelerated fibrosarcoma
RANKL	nuclear factor kappa-B ligand
RAS	rat sarcoma
RNA	ribonucleic acid
RPM	revolutions per minute

RR-MM	relapsed/refractory MM
SAMP	self-associated molecular pattern
sCR	stringent complete response
SD	standard deviation
SDF-1	stromal cell-derived factor 1
SEM	standard error of mean
sgRNA	single guide RNA
SHP	Src homology-containing tyrosine phosphatases
SIA	sialyltransferase inhibitor 3Fax-PeractylNeu5AC
Siglec	sialic acid-binding immunoglobulin-like lectin
Siglec-7L	Siglec-7 ligand(s)
Siglec-9L	Siglec-9 ligand(s)
SLAMF7	signalling lymphocyte activation molecule F7
SMH	somatic hypermutation
SMM	smouldering MM
SNA	*Sambucus nigra agglutinin*
SS	single stain
SSC	side scatter
STAT3	signal transducers and activators of transcription 3
TCR	T cell receptor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TME	tumour microenvironment
TNF-α	tumour necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
Tras	Trastuzumab
T-reg	regulatory T cell
TriKE	tri-specific killer engager
ULBP	UL-16 binding protein
VCAM-1	vascular cell adhesion protein 1
VEGF	vascular endothelial growth factor
VEGF-R1	vascular endothelial growth factor receptor 1

VEGF-R2	vascular endothelial growth factor receptor 2
ZAP-70	zeta-chain-associated protein kinase 70

1. Introduction

1.1 Multiple Myeloma

1.1.1 Historical Overview

Although Multiple Myeloma (MM) has likely existed for much longer, the first reported case was in 1844 and involved a thirty nine-year old woman – Sarah Newbury - who presented with bone pain and fatigue [1]. Upon autopsy, abnormal bone marrow (BM) was observed and was consistent with what was reported in probably the most famous case of MM, in Thomas Alexander McBean. Autopsy of Mr. McBean in 1846 revealed the presence of round or oval cells that were up to twice as large as an average blood cell, that presented with one or two nuclei and a brightly coloured nucleolus. These cells were observed within the bone marrow, and it was noted that bones such as rib bones were brittle and weak [2]. In 1875 the term plasma cell was coined by the German anatomist Waldeyer, but likely referred to mast cells at the time. Ramon y Cajal and Marschalko contributed to the best early descriptions of plasma cells, noting them to contain a spherical or irregular cytoplasm and eccentric nucleus positioning [3, 4].

In 1900, Wright proposed that MM cells were plasma cells, or direct descendants of plasma cells [5]. In 1928, a report on over four hundred cases of MM reported consistent fractures of the bones, anaemia and chronic renal disease in afflicted patients. The report also first reported hyperproteinemia (increased protein concentration) in urea of MM patients [6]. Upon the invention of the bone marrow aspirate (BMA) technique in 1929 by Arinkin, increased recognition of MM was possible [7]. As proof, three cases of MM had been reported in Mount Sinai Hospital, New York in a nineteen-year period up until 1935. However, upon availability of BMA, thirteen cases of MM were reported in the next two and a half years [8]. In 1961, Waldenström presented the idea of monoclonal vs polyclonal gammopathies, and detailed that MM patients often presented with higher levels of monoclonal protein (M-protein) but had no other symptoms of the disease (this eventually became known as malignant gammopathy of undetermined significance (MGUS) - a precursor condition of MM). Conversely, symptomatic MM patients presented with high levels of M-protein, while patients presenting with higher level of polyclonal proteins were likely to suffer from non-malignant maladies such as inflammation or infection [9].

Initial therapies for MM were simple, with Sarah Newbury being treated with rhubarb and orange peel infusions [1]. Thomas Alexander McBean was treated with quinine and steel and showed a good response [2, 10]. Urethane was used as the frontline therapy for MM for more than fifteen years, first being introduced in 1947 by Alwall. Urethane resulted in reduced serum globulin, a decrease in bone marrow cells and an abolishment of proteinuria [11]. However, when a study in 1966 treated MM patients with urethane or a control, no overall improvements or survival was observed [12]. Melphalan, a chemotherapy drug, showed promise producing responses in 78% of MM patients, including newly diagnosed (ND MM) and previously treated MM patients [13-15]. Corticosteroids were also used, with varying results. The most prominent, prednisone, showed promise as a single agent and when combined with melphalan contributed to a better therapeutic response than melphalan alone [16]. In 1974, the M-2 protocol was established, where MM patients were treated with carmustine, cyclophosphamide, melphalan, vincristine and prednisone. Separate studies showed that this regimen resulted in 60% and 87% responses in MM patients, respectively [17, 18]. However, another study demonstrated that this regimen did not produce longer-lasting responses and did not improve survival compared to melphalan and prednisone treatment [19].

Stem cell transplantation was first introduced in 1957, where patients were treated with irradiation or chemotherapy after which allogeneic bone marrow cells were intravenously infused [20]. In MM the first successful syngeneic bone marrow transplantation was recorded in 1982, after which bone marrow transplant studies were conducted and showed promise [21]. In 1987, Barlogie described treatment of MM using melphalan, total body irradiation and autologous bone marrow transfer, and subsequently developed therapies using autologous transplantation which contributed to the standard of care of MM for many years [22].

Treatments for MM have improved dramatically in the last couple of decades, with the addition of several novel new drugs including proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) contributing to a current overall five-year survival rate for MM of marginally over 50% compared to 34% in 1998 and approximately 25% in the late 1960s [23]. These are discussed in detail in **1.1.7**, while a historical timeline of MM with notable events is presented in Figure 1.1.

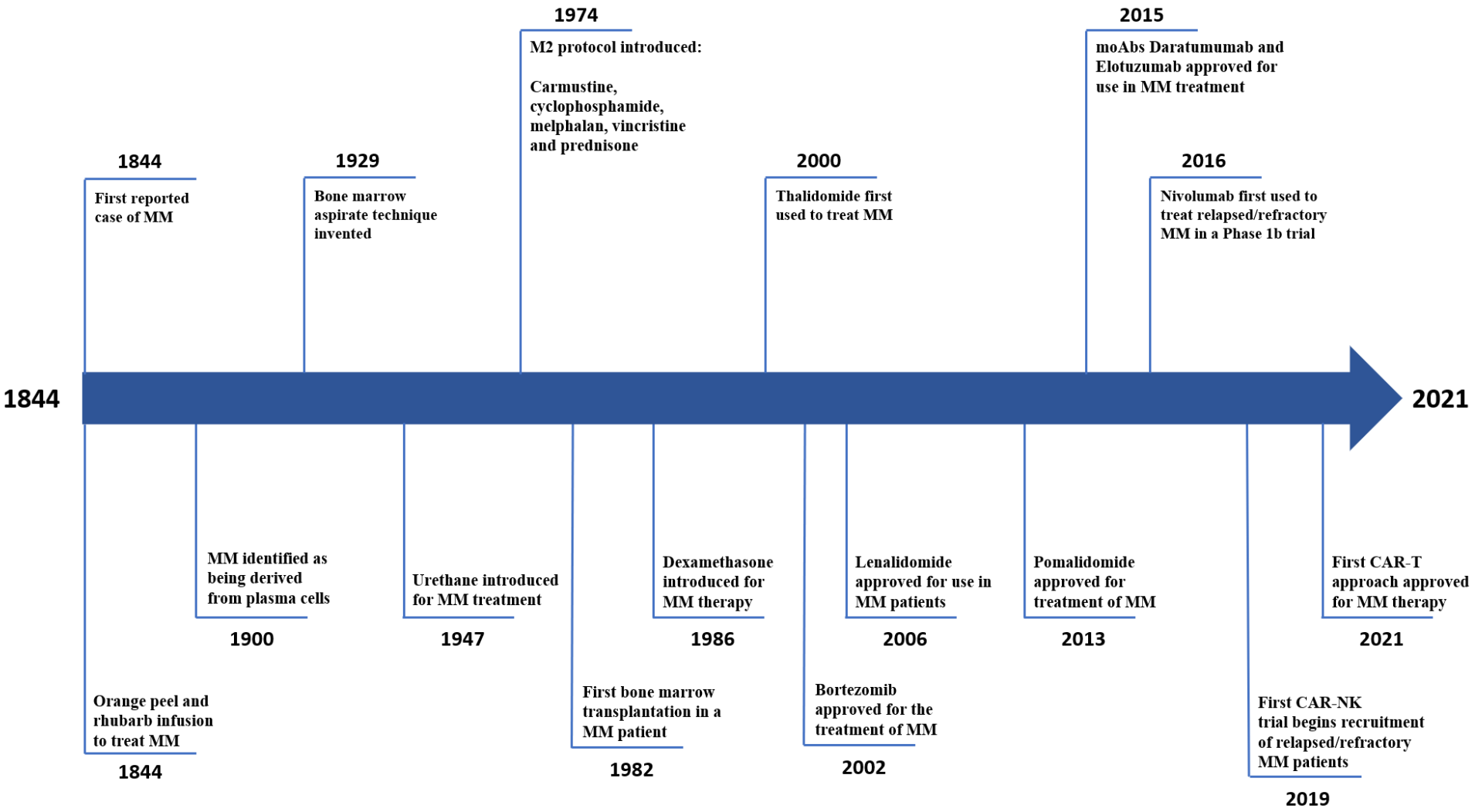


Figure 1.1 Historical overview of major advances in the field of MM research

1.1.2 Epidemiology of MM

MM is the second most common blood cancer worldwide, after lymphoma [24]. In 2020, the Global Cancer Observatory reported the diagnosis of approximately 175,000 new cases of MM diagnosed that year, with males accounting for around 98,000 cases while females accounted for around 77,000 cases. The Global Cancer Observatory also reported that, in 2020, 117,000 deaths were recorded due to MM, accounting for 1.2% of cancer-related deaths in the world that year. In the Western world, the incidence rate of MM is considered to be 5 in 100,000 with the median age range of patients diagnosed between 66-70. Approximately 37% of patients will be under the age of 65 [24, 25]. In people younger than 30 years old, the incidence rate for MM is extremely low with a reported frequency of 0.02-0.03% with males in this category appear slightly more susceptible [26]. MM is not considered to be a hereditary disease although studies have shown that relatives of MM patients are slightly more susceptible to developing MGUS, MM, Waldenström macroglobulinemia and chronic lymphocytic leukaemia [27]. MM is twice as common amongst African Americans compared to European Americans, and three times as common in the same populations in people aged below 50 [28, 29]. This is attributed to increased prevalence of the MM precursor condition MGUS amongst African Americans compared to European Americans. The age of disease onset was also reported to be younger in African Americans compared to European Americans [29]. Incidence of MM appears to be higher in the Western world compared to Asia and Africa, but is likely due to the diagnostic capabilities available in more advanced regions. In 2016, a marked 126% increase in the incidence of MM globally was documented and attributed to both an aging and growing worldwide population [30]. Risk factors such as obesity, chronic inflammation, exposure to pesticides and radiation as well as organic solvents are linked with MM [31].

Advancements in science and therapies have significantly improved the outlook for MM patients over the last two decades. In 2008, a study revealed that the median survival of relapsed/refractory MM (RR MM) patients prior to 2000 was 12 months, which increased to 24 months post-2000 and has likely increased further since then [32]. Furthermore, five-year relative survival of MM patients increased from 34% in the 1989-1992 period of diagnosis to 56% in the 2001-2005 period of diagnosis [33]. Another study reported that the five-year

relative survival rates of MM patients from 2005-2011 was 49% compared to 27% from 1987-1989 and 27% from 1975-1977 [34]. The 2005-2011 period was influenced by the novel treatments using the PIs bortezomib and the IMiDs thalidomide and lenalidomide. Treatment of RR MM patients with one of these drugs resulted in a doubling of survival post relapse [35]. Survival rates of MM patients vary, depending on the stage of the disease upon diagnosis. For example, patients with localized MM have a 74.8% five-year survival rate, however this only equates to 5% of all diagnosed MM patients. The remaining 95% of patients will have systemic myeloma, for which the five-year survival rate is 52.9%, as reported in as study by the SEER Cancer Statistics Review (https://seer.cancer.gov/csr/1975_2016/).

Despite the promising advances in MM therapy, concern remained as to how beneficial recently introduced therapies are to older MM patients. Clinical trials for MM often exclude older patients due to health issues rendering them unsuitable for trials. Studies showed that patients over 60 years old did not have an improved five-year survival rate, while improvements in ten year-survival rates were observed in patients under the age of 65 [36, 37]. Furthermore, a study in 2010 showed that in a comparison of patients in the Netherlands over the age of 65 diagnosed in 1989-1992 and 2001-2005, no improvement in five-year survival rate had occurred [33]. At the time, it was assumed that this was due to older patients being ineligible for autologous stem cell transplants (ASCTs). However, more recent studies have suggested that patients aged 65-79 have improved survival rates compared to earlier time ranges, but that patients aged over 80 do not seem to have improved survival rates [38]. The more recent improvements in survival of the 65-79 age range are attributed to the availability of novel drugs that benefit ASCT-ineligible MM patients.

Despite recent advancements in MM therapies, most patients will face relapses and will ultimately succumb to the disease or related health-complications.

1.1.3 Diagnosis of MM

MM patients will often present with fatigue and bone pain. Fatigue is induced by anaemia, reported in 75% of MM patients. Bone lesions can be detected in approximately 80% of patients and can be observed using X-rays, magnetic resonance imaging and

computerised tomography scans. Low-dose full-body computerised tomography scans are recommended, due to rapidity and better sensitivity compared to plain radiography [39]. Also observed upon diagnosis of MM is hypercalcemia (in 15% of patients) and elevated serum creatinine (in 20% of patients) [25]. BMAs will routinely be taken, to allow assessment of MM cell infiltration, and flow cytometry is a common technique used to assess MM cell frequency within the BM. Although not common, isolated purified MM cells can be examined for the presence of high-risk genetic lesions, such as t(4;14), t(14;16), and del(17p) [40]. A high concentration of a specific M-protein is observed in the vast majority of patients in routine blood tests and is indicative of MM presence. M-protein, produced by MM cells, can be thoroughly further tested for using the serum free light chain assay in combination with immunofixation in serum protein electrophoresis [41]. In 2014 the International Myeloma Working Group defined that MM patients are classified as having > 60% of bone marrow plasma cells, and serum free light-chain ratio of > 100. Furthermore, active MM patients will have at least two focal lesions of the skeleton [42]. During diagnosis, patients will be assessed using the CRAB mnemonic, which is a summary of the most common presenting clinical manifestations in MM patients: calcium elevation, renal impairment, anaemia and bone disease. The CRAB mnemonic helps to determine disease stage upon diagnosis and furthermore, analysis of patients presenting with CRAB factors revealed that patients presenting with hypercalcemia and bone disease was associated with worse prognosis [43].

1.1.4 Monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM) and their management

MM is known to develop from a condition known as monoclonal gammopathy of undetermined significance (MGUS). MGUS is an pre-malignant, asymptomatic precursor to MM, and is observed in 3-4% of adults over the age of 50 in America [44]. MGUS was first reported in 1978. In 1980, another more advanced, but still not active, MM disease condition was described when six patients met the criteria for MM but the disease was not observed to be aggressive at the time of diagnosis. This condition was termed smouldering multiple myeloma (SMM). Only 10% of MM patients will have a history of MGUS due to the asymptomatic nature of the condition. MGUS is defined by the concentration of < 3g/dL of

M-protein and < 10% of bone marrow plasma cells. SMM can be diagnosed by the presence of either > 3g/dL of M-protein in the serum or 10-60% of bone marrow plasma cells but no defining symptoms of myeloma as defined by the CRAB criteria. Both MGUS and SMM will have absence of end-organ damage, also determined by the CRAB criteria [45].

The median age of diagnosis of MGUS is approximately 70 years old while < 2 % of all MGUS cases are recorded in patients aged less than 40 years old, indicating that MGUS is a disease of the elderly [44, 46]. MGUS has a risk of transitioning to MM of 1% every year, while SMM has a higher risk of transition into MM of approximately 10% per year for the first five years, decreasing to 3% for the next five years and 1% for the years thereafter. The risk of transition from MGUS to MM does not decrease over time, and lifetime transition to active MM for MGUS patients is approximately 11% [47]. Determining the risk of progression to full MM in MGUS patients is difficult, however the size of the M-protein was the most important predictor of progression in a study of over 1,384 MM patients. This study revealed that MGUS patients with an M-protein level of 25g/l risked progressing to MM or a related disorder at a rate of 49%, while MGUS patients with a maximum M-protein level of 5g/l risked progression at a rate of 14% [48]. The importance of M-proteins has also been emphasised when a study found that an increase in M-protein level was the single most indicative risk factor for expression [49]. Further analysis of the 1,384 MM patient cohort have revealed that IgM and IgA MGUS patients have an increased likelihood of transitioning to MM compared to IgG MGUS patients, while a separate study confirmed that IgA MGUS patients also have an increased likelihood of progression compared to IgG and IgM MGUS patients [48, 50].

SMM consist of two main subsets of patients: those who have a biological premalignancy (MGUS) and biological malignancy (MM) but have not yet developed MM-defining criteria as defined using the CRAB criteria [42]. Therefore, patients with SMM can be very stable for many years or transition to full MM within a couple of years of diagnosis. In comparison to the risk of progression to MM from MGUS, SMM patients with IgA M-protein are also at risk of progressing quicker to MM than patients with IgG and IgM M-protein [51]. Similarly to MGUS, SMM patients with an increasing M-protein level are at a higher risk of progressing to MM than those who have stable levels of M-protein [52]. Flow cytometry can be used to determine the prognosis of SMM patients, with malignant plasma cells expressing an aberrant phenotype, including decreased expression of CD38, increased expression of

CD56 and absence of one or both of CD19 and CD45 [53-55]. SMM patients with an aberrant plasma cell phenotype similar to that of MM cells (> 60% of SMM patients) were determined to be at a higher risk of progression to MM compared to those with less aberrant phenotypes [53].

Most patients with SMM will transition into having MM and will require treatment. Both MGUS and SMM patients should be monitored regularly to facilitate early recognition of transition to full MM, and the current standard of care for both MGUS and SMM is observation until transition to MM is observed. However, in 2014, the International Myeloma Working Group declared that SMM patients with high-risk of progression were to be considered to have active and were to be eligible for chemoprevention trials. Other SMM patients will undergo serum protein electrophoresis, a complete blood count, quantification of calcium and creatinine levels and urine collection for electrophoresis and immunofixation upon initial diagnosis. Additionally, a baseline biopsy of BM should be taken along with a full skeletal survey. Finally, magnetic resonance imaging scans of the spine and pelvis should be undertaken to identify bone lesions. This should be repeated regularly, every 3-4 months after diagnosis, but the rate of testing can be slowed to 6-12 months if the disease appears to be stable. If evidence of progression is obtained in the tests outlined above, a skeletal survey should be undertaken [45].

1.1.5 Generation of plasma cells and transformation into MM clones

Plasma cells are the cell of origin for MM cells. Plasma cells are antibody-producing cells which represent the terminal differentiation of B lymphocytes and are located primarily within the bone marrow, spleen, lymph nodes, from where they can secrete antibodies which can easily transfer into the circulatory system [56]. Antibodies are vital proteins involved in the immune system, and facilitate the clearance of pathogens or toxins by binding to unique antigens expressed on the invading cell surface, triggering the immune system to destroy the pathogen or toxin. Furthermore, antibodies can recruit cells of the innate immune system, such as Natural Killer (NK) cells and T cells to aid in pathogen clearance [57].

As mentioned previously, plasma cells are the terminal differentiation of B lymphocytes. The initial stages of this final differentiation occur within the bone marrow, where rearrangements of the heavy chain Ig gene – *IGH* occurs. *IGH* is a large gene, roughly 2Mb in size, and contains four major domains; the variability domain (*VH*, over 100 deoxyribonucleic acid (DNA) segments), the diversity domain (*DM*, 27 DNA segments), the joining domain (*JH*, 7 DNA segments) and the constant domain (9 DNA segments). Firstly, DNA deletions result in one *DH* domain combining with one of up to six *JH* domains in a stochastic manner (*DH-JH*). Should the combination be molecularly productive, the pre-B cell then combines this *DH-JH* segment with a *VH* segment, facilitated by a recombinase enzyme. If this combination is successful, and the pre-B cell is considered productive, then the light chain genes IGLκ and IGLλ will be rearranged. If successful, the mature B cell produces IgMκ, located at the cell surface. The mature B cells then egress from the bone marrow, and locate to secondary lymphoid organs to continue maturation. While the development of the B cell until now was antigen independent, the subsequent development is antigen dependent and is facilitated by T cells and dendritic cells. Somatic hypermutation (SMH) occurs in plasma cells within the germinal centres of these secondary lymphoid organs, and involves the enzyme activation-induced deaminase, which causes random mutations in the VDJ gene segments. If these mutations improve the affinity of the antibody for its antigen produced by the individual B cell, the B cell is allowed to survive, while other B cells will be eliminated by apoptosis. A final mutation occurs in the secondary lymphoid organs, known as class switch recombination (CSR). Again utilising activation-induced deaminase, DNA segments known as switch regions will be combined with interswitch DNA being deleted. After this, the B cell will competently produce either an IgG, IgA or IgE immunoglobulin and relocate to the BM where it will finally differentiate into a memory B cell or a long-lived plasma cell [(58)].

MM is considered genetically complex and require the occurrence of multiple genetic events to lead plasma cells to transform into MM cells. The initial events, or “hits”, are thought to occur in the secondary lymphoid organs. MM cells are observed to have high levels of somatic mutations, occurring during the SMH stage of B cell development. Furthermore, MM predominantly consists of IgA and IgE-producing MM cells, indicating that genetic abnormalities are also likely gained during the CSR process, again occurring in germinal centres of secondary lymphoid organs [(58)]. Genetic aberrations in MM can be classified as

being either primary or secondary events. Primary events lead to the formation of long-lived plasma cell clones, while secondary events lead to MM disease progression [59]. MM can be divided into two classes; hyperdiploidy and non-hyperdiploidy MM which are defined by the presence of certain primary events [60]. Hyperdiploidy MM occurs in approximately 55% of MM patients. Analogous to other B cell malignancies, chromosomal translocations are found in a large percentage of MM cells, which is known as non-hyperdiploidy MM, and accounts for approximately 45% of MM cases [61]. These translocations involve the IgH locus during aberrant CSR and result in the juxtapositioning of IgH enhancers to one of five oncogenes; *4p16* (*MMSET* and *FGFR3*), *6p21* (*CCN D3*), *11q13* (*CCN D1*), *16q23* (*c-MAF*) and *20q11* (*MAFB*) [62]. These translocations can either occur near or within switch regions but can sometimes can be found near or within VDJ regions, suggesting both CSR and somatic hypermutation can lead to the formation of these translocations [63]. The most common translocation observed is t(11;14), which is observed in 15-20% of MM patients and results in the overexpression of Cyclin D1 (CCND1) [64]. The second most common translocation observed is t(4;14), in 12-15% of MM patients, leading to the upregulation of oncogenes Fibroblast growth factor receptor 3 (*FGFR3)* and Multiple Myeloma SET domain (*MMSET)*. While upregulation of the FGFR3 protein is observed in approximately 70% of MM patients, the most common target of this translocation is *MMSET*, resulting in the increased expression of the histone methyl-transferase protein MMSET which methylates several proteins within the genome [65]. These represent the main translocations observed within MM patients, although other translocations such as t(14;16), t(14;20) and t(6;14) are infrequently observed dysregulating the oncogenes Musculoaponeurotic fibrosarcoma (*MAF),* Musculoaponeurotic fibrosarcoma oncogene homolog B *(MAFB)* and Cyclin D3 (*CCND3),* respectively [66-68]. The overexpression of cyclin D genes is a key molecular abnormality observed in MM [68]. Hyperdiploidy MM is associated with better prognosis, and is associated with trisomies of genes 3, 5, 7, 9, 11, 15, 19 and 21, with a low rate of *IGH* translocations [69]. While the exact reasons for non-hyperdiploidy are not well understood, it is hypothesized that a single catastrophic mitosis event could result in the acquisition of extra copies of the above chromosomes [70]. Similar to non-hyperdiploidy MM, the upregulation of cyclin D genes is also observed in hyperdiploidy MM - ultimately dysregulating the G1/S cell cycle phase transition point. These primary events result in the formation of long-lived plasma cell clones, but secondary events are required for the formation of malignant MM cells.

These secondary events are found primarily in SMM and MM patient-derived MM cells. These events include secondary translocations, acquired mutations, epigenetic changes and copy number variations [69]. Secondary translocations are CSR-independent and happen after the B cell has matured in the germinal centres of secondary lymphoid organs. One of the most dysregulated targets of secondary translocations is MYC Proto-Oncogene, BHLH Transcription Factor (*MYC)* which is rarely observed in MGUS but is more frequent in MM and more frequent again in advanced disease [71]. Increased MYC expression results in increased rates of DNA replication, making translocations targeting *MYC* indicators of poor prognosis and indicative or higher tumour burden [72].

Activating mutations of *RAS* and *BRAF* have also been observed in MM, and are observed in roughly 15% of ND and RR MM patients. Mutations in rat sarcoma (*RAS)* and rapidly accelerated fibrosarcoma (*RAF)* are linked with reduced endoplasmic reticulum (ER) stress and promote MM cell survival as well as conferring resistance to PI-based treatments [73].

Also recorded in approximately 50% of MM cases is an active nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway. The NFκB pathway is activated in MM cells through abolishment of pathway suppressors due to genetic deletions, or pathway hyperactivity due to translocations or copy number variants [74, 75]. The NFκB pathway facilitates MM cell survival and proliferation, contributing to the presence of long-lived MM cell clones. Interestingly, the NFκB pathway may be active within surrounding residential cells in the bone marrow resulting in the production of cytokines such as interleukin (IL) -6 , that provide key survival signals to MM cell clones [76].

IL-6 has also been implicated in activating the JAK-STAT pathway within MM cells. The implication of this is hyperactivity of signal transducers and activators of transcription 3 (STAT3), a transcription factor which results in high expression of B-cell lymphoma-extra large (Bcl-xL), an important anti-apoptotic protein which has been shown to correlate with chemotherapy resistance in MM [77, 78]. Also important to myelomagenesis is the phosphatidylinositol-3 kinase (PI3K) pathway. Signals induced by cytokines and chemokines such as IL-6 and insulin-like growth factor (IGF-1), result in the phosphorylation of AKT Serine/Threonine Kinase (AKT). AKT then activates downstream targets, resulting in enhanced

cell proliferation and resistance to apoptosis and is observed in approximately 50% of MM patients [79].

Other secondary genetic events are observed with less frequency but, in general, most common primary and secondary events have been well documented and can indicate the prognosis for each patient, depending on the primary and secondary events which are present within the genetic profile of MM.

1.1.6 The bone marrow microenvironment and its contribution to MM progression

BM is semi-solid tissue which occupies the hollow spaces at the centre of spongy or cancellous bone. Situated in the BM are blood vessels and interstitial cells which take on the main role of the bone marrow: haematopoiesis - the formation of all blood cells from haematopoietic stem cells [80]. Within the bone marrow microenvironment (BME) is located a cellular and non-cellular component. The cellular component consists of a milieu of cell types including mesenchymal stromal cells, endothelial cells, osteoblasts, osteoclasts, fibroblasts, hematopoietic cells as well as immune cell types. The non-cellular component consists of liquids and the extracellular matrix, and is rich in chemokines and cytokines produced by BM-residing cells [81]. Homeostasis of the BME is critical for the proliferation, differentiation and metabolism of normal cells, and abnormal BME conditions have been shown to lead to tumorigenesis and bone lesion development [82]. Studies into the role of the BM and BME have shown that the BME can support the expansion and differentiation of malignant plasma cells while also contributing to drug resistance, demonstrating that the BM is an important contributor to MM disease progression (Figure 1.2) [83, 84].

In MM, malignant plasma cells accumulate within the BM. This is mediated by the C-X-C chemokine receptor type 4 (CXCR4) receptor on MM cells which binds to stromal cell-derived factor 1 (SDF-1) and leads to migration to the bone marrow matrix, notably the stromal compartment [85]. Both the cellular and non-cellular compartments of the bone marrow contribute to the survival of MM cells. However, the immune system within the BME is capable of mounting a strong anti-tumour response, mainly driven by NK and T cell effector cells. In order for progression of MM to occur, a strongly immunosuppressive environment is

created within the tumour microenvironment (TME) of the BM and leads to immune-escape by MM cells. Some of the most important BM-residing cell types within the cellular compartment which contribute to MM disease progression and their survival are discussed below, but other types exist and have been researched and reviewed elsewhere extensively [81, 83, 86].

Bone marrow stromal cells (BMSCs) are important contributors to MM progression [87]. Interactions between BMSCs, their extracellular matrix (ECM) and MM cells have been shown to result in aberrant proliferation, growth and invasion of MM cells as well as contributing to drug resistance. Additionally, these interactions also lead to the formation of lytic bone lesions and angiogenesis. Notably, binding of MM cells to BMSCs causes secretion of the cytokine IL-6. IL-6 promotes the proliferation of MM cells as well as regulating apoptosis, contributing to the development of MM within the BM [82]. Furthermore, IL-6 stimulates the secretion of vascular endothelial growth factor (VEGF) by MM cells, contributing to the progression of MM [88]. BMSCs from within the MM TME have been shown to bind to MM cells with higher affinity than normal healthy BMSCs, which can contribute to the retention of MM cells within the BM [83]. BMSCs are known to contribute to the initiation of tumours and self-renewal of MM cells by aberrantly secreting growth differentiating factor 15 (GDF15) [89]. BMSCs also express several pro-angiogenic factors such as VEGF, basic fibroblast growth factor (bFGF), angiopoietin 1, platelet-derived growth factor (PDGF) and hepatocyte growth factor (HGF), contributing to angiogenesis within the tumour site [90]. More recently, studies have observed that BMSCs secrete exosomes which are inherited by MM cells, and are linked to disease progression and drug resistance [91, 92].

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells which develop into granulocytes, macrophages or dendritic cells. However, this differentiation is halted in cancer, leading to the development of a large population of MDSCs [93, 94]. In MM, these MDSCs accumulate within the BM [95]. MDSCs regulate T cell proliferation by secreting immunosuppressive factors such as arginase, nitric oxide and reactive oxygen species [96]. Additionally, MDSCs have been shown to facilitate the development of regulatory T-cells (T-regs) in-vivo, leading to further immunosuppression [97].

T-regs are a subset of $CD4^+$ T cells, and are recruited to BME by CXCR4 signalling. T-regs regulate the functions of both antigen-presenting cells and effector T cells through cell-

cell interactions or by secreting anti-inflammatory cytokines such as IL-10 or transforming growth factor-beta [83]. Due to the suppressive nature of T-regs, high expression of T-regs is considered as a marker of poor prognosis due to their immunosuppressive effects [98]. In MM, patients with high T-reg numbers is shown to lead to shorter time to disease progression and higher levels of functional T-regs have been recorded in the PB of MM patients [83].

As mentioned previously, both the cellular and non-cellular compartments contribute to the progression of MM. The role of IL-6 has been discussed, but other important factors such as VEGF and IGF-1 are also key to MM progression. VEGF secretion by MM cells can be triggered by a number of different factors or cytokines including, but not limited to, IL-6, bFGF and tumour necrosis factor-α (TNF-α). VEGF secretion results in angiogenesis which is triggered by the binding of VEGF to its receptors VEGFR-1 and VEGFR-2 on epithelial cells, resulting in the growth, migration, differentiation and survival of BMSCs and epithelial cells [99]. Additionally, an increase in vascularization within the BM can contribute to the development of MGUS into MM, and is indicative of a poor prognosis overall [99, 100]. VEGF can also stimulate activation of osteoclasts [101]. Increased osteoclast activity is also stimulated by factors secreted by MM cells such as receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage inflammatory protein 1-alpha (MIP1α). Increased activity of bone-dissolving osteoclasts compared to the activity of bone-forming osteoblasts leads to the formation of lytic bone lesions localized at the sites of MM tumours within the BM [102]. Furthermore, differentiation of osteoblast precursor cells is inhibited by dickopff1 (DKK1) secreted by MM cells, resulting in bone degradation by increased levels of osteoclastic activity in the local environment [103].

Similarly to VEGF, IGF-1 has also been directly implicated in MM disease progression. The IGF system may compensate for the IL-6 signalling pathway and can contribute to the proliferation and survival of IL-6-independent MM cells [104]. The IGF system is made up of six insulin-like growth factor binding proteins and the cascade initiated by IGF binding leads to the promotion of MM cell survival and proliferation. There is a high level of IGF-1 within the BM, secreted by both BMSCs and osteoblasts (OBs), influencing MM cell growth as well as DNA synthesis as well as cell proliferation and survival [105]. IGF-1-mediated MM survival and proliferation is mediated by the PI3K/AKT and MAPK pathways, where survival is promoted by the stimulation of the PI3K/AKT pathway by IGF-1 receptor (IGF-1R) while the MAPK

pathway stimulation of IGF-1 is responsible for the proliferation of MM cells [106, 107]. There is also evidence to suggest that IGF-1 may activate the Janus kinase pathway, important in the regulation of signal transduction throughout the MM cell [108].

The hypoxic nature of the BM must also be discussed. Hypoxia is a common feature in both solid and haematological cancers, but the bone marrow is considered to be highly hypoxic in nature, with oxygen levels within the range of <1-6% [109]. In MM, hypoxia plays a crucial role in the progression of MM. MM cells under hypoxia have active epithelial mesenchymal transition (EMT) machinery , a decreased expression of E-cadherin and interact physically less with surrounding BM-residing cells. This leads to egress of MM cells from the bone marrow, resulting in metastasis. Furthermore, hypoxia also upregulates the expression of CXCR4, potentiating the ability of MM cells to migrate to novel BM niche sites where new tumours can be established [110]. Hypoxia upregulates expression of hypoxia-inducible factor 1 subunit alpha (HIF1α) within MM cells, and can potentially facilitate angiogenesis induced by secretion of pro-angiogenic cytokines such as VEGF, IL-8 and IL-10 from MM cells [110, 111]. Hypoxia has also been demonstrated to lead to tumour initiation, with MM cells cultured under hypoxia prior to injection to a mouse model leading to greater tumour burden compared to injected MM cells pre-cultured under normoxic conditions [112].

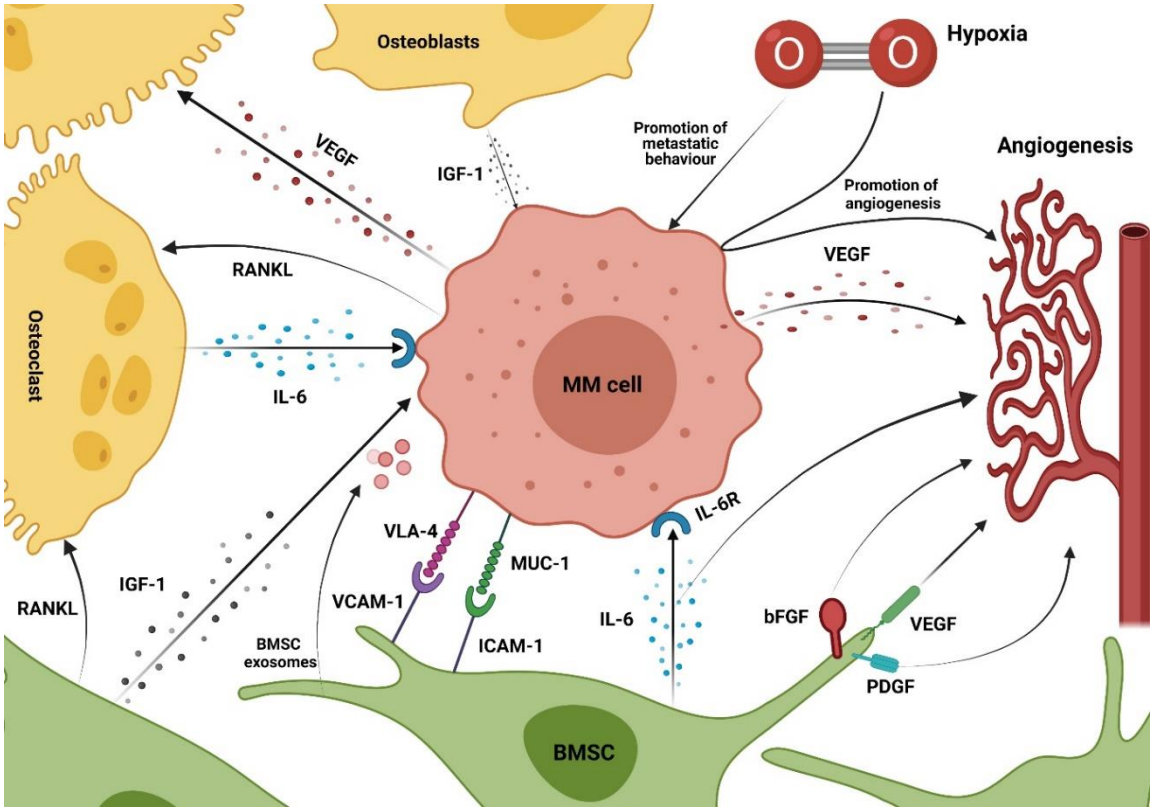


Figure 1.2 Interactions between MM cells and BM-residing cells contribute to disease development and progression. MM cells interact with BMSCs, osteoblasts, osteoclasts and the hypoxic BM microenvironment to promote survival, proliferation and metastasis, contributing to MM progression.

Taken together, it is evident that the BME is highly supportive of MM cell proliferation, survival and metastasis. Several therapeutic strategies have been tested to determine the effect of targeting the BME in MM. For example, the prodrug 2-nitroimidazole-based nitrogen mustard (TH-302) was used in MM. Activated by hypoxic conditions, TH-302 was used in a murine model where it stimulated apoptosis of MM cells and was shown to work in combination with the PI bortezomib [113, 114]. Drugs are also available which target BMSC-MM cell interactions. Bortezomib can also be used to inhibit BMSC-MM cell adhesion, inhibiting the secretion of pro-proliferative and pro-survival cytokines and factors [115]. Targeting BMSC-MM cell interactions is also the strategy utilised by the CXCR4 inhibitor AMD3100 and sensitizes MM to other drugs by preventing BMSC-MM cell interactions [116]. As well as inducing immune-mediated MM therapy, cyclophosphamide treatment reduces the amount of T-regs and in a MM murine model cyclophosphamide treatment enhanced survival and improved recurrence times [117].

1.1.7 Recent improvements in MM therapies

As stated previously, advancements in the treatments available for MM has resulted in a better outlook for patients. The International Myeloma Working Group have defined response criteria which are recognized globally, and are used to compare the results of separate clinical trials. Stringent complete response (sCR) is defined as an absence in clonal plasma cells within the bone marrow, and a normalization of serum light chain levels [118]. The next best level of response is termed complete response (CR), and involves < 5% plasma cells in the bone marrow, and negative serum. Achievement of CR leads to longer progression-free survival times in MM and is linked with enhanced overall survival (OS), however attention remains focused in increasing the overall survival as well as lengthening progression free survival (PFS) times [119]. MM patients who achieve CR have been observed to have longer OS, however this was only observed in MM patients treated with newer therapies compared to older strategies [119].

In the past 20 or so years, new treatments have become available for MM treatment. This started with the discovery of the potency of thalidomide, which was initially used as a sedative and to treat morning sickness in pregnancies in the 1960s [120]. However, thalidomide was discovered to be an anti-angiogenic agent, suppressing the formation of new blood vessels. In 2000, initial use of thalidomide to treat MM resulted in 34% of MM patients entering CR after, making it the first single agent anti-MM drug to become available in more than 30 years [121]. The initial study used thalidomide to treat RR MM patients, however thalidomide has since shown activity in MM patients in all disease stages. Additionally, when combined with steroids a response rate of approximately 50% is observed in MM patients, which increases to approximately 65% when combined with the chemotherapeutic agent cyclophosphamide [122]. More recently Pomalidomide, a newer second-generation derivation of thalidomide, has demonstrated efficacy in treating RR MM, as both a single agent and in combination with the steroid dexamethasone led to an increased response rate and longer PFS in MM, compared to dexamethasone alone [123, 124].

After the introduction of thalidomide, several analogues were created with the aim of enhancing the efficacy of the drug and lessen toxicity observed with thalidomide. Several analogues were initially synthesized, and lenalidomide was chosen for further studies.

Lenalidomide, a four amino acid-substituted analogue of thalidomide, was approved for the treatment of RR MM in the USA in 2006 [125]. When combined with ND MM patients, lenalidomide was considered a more potent elicitor of response than dexamethasone alone [126]. Furthermore, studies have also showed that lenalidomide and dexamethasone treatment of RR MM results in enhanced time until progression compared to dexamethasone alone [127]. More recently lenalidomide and dexamethasone treatment, and subsequent lenalidomide maintenance treatments, of SMM patients categorised as having "high-risk" SMM delayed progression to full MM and increased OS [128].

Shortly afterwards, the PI bortezomib was presented. PIs prevent protein degradation by proteasomes within the cell, and cause apoptosis which is more pronounced in malignant or transformed cells [129]. Boronic acid-derived inhibitors were invented to target proteasomes, and were more specific in targeting malignant cells than early inhibitors were demonstrated to be. Bortezomib, a boronic acid-derived PI with potent cytotoxic and anti-growth properties showed promise in an initial clinical trial, where patients with advanced haematological malignancies were treated [130]. In MM, bortezomib showed promise in an initial Phase 1 clinical trial, and in a separate Phase 2 trial approximately 33% of MM patients responded to bortezomib treatment with an average response duration of one year [130, 131]. The results of this Phase 2 trial led to FDA approval for bortezomib as a treatment for MM in 2003.

The availability of these drugs significantly improved the outcome for MM patients, prolonging PFS times and five year-survival rates. However, of late, attention has focused on the promising potential of immunotherapies in the treatment of MM. One of the most important additions to therapies available for MM are monoclonal antibodies (moAbs), which have demonstrated potential as both single agents as well as in combinations with other drugs. moAbs can induce killing of MM cells by inducing antibody-dependent effector cell cytotoxicity and antibody-induced complement cytotoxicity as well as directly inducing apoptosis [132]. moAb therapy, as well as two of the most promising moAbs; Elotuzumab and Dara are discussed in detail in **4.1.**

Another promising immunotherapeutic approach is targeting of immune checkpoints. The most notable research into immune checkpoints thus far revolves around the programmed death receptor (PD-1). PD-1, a transmembrane protein is expressed by T-cells,

NK cells, B cells and activated monocytes. PD-L1 and PD-L2 - ligands for PD-1 - are primarily expressed by antigen presenting cells (APCs) and, upon binding, inhibit T cell proliferation, inhibit cytotoxicity and promote apoptosis [133]. However, tumour cells can hijack this axis by expressing PD-L1 and PD-L2 as a mechanism of resistance to T cell-mediated immunosurveillance [134]. PD-L1 has been documented to be upregulated by many different types of cancer but blockade of PD-1/PD-L1 interactions has shown promising results in solid tumours [135, 136]. In MM, increased PD-1 expression has been recorded on T cells of afflicted patients, and PD-L1 expression has been recorded on MM cells while its expression is absent on normal healthy plasma cells [137, 138]. Utilisation of nivolumab, an anti-PD-1 antibody, proved underwhelming at first, with little therapeutic response observed in nivolumab-treated RR MM [139]. While this indicates that immune checkpoints as a monotherapy may not offer a response, combination approaches using nivolumab and lenalidomide resulted in enhanced T cell and NK cell activation while simultaneously reducing PD-1 expression on T cells [140].

One of the most prominent immunotherapeutic strategies to combat cancer of late is chimeric antigen receptor T cells (CAR-T cells). This approach utilises tumour-infiltrating T cells, which are isolated, expanded and modified to express a specific receptor recognizing antigens displayed by target cells [141]. Upon reinfusion these T cells demonstrated enhanced tumour specificity against MM than peripheral blood (PB) lymphocytes from the same patients [142]. MM patients, including both ND and RR MM, responded positively with prolonged PFS when infused with bone marrow-infiltrating lymphocytes were harvested, expanded and reinfused [143]. Another CAR-T approach utilised in MM is the CD19-CAR-T method, where T cells express chimeric receptors targeting CD19. Initial trails using the CD19-CAR-T in MM patients with prior lines of therapy were promising with one patient entering CR, while other patients experienced an extended period of remission [144]. CAR-T approaches utilising a B cell maturation antigen (BCMA) chimera have also demonstrated promising potential, with some heavily pre-treated MM patients responding to the therapy in a Phase 1 trial. One patient was observed to enter sCR, while three others of the 12 patients enrolled in total demonstrated stable disease [145]. Further trials utilising CD19-CAR-T and BCMA-CAR-T approaches are currently being conducted and should reveal the true potential of using a CAR-T cell bases strategy to treat MM [146].

When dealing with some of the most common symptoms of MM, such as hypercalcaemia and bone lesions, bisphosphonates represent a promising therapy. Bisphosphonates such as pamidronate, zoledronic acid and clodronic acid represent methods of treating bone pain as well as potentially preventing the reoccurrence of bone lesions in later stages of MM, and guidelines for their use in treating MM in the clinic have been published by the American Society of Clinical Oncology [147-149].

Despite the recent advances in therapies available for MM, and the promise that they show in generating responses, particularly in heavily pre-treated RR MM patients, relapse is inevitable for MM patients and often is accompanied with worsened health problems. MM patients will inevitably perish from the disease, or from side-effects associated with it. Furthermore, a lot of MM treatments can have significant side-effects and may only be suitable for a subset of MM patients. Therefore, there is still significant imperative for generating novel therapeutic approaches to target MM and to elucidate mechanisms of resistance which may enhance our understanding of this difficult-to-treat malignancy.

1.1.8 Mechanisms of resistance in MM

MM is an extremely difficult-to-treat disease, with patients who enter remission after treatment facing an inevitable relapse with worsened health conditions. While the BME contributes to MM progression and drug resistance, MM cells have also developed potent mechanisms of resistance to clinical therapies as well as the hosts own immune system [150].

MM cells develop resistance to chemotherapies in an efflux pump-dependent manner, which was elucidated by Turner et al. in 2006. After treatment with doxorubicin and topotecan, expression of the efflux pump APT-binding cassette transporter G2 (ABCG2) was observed to increase on MM cell lines. This was observed on MM cells in log phase compared to non-replicating MM cells [151]. Similarly, patient-derived MM cells taken at relapse were observed to have increased ABCG2 expression after treatment with topotecan and melphalan. In the same study, methylation-specific polymerase chain reaction (PCR) analysis revealed that treatment of MM cells with topotecan resulted in the demethylation of a regulatory promoter of ABCG2, thereby increasing the expression of ABCG2 [151]. Additionally,

further studies have shown that treatment of MM cells with chemotherapeutic agents such as doxorubicin and dexamethasone results in increased expression of another efflux pump, P-glycoprotein [152].

Alkylating agents such as melphalan and cyclophosphamide are used as palliative care in MM, and operate by forming crosslinks between DNA strands, thereby inhibiting the synthesis and replication of DNA, ultimately preventing cell replication. MM cells have developed mechanisms of resistance against alkylating agents, including increased repair rate of DNA strand cross links. This was hypothesized, and ultimately proven, to be due to the Fanconi anaemia (FA) pathway. When the FA pathway was inhibited using siRNA, melphalan resistance in MM cells was overcome. Furthermore, overexpression of FA genes resulted in increased MM cell survival when treated with melphalan [153]. More recently melanoma antigen gene family member A1 (*MAGE-A)* was implicated in promoting resistance to melphalan as well as bortezomib by expressing Type I Melanoma Antigen Genes of the *MAGE-A* family (MAGE-A), which regulates the expression of the pro-apoptotic Bcl-2 protein [154].

Resistance to thalidomide analogues (also referred to as IMiDs) has also been observed in MM. Cereblon (CRBN), expressed ubiquitously, is required for the anti-MM activity of IMiDs. CRBN forms a complex with DNA binding protein 1 (DDB1) and Cullin 4A (CUL4A) which ubiquitinate and ultimately result in the degradation of lymphoid transcription factors Ikaros and Aiolos [155]. Decreased expression of CRBN is associated with IMiD resistance and investigations into lenalidomide-resistant MM cells resulted in the documentation of three separate CRBN mutations, which were not observed in RR MM patients and which resulted in lenalidomide resistance in MM cells in vitro [156].

PIs have been approved to treat both ND and RR MM and have been strongly involved in clinical practice in recent times. The earliest mechanisms of resistance to PIs by MM were reported to be mutations within proteasome subunits, or abnormal expression of subunits, resulting the in prevention of PIs locating and binding to subunits. Proteasome 20S Subunit Beta 5 (PSMB5), a component of the proteasome complex has been documented to be upregulated in MM, and contributes to bortezomib resistance. Additionally, the G322A mutation within *PSMB5* has also been confer resistance to bortezomib, by preventing unfolded protein build-up and apoptotic signalling [157, 158]. Resistance to bortezomib has also been linked with the chromosomal instability gene NIMA related kinase 2 (*NEK2)*. High NEK2

expression resulted in bortezomib resistance by activating efflux pumps, which was abrogated when *NEK2* was downregulated. Down-regulation of *NEK2* also resulted in an induction of bortezomib-mediated growth inhibition [159]. Notch signalling has also been implicated in bortezomib resistance. The Notch ligand DLL-1, expressed by BMSCs, binds to Notch receptors expressed by MM cells and signalling through the Notch2 receptor was demonstrated to induce resistance to bortezomib by upregulating the expression of CYP1A1: an enzyme involved in drug metabolism [160]. Finally, heat shock proteins have also been observed to contribute to bortezomib resistance, although the exact molecular mechanism is currently unknown. Overexpression of one heat shock protein: Hsp27, in a bortezomib-sensitive MM cell line resulted in the development of bortezomib resistance while the silencing of Hsp27 in a separate, bortezomib-resistant MM cell line [161].

MM has also developed potent mechanisms of resistance to immunotherapies. In particular, MM can evade Dara-mediated therapy using multiple strategies. Previously it has been shown that increasing CD38 expression on MM cells results in enhanced efficacy of both antibody-dependent cellular-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) induced by Dara, and contributes to a better response in MM patients [162, 163]. However, MM patients treated with Dara were observed to have decreased levels of CD38, which was also observed in MM cells upon disease progression [163]. Aberrant CD38 expression is one of the most common mechanisms of resistance to the moAb, allowing the proliferation of $CD38^{low}$ MM clones. Additionally, ADCC induced by Dara was also observed to be regulated by the upregulation of Survivin, an anti-apoptotic protein in MM cells after interactions with local BMSCs [164]. MM are also able to evade antibody-dependent cellular phagocytosis (ADCP) mediated by macrophages. Integrin-associated protein (CD47), is expressed in low amounts in normal healthy plasma cells, is highly expressed by malignant MM cells [165]. CD47 forms a complex with signal-regulatory protein alpha which acts as a "don't eat me" signal, preventing ADCP against the Dara-coated MM cell [166]. Finally, MM cells can overexpress complement-inhibitory proteins CD55 and CD59, in order to inhibit CDC mediated by Dara. Indeed, MM cells from patients with progressing disease were observed to have increased levels of both CD55 and CD59, indicating that overexpression of these proteins can contribute to Dara resistance [163].

While MM cells have evolved mechanisms of resistance to drug-based therapies, they also possess mechanisms of resistance to immunosurveillance mediated by the hosts own immune system. To evade T-cell mediated immunosurveillance, MM cells overexpress PD-L1 to suppress T cell proliferation and cytotoxicity [167, 168]. While malignant cells can lose expression of major histocompatibility complex I (MHC class I) molecules to evade $CD8^+$ T cell mediated immunosurveillance, this does not appear to be the case in MM, with patient MM cells observed to have high levels of MHC class I molecules [169, 170]. However, in early stages of MM, lower levels of MHC class I molecules are expressed by MM cells, potentially sensitizing them to NK cell-mediated immunosurveillance which relies heavily on recognizing MHC class I cells as markers of "self" and targeting cells without MHC class I expression for destruction [171]. However, MM cells also possess mechanisms of resistance to counter NK cell-mediated immunosurveillance. MM cells frequently overexpress ligands for inhibitory receptors. Examples of this include high levels of CD155 expression on MM cells, which binds to the inhibitory receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT) as well as CD96, which has been shown to be a regulator of NK cell cytokine secretion against several cancer cell types [172].

Whilst these mechanisms of resistance are understood in MM, it is likely that alternate mechanisms of resistance to both therapies and the hosts own immune system exist and remain to be elucidated. Recently, attention has focused on the role of the hypersialylated cancer cell surface in facilitating evasion of immunosurveillance.

1.1.9 The importance of the hypersialylated MM cell surface in facilitating MM survival

The surface of MM cells is known to be hypersialylated, with sialic acids observed terminating cell surface expressed glycoproteins and glycolipids. Sialic acids are nine-carbon monosaccharides, and are attached to underlying glycan chains via a glycosidic bond formed by the action of a family of Golgi apparatus-bound sialyltransferase enzymes. There are currently 20 documented sialyltransferases known to be expressed by human cells [173]. Over 50 forms of sialic acids have been observed in nature, the most common of which is *N*-acetylneuraminic acid (Neu5Ac). Sialic acids are synthesized in the cytosol where Neu5Ac is synthesized from uridine 5'-diphosphate N-acetylglucosamine in a stepwise process involving

three separate enzymes, after which Neu5Ac is transported to the nucleus where cytidine-5'-monophosphate (CMP)-sialic acid synthase creates CMP-Neu5As, the active form of Neu5Ac. In mammals, CMP-Neu5Ac is transformed into *N*-glycolylneuraminic acid (Neu5Gc) by CMP-Neu5Ac hydroxylase (CMAH) before being transported to the Golgi apparatus where it is attached to glycoproteins and glycolipids by sialyltransferases [(174)]. However, in humans *CMAH* is inactive, resulting in high levels of Neu5Ac being prevalent in humans which is then attached to glycoproteins and glycolipids [(175)].

The glycosidic bonds binding sialic acids to underlying glycan chains occurs in α2-3, α2-6, or α2-8 linkages, depending on the sialyltransferase involved in the process. Sialyltransferases create glycosidic bonds between the C2 carbon in sialic acids and C3, C6 and C8 hydroxyl residues of an accepting glycan molecule. Thus, sialyltransferases can be divided into sub-groups depending on the hydroxyl residue on the receiving glycan: ST3, ST6 and ST8 sialyltransferases [(176)]. Specifically, sialic acids can be attached to galactose in α2,3 or α2,6-glycosidic linkages. Sialic acids can also be attached to *N*-acetylgalactosamine or *N*-acetylglucosamine in α2,6 bonds or to existing sialic acid residues on the target glycan via α2,8 bonds [(177)].

Aberrant cell surface glycosylation is considered a hallmark of cancer [(178)]. Generation of a hypersialylated tumour cell surface is due, in large part, to aberrant sialyltransferase activity which has been reported in several cancer types. Indeed, overexpression of approximately half of the 20 existing human sialyltransferases implicated in a myriad of cancer types [(179)]. While hypersialylation is primarily associated with the increased activity of sialyltransferases, this is not potentially the only reason for the development of a hypersialylated MM cell surface. Sialidases, enzymes which are responsible for the removal of sialic acids are found within human cells. Four of these enzymes exist; neuraminidase 1-4, and their expression has been demonstrated to be dysregulated in cancer [(180)].Finally, the local availability of Neu5Ac in the adjacent cellular microenvironment may also influence the synthesis and subsequent expression of sialic acids on the cancer cell surface [(181)]. Although unlikely to contribute to hypersialylation as significantly as upregulated sialyltransferases, these must be mentioned as potential contributors to the hypersialylation development.

Hypersialylation plays a key role in the survival and progression of cancer and ST6GAL1 appears to be one of the most prominent sialyltransferases in facilitating this. The Fas death

receptor, which activates apoptosis within the tumour cell when bound to its concomitant ligand Fas-L, was found to be sialylated by ST6GAL1, abolishing its ability to induce apoptosis in cancer cells by preventing the formation of the death-inducing signalling complex [182]. Additionally, sialylation has also been demonstrated to mediate resistance to anoikis; cell death induced following detachment of the cell from surrounding cells or the ECM [183]. Hypersialylation has also been implicated in facilitating cancer metastasis, potentiating the ability of cancer cells to migrate to surrounding niche sites where new tumours can be established [184]. Furthermore, hypersialylation has also been shown to confer drug resistance, with high expression of ST6GAL1 reported to facilitate resistance to the chemotherapeutic drug cisplatin. High levels of ST6GAL1 were also observed in cisplatin-resistant ovarian cancer cells compared to cisplatin-sensitive cancer cells and knockout (KO) of ST6GAL1 resulted in sensitization of cisplatin-resistant ovarian cancer cells to cisplatin [185]. Furthermore, ST6GAL1 was also implicated in facilitating resistance to radiation therapy, which was reversed when a sialidase enzyme was expressed in the colon cancer cells or upon ST6GAL1 KO [186].

In MM, the importance of hypersialylation was demonstrated when a study showed that high expression of ST3GAL6 correlated with inferior survival. Subsequent ST3GAL6 KO was observed to decrease tumour burden and increase survival in a murine model, and was also shown to reduce interactions between MM cells and BMSCs as well as reducing the homing of MM cells to the BM [187]. Further studies revealed that ST3GAL6 also generated sialic acid-derived moieties capable of binding to E-selectin. E-selectin is an adhesion receptor expressed by endothelial cells, and interactions between E-selectin and MM cells results in MM cell rolling and eventual migration to the BM [188]. While the role of hypersialylation in facilitating metastasis in MM is understood, little is known about the role of the hypersialylated MM cell surface in facilitating immune-evasion.

Certain sialic acid-derived moieties have the ability to bind to sialic acid-binding immunoglobulin like lectins (Siglecs). Siglecs are a family of transmembrane receptors, primarily expressed by cells of the immune system containing both activating and inhibitory receptors. Of interest in particular is the role of sialic acid-derived Siglec ligands in regulating immune responses upon binding to Siglec receptors. This postulates sialic acids, or certain derivations, as potential markers of “self”, which was first proposed by Varki in 2011 [189]. Several groups have previously demonstrated, by employing recombinant Siglec-7 and Siglec-

9 Fc chimeras to screen primary cancer cells and cancer cell lines, that a multitude of different cancerous cell types are heavily decorated with sialic-acid derived ligands for Siglec-7 (Siglec-7L) and Siglec-9 (Siglec-9L) [190-193]. Cancer cells have been demonstrated to hijack this axis by highly expressing Siglec ligands to promote a dampened immune response (Figure 1.3). Of particular relevance for this project, Siglec-7 and Siglec-9 - inhibitory receptors expressed by NK cells - have been shown to inhibit NK cell-mediated cytotoxicity against several cancer types. An overview of Siglec-7 and Siglec-9 is discussed in **1.2.7** while the potential role of the hypersialylated MM cell surface, Siglec-7 and Siglec-9 in facilitating evasion of NK cell mediated immunosurveillance is discussed in **3.1.**

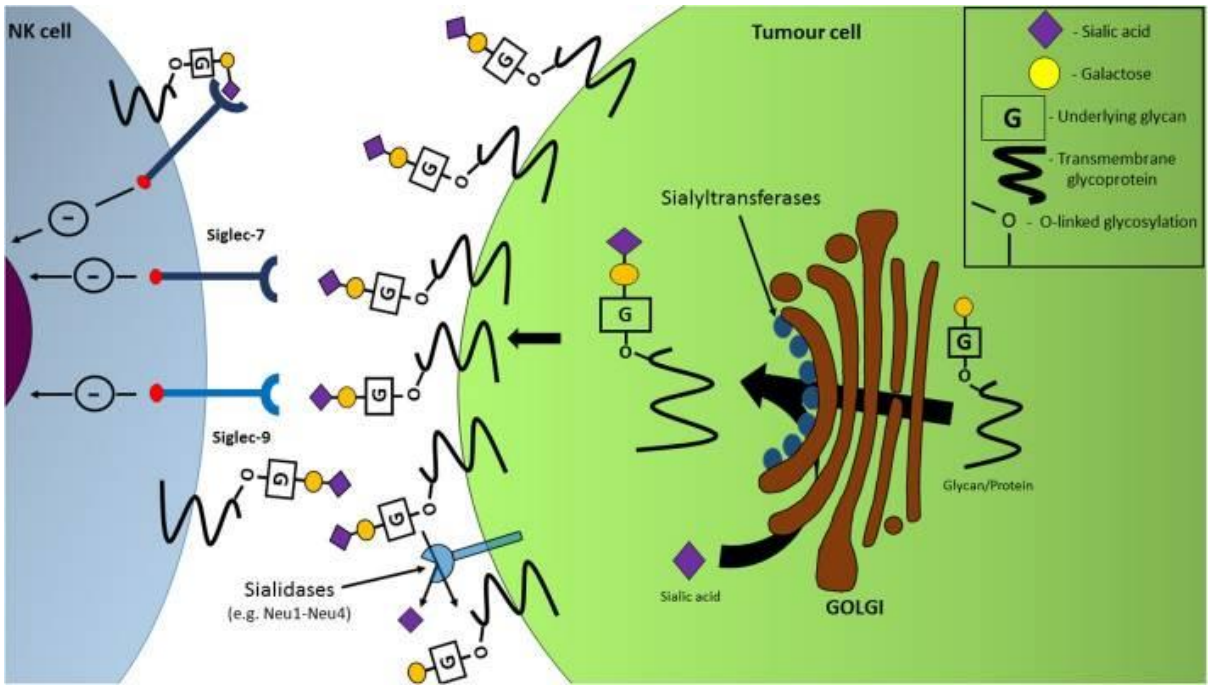


Figure 1.3 Sialic acid-terminated glycans and glycoproteins presented by MM cells are capable of binding to Siglec-7 and Siglec-9 receptors expressed by NK cells. Figure adapted from Daly et al., 2019.

1.2 Natural Killer cells

Natural Killer (NK) cells are cytotoxic lymphocytes with an innate ability to recognize and destroy virally infected, genetically damaged/stressed and malignantly transformed cells.

NK cells were initially observed to be lymphoid cells capable of lysing tumour target cells without the need for prior stimulation or activation [194, 195]. Further studies revealed NK cells to be capable of both cytotoxicity and cytokine secretion, used to recruit and activate effector cells of the adaptive immune response [196]. As part of the innate immune system, NK cells have a key role in immunosurveillance recognizing and destroying tumour cells. In humans NK cells are defined as $CD56^{+}/CD3^{-}$ lymphocytes, constituting between 5-15% of PB-mononuclear cells (PBMCs) [197]. NK cells can also be found in the breast, skin, liver, lung, uterus, kidney, gut and joints. In the liver, NK cells represent between 20-30% of hepatic lymphocytes, and constitute approximately 10% of lymphocytes in the lung [198]. The innate ability of NK cells to lyse target cells and secrete pro-inflammatory cytokines places them right in the front line in defence of the host system.

1.2.1 Generation of NK cells in the human body

The development of NK cells takes place primarily within the BM, which is critically important to development. As with B and T cells, NK cells are derived from hematopoietic stem cells (HSCs) in the $CD34^{+}$ compartment within the BM. HSCs develop into multi-potent progenitor (MPP) which can subsequently develop into a common lymphoid progenitor (CLP), from which B cells, T cells as well as NK cells can be formed [199]. These early pathways of development are not well elucidated; however, it is likely a host of cytokines and transcription factors within the BM are required for the differentiation into either B cells, T cells or NK cells. Transcription factors such as Ikaros, Stat5, E2A, Bc111a and others are known to be required for these early steps of differentiation [200]. From the CLP, at least three other differentiation steps are required to generate mature NK cells (mNK cells). The NK progenitor (NKp) cell develops from the CLP, and subsequently gives rise to the immature NK (iNK) cell. At this stage of development, germline encoded receptors such as NKp46, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), CD94, NK1.1 and natural killer group 2 member D (NKG2D) are expressed. Further receptors are expressed upon the transition from the iNK cell to the mNK cell. These receptors include CD16, Ly49 and CD49b. As the NK cell becomes more mature, TRAIL and CD27 expression becomes downregulated and the expression of several maturation markers become upregulated. During the maturation process the NK cell acquires

functional competence, including the capacity for both cytotoxicity and cytokine secretion. Importantly, chemokine receptors which facilitate the egress of NK cells from the BM into the periphery become expressed. mNK cells leave the BM and continue the maturation process whilst in circulation. At this stage, co-expression of markers such as killer cell lectin like receptor G1 (KLRG1), CD11b and CD27 occurs and mNK cells are considered fully mature upon co-expression of these receptors (Figure 1.4) [201]. As mentioned previously, several key transcription factors are required for the development of NK cells, and their role as well as implication upon failure of expression of the factors has been summarised in detail by Sun [199]. As important in NK cell development as transcription factors are cytokines. Notably, IL-2, IL-7 and IL-15 are all key cytokines needed for the competent development and maturation of NK cells. Other IL cytokines such as IL-12, IL-18 IL-27 and IL-35 are also thought to be critically involved in this process [202].

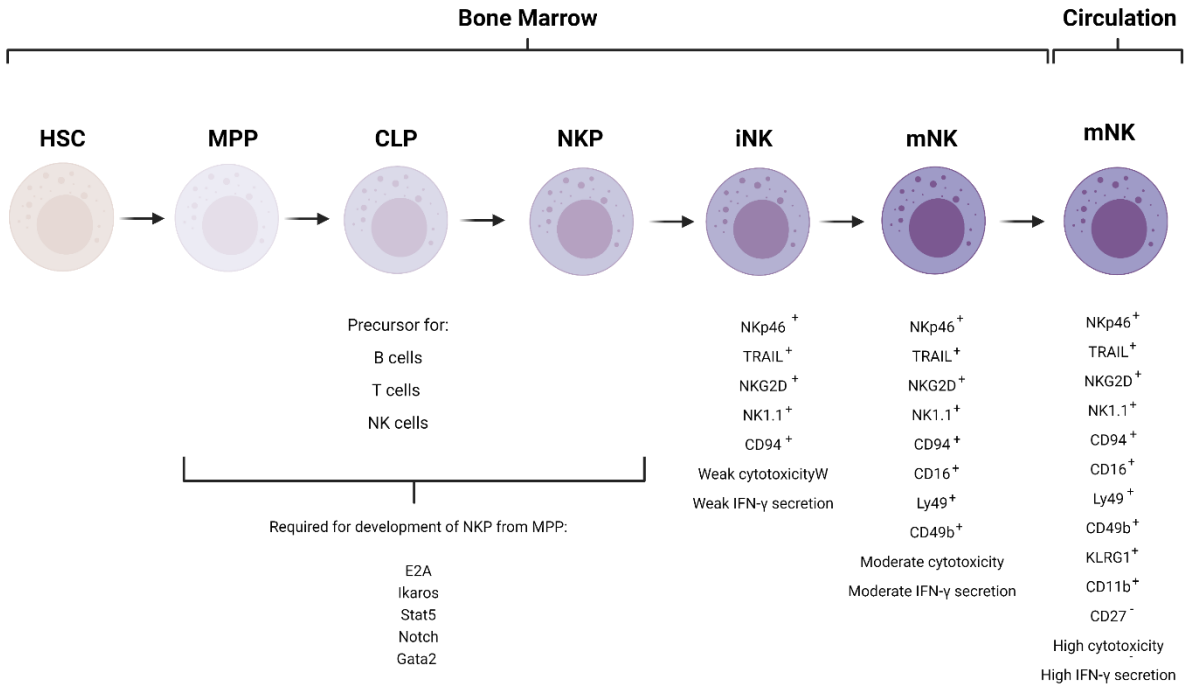


Figure 1.4 NK cell development, including important receptor acquisition, from HSC to a circulating mNK cells.

1.2.2 NK cell subsets

NK cells are classified by the expression of CD56 and the lack of expression of CD3. Within this, NK cells can be further divided into two subsets based on intensity of CD56

expression and the expression of the CD16 receptor: $CD56^{high}CD16^{\pm}$ and $CD56^{low}CD16^{high}$. $CD56^{high}CD16^{\pm}$ are predominantly located within secondary lymphoid tissues and constitute approximately 5% of total NK cells, while $CD56^{low}CD16^{high}$ NK cells represent above 90% of the total population and are the predominant NK cells observed within the circulation. $CD56^{low}CD16^{high}$ NK cells are highly cytotoxic, expressing high levels of the maturation antigen CD57, as well as a large amount of inhibitory killer cell immunoglobulin-like receptors (KIRs) and perforin. In contrast, $CD56^{high}CD16^{\pm}$ are not highly cytotoxic, but are highly potent cytokine producers, capable of recruiting and activating effector cells of the adaptive immune response. Based on receptor expression, other minor subsets of NK cells have been detailed, but constitute minor populations compared to the numbers of $CD56^{high}CD16^{\pm}$ and in particular $CD56^{low}CD16^{high}$ [203-205]. NK cell subsets can differ based on the location of the NK cell within the host system. Tissue-residing $CD56^{high}$ NK cells express adhesion receptors such as CD49a and CD103 which help to retain the NK cell within the tissue, while $CD56^{high}$ NK cells within the PB express CD62L, CXCR4, C-C chemokine receptor type 7 (CCR7) and CXCR3 which help to relocate NK cells to secondary lymphoid tissues, inflamed tissues and tumour sites. $CD56^{low}CD16^{high}$ NK cells have low expression of CD62L and do not express CCR7, however do express CXCR2, CXCR3, CXCR4 and CX3CR1; chemokine receptors which facilitate NK cell migration to sites of inflammation [206].

1.2.3 Surface expressed receptors control NK cell functions

NK cells possess an innate ability to target and destroy invading pathogens, virally infected or malignantly transformed cells. NK cells do not need prior activation or stimulation to activate their cytotoxic potential. Rather, they are activated by the results of an influx of signals generated by surface expressed activating and inhibitory receptors (Figure 1.5). An overall net stimulus is generated from interactions between these receptors and their cognate ligands expressed by the target cell encountered by the NK cell. If an overall inhibitory signal is generated the target cell is released, however if an overall activating signal is generated the NK cell is activated and secretes perforin and granzymes located within lytic granules which trigger apoptosis of the target cell [207]. Perforin forms pores in the target cell membrane, facilitating entry of granzymes which cleave caspases and initiate apoptosis [208].

To prevent indiscriminate killing this process is tightly regulated, and takes place in four steps. Initially, an immune synapse is formed between the NK cell and the target cell. Following establishment of the synapse, the microtubule-organizing centre and lytic granules within the NK cell migrate toward the synapse which then anchor with the plasma membrane at the location of the synapse and eventually fuse with the membrane resulting in the secretion of perforin and granzymes [208]. Once the contents of the lytic granule are secreted, the lytic granule membrane fuses with the plasma membrane and markers such as CD107a which were previously expressed on the interior of the lytic granule become expressed on the NK cell surface. By analysing the expression of CD107a, it is possible to determine whether the NK cell has been activated and secreted lytic granules [209, 210].

1.2.3.1 Inhibitory receptors

Inhibitory NK cell receptors deliver an inhibitory signal to the NK cell via their cytoplasmic tail-located immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon binding to the cognate ligand, ITIMs recruit and activate intracellular phosphatases Src homology-containing tyrosine phosphatases (SHP-1 and SHP-2) as well as lipid phosphatase SH2 domain-containing inositol-5-phosphatase (SHIP) [211]. Once phosphorylated and activated, these phosphatases then dampen activation pathways within NK cells, regulating activation, cytotoxicity and cytokine release [207]. Several classical inhibitory receptors have been defined, and are well understood, however the role of several other potential inhibitory receptors remains to be fully elucidated.

Of the inhibitory receptors expressed by NK cells, the killer cell immunoglobulin-like receptors (KIRs) are the most influential and studied. KIRs are type I transmembrane proteins containing short or long cytoplasmic tails and two or three IgG-like domains. Long cytoplasmic tails are associated with inhibitory KIRs, while shorter cytoplasmic tails are observed in activating KIRs. Inhibitory KIR receptors include KIR2DL1-3, KIR2DL5 and KIR3DL1-3 which bind to human leukocyte antigen (HLA)-A, HLA-B and HLA-C molecules. HLA molecules represent markers of self in humans, and binding to KIR receptors results in a strong inhibitory signal being generated within the NK cell [212]. Tumour cells will often lose expression of HLA molecules in an attempt to evade T cell-mediated immunosurveillance, however this

sensitizes them to NK cells, making NK cells crucial in the recognition and destruction of malignant cells in the body [213]. The process by which KIR receptors stimulate NK cells to destroy target cells lacking HLA expression is referred to as the "missing-self hypothesis", and is one of the main mechanisms utilized by NK cells to mediate immunosurveillance [214, 215]. CD94-natural-killer group 2, member A (NKG2A-CD94) is another classical ITIM-containing inhibitory dimeric receptor expressed by NK cells, which also recognize the HLA molecule HLA-E [216]. Due to the ubiquitous expression of HLA-E on tumour cells, NKG2A-HLA-E binding has been targeted and has demonstrated promising results [217].

However, the role for several non-classical NK cell receptors in regulating NK cell functions cannot be discounted, and has been examined recently. In particular, receptors such as PD-1, TIGIT and CD96 have been attracting attention. While PD-1/PD-L1 interactions are predominantly associated with the regulation of T cell functions, PD-1 can also be expressed on NK cells in cancer patients, potentially influencing NK cells activities [217]. TIGIT is another inhibitory receptor, the targeting of which has shown promise as an anti-cancer immunotherapy. TIGIT was originally introduced as a T cell receptor, but expression is also observed on a subset of NK cells in both healthy donors and in cancer patients. Ligands for TIGIT include CD155, CD111 and CD112. TIGIT has been observed to regulate both NK cell cytotoxicity and cytokine secretion [218-220]. CD96, again expressed by a subset of NK cells, also recognizes CD155 as a preferred ligand, but can bind to CD111 and CD112. The role of CD96 in NK cells has been controversial with early studies suggesting CD96 to be an activating NK cell receptor, however more recent studies have demonstrated CD96 to be an inhibitory receptor in the context of cancer [221, 222].

With increased attention being given to the role of the hypersialylated tumour cell surface, the spotlight has recently been focusing on the role of Siglec receptors in regulating NK cell functions against cancer cells. NK cells express both Siglec-7 and Siglec-9, with Siglec-7 expression being expressed on nearly all NK cells while Siglec-9 expression appears to be restricted to a subset of $CD56^{dim}$ NK cells. Siglec-7, Siglec-9 and their role in regulating NK cell cytotoxicity are discussed in further detail in **1.2.7** and **3.1.**

1.2.3.2 Activating receptors

While inhibitory NK cell receptors regulate NK cell responses and prevent the targeting of cells of "self", activating receptors are also expressed to stimulate cytotoxicity and cytokine secretion upon encountering a malignantly transformed cell or invading pathogen. While inhibitory receptors contain an intracellular ITIM motif which stimulates a regulatory effect on NK cell activities upon binding of the receptor to a ligand, activating receptors possess an intracellular tyrosine-based activating motif (ITAM). This is not always the case however, as some activating receptors do not possess ITAMs, but instead bind to, and activate, ITAMs expressed by adaptor proteins via transmembrane positively charged amino acids such as lysine and arginine [207, 223]. Upon binding to the cognate ligand and subsequent receptor clustering, ITAMs are phosphorylated by Src family protein tyrosine kinase (PTK). Upon phosphorylation of two sites within the ITAM, members of the Syk family PTK phosphorylate a number of substrates which form a membrane-proximal scaffold which in turn recruits effector molecules, leading to calcium signalling and RAS activation which stimulates the extracellular-signal-regulated kinase pathway (ERK) ultimately resulting in NK cell activation.

One of the most prominent NK cell-activating receptors in the context of cancer is NKG2D. This C-type lectin-like type II transmembrane protein, expressed as a homodimer, is observed on all NK cells [197]. In humans, two classes of NKG2D ligands are expressed: MHC class I chain-related proteins A/B (MIC A/B) and UL-16 binding proteins 1-6 (ULBP 1-6). These ligands are expressed in low levels by healthy cells, however upon malignant transformation their expression becomes highly upregulated and is observed across a wide range of cancer types including leukaemia, glioma and melanoma and breast cancer [224]. Expression of NKG2D ligands is influenced by a number of factors and can vary within disease stages for each individual cancer. Therapeutic agents such as PIs and histone deacetylase inhibitors have been shown to stimulate increased NKG2D ligand expression in cancer cells [225, 226].

Other notable activating receptors consist of the natural cytotoxicity receptors (NCRs), an immunoglobulin superfamily of activating receptors expressed by NK cells. NCRs recognize a host of ligands, including ligands expressed on human cells as well as parasitic, bacterial and virally-derived ligands [227]. Three types of NCR are expressed by human NK cells: NKp46, NKp44 and NKp30 [197]. While NKp44 is expressed only on activated NK cells, NKp46 and

NKp30 are expressed on both resting and activated NK cells. Upon binding to their cognate ligands, NCRs recruit ITAM-containing adaptor proteins such as FcεRI-γ and CD3-ζ [227].

2B4 (CD224) is another transmembrane, adaptor protein-dependent receptor expressed by human NK cells. However, 2B4 is distinct to receptors such as KIRs and NCRs in that it can act as both an activating and inhibiting receptor. Studies have shown that 2B4 acts as an inhibitory receptor in murine NK cells, while functioning as an activating receptor in human NK cells [228-230]. However conflicting data has shown that the cytolytic functions of human NK cells from patients with X-linked lymphoproliferative disease was negatively regulated by interactions between 2B4 and CD48. CD48 acts as a ligand for 2B4 and is expressed on hematopoietic cells, but its expression has been recorded to increase on human B cells infected with Epstein-Barr Virus (EBV) [231].

One of the most important activating NK cell receptors is the FcγRIIIA receptor (CD16). CD16, also expressed by macrophages and mast cells, is expressed by mature $CD56^{low}CD16^{high}$ NK cells as well as minor subsets of $CD56^{high}CD16^{\pm}$ NK cells. CD16 expression is key for NK cells to mediate ADCC. CD16 recognizes the Fc chain of antibodies, which can be produced naturally via the host's immune system, but can also be artificially introduced as part of moAb therapy . Therefore, NK cells are critical effectors of moAb-mediated therapy. In order for this to function efficiently, the target antigen needs to be expressed by target cells, the antibody needs to be bound to the target antigen and the NK cell encountering the antibody-decorated target cells requires CD16 expression. Approximately 15% of humans contain NK cells with a mutated version of CD16. In this allele, a single nucleotide polymorphism (F158V) results in a high-affinity version of CD16 (haCD16). The haCD16 allele is a more potent inducer of NK cell cytotoxicity, and studies have shown that NK cells expressing a recombinant version of this mutated CD16 receptor elicit stronger ADCC [232]. In particular, the NK cell line KHYG-1 transfected with haCD16 demonstrated potentiated ADCC of $CD38^{+}$ MM cell lines in the presence of the anti-CD38 moAb Dara [233]. Thus, CD16 represents a vitally important NK cell receptor, for the basal function of NK cells within the innate immune response and as effectors of emerging moAb-based immunotherapies. In MM, as discussed in **4.1**, moAbs such as Dara and Elotuzumab represent one of the most recent advances in MM therapy, and thus NK cells are critical effector cells in currently clinically used MM therapies.

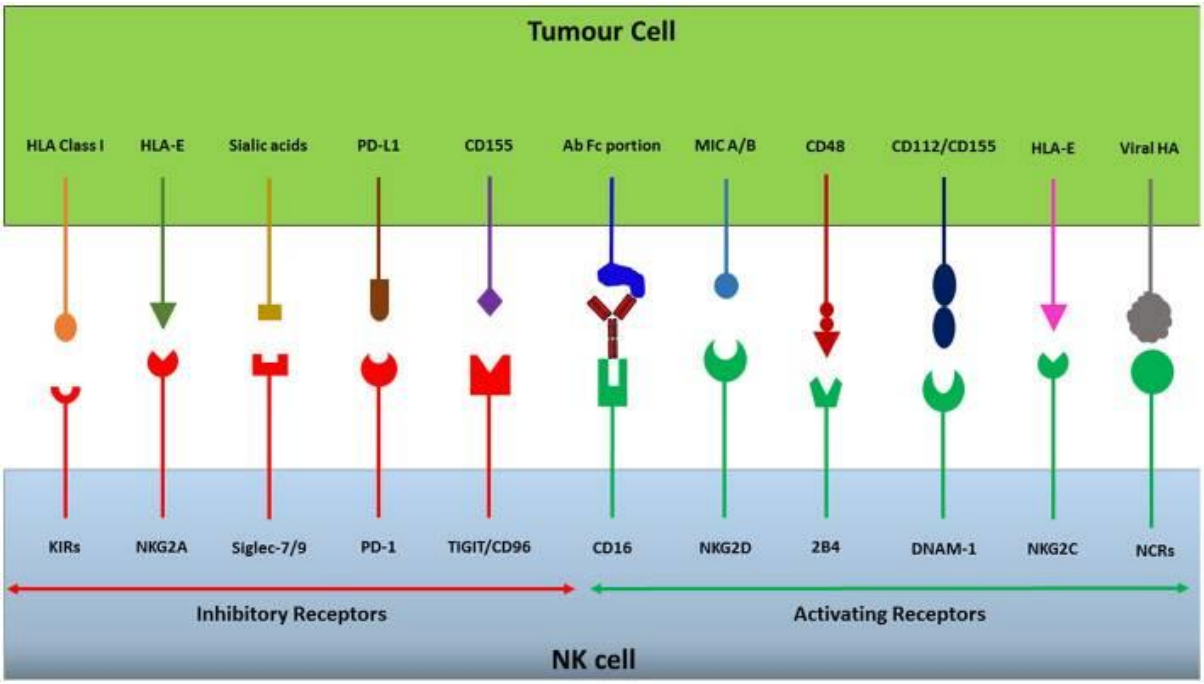


Figure 1.5 Signalling from surface-expressed activating and inhibitory NK cell receptors determine the fate of target cells. Adapted from Daly et al., 2019.

1.2.4 Further mechanisms by which NK cells kill target cells

Aside from receptor-dependent NK cell activation or inhibition, NK cells can clear target cells using several alternate mechanisms. One of these is death receptor-induced target cell lysis. Expression of death receptor ligands by NK cells such as TRAIL, TNF and TNF receptor ligand-Fas ligand (FasL) [(234)]. Upon engagement of these ligands to their cognate death receptors on target cells, the death receptor undergoes a conformational change, leading to the recruitment and activation of adaptor molecules and the initiation of apoptotic pathways within the target cell [(235)].

As described in **1.2.3**, upon NK cell activation, lytic granules are secreted containing perforin and granzymes which result in target cell apoptosis. However, lytic granules have death-inducing capacity beyond perforin and granzymes. Lytic granules can be uptaken by tumour cells and contain, amongst others, the tumour suppressor miR-186 which has been implicated in NK exosome-induced toxicity against neuroblastoma cells [(236)]. Lytic granules derived from NK cell line NK-92 were observed to express FasL and demonstrated anti-

melanoma effects, including reduced tumour cell viability and regulating proliferation. Additionally, melanoma tumours injected with these exosomes had decreased size compared to a control-treated group [237].

Finally, activated NK cells secrete a wide range of cytokines including TNF-α, interferon gamma (IFN-γ), IL-10 and IL-5 and chemokines including MIP-1α, MIP-1β and IL-8 [238-240]. One of the most influential cytokines secreted by NK cells is IFN-γ. IFN-γ secretion has been demonstrated to be activated by NKG2D-mediated signalling, and has anti-tumour, anti-viral and anti-bacterial capacity. IFN-γ upregulates the expression of TRAIL, Fas-L and stimulates anti-tumour activity by NK cells [197, 241, 242]. IFN-γ expression also enhances NK cell cytotoxicity of cancer cells by promoting the expression of vascular cell adhesion protein 1 (VCAM-1), promoting the binding of NK cells to the target cell encountered [243]. IFN-γ secretion can also be triggered by IL-1, IL-18, IL-12 and TNF-α secreted by DCs, neutrophils and macrophages [244, 245]. IL-10, also secreted by NK cells, induces NK cell cytotoxicity, proliferation and TNF-α secretion when combined with IL-18 [246]. IL-12 also contributes to anti-cancer activity, and has been recorded to promote regression of melanoma cells in an NK cell-dependent manner . Additionally, IL-21, also secreted by NK cell as well as T cells, induces NK cell activation in vivo against melanoma and renal cell carcinomas in afflicted patients. Furthermore, IL-21 has also been demonstrated to reject several tumours established in murine models in an NKG2D-dependent manner [247, 248].

NK cells can also influence the adaptive immune response. IFN-γ secreted by NK cells matures DCs (mDCs), and cross-talk between NK cells and DCs activates both cell types. IL-12 secreted by NK cell-activated DCs has been shown to activate and enhance responsiveness of CD8 T cells against [249, 250]. NK cells have also been observed to eliminate immature DCs (iDCs) and tolerate mDCs, which contributes to the activation of T cells as iDCs favour the regulation of CD8 T cell activity [251].

1.2.5 NK cell licensing

NK cells acquire functional competence by interactions between KIRs and HLA molecules. This process is known as NK cell licensing or education. NK cells acquired from

murine models lacking MHC class I molecules (the murine analogue of HLA molecules), or murine NK cells lacking Ly49 (murine NK cell MHC class I-binding inhibitory receptor) were unable to mediate lysis of MHC class I-expressing target cells [252]. This leads to the hypothesis that NK cells which engage MHC class I or HLA molecules during development develop fully functional maturity and are capable of recognizing and destroying HLA/MHC class I molecule deficient target cells upon maturation (Figure 1.6). Transgenic expression of MHC class I molecules and HLA molecules resulted in the licensing of murine ($Ly49^+$) and human (KIR^+) NK cells, respectively [253, 254]. This results in the development of two NK cell subsets: licensed NK cells capable of lysing an HLA-deficient target cell but tolerant of HLA-expressing target cells, and unlicensed NK cells with minimal cytotoxic capacity and chance of targeting surrounding HLA-expressing cells of "self". The presence of an ITIM in the cytoplasmic tail of KIRs is crucial for the licensing process which is dependent on ITIM-mediated signalling [255]. SHP-1 is not thought to be involved in NK cell licensing but cannot fully be discounted, whereas SHIP is not required for the licensing, and although SHP-2 remains a candidate lack of SHP-2 expression leads to embryonic lethality [255-257]. Therefore, it is possible that KIR-mediated licensing can be mediated by the recruitment of a currently unknown molecule to the ITIM upon initial binding of HLA molecules.

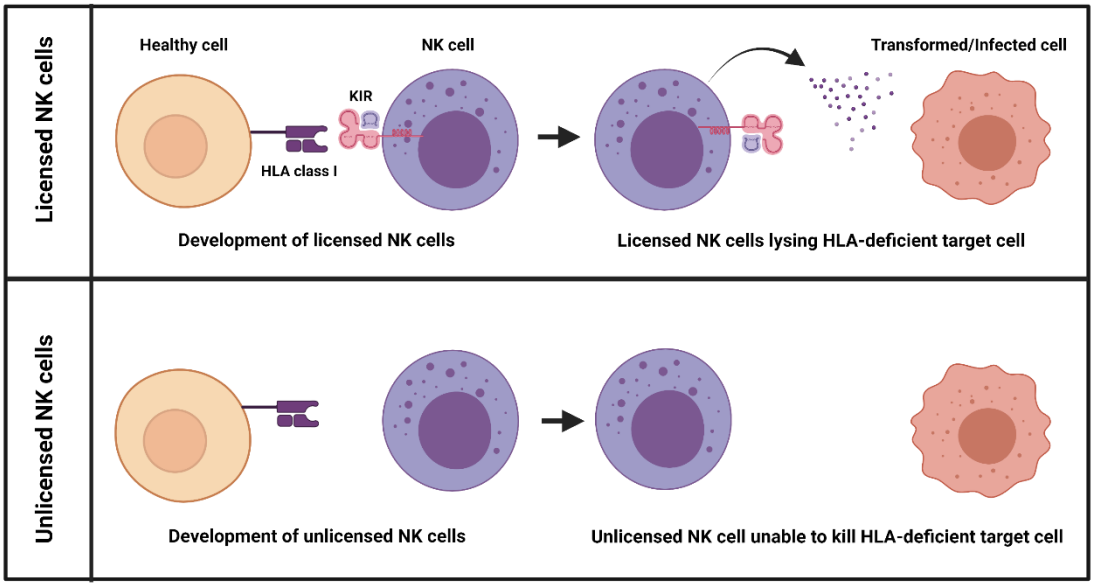


Figure 1.6 NK cell licensing is critical for the development of fully functional NK cells capable of carrying out functions such as cytotoxicity.

As NK cells express a large panel of inhibitory receptors aside from KIRs, the role of licensing of NK cells through non-classical inhibitory receptors has also been investigated. NKG2A is also thought to be involved in NK cell licensing, and has been shown to educate NK cells in the foetus whereas KIRs are unable to license foetal NK cells [258, 259]. Additionally, NKG2A has been shown to educate NK cells in the PB. However, the exact mechanism behind NKG2A-mediated licensing of NK cells is not currently understood. Furthermore, expression of the inhibitory Ly49A receptor has been shown to correlate with NK cell maturation in an ontogenic setting, indicating that Ly49A-mediated licensing of NK cells may be critical to ensure acquisition of functional capacity [260]. Furthermore, mature unlicensed NK cells derived from MHC class I-deficient mice were injected into a murine model and licensing was observed in a Ly49-dependent manner [261]. Originally NK cell licensing was thought to take place within the BM, however studies in murine models have demonstrated that this is not the case. Instead, NK cell licensing seems to be a very fluid process, capable of happening whilst NK cells are in circulation. Nevertheless, licensing is an important part of NK cell maturation and acquisition of fully functional capacity.

1.2.6 NK cells in cancer therapy

NK cell based-therapies for the treatment of cancer have emerged as potential novel strategies in recent years (Figure 1.7). As mentioned in **1.2.3.2**, NK cells are critical effectors of moAb-based therapies and exert potent ADCC upon encountering opsonised cancer target cells. However, this is not the only approach utilised to harness NK cells against cancer. Interruption of signalling by inhibitory receptors using blocking antibodies has been widely utilised in both pre-clinical and clinical research to enhance NK cell functions against cancer. For example, blockade of NKG2A using the blocking antibody monalizumab has shown promise against squamous cell carcinoma of the head and neck when combined with the anti-epidermal growth factor receptor (EGFR) antibody cetuximab [262]. This study demonstrates the power of combinatory treatments to induce NK cell anti-cancer activity by combining ADCC, stimulated with cetuximab, and blockade of inhibitory receptors. Monalizumab is also in clinical development against several different tumour types and considering the prevalence of HLA-E on tumour cell types, represents a promising potential NK cell-based anti-cancer

therapy [263]. Other blocking antibodies targeting PD-1, KIR2D, TIM-3, CD96 and TIGIT are currently under clinical investigation to enhance NK cell-mediated anti-cancer functions. While these studies are predominantly early Phase 1 or Phase 2 trials, blocking antibodies have demonstrated tolerability in patients. Further trials will demonstrate the therapeutic value of these blocking antibodies, however evidence suggests that combinatory approaches might elicit a stronger therapeutic response [264]. These studies have been carried out in solid tumours to-date.

In haematological malignancies, NK cells also hold promise. Blockade of KIR2D, this time using the anti-KIR2D antibody lirilumab, potentiates NK cell cytotoxicity against acute myeloid leukaemia (AML). Lirilumab was well tolerated by both AML and MM patients, with no autoimmunity or toxicity being observed. The potential of a combinatory approach to enhance NK cell cytotoxicity is further demonstrated in MM, where lirilumab did not induce a response in MM patients, but elicited a good response when combined with lenalidomide in RR MM patients [264-266]. Lirilumab is also currently being tested in combination with other agents such as immune checkpoints (nivolumab (anti-PD1) in MM, non-Hodgkin's lymphoma and Hodgkin's lymphoma) and moAbs such as rituximab [264, 266]. While moAbs have demonstrated success against haematological malignancies, their efficacy against solid cancers has been limited. As a result of this, antibodies were designed to target two antigens simultaneously to induce stronger effector cell-mediated responses. These are classed as bispecific antibodies (BsAbs). BsAbs contain a tumour-targeting single-chain variable fragment linked to an anti-CD3 or anti-CD16 agonistic fragment designed to stimulate signalling by CD3 or CD16 on T cells and NK cells, respectively [267]. The most notable results of using BsAbs to-date have been observed using T cells, where bispecific molecules such as catumaxomab and blinatumomab and ionotuzumab have been used to treat B cell lymphoblastic leukaemia-afflicted patients which improved overall survival and remission, respectively [268, 269]. However, one of the drawbacks of using BsAbs to stimulate T cell-mediated anti-cancer functions is the resulting increase in cytokines which can result in capillary leak or respiratory distress. As a result, NK cells have become prominent target effectors of BsAbs-induced cancer therapy, with many ongoing trials evaluating the efficacy of BsAbs within the context of breast cancer, for example [267]. In MM, early-phase clinical trials have targeted BCMA, orphan G protein-coupled receptor, class C group 5 member D

(GPRC5D) and Fc receptor-homolog 5 (FcRH5) and demonstrated tolerability and early indications response inducement in treated MM patients. Further studies are also investigating the efficacy of BsAbs in MM in combination with other agents such as pomalidomide, dexamethasone and anti-PD1 antibodies [270].

Whilst CAR-T cell therapy is attracting significant attention for the treatment of cancer, and has been approved by the FDA in the case of acute lymphoblastic leukaemia (ALL) and B cell lymphomas, NK cells represent a competitive alternative to T cells in this regard. Clinically relevant numbers of NK cells can be accrued quickly compared to T cells, and whilst CAR-T cells can induce graft vs host disease (where the host system recognizes CAR-T cells as foreign and destroys them) NK cells do not [271]. Furthermore, obtaining sufficient numbers of T cells from heavily treated cancer patients can be difficult, whereas NK cells can be acquired from several different sources such as peripheral blood, umbilical cord blood and haematopoietic stem cells [272]. NK cells can also be sourced from cell lines, representing a potential "off-the-shelf" product [273-275]. Finally, the capacity for NK cells to recognize and destroy tumour cells independently of CAR indicates that they might be less susceptible to downregulation of CAR ligand expressed by tumour cells, as has been observed in certain CAR-T cell settings [273]. Several mechanisms can be utilised to express CARs on NK cells, including viral methods such as lentiviral and retroviral-based transductions and non-viral methods including electroporation and transposons. CAR-NKs have been used to target both solid cancers and haematological malignancies in pre-clinical investigations and has been shown to enhance NK cell cytotoxicity [276-278]. More recently, CAR-NK cells have been designed to combat MM. CD2 subset 1 (CS1), a surface protein highly expressed by MM cells has been used as a target antigen for CS1-CAR-NK cells, which demonstrated enhanced cytotoxicity and IFN-γ secretion when co-cultured with CS1-expressing MM cell lines in-vitro and demonstrated enhanced activity against primary MM tumours. Furthermore, NK-92 NK cells expressing a CS1-CAR prolonged survival of mice xenograft models with established MM tumours, and enhanced clearance of human MM cells [279]. Additional studies have demonstrated the potential of the NK-92 cell line as a promising candidate for the development of CAR-NK-based therapies. NK-92 cells, modified to be IL-2 independent (NK-92MI), expressing an anti-CD138 CAR showed enhanced cytotoxicity against both MM cell lines and primary MM cells. Potentiated activity of these CD138-CAR-NK-92 cells was observed by enhanced CD107a expression and increased

secretion of granzyme-B and IFN-γ were observed when after co-culture with MM cells [280]. Finally, NKG2D-CAR-NK and BCMA-CAR-NK cells have been developed and demonstrate enhanced activity against MM, with clinical trials currently recruiting patients with RR MM for treatment with BCMA-CAR-NK-92 [281]. Challenges do exist with CAR-NK cell therapy, particularly regarding NK cell persistence in the human body although patients treated with CAR-NK cells have been observed to retain CAR-NK cells for up to one-year post-transfusion. Further results from ongoing CAR-NK clinical trials will help to elucidate the capability NK cells from different sources to persist within humans [282]. Furthermore, issues with CAR-NK cell viability and capabilities post-thaw can limit the efficacy of the treatment, and T cell uptake of genomic material, particularly when viral based-transduction is used, is more efficient compared to NK cells [283].

In order to combat these issues, bi- and tri-specific killer engagers (BiKEs and TriKEs) are being researched as a complementary approach to further maximise NK cell-mediated anti-tumour capabilities. BiKEs and TriKEs contain two and three antibody variable fragments respectively, allowing the simultaneous targeting of multiple target antigens expressed by a target tumour cell [284]. In BiKEs, one of these antibody fragments recognizes and binds to CD16, stimulating NK cell activity while the other variable fragment recognizes a target antigen expressed by the tumour cell. TriKEs can elicit more potent NK cell-mediated cytotoxicity, by containing two distinct antibody fragments recognizing target antigens as well as the CD16 engager fragment [285]. Further generations of TriKEs have incorporated IL-15 into the TriKE, to drive NK cell proliferation in-vivo as well as stimulating cytotoxicity. IL-15 is favoured over IL-2 to enhance NK cell proliferation and subsequent activation as IL-2 can potentially lead to systemic vascular leak and the activation of $CD25^+$ regulatory T cells [286, 287]. The final function of both BiKEs and TriKEs is to drive the formation of an immunological synapse between the NK cell and target cell, facilitating cytotoxicity [285]. One of the most common antigens targeted by BiKEs and TriKEs in haematological malignancies is CD33, expressed by both AML and MM cells. TriKEs targeting CD33 on MM cells have demonstrated enhanced cytotoxicity compared to CD33-targeting BiKEs and were observed to enhance not only cytotoxicity but proliferation, cytokine release and survival of NK cells in vitro [288]. Furthermore, in a xenograft mouse model, adoptively-transferred NK cells in combination with the same TriKE led to significant reduction of engrafted $CD33^+$ MM tumours and

increased NK cells in the blood after TriKE-mediated stimulation [288]. Clinical trials using BiKEs and TriKEs are currently ongoing, and should shed more light on their future clinical potential.

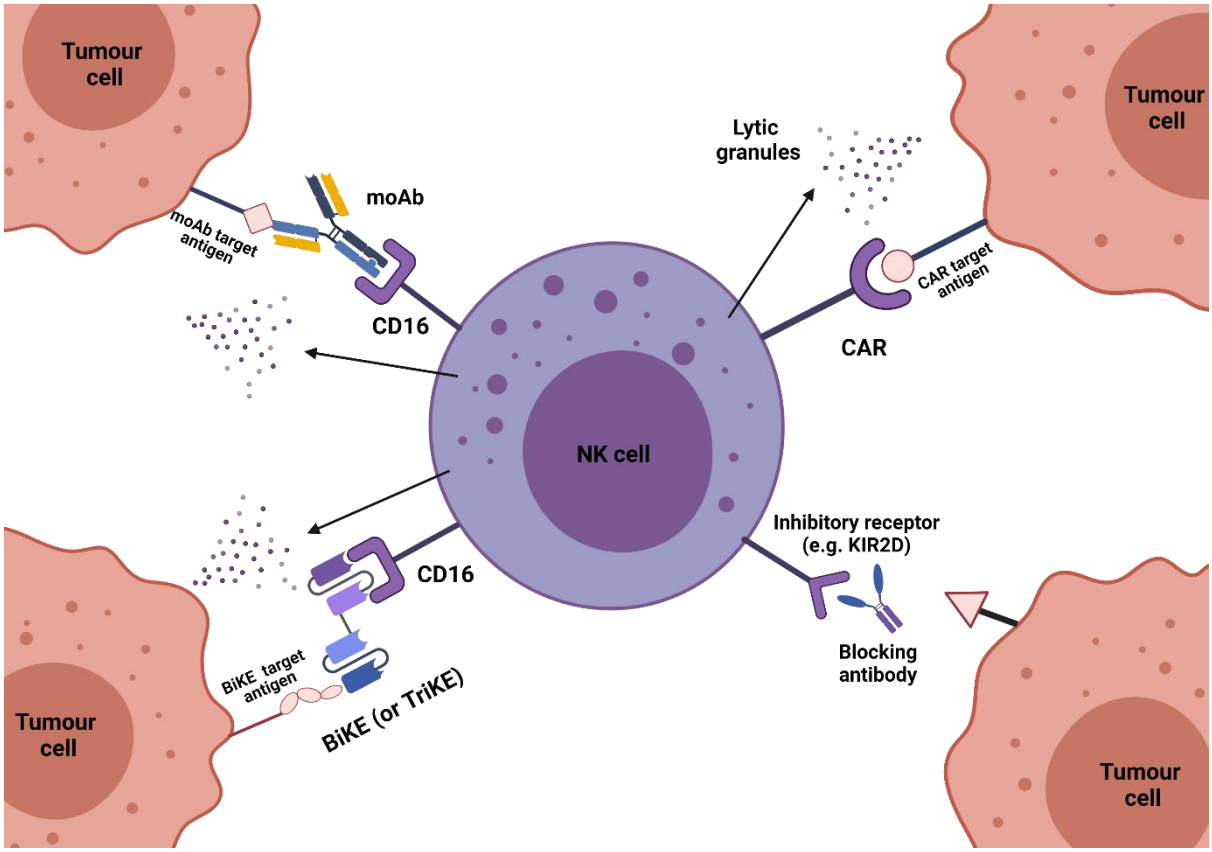


Figure 1.7 NK cell-based immunotherapies currently in development or in clinical use to treat cancer.

NK cells therefore represent a promising tool for novel immunotherapies targeting both solid tumours and haematological malignancies. However, tumour cells possess mechanisms of resistance to NK cell-mediated immunosurveillance, which could be targeted in combination with novel NK cell-based therapies such as CAR-NK cells or BiKEs/TriKEs to further enhance their anti-cancer capabilities.

1.2.7 Siglec expression in NK cells

I-type lectins were originally classified as members of the Immunoglobulin-like protein superfamily capable of binding to glycans. Siglecs are a sub-type of I-type lectins with an affinity for sialic acid-derived moieties. Nicoll et al. were the first group to classify Siglecs in the late 1990s [289]. Siglecs are a 15-member family of surface receptors, containing both activating and inhibitory members. Siglecs can bind to sialic acids using a N-terminal V-set domain, facilitated in main by a carbohydrate-binding arginine residue. Siglecs also have a varying number of C2 -set immunoglobulin domains [290]. Siglecs can be subdivided into two groups: CD33-related Siglecs consisting of Siglec-3, 5, 6, 7, 8, 9, 10, 11, 14 and 16 and non-related Siglecs which consists of Siglec-1, 2, 4 and 15 . CD33-related Siglecs share a 50-99% sequence similarity, with some members being highly similar to each other, while the non-related Siglecs are more distinct and only share a 25-30% sequence identity [291]. Apart from Siglec-6 which is expressed by mast cells, human Siglecs are expressed on hematopoietic and immune cell types. Expression of some of these Siglecs is restricted to certain cell types. For example, Siglec-1 (sialoadhesion) is expressed only by macrophages. For the most part, CD33-related Siglecs are expressed by immune cell types, particularly in cells of the innate immune system [291]. The fact that each Siglec has its own unique sialic acid specificity suggests that Siglecs have their own distinct roles in each cell type they are expressed by. Siglec-2 and CD-33 related Siglecs contain an ITIM motif and are receptors with an inhibitory function, whereas Siglec-14 recruits DAP12 upon ligand binding – an ITAM-containing adaptor protein capable of both activating and inhibitory signalling [292]. Expression of two inhibitory Siglec receptors has been recorded on NK cells: Siglec-7 and Siglec-9.

Siglec-7 (p75/AIRM), a 75kDa transmembrane protein, expression was first recorded on NK cells by Falco et al. in 1999 [293]. Siglec-7 is also highly expressed by monocytes and a minor subset of $CD8^+$ T cells [289, 294]. Siglec-7 preferentially binds to α2,8-disialyl sialic acid residues, but also bind to α2,6-sialic acid-derived residues [295]. Despite being unable thus far to generate a crystallographic structure of Siglec-7-Siglec-7 ligand (Siglec-7L), the crystallographic structure of Siglec-7 has been solved, and comparison between Siglec-7 and Siglec-1 have allowed the identification of key residues needed for successful sialic acid-binding. A partial opening of the β-sandwich due to the lack of a stabilizing inner-sheet

disulphide reveals the Siglec-7L binding site containing basic residues such as Arg-23, Arg-120, Arg-124 and Lys-135 which are required for the binding of the negatively charged sialic acids. The most critical residue is Arg-124, which binds to the carboxyl group presented by the terminal sialic acid [296]. Siglec-7 contains one intracellular ITIM, defining it as a sialic acid-binding inhibitory NK cell receptor [290]. Studies of T cell receptor (TCR) signalling revealed that, upon TCR stimulation, Siglec-7 recruits and ITIM-located tyrosine residue phosphorylation recruits and activates SHP-1 leading to reduced downstream signalling events such as reduced phosphorylation of zeta-chain-associated protein kinase 70 (ZAP-70), a critical T cell signalling molecule (Figure 1.8) [294]. Siglec-7 is highly expressed by mature $CD56^{dim}CD16^{+}$ NK cells, and Siglec-7^{+} NK cells express higher levels of activating receptors such as CD16, DNAX accessory molecule (DNAM-1) and CD38 while expressing lower levels of inhibitory receptors such as NKG2A and CD158b. Furthermore, NK cell stimulation using phorbol 12-myristate 13-acetate (PMA) resulted in increased CD107a expression and IFN-γ by Siglec-7^{+} NK cells compared to Siglec-7^{-} NK cells as well indicating that Siglec-7 defines a mature, potent NK cell [297].

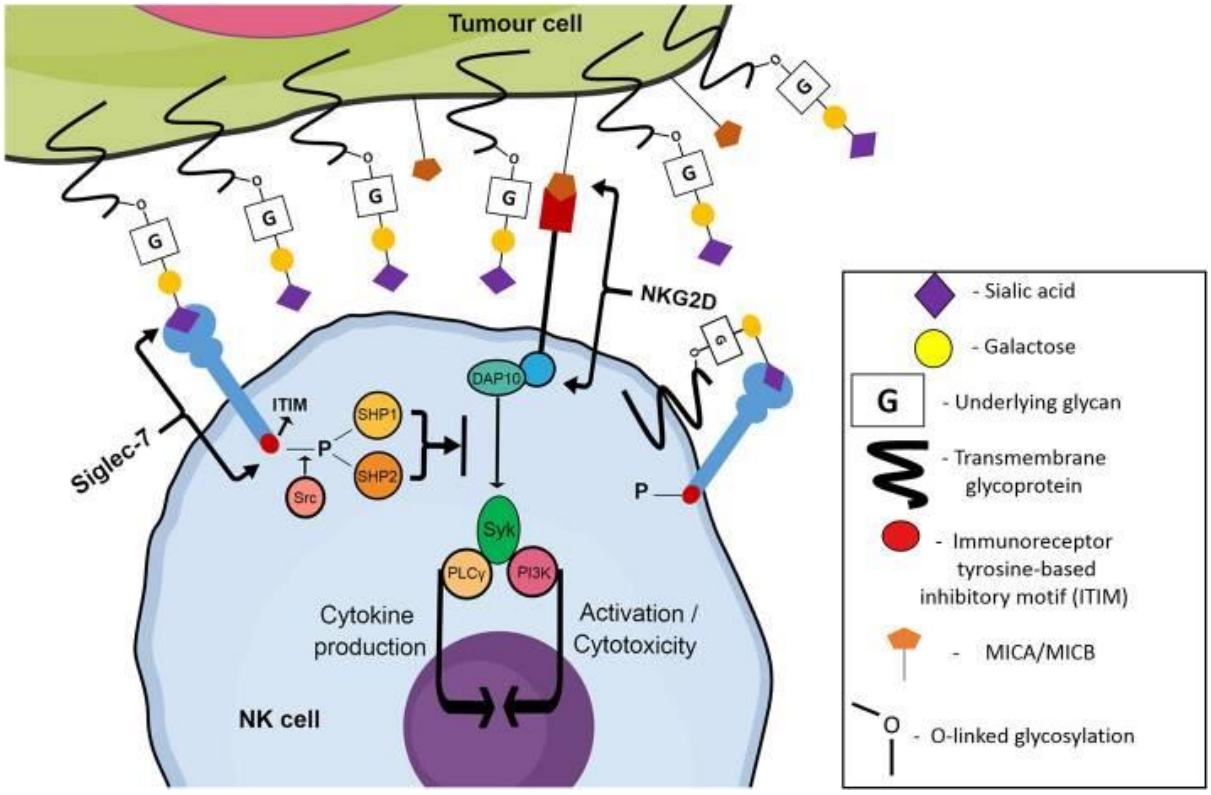


Figure 1.8 Siglec-7 regulates NK cell cytotoxicity against MM cells. The inhibitory Siglec-7 receptor expressed by NK cells recruits and activates intracellular phosphatases which dampen activation pathways within the NK

cell upon binding to their sialic acid-derived ligand presented by MM cells, preventing destruction of the MM cell. Adapted from Daly et al., 2019.

Siglec-9 was first described by Xhang et al. in 2000 and has an 84% sequence similarity to Siglec-7, and a 74% similarity to the V-set domain [(295)]. Siglec-9 is expressed by monocytes, neutrophils, in minor populations of B and T cells [(290)]. Siglec-9 is also expressed on a subset of $CD56^{dim}$ NK cells, but is less frequently expressed by NK cells in general compared to Siglec-7. Siglec-9 expression is virtually absent on $CD56^{high}$ NK cells [(190)]. Similar to Siglec-7, Siglec-9 possesses one intracellular ITIM motif located proximally to the cell membrane. However, Siglec-9 also possesses an ITIM-like motif located distally from the cell membrane which is capable of weakly interacting with phosphatases SHP-1 and SHP-2, but is not labelled as a classic ITIM due to a dissimilar amino acid sequence in the motif [(298)]. As with Siglec-7, Siglec-9 has demonstrated an ability to negatively regulate TCR signalling. In neutrophils, Siglec-9 has been shown to induce cellular apoptosis upon ligand binding which could be enhanced in the presence of IFN-γ, IFN-α and granulocyte/macrophage colony stimulating factor (GM-CSF). Siglec-9 has also been shown to prevent polarization of tumour-infiltrating macrophages into a cellular growth-promoting pro-tumorigenic M2 state [(192)]. Treatment of neutrophils resulted in a rapid tyrosine phosphorylation of Siglec-9, which enhanced Siglec-9 dependent death [(299)]. Siglec-9 recognizes α2,3-linked sialic acids, and the difference in preference between Siglec-7 and Siglec-9 is due to a six amino acid stretch located in the C-C' loop of both receptors. It is hypothesized that the difference in the side chains of amino acids within this loop affects the individual preferences for sialic acids within the Siglec family [(296)]. The key residues for Siglec-9-Siglec-9 ligand (Siglec-9L) binding are Ala-66, Thr-68 and Asp-71 . Siglec-9 recognizes Mucin 1 (MUC-1) as a ligand, which is sialylated in cancer and promotes the expression of PD-L1 of macrophages and promotes the development of a tumour-associated macrophage-like phenotype.

Siglec-7 and Siglec-9 have both been implicated in facilitating tumour cell evasion from NK cell-mediated immunosurveillance, and represent promising novel immune checkpoints which could be targeted in future immunotherapies involving NK cells. The role both Siglec-7 and Siglec-9 in facilitating immune-evasion is discussed in detail in **3.1**.

2. Methodology

2.1 Multiple Myeloma and target cell culture

MM1S, H929, JJN3 (MM cell lines) and K562 (Gold standard target erythroleukemia cell line for NK cell studies) were used throughout this project and were obtained from ATCC. These cell lines were cultured as outlined in **Table 2.1**.

Table 2.1. Culture conditions for MM cell lines used in this project.

Cell line	Doubling time (hours)	Seeding density (per ml)	CO_2 (%)	Temp. (°C)
MM1S	60-75	500,000	5	37
H929	40-50	400,000	5	37
JJN3	40-50	400,000	5	37
K562	24-36	200,000	5	37

All cell lines were cultured in RPMI1640 (Sigma Aldrich) including 10% foetal bovine serum (FBS)(Sigma Aldrich), 100IU/ml penicillin and 100µg/ml streptomycin (both ThermoFisher Scientific). MM1S and K562 were cultured in tissue culture dishes (100mm x 20mm, Sarstedt), while H929 and JJN3 were cultured in T25 culture flasks (Sarstedt) in a horizontal position. All cell lines were passaged every 48 hours.

Where MM cell lines were co-cultured under hypoxia, MM cells were seeded at the density described in **Table 2.1** in 6-well plates in duplicate, after which cells were cultured in a InvivO2 Physiological Cell Culture Workstations hypoxia chamber (Baker) for 72 hours, prior to being collected and stained for Siglec-7L and Siglec-9L expression as described in **2.5**.

2.2 Natural Killer cell line culture

KHYG-1 and NK-92 NK cell lines were used at the beginning of the project. KHYG-1 were kindly provided by Dr. Armand Keating (University of Toronto). These cell lines were cultured as outlined in **Table 2.2**.

Table 2.2 Culture conditions for NK cell lines used in this project.

Cell line	Doubling time (hours)	Seeding density (per ml)	CO_2 (%)	Temp. (°C)
KHYG-1	36-48	400,000	5	37
NK-92	40-50	400,000	5	37

KHYG-1 was cultured in RPMI1640 including 10% FBS, 500IU/ml IL-2 (Peprotech), 100IU/ml penicillin and 100µg/ml streptomycin. NK-92 were cultured in Lonza X-VIVO 10 media (Lonza), 20% Human AB serum (Sigma Aldrich), 500U/ml IL-2, 100IU/ml penicillin and 100µg/ml streptomycin. Both cell lines were cultured in T25 culture flasks in a horizontal position and were passaged every 48 hours.

Where NK cells were co-cultured under hypoxia, NK cells were seeded at the density described in **Table 2.1** in 6-well plates in duplicate, after which cells were cultured in a InvivO2 Physiological Cell Culture Workstations hypoxia chamber for 72 hours, prior to being collected and stained for Siglec-7/9 as described in **2.8.**

2.3 Primary NK cell isolation and expansion

Fresh peripheral blood was supplied by consenting informed healthy donors on the day of isolation. Peripheral blood was collected from donors at University Hospital Galway. Blood was collected and stored at 4 °C until isolation in ethylene diamine tetra-acetic acid (EDTA)-containing vacutainer tubes (VACUETTE) to prevent clotting. Blood was pooled from Vacutainer tubes and diluted to a final volume of 35ml using PBS (Sigma) if needed, before being slowly layered on top of 15ml Ficoll-Paque (GE Healthcare) in a 50ml tube. The blood was then centrifuged (ThermoFisher Scientific) at 2,000 revolutions per minute (RPM) for 20 minutes, using an acceleration and brake of 1,1. After spinning the peripheral blood mononuclear cells (PBMCs) formed a thin pale layer known as the “buffy layer”. The buffy layer was completely collected using a Pasteur pipette (Sarstedt) and transferred to a new 50ml tube. PBMCs were then washed twice with PBS before being counted using a disposable

haemocytometer (NanoEntek). After determining the total PBMC number cells were resuspended in MACS buffer (PBS including 2% FBS and 1mM EDTA) and stained using the NK cell isolation kit as per the supplier's instructions (Miltenyi). After staining PBMCs were passed through an LS column (Miltenyi) mounted on a Midimacs separator magnet (Miltenyi) where NK cells were eluted into 15ml collection tubes due to negative magnetic selection. After rinsing the LS column using MACS buffer twice isolated NK cells were centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9 after which the supernatant was removed using a P1000 pipette, being careful to avoid disturbing the cell pellet. NK cells were then resuspended in 2ml of NK MACS expansion media (NK MACS media (Miltenyi) including 5% Human AB serum, 500IU/ml IL-2 and 1% supplement (Miltenyi)) before being counted and seeded according to the cell number as outlined in **Table 2.3**. Purity was determined by staining for CD56 and CD3 expression on isolated cells, NK cells were determined as being $CD56^{+}/CD3^{-}$. Cell viability was determined using propidium iodide (P. Iodide), 100µg/ml P. Iodide in milliQ H_2O) staining.

Table 2.3 Protocol used for initial culture of freshly isolated primary NK cells

NK cell number	Volume of expansion media used (ml)	Plate used for initial culture
$< 1x10^6$	2	24-well
$1\text{-}2x10^6$	2.5	12-well
$> 2x10^6$	3	6-well

For the initial 72 hours after seeding NK cells were monitored daily. Fresh NK expansion media was added to the cells after 72 hours and 25% of the initial seeding volume was used to replenish media lost to evaporation. NK cells typically began expanding 5-6 days after isolation, and were cultured at $5x10^5$ cells/ml after this point. Primary NK cells were cultured in 100x20mm dishes once expansion had been confirmed by cell count number. NK cells typically stopped expanding approximately 3 weeks after isolation, after which cultures were destroyed using Virkon (LANXESS).

2.4 Isolation of primary MM cells

Bone marrow aspirates (BMAs) were provided by consenting informed patients. BMAs were collected from patients at University Hospital Galway, and stored at 4°C until required for isolation in EDTA-containing vacutainer tubes. The BMA was pooled together in a 50ml tube and diluted 1:1, or until the volume was at least 15ml. The blood was then layered on top of 15ml Ficoll-Paque and subsequently centrifuged at 2,000 RPM for 20 minutes using an acceleration and break of 1,1. PBMCs were presented as a buffy layer and were collected using a Pasteur pipette before being transferred to a fresh 50ml tube where PBS was added to create a final volume of 50ml to perform a wash. The PBMCs were centrifuged at 1,200 RPM for 5 minutes using an acceleration and break of 9,9, after which the supernatant was removed, the cells resuspended in PBS and then counted using a disposable haemocytometer. After cell number was determined, cells were stained and isolated using a EasySep CD138 positive selection kit II (STEMCELL technologies) as per the supplier's instructions. After isolation, primary MM cells were stained immediately if used for flow cytometry. Purity was determined by staining with anti-CD38 and anti-CD138 antibodies, and $CD38^{+}/CD138^{+}$ cells were considered to be MM cells. Cell viability was determine using P. Iodide staining.

2.5 Staining of primary MM cells and MM cell lines for Siglec-7L and Siglec-9L

Primary MM cells, MM1S, H929 and JJN3 were collected and counted before being centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Cells were resuspended at a concentration of $5x10^5$ cells/ml in FACS buffer (PBS including 2% FBS, 1mM EDTA and 0.1% sodium azide). 100µl of the cell suspension was transferred to FACS tubes (Sarsted), and 1.25µl or 2.5µl of recombinant Siglec-7 Fc chimera and recombinant Siglec-9 Fc chimera (R&D Systems) was added, respectively. For controls, cells were stained with a ChromPure Human IgG, Fc fragment control (hereafter referred to as Fc chain, Jackson Immunoresearch) control at the same volume as was used for recombinant Siglec-7 or Siglec-9 Fc chimeras Cells were vortexed briefly and incubated on ice in the dark for 30 minutes. 300µl of FACS buffer was then added to the tubes which were then centrifuged at 2,000 RPM for 3 minutes using an acceleration and brake of 9,9 (this is referred to hereafter as being

"washed"). Supernatant was discarded and cells were suspended in 100µl of FACS buffer containing 1:400 APC AffiniPure F(ab)2 fragment goat anti-human IgG, Fc-γ fragment specific (Jackson Immunoresearch) for 15 minutes on ice in the dark. Cells were then washed after which cells were resuspended in 250µl of FACS Buffer before being kept on ice in the dark until required for flow cytometry. Just before samples were run, 2µl of PI was added to each tube before vortexing and incubation on ice for 2 minutes. Gates to determine Siglec-7L$^+$ and Siglec-9L$^+$ cells were set according to Fc chain-stained control cells. Mean Fluorescence Intensity (MFI) of Siglec-7L$^+$ and Siglec-9L$^+$ cells were also determined.

Samples were run on a BD FACS Canto II flow cytometer (BD Biosciences), located at the NUIG Flow Cytometry Core Facility. Samples analysed using flow cytometry from hereafter were run on the BD FACS Canto II unless otherwise stated. Qualitative control maintenance was carried out on the BS FACS Canto II flow cytometer weekly by a qualified technician. Data files were analysed using FlowJo v.7 software (Tree Star Inc.).

2.6 Staining of primary MM cells and MM cell lines to determine sialic acid expression

Cells of interest were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was discarded and cells were resuspended at 5x10^4 cells/ml of FACS buffer.

Cells were stained with either 1:10,000 or 1:20,000 of *Maackia amurensis agglutinin* (MAA, targeting α2,3-linked sialic acids, Vector Laboratories) or *Sambucus nigra agglutinin* (SNA, targeting α2,6-linked sialic acids, Vector Laboratories) lectins for 30 minutes on ice in the dark. Cells were then washed with 300µl FACS buffer before being centrifuged at 2,000 RPM for 3 minutes using an acceleration and brake of 9,9. Supernatant was removed and cells were stained 5µl of APC-tagged Streptavidin (BD Pharmingen) in FACS buffer (stock solution was made by diluting 7µl of APC-Streptavidin in 243µl FACS buffer). Cells were incubated for 15 minutes on ice in the dark before being washed again with FACS buffer. Supernatant was discarded and cells were resuspended in FACS buffer. Cells were kept on ice in the dark until required for flow cytometry.

As controls, cells were stained with either MAA, SNA or APC-tagged streptavidin alone to determine background positivity. Gates were set based on APC-tagged streptavidin-stained cells alone.

2.7 Siglec-7L pulldown and mass spectrometry analysis to elucidate ligand identity*

2.7.1 MM ligand pulldown

$1x10^7$ MM1S, JJN3 and H929 MM cells were lysed in ice cold 0.1% Nonidet P-40 containing 1 mM EDTA and 1× Halt Protease Inhibitor mixture (Thermo Fisher Scientific) and rotated at 4 °C for 1 hour. The insoluble fraction was then cleared by centrifugation at 18,000 × g for 15 minutes. Protein concentration was determined by bicinchoninic acid (BCA) assay, and lysates were diluted further in lysis buffer to 500 µL with a final protein concentration of 1 µg/mL. Lysate was split into two 500-µL aliquots; one aliquot was left untreated, while the other was treated with 100 nM neuraminidase (sialidase from *Vibrio* cholerae, NEURA, Roche) for 1 hour. In parallel, 5 µg Siglec-7 Fc chimera was mixed with 50 µL Protein G Dynabeads in 250 µL PBS and rotated at room temperature for 1 hour. Beads were isolated with a magnet and washed once with PBS. Treated and untreated cell lysates were then added to the beads and incubated overnight at 4 °C with rotation. Following pulldown, flow-through was removed and beads were washed twice with ice cold 0.1% Nonidet P-40 and twice with ice cold 50 mM ammonium bicarbonate. Trypsin (1 µg, Promega) and GluC (1 µg, Progema) were then added to beads in 100 µL 50 mM ammonium bicarbonate, and beads were incubated overnight with shaking at 37 °C. The following day, digestion was acidified by adding formic acid to beads to a final concentration of 2%, followed by shaking at 37 °C for 30 minutes. Supernatant was then removed; beads were washed once with 100 µL 2% formic acid, and this wash was pooled with the original supernatant. The digested sample was dried down in a speedvac and resuspended in 10 µL 0.1% FA.

2.7.2 Mass Spectrometry of pulldown proteins

Peptides were separated over a 25 cm EasySpray reverse-phase LC column (75 µm inner diameter packed with 2 µm, 100 Å, PepMap C18 particles, Thermo Fisher Scientific). The mobile phases (A: water with 0.2% formic acid and B: acetonitrile with 0.2% formic acid) were driven and controlled by a Dionex Ultimate 3000 RPLC nano system (Thermo Fisher Scientific). An integrated loading pump was used to load peptides onto a trap column (Acclaim PepMap 100 C18, 5 um particles, 20 mm length, Thermo Fisher Scientific) at 5 µL/minute, which was put in line with the analytical column 5 minutes into the gradient. The gradient was held at 0% B for the first 6 minutes of the analysis, followed by an increase from 0% to 5% B from 6 to 7 minutes, and increase from 5 to 25% B from 7 to 66 minutes, an increase from 25% to 90% from 66 to 70 minutes, isocratic flow at 90% B from 70 to 75 minutes, and re-equilibration at 0% B for 15 minutes for a total analysis time of 90 minutes. Eluted peptides were analyzed on an Orbitrap Fusion Tribrid MS system (Thermo Fisher Scientific). Precursors were ionized was ionized using an EASY-Spray ionization source (Thermo Fisher Scientific) held at +2.2 kV relative to ground, the column was held at 40 °C, and the inlet capillary temperature was held at 275 °C. Survey scans of peptide precursors were collected in the Orbitrap from 350-1350 Th with an AGC target of 1,000,000, a maximum injection time of 50 ms, RF lens at 60%, and a resolution of 60,000 at 200 m/z. Monoisotopic precursor selection was enabled for peptide isotopic distributions, and precursors of z = 2-5 were selected for data-dependent MS/MS scans for 2 seconds of cycle time. Dynamic exclusion was set to exclude precursors after being selected once for an exclusion time of 30 seconds with a ±10 ppm window set around the precursor monoisotope. An isolation window of 1 Th was used to select precursor ions with the quadrupole, and precursors were fragmented using a normalized HCD collision energy of 30. MS/MS scans were collected with an AGC target of 100,000 ions, with a maximum accumulation time of 54 ms and an Orbitrap resolution of 30,000 at 200 m/z. The same method was used for both untreated and sialidase treated samples. Raw data were processed with MaxQuant version 1.6.2.10 and tandem mass spectra were searched with the Andromeda search algorithm. 20 ppm, 4.5 ppm, and 20 ppm were used for first search MS1 tolerance, main search MS1 tolerance, and MS2 product ion tolerance, respectively. Oxidized methionine and deamidated asparagine were set as variable modifications, and carbamidomethylation of cysteine was set as a fixed modification. Cleavage specificity was

set to Trypsin/P with 2 missed cleavage allowed. Peptide spectral matches (PSMs) were made against a human protein database (reviewed entries only, 20,416 entries total) downloaded from Uniprot. Peptides were filtered to a 1% false discovery rate (FDR) using a target-decoy approach, and a 1% protein FDR was applied. Proteins were quantified and normalized using MaxLFQ, and the match between runs feature was enabled. Label free intensity values were log2 transformed and plotted using Perseus version 1.6.2.2.

** This work was kindly carried out as part of a collaboration with Prof. Carolyn Bertozzi, at Stanford University. The protocol detailed in **2.7.1** and **2.7.2** was carried out by Dr. Jessica Stark and Dr. Nicholas Riley in order to identify Siglec-7 ligands. MM1S, JJN3 and H929 frozen cell pellets were prepared and sent to Dr. Stark at Stanford University in preparation for this assay.*

2.8 Staining of primary NK cells or NK cell lines for Siglec-7 and Siglec-9 expression

Primary expanded NK cells, KHYG-1 or NK-92 were collected and counted before being centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Cells were resuspended in FACS buffer at $5x10^5$ cells/ml and 100µl of cells were transferred to FACS tubes. NK cells were then stained with Siglec-7 or Siglec-9 antibodies (Miltenyi). Cells were vortexed briefly and incubated on ice in the dark at 4°C for 20 minutes. Cells were then washed and resuspended in 250µl FACS buffer before being kept on ice in the dark until required for flow cytometry. Just before samples were run, P. Iodide was added to each tube before vortexing and incubation on ice for 2 minutes. Gates to determine Siglec-7^+ and Siglec-9^+ positive cells were set based on unstained NK cell controls.

Where NK cells from MM patient BMAs were analysed for Siglec-7 expression NK cells were isolated as described in **2.4**, and then stained as outlined above.

2.9 Analysis of Siglec-7, Siglec-9 and CD96 expression on immune cell subsets from MM patient BMAs compared to healthy donor-derived PBMCs

$CD138^-$ fractions isolated from MM patient-supplied BMAs were kindly provided by the Blood Cancer Biobank Ireland as frozen pellets. As a control for this experiment PBMCs from the PB of healthy donors were used and were stained under the same conditions.

Cells were thawed, washed and resuspended in RPMI1640 including 10% FBS before being counted. Aliquots of $2x10^5$ cells were transferred to V-bottomed 96-well plates (as required per the assay outline including single stains (SS) and Fluorescence Minus One (FMO), Sarstedt) which were then centrifuged at 2,000 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was discarded and cells were resuspended in FACS buffer containing antibodies designed in two separate panels to allow the identification of NK cells, $CD4^+$ and $CD8^+$ T cells as well as monocytes/macrophages. The panels and antibodies are detailed in **Table 2.4**.

Table 2.4 Flow cytometry staining panels used to determine Siglec-7, Siglec-9 and CD96 expression on human immune cell subsets from healthy donors and MM patient samples.

Panel 1 (For analysis of NK cells, $CD4^+$ and $CD8^+$ T cells)				
Antibody	**Clone**	**Supplier**	**Fluorochrome**	**Catalogue number**
SYTOX	N/A	ThermoFisher	BV421	S34857
CD56	5.1H11	Biolegend	Pe/Cy7	362510
CD3	UCHT1	Biolegend	PerCP/Cy5.5 or BV510	300430 or 317332
CD4	A161A1	Biolegend		
CD8	SK1	Biolegend	Pe, PerCP/Cy5.5 orBV510	344706, 344708 or 344732
Siglec-7 (CD328)	REA214	Miltenyi	APC	130-101-016
Siglec-9 (CD329)	REA492	Miltenyi	FITC	130-107-651
CD96	REA195	Miltenyi	PE	130-101-036
Panel 2 (For analysis of monocytes/macrophages)				
Antibody	Clone	Supplier	Fluorochrome	Catalogue number
SYTOX	N/A	ThermoFisher	BV421	S34857
CD56	5.1H11	Biolegend	PE/Cy7	362510
CD3	UCHT1	Biolegend	PerCP/Cy5.5 or BV510	300430 or 317332
CD11b	ICRF44	Biolegend	APC/Cy7	301342
CD14	HCD14	Biolegend	Pe or BV421	325606 or 325628
Siglec-7 (CD328)	REA214	Miltenyi	APC	130-101-016
Siglec-9 (CD329)	REA492	Miltenyi	FITC	130-107-651
CD96	REA195	Miltenyi	PE	130-101-036

Compensation and gate setting was carried out using SS and FMOs. Expression of markers of interest was carried out on viable single cells. Overall percentage of cells positive for markers of interest was recorded, and MFI of positive cells was also determined using FlowJo.

NK cells were determined as being $CD56^+/CD3^-$, $CD4^+$ T cells were determined as being $CD56^-/CD3^+/CD4^+$, $CD8^+$ T cells were determined as being $CD56^-/CD3^+/CD8^+$ and monocytes/macrophages were determined as being $CD56^-/CD3^-/CD11b^+/CD14^+$.

2.10 MM or NK cell surface desialylation

Two separate methods of cell surface desialylation were used in this project; the sialidase enzyme NEURA (derived from *Vibrio cholerae*) or a cell permeable specific sialic acid analogue 3Fax-Peracetyl Neu5Ac (SIA, Calbiochem) which acted as a sialyltransferase inhibitor.

2.10.1 Neuraminidase-mediated cell surface desialylation

Cells to be desialylated were collected, counted and two aliquots of $1.25x10^6$ cells were transferred to 15ml tubes. Cells were then centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Cells were resuspended in 225µl of appropriate culture media without serum and treated with either 0.1IU/ml of NEURA or 1X glycobuffer (GLYCO, 50mM sodium acetate and 5mM $CaCl_3$) which acted as a control. Cells were vortexed briefly and then incubated at 37°C for 45 minutes. 4.75ml of appropriate culture media without serum was added to the cells which were centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed and cells were washed again in 5ml of appropriate media without serum. Supernatant was again discarded after which cells were used as desired. To determine the efficacy of NEURA treatment, cells were stained for Siglec-7L and Siglec-9L as outlined in **2.5** or sialic acid expression as outlined in **2.6.**

2.10.2 Sialyltransferase inhibitor-mediated cell surface desialylation

Cells to be desialylated were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Cells were then seeded into 6-well plates (Sarstedt) at the concentrations outlined in **Table 2.1** in a final volume of 3ml. Cells were treated with 300µl of the cell permeable sialic acid analogue 3Fax-Peracetyl Neu5Ac (hereafter referred to as SIA) to inhibit sialyltransferase activity. Control cells were treated with same volume of dimethyl sulfoxide (DMSO, Sigma Aldrich). Cells were cultured at 37°C for 72 hours after which cells were collected and used as needed in further experiments. To determine the efficacy of SIA treatment, cells were stained for Siglec-7L and Siglec-9L as outlined in **2.5** or sialic acid expression as outlined in **2.6.**

2.11 Cytotoxicity assays

Primary NK cells or NK cell lines were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed and NK cells were resuspended in 500µl of PBS containing 1% BSA. 1µl of a stock CellTrace™ CFSE Cell Proliferation Kit (stock prepared according to supplier's instructions, ThermoFisher Scientific) was added to NK cells which were vortexed and incubated at 37°C for 5 minutes. 2ml of ice-cold culture media was added to the NK cells which were incubated on ice in the dark for 6 minutes. 3ml of culture media was added to the NK cells which were then centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was carefully discarded and NK cells were resuspended at a pre-determined concentration to match the maximum Effector : Target (E:T) cell ratio used in the assay as outlined in **Table 2.4**. NK cells were seeded into flat bottomed 96-well plates (Sarstedt), along with cytotoxicity media (RPMI1640 including 10% FBS and 500IU/ml IL-2) to create a volume of 100µl. 100µl of target cells were then added to create a final volume of 200µl in each well. As controls, target cells were seeded alone. All conditions were carried out in duplicate. NK cells were also seeded alone to allow for gating to be set between $CFSE^-$ (target cells) and $CFSE^+$ (effector cells) during analysis.

Table 2.5 Example of cytotoxicity assay setup using E:T ratios ranging from 0.5:1 to 20:1

E:T Ratio	Target cells used	Volume of target cells (µl)	Effector cells used	Volume of effector cells (µl)	Supplemental media to create final volume of 200µl
1:0	0	0	$4x10^6$	100	100
0:1	$2x10^4$	100	0	0	100
0.5:1	$2x10^4$	100	$1x10^4$	3.625	96.375
1:1	$2x10^4$	100	$2x10^4$	6.25	93.75
2.5:1	$2x10^4$	100	$5x10^5$	12.5	87.5
5:1	$2x10^4$	100	$1x10^6$	25	75
10:1	$2x10^4$	100	$2x10^6$	50	50
20:1	$2x10^4$	100	$4x10^6$	100	0

All cytotoxicity assays for this project were carried out for 4 hours at 37°C in flat bottomed 96-well plates in a tissue culture incubator. After 4 hours cells were collected and transferred to individual FACS tubes containing 300µl of FACS buffer. The cells were then centrifuged at 2,000 RPM for 3 minutes using an acceleration and brake of 9,9. Supernatant was discarded and cells were resuspended in 300µl of FACS buffer and kept on ice in the dark until required for flow cytometry. Cell viability was determined using PI staining as outlined in **2.5**. NK cells were determined as being $CFSE^+$ while target cells were determined as being $CFSE^-$. Primary expanded NK cells, IL-2 activated NK cells (isolated and cultured overnight in RPMI1640 including 10% FBS and 500IU/ml IL-2), naïve NK cells (isolated and cultured overnight in RPMI1640 including 10% FBS) as well as KHYG-1 and NK-92 were all used in cytotoxicity assays during this project.

2.12 IL-2 activated NK cell degranulation assay

NK cells were isolated from buffy coats supplied by healthy consenting informed donors as described in 2.4 and cultured overnight in RPMI1640 including 10% FBS and 500IU/ml IL-2. Thus, these cells were classified as being "IL-2 activated". NK cells were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. $4x10^5$ NK cells were co-cultured with target cells K562, H929 and JJN3 which were pre-treated with NEURA or glycobuffer as outlined in **2.10.1** at a 1:1 E:T ratio in a total volume of 200µl in a U-bottomed 96-well plate (Sarstedt) for 1 hour. Cells were collected, transferred to V-bottomed 96-well plates and washed twice using FACS buffer. As controls for this assay, NK cells were seeded alone and were stained or unstained using the panel of antibodies detailed in **Table 2.6**.

Table 2.6 Staining panel for NK cell degranulation after short-term co-culture with sialylated or desialylated target cells

Panel 1 (For analysis of NK cells, $CD4^+$ and $CD8^+$ T cells)				
Antibody	**Clone**	**Supplier**	**Fluorochrome**	**Catalogue number**
Live/Dead	N/A	ThermoFisher	V525	L34957
NKG2A	Z199	Beckman Coulter	Pe/Cy7	B10246
KIR3DL1	DX9	Biolegend	BV421	312714
KIR2DL1/DS1	EB6B	Beckman Coulter	PE	AO9778
KIR2DL2/3	DX27	Biolegend	UV379	N/A
Siglec-7 (CD328)	REA214	Miltenyi	APC	130-101-016
Siglec-9 (CD329)	REA492	Miltenyi	FITC	130-107-651
CD107a	H4A3	Biolegend	BV780	328644
CD56	NCAM16.2	BD Biosciences	BUV737	612767

Cells were stained with the above panel for 30 minutes in a final volume of 50µl for 30 minutes in the dark on ice. Cells were washed using FACS buffer before being resuspended in 250µl FACS buffer. BD compensation beads (BD Biosciences) were used to create a compensation matrix on FlowJo v.7 which was then applied to all samples to ensure the correct compensation was used. Gates were set using SS and FMOs as appropriate. Samples were run on the BD Fortessa flow cytometer at the MedH Flow Cytometry Core Facility, Karolinska Institutet, Stockholm.

2.13 Expanded NK cell degranulation and intracellular cytokine staining assay

K562, H929 and JJN3 were treated with neuraminidase or glycobuffer as outlined in **2.10.1**. While target cells were being treated with neuraminidase expanded NK cells were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. NK cells were then stained with CFSE as outlined in **2.11**. NK cells were co-cultured with target cells at a 1:1 E:T ratio using $1x10^5$ target and effector cells in a flat bottomed 96-well plate in a final volume of 200µl of cytotoxicity media. As controls NK cells were seeded on their own with or without stimulation using PMA/Ionomycin according to supplier's instructions (without Brefeldin, Biolegend). All conditions were carried out in duplicate. Immediately after co-culture was initiated all wells were stained with 4µl of anti-CD107a (Biolegend) and mixed well before being incubated at 37°C for one hour. After one hour, BD Golgi-plug Protein Transport Inhibitor (BD Biosciences) was added to each well to create a 1:1,000 dilution (BD Biosciences). All wells were mixed well again and incubated at 37°C for a further 3.5 hours. Cells were then collected and transferred to FACS tubes containing 300µl FACS buffer. Cells were then centrifuged at 2,000 RPM for 3 minutes using an acceleration and brake of 9,9. Supernatant was discarded and cells were then fixed and permeabilised using eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) according to supplier's instructions. Cells were then stained with antibodies detailed in **Table 2.7**.

Table 2.7 Staining panel for IFN-γ and TNF-α in expanded NK cells

Antibody	Clone	Supplier	Fluorochrome	Catalogue number
TNF-α	MAb11	BD Biosciences	BV650	563418
IFN-γ	B27	BD Biosciences	Pe-Cy7	560924

After staining cells were washed with 300µl permeabilization buffer. Supernatant was discarded and cells were resuspended in 300µl FACS buffer before being kept on ice in the dark until required for flow cytometry. NK cells were determined as being $CFSE^+$. Compensation and gates to determine $CD107a^+$, TNF-α^+, IFN-γ^+ NK cells were determined using SSs, FMO controls and NK cells stimulated using PMA/Ionomycin. Unstained cells were also used to set preliminary gates. NK cells alone were used to determine background positivity for CD107a, TNF-α and IFN-γ. Expression of CD107a, TNF-α and IFN-γ was compared in single viable NK cells co-cultured with desialylated target cells compared to NK cells co-cultured with sialylated control target cells.

2.14 Disruption of Siglec-7 expression on primary expanded NK cells using CRISPR/Cas9

Single guide ribonucleic acid (sgRNA) designed to target *Siglec-7* were purchased from Synthego ("Gene KO Kit V2"). sgRNA was suspended in 15.2µl of sterile TE buffer (Synthego) to create a 100µM stock solution and 2.5µl aliquots were stored at -20°C until use. On the day of CRISPR gene editing sgRNA was thawed on ice and then mixed with 2.5µl of Cas9 enzyme (50pM) to create a sgRNA-Ribonucleicprotein (guide-RNP) complex with a molar ratio of guide-RNP:Cas9 of 5:1. The suspension was mixed thoroughly and left for 15 minutes at room temperature. As a negative control, 5µl of electroporation buffer was added to a separate Eppendorf tube ("Mock" NK cells).

Primary NK cells from were isolated and expanded as detailed in **2.3** for 6-8 days prior to transfection. During the 15-minute guide-RNP complex incubation expanded NK cells were collected, counted and $4x10^6$ NK cells were transferred to a 15ml tube. Cells were centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed,

with care taken not to disturb the small pellet. Cells were then resuspended in 5ml MaxCyte electroporation buffer (MaxCyte) and centrifuged again using the same settings. Supernatant was then discarded and the cell pellet was dislodged by gentle flicking of the 15 ml tube. 30µl of electroporation buffer was then added to the cells which were slowly resuspended. Due to residual buffer in the tube the final volume became at least 40µl. 20µl of cells were then transferred to the tube containing the guide-RNP complex or electroporation buffer and mixed thoroughly but gently. The cell suspension-guideRNP mixture and mock NK cell suspension was left to stand for 2 minutes at room temperature before being transferred to OC 25x3 transfection cuvettes (MaxCyte) and transfected using the MaxCyte GT transfection system (pre-programmed NK cell electroporation programme 4, MaxCyte). Special care was taken to ensure no air bubbles were present at the bottom of the cuvettes prior to transfection, by dragging a P10 pipette tip gently across the bottom of the cuvette. After transfection NK cells were transferred to 24-well plate wells containing 500µl of NK expansion media without antibiotics and incubated at 37°C for 20 minutes. Finally, 1.5ml of expansion media was added to the wells which were then cultured for 5-7 days. Siglec-7 knockout (Siglec-7^{KO}) was determined by flow cytometry.

**CD38, CD96 and TIGIT were also targeted for KO using the same protocol as above during this project.*

2.15 Determining the expression of potential therapeutic antigens after MM cell surface desialylation

MM cell lines MM1S and H929 were desialylated using SIA, or DMSO as a control, as described in **2.10.2**. $5x10^4$ cells were taken per sample and were stained for the expression of immunotherapeutic target antigens CD38, BCMA, SLAMF7, MUC1 to compare the expression of each on desialylated MM cells to sialylated controls. The antibodies used for this assay are detailed in **Table 2.8**. All samples were stained with each antibody individually, no SSs or FMOs were used.

Table 2.8 Antibodies used to determine expression of immunotherapeutic target antigen expression following MM cell surface desialylation.

Antibody	Clone	Supplier	Fluorochrome	Catalogue number
CD38	HIT2	BD Biosciences	FITC	564498
BCMA	19F2	Biolegend	APC	357506
SLAMF7	162	AbD Serotec	Alexa Fluor 647	MCA4645A647
MUC1	HMFG2	BD Biosciences	Alexa Fluor. 647	566590

2.16 Determining the expression of NKG2D ligands on MM cells prior to and after desialylation using neuraminidase

MM1S, H929, JJN3 and K562 were desialylated using neuraminidase, or glycobuffer as a control as outlined in **2.10.1**. Cells were then stained for MHC class I chain-related protein A and B (MICA/B) and UL16-binding protein 1-6 (ULBP1-6) using antibodies detailed in **Table 2.9**.

Table 2.9 Antibodies used to determine expression of NKG2D ligands by MM cells

Antibody	Clone	Supplier	Fluorochrome	Catalogue number
MICA/B	6D4	Miltenyi	FITC	130-106-100
ULPB-1	170818	R&D Systems	APC	FAB1380P
ULBP-2/5/6	165903	R&D Systems	PE	FAB1298P
ULBP-3	166510	R&D Systems	PE	FAB1517P
ULBP-4	709116	R&D Systems	APC	FAB6285P

$5x10^4$ cells were examined per sample. Only single, viable cells were examined. Gates were set using unstained cells of the same cell line.

2.17 Cytotoxicity assays using desialylated MM cells combined with Daratumumab treatment

Daratumumab (Dara) was kindly provided by University Hospital Galway. H929 and JJN3 MM cells were desialylated using SIA as outlined in **2.10.2**. After treatment for 72 hours with SIA or DMSO, cells were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Cells were resuspended at $1x10^6$ cells/ml and 2ml of cells was transferred to a new 15 tube where they were treated with either Dara or PBS (of the same volume used in Dara treated cells) for 30 minutes at room temperature. 3ml of RPMI1640 including 10% FBS was added to the tubes which were then centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was discarded and cells were resuspended in RPMI1640 including 10% FBS and 500IU/ml IL-2 at a concentration of $2x10^5$ cell/ml. 100µl aliquots were then transferred to 96-well plates to initiate cytotoxicity assays with primary expanded NK cells as outlined in **2.11**. The E:T ratios used in this assay were 0.5:1 and 1:1. Cell viability was determined using PI.

CD16 expression on primary expanded NK cells used in assays involving Dara was determined to confirm the capacity of NK cells to carry out antibody-dependent cellular-mediated cytotoxicity.

2.18 ATRA treatment of JJN3 to up-regulate CD38 expression

JJN3 cells were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed and cells were resuspended in culture media at $4x10^5$ cells/ml. 3ml of cells were transferred to a 6-well plate for treatment with either all-*trans* retinoic acid (ATRA) or DMSO as a control. Cells were treated with 10nM ATRA or same volume DMSO and cultured for 72 hours before being collected, counted and stained for CD38 to confirm up-regulation of expression.

Where needed, JJN3 were also treated with both ATRA and SIA (10nM and 300µM, respectively) or ATRA alone (10nM), SIA alone (300µM) and DMSO (total volume of ATRA and SIA combined) alone. Cells were also cultured for 72 hours for this combined treatment. CD38 expression was measured on each treatment to confirm the efficacy of ATRA and SIA.

When cells treated as above were used in cytotoxicity assays, cells were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9 before being resuspended in RPMI1640 including 10% FBS and 500IU/ml IL-2 at $2x10^5$ cells/ml before being used in cytotoxicity assays as outlined in **2.11**. The E:T ratios used in this assay were 0.5:1 and 1:1. Cell viability was determined using PI.

2.19 Cytotoxicity assays using Siglec-7^{KO} NK cells in combination with Dara treatment of MM cells

Siglec-7^{KO} and mock NK cells were generated as described in **2.14**. MM1S MM cells were treated with Dara as described in **2.17**. Siglec-7^{KO} and mock NK cells were co-cultured with MM1S separately treated with PBS and Dara. Cytotoxicity assays were set up as described in **2.11**. E:T ratios of 1:1, 2.5:1 and 5:1 was used for this assay.

CD16 expression was evaluated on mock and Siglec-7^{KO} NK cells on the day of the cytotoxicity assay to ensure that Siglec-7^{KO} did not impact CD16 expression.

2.20 Enzyme-linked immunosorbent assay (ELISA)

Target cells of interest were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed and cells resuspended at $8x10^5$ cells/ml in RPMI1640 including 10% FBS and 500IU/ml IL-2. 500µl of target cells were then co-cultured with NK cells of interest at a 1:1 E:T ratio for 4 hours in a final volume of 1ml of RPMI1640 including 10% FBS and 500IU/ml IL-2. NK cells were not stained with CFSE.

After 6 hours cells were collected. transferred to a 2ml Eppendorf tube centrifuged at 2,000 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed carefully using a P200 pipette, with care being taken not to disturb the cell pellet. The supernatant was centrifuged again using the same conditions, with supernatant again being removed and transferred to new Eppendorf tubes. Supernatants were then stored at -20°C

until needed. Controls for this experiment were NK cells cultured alone to determine basal levels of TNF-α and IFN-γ secretion.

Supernatants were analysed for the concentration of TNF-α and IFN-γ using Human TNF-α and IFN-γ Standard ABTS ELISA development kits (Peprotech). Protocols carried out were as per the supplier's instructions. Briefly, capture antibody was diluted with PBS to a concentration of 1µg/ml after which 100µl was added to each well to be used in the ELISA in a flat-bottomed 96-well ELISA plate and incubated overnight at room temperature. The wells were aspirated and then washed 4 times with 300µl wash buffer (0.05% Tween-20 in PBS) before 300µl of block buffer was added to each well and incubated for one hour at room temperature after which the wells were aspirated and then washed 4 times with wash buffer. The standard for TNF-α and IFN-γ was diluted from 3000pg/ml to 0 in diluent (PBS containing 0.05% Tween-20 and 0.1% BSA), after which 100µl of each concentration was added in triplicate to appropriate wells in the 96-well ELISA plate. The plate was incubated for 2 hours at room temperature after which the wells were aspirated and washed 4 times with wash buffer. Detection antibody was diluted in diluent to create a concentration of 0.5µg/ml after which 100µl was added to each well of the plate and incubated at room temperature for 2 hours. The wells were then aspirated and washed 4 times using wash buffer. 5.5µl of Avidin-horse radish peroxidase (HRP, Peprotech) was diluted 1:20,000 in diluent after which 100µl was added to each well and incubated at room temperature for 30 minutes. The wells were aspirated and washed 4 times with wash buffer. 100µl of the appropriate substrate solution (ABTS liquid substrate) was added to each appropriate well in duplicate, as per assay design. Colour development was monitored and the plate was analysed using an ELISA plate reader at 405nm with wavelength correction set at 600nm. Optical density (OD) of 600nm was subtracted from the OD value of 405nm for each individual well. Values obtained were then plotted against a standard curve generated using the readings for the standard-containing wells.

When analysing the results, the values obtained at 600nm were subtracted from the values obtained using the 450nm values, which were then compared to the standard to obtain a concentration of either TNF-α or IFN-γ present in each sample.

2.21 Cell culture under hypoxia

MM cell lines MM1S, H929 and JJN3 were collected, counted and centrifuged at 1,200 RPM for 5 minutes using an acceleration and brake of 9,9. Supernatant was removed and the cells were resuspended in culture media at the concentrations outlined in **Table 2.2**. Cells were seeded into normal culture flasks/dishes and incubated in a Galaxy 170 R hypoxia chamber for 72 hours (New Brunswick, Eppendorf). After 72 hours cells were collected, counted and centrifuged. Supernatant was removed and cells were resuspended at a concentration desired for future assays.

KHYG-1 and expanded primary NK cells were also cultured under hypoxia in the same manner as described above and using the seeding concentrations outline in **Table 2.3** and in **2.3** for KHYG-1 and expanded primary NK cells, respectively. Cells were collected, counted and centrifuged. Supernatant was removed and NK cells resuspended at a concentration desired for further assays.

2.22 Flow cytometry analysis

During analysis of samples using flow cytometry a number of similar gating strategies were used across all samples. Initially forward scatter (FSC, x-axis) vs side scatter (SSC, y-axis) gating was used to identify cell subsets and exclude cellular debris, dust etc located toward the bottom left corner of the FSC vs SSC plot. Single cells were then identified using FSC-area (FSC-A, x-axis) and FSC-height (FSC-H, y-axis), doublets were excluded. Viable cells were then gated for, using FSC-H vs cell viability stain (e.g., P. Iodide (x-axis) vs FSC-H (y-axis)). Expression of markers of interest were then recorded using marker of interest (x-axis) vs FSC-H (y-axis). A minimum of 7,500 events of interest were recorded for all samples analysed using flow cytometry. Solely viable cells were analysed in this project when analysing expression of markers of interest, dead cells were excluded using various stains depending on the panel design. All antibodies used in this project were carefully titrated using positive or negative controls before being used in full experiments. Where FMOs were used, cells were stained with the same volume of antibodies using in full stains, with each fluorochrome excluded in

order to determine the best gate setting. SSs were used to help carry out compensation on the cytometer in the absence of compensation beads.

2.23 Ethical Approval

Peripheral blood was sourced from informed, consenting healthy donors for the isolation of primary NK cells. Ethical approval is as follows: NUI Galway – C.A 1805, Karolinska Institutet - Dnr 2006/229-31/3.

Bone Marrow Aspirates were provided by informed consenting patients at University Hospital Galway. Ethical approval is as follows: NUI Galway - C.A 662.

This study was developed in accordance with the ethical principles outlined in the Declaration of Helsinki and followed obligations as set out by the International Conference on Harmonisation's Good Clinical Practice Guidelines (ICH GCP).

2.24 Statistical analysis

Data was analysed using GraphPad Prism v7.0. Comparisons between two groups (for example between mock and Siglec-7^{KO} NK cells from individual donors) were analysed using Student's paired t-test. Comparisons when analysing data from cell lines only were analysed using Student's unpaired t-test. Comparisons between more than two groups were analysed using repeated measure one-way ANOVA. Statistical significance was considered at $p<0.05$. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.01$, **** denotes $p<0.0001$.

Chapter 3:
Investigating the potential for the hypersialylated MM cell surface to facilitate evasion from NK cell-mediated immunosurveillance

3.1 Introduction

One of the hallmarks of cancer is aberrant glycosylation, and in MM a dense layer of sialic acids is found terminating glycoproteins on the cell surface. As discussed in **1.1.9**, this is mediated by the action of a family of Golgi-bound sialyltransferases, which attach nine-carbon monosaccharide sialic acids to glycoproteins via a glycosidic bond [300]. Terminating glycoproteins, sialic acids are strategically placed to be heavily involved in cell-cell interactions and are hypothesized to act as self-associated molecular patterns (SAMPs: a marker of "self") [301]. Aberrant sialyltransferase activity is reported in malignantly transformed cells, with overexpression of approximately half of the 20 existing human sialyltransferases being implicated in a myriad of cancer types [179]. In the case of MM, increased sialyltransferase activity has been recorded in PBMCs and MNCs from MM patients when compared to both healthy donors and patients with MM precursor conditions such as MGUS [302]. More recently, studies have implicated expression of sialyltransferases such as ST3GAL6 in inferior survival for MM patients. shRNA-mediated knockdown of ST3GAL6 resulted in decreased expression of α2,3-linked sialic acids and interrupted homing of MM cells to the BM. Furthermore, ST3GAL6 knockdown in MM cell lines introduced to a murine model resulted in prolonged survival and decreased tumour burden [187]. Hypersialylation of the MM cell surface can also promote metastasis, with polysialylation thought to promote tumour cell detachment from the tumour mass and increase invasive potential [303]. Additionally, hypersialylation can promote drug resistance, with ST8Sia4 expression in chronic myeloid leukaemia (CML) promoting multi-drug resistance [179]. Therefore, the role of hypersialylation in promoting cancer survival and progression is apparent, particularly in MM considering increased sialyltransferase activity recorded in PBMCs and MNCs throughout disease progression and the implication of specific sialyltransferases in MM progression and survival [187, 302].

Although hypersialylation and sialyltransferase expression has been implicated in MM cell survival and overall disease progression, the role of hypersialylation in facilitating evasion of NK cell-mediated immunosurveillance had previously not been addressed. As mentioned in **1.1.9**, several groups have previously demonstrated that a multitude of different cancerous cell types are heavily decorated with sialic-acid-derived Siglec-7L and Siglec-9L, capable of binding to concomitant NK cell inhibitory receptors Siglec-7 and Siglec-9 [190-193]. Furthermore,

targeted desialylation of several cancerous cell lines and individual blockade of Siglec-7 and Siglec-9 on NK cells using anti-Siglec-7/9 moAbs has been demonstrated to enhance NK cell-mediated cytotoxicity [190, 304, 305]. In MM this is particularly interesting, as NK cells from cancer patients can display an exhaustive phenotype and NK cells from MM patient samples have been shown to be hypofunctional [306-308]. Targeting mechanisms by which MM, and cancer in general, regulates NK cell activity should be prioritised considering the role of NK cells in recent cellular-based immunotherapies and the promise they hold for future therapies, as discussed in **1.2.6**.

In order to begin this project, preliminary experiments were to be carried out in order to complement existing literature profiling the expression of Siglec-7L and Siglec-9L on several cancer types [190]. The easiest method to profile Siglec-7L and Siglec-9L expression is to employ recombinant Siglec-7 and Siglec-9 chimeras to stain MM cells. However, while the identify of Siglec-7L has been elucidated in leukaemia where mass spectrometry of Siglec-7 Fc chimera-binding proteins revealed the glycoprotein CD43 to be a prominent Siglec-7L, the nature and identity of Siglec-7L or Siglec-9L in MM had not previously been investigated [309]. A limitation of using recombinant Siglec-7 and Siglec-9 chimeras is that staining can only determine the presence or absence of Siglec-7L and Siglec-9L, and not their identity. Elucidating the identity of these ligands in MM, if expressed, could present novel glycoproteins as targets to enhance NK cell-mediated cytotoxicity which could be targeted using blocking antibodies to abolish interactions between the protein and Siglec-7/9, for example. Furthermore, if multiple Siglec-7L or Siglec-9L are identified, then a predominant ligand could be identified by determining the affinity with which the ligands bind to their concomitant Siglec receptor. Identification of Siglec-7L and Siglec-9L would allow screening for these individual ligands on MM cells from patient samples and subsequent analysis of their expression throughout disease progression or in response to certain frontline MM treatments.

In this chapter primary MM cells, isolated from both ND and RR patient BMAs, and MM cells lines were screened for the expression of Siglec-7L and Siglec-9L using recombinant Siglec-7 and Siglec-9 Fc chimeras, as used in several other cancer cell types. The levels of expression of Siglec-7L and Siglec-9L were compared between MM cell lines by measuring the MFI of Siglec-7L$^+$ or Siglec-9L$^+$ MM cells. Additionally, mass spectrometry of Siglec-7 binding

proteins, purified after incubation of MM cell lysates with magnetic bead-Siglec-7 Fc chimera complexes, was also carried out to elucidate the identify of Siglec-7L expressed by MM cells.

While the expression of Siglec-7L or Siglec-9L on MM cells had not previously been assessed, expression of Siglec-7 and Siglec-9 on NK cells has been well documented, with Siglec-7 being present on nearly all NK cells while Siglec-9 expression is limited to a subset of $CD56^{dim}$ NK cells [289, 297, 298, 310, 311]. Although the expression profile of Siglec-7 and Siglec-9 on NK cells within healthy humans is understood, the impact of the MM TME on Siglec expression cannot be underestimated. For example, hypoxia has been demonstrated to impair NK cell-mediated killing of MM cell lines. Furthermore, NK cells cultured under hypoxia were observed to have decreased levels of NKG2D and CD16 expression compared to NK cells from the same donor cultured in normoxia, highlighting the potential for NK cell receptor profiles to be influenced by the tumour microenvironment [312]. Therefore, it is necessary to immunophenotype NK cells taken directly from the MM TME and compare the expression of potentially important inhibitory receptors such as Siglec-7 and Siglec-9 to expression on NK cells from the circulation of healthy donors. It is possible that hypofunctional NK cells may have increased Siglec expression due to the myriad of influential TME factors, discussed in **1.1.6**. Additionally, it is important to consider that Siglec expression is not limited to NK cells and that expression of Siglecs is also observed on several immune cell types such as monocytes, macrophages, dendritic cells and eosinophils [291]. Additionally, Siglec-9 expression on tumour infiltrating $CD4^+$ and $CD8^+$ T cells was reported to be increased in non-small cell lung cancer, demonstrating the potential for abnormal Siglec expression in immune cells in cancer patients [313]. Thus, information on the expression of Siglec-7 and Siglec-9 on immune cell subsets such as $CD4^+$ and $CD8^+$ T cells as well as monocytes/macrophages would also be valuable to the field of Siglec research in MM.

In this chapter the expression of Siglec-7 and Siglec-9 on primary NK cells, and on routinely used NK cell lines was measured. $CD138^-$ fractions from MM patient BMAs were also analysed and Siglec-7 and Siglec-9 expression on NK cells, $CD4^+$ T cells, $CD8^+$ T cells and monocytes/macrophages was recorded and compared to expression on the same immune cell types isolated from the PB of healthy donors. Percentage overall positive cells was recorded, as was MFI of positive cells to provide an in-depth analysis of the expression profile of Siglec-

7 and Siglec-9. Furthermore, expanded NK cells were cultured under normoxia and hypoxia to determine the effect of these conditions on Siglec-7 and Siglec-9 expression.

As well as recording Siglec-7L and Siglec-9L on cancer cells, previous studies have shown that hypersialylation of several tumour cell types has been shown to protect cancer cells from NK cell-mediated immunosurveillance. Desialylating agents such as sialidases and sialic acid analogues acting as sialyltransferase inhibitors have been employed to successfully potentiate NK cell-mediated cytotoxicity against desialylated cancer cells compared to untreated controls. Treatment of leukaemia and breast cancer cell lines with the sialidase NEURA abolished sialic acid expression on the cell surface and simultaneously enhanced the cytotoxicity of PB-derived NK cells in cytotoxicity assays compared to untreated K562 and HeLa controls. Conversely, the lymphoblastoid cell line 721.221, which was observed not to express Siglec-7L and Siglec-9L was not sensitized to NK cells upon treatment with NEURA [(190)]. An alternative, but effective, means of achieving tumour cell surface desialylation is to use the sialyltransferase inhibitor SIA. Nanoparticles loaded with SIA have previously been utilised to desialylate melanoma cells within a murine model and treatment of mice with SIA led to decreased cancerous nodes per lung compared to mice treated with PBS alone [(314)].

In the case of MM, no research had been done into investigating the role of hypersialylation in facilitating evasion of NK cell-mediated immunosurveillance. Desialylation of MM cells followed by co-culture with NK cells, is a useful preliminary assay to complement existing research carried out to elucidate the role of hypersialylation in facilitating cancer cell evasion of NK cell-mediated immunosurveillance in different cancer types. As well as being able to measure NK cell-mediated lysis of target cells following desialylation, NK cell activity can also be determined by measuring CD107a expression [(209, 210)]. CD107a is located on the inner surface of lytic granules located within NK cells which becomes expressed on the cell surface upon NK cell activation when merging of the lytic granule membrane with the cellular membrane occurs upon secretion [(209, 210)]. Previously, desialylation of leukaemia and breast cancer cells was demonstrated to strongly enhance CD107a expression on NK cells co-cultured with desialylated target cancer cells, which supported the data showing increased NK cell-mediated lysis of the same cancer cells upon desialylation [(190)].

It is also important to note that cytotoxicity is only one function of NK cells. As mentioned in **1.2.4**, NK cells can also secrete cytokines including, but not limited to, TNF-α and IFN-γ. IFN-γ secretion by NK cells results in a pro-inflammatory state being generated, leading to the activation and recruitment of the other immune cell subsets of the innate immune response [315, 316]. TNF-α can enhance the effects of IL-2 on NK cells, inducing NK cell differentiation and activation [317]. Additionally, TNF-α has also been shown in-vitro to potentiate NK cell cytotoxicity against target cell lines, and may enhance NK cell activity in-vivo [317]. While enhanced NK cell-mediated cytotoxicity against desialylated MM cells is important in terms of advancing this approach towards in-vivo models or the clinic, it is also important to determine whether desialylation could potentially result in an enhanced immunotherapeutic response aside from NK cell-mediated cytotoxicity.

In this chapter, the effect of desialylation, achieved using either a sialidase enzyme or sialyltransferase inhibitor, on NK cell-mediated cytotoxicity was assessed using Naïve, IL-2 activated and expanded primary NK cells and the commonly used NK cell line KHYG-1. As well as determining the effect of desialylation on NK cell cytotoxicity, NK cell activation was recorded by measuring CD107a expression on NK cells co-cultured with sialylated and desialylated target cell lines. Finally, in order to assess the effect of desialylation on NK cell cytokine secretion, TNF-α and IFN-γ levels were recorded in expanded primary NK cells after co-cultures with desialylated or sialylated MM cell lines.

Finally, it has been hypothesized that hypersialylation of tumor cells can mask ligands for the activating NKG2D receptor expressed by NK cells [173]. NKG2D allows NK cells to recognize virally infected, stressed or malignantly transformed cells, which have increased expression of its cognate ligands MIC A/B and ULBP-1-6 [318]. Furthermore, increased binding to NKG2D Fc chimeras has been observed in breast cancer upon desialylation [305]. Should desialylation of MM cells lead to enhanced NK cell-mediated cytotoxicity, it is important to understand the mechanisms for increased NK cell cytotoxicity upon tumour cell surface desialylation, and to explore whether additional mechanisms can potentially contribute to enhanced NK cell cytotoxicity aside from abolishment of Siglec-7/9 signalling.

As hypersialylation has been theorised to mask ligands for the activating NK cell receptor NKG2D, in the final section of this chapter the expression of NKG2D ligands MIC A/B and ULBP-1-6 were measured on desialylated target cell lines and compared to expression on untreated controls to observe the effect, if any, of desialylation on NKG2D ligand expression.

3.2 Hypothesis and Objectives

3.2.1 Hypothesis

I. MM cells are highly decorated with sialic acid-derived ligands for Siglec-7 and Siglec-9 inhibitory receptors expressed by NK cells

II. Hypersialylation facilitates immune-evasion of NK cell-mediated cytotoxicity which can be enhanced upon targeted desialylation

3.2.2 Objectives

I. Determine the potential for NK cell regulation by the hypersialylated MM cell surface through Siglec-Siglec ligand interactions by profiling the expression of Siglec-7/9L and their cognate receptors on MM and NK primary cells and cell lines, respectively.

II. Determine the role of hypersialylation on facilitating evasion of NK cell-mediated anti-cancer capabilities such as cytotoxicity and cytokine release by carrying out co-cultures of primary NK cells with desialylated MM target cell lines

3.3 Study design

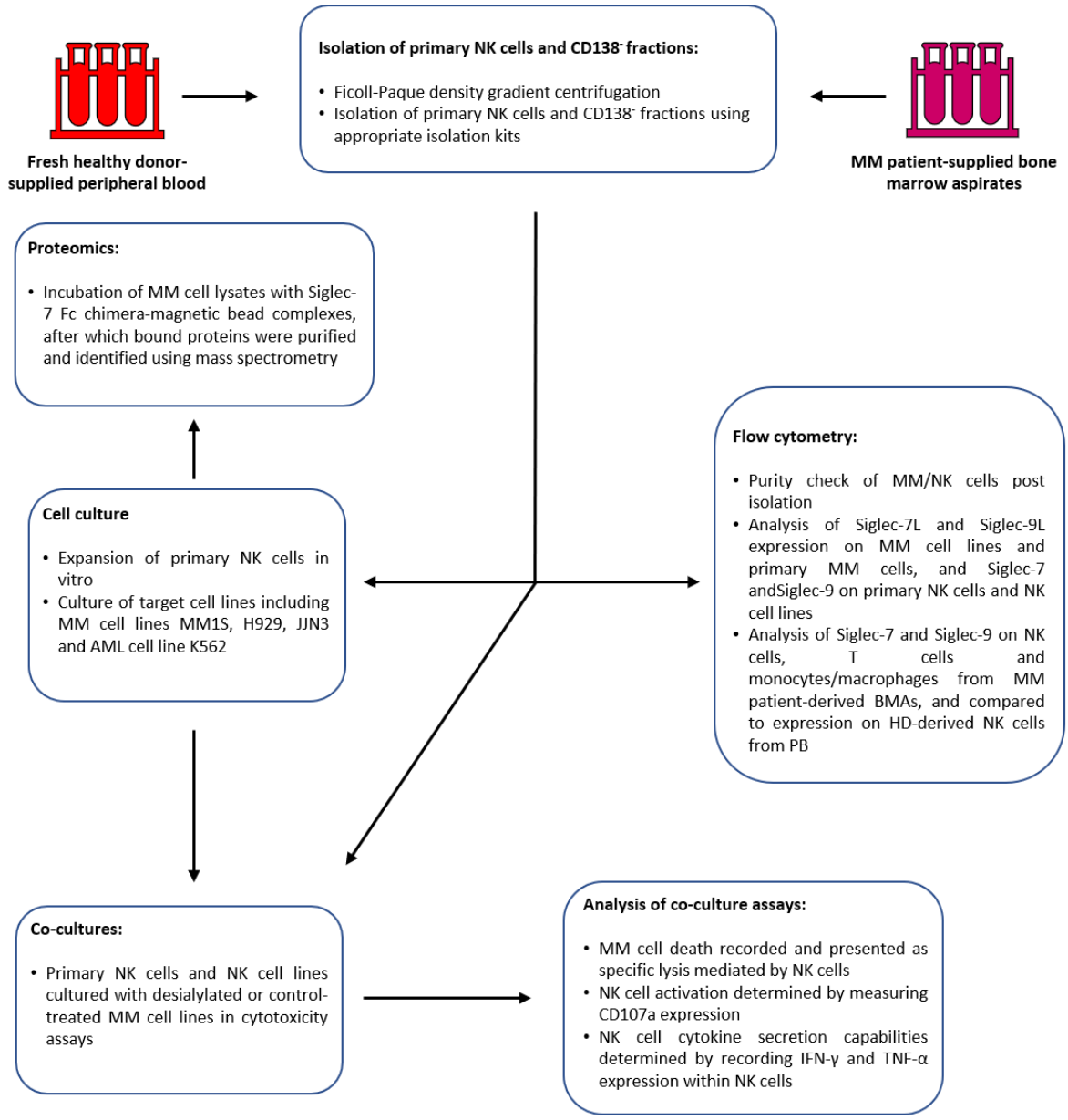


Figure 3.1 Schematic representation of the experimental work carried out in this chapter.

An overview of the workflow that was carried out for this chapter. Primary samples were obtained from healthy donors or MM patients and MM/NK/CD138- fractions were subsequently isolated using appropriate isolation kits. Primary MM and NK cells were analysed for the expression of Siglec ligands and receptors, respectively. Primary MM cells were stained immediately, while primary NK cells were used immediately in co-culture or immunophenotyping assays or were expanded for future experiments. Co-culture assays were used to examine both target cell death or measure NK cell activation and cytokine release in response to encountering desialylated target cells. Readouts and data were gathered using flow cytometry analysis. Finally, lysates of MM cell lines were incubated with Siglec-7 Fc chimeras to pulldown binding proteins which were subsequently identified using mass spectrometry.

3.4 Results

3.4.1 Siglec ligands, and their cognate receptors, are expressed by primary MM and NK cells, as well as MM and NK cell lines, respectively.

Using recombinant Siglec-7 and Siglec-9 Fc chimera staining, strong expression of Siglec-7L and Siglec-9L was observed on primary MM cells isolated from patient-derived BMAs including ND (84.9±16.0% and 77.4±18.4%, respectively) and RR (36.25±3.2%, 81.25±15.2%, respectively) patients (**Figure 3.2A**). Additionally, Siglec-7L and Siglec-9L expression was recorded on MM cell lines MM1S (92.7±2.3%, 97.1±0.7%, respectively), H929 (84.7±4.8%, 97.8±1.0%, respectively) and JJN3 (85.8±2.7%, 98.5±0.5%, respectively, **Figure 3.2B**). When analysing the expression of Siglec-7L and Siglec-9L, mean fluorescence intensity (MFI) of Siglec-7L and Siglec-9L positive MM cells was determined and MM1S were confirmed to have the highest expression of Siglec-7L (MFI 31,861 ± 8015) followed by JJN3 (MFI 18,181 ± 5,737) and H929 (MFI 9,467 ± 621, **Figure 3.3A**). All three cell lines were observed to have comparable levels of Siglec-9L expression (MFI 7,841 ± 1,130 for H929, 7,523 ± 3,715 for MM1S, 5,859 ± 4,373 for JJN3, **Figure 3.3B**).

Immunophenotyping using anti-Siglec-7 and anti-Siglec-9 antibodies revealed that IL-2 activated primary NK cells were observed to have strong expression of Siglec-7 (90.7 ± 6.4%) and partial expression of Siglec-9 (59 ± 4.9%), correlating with existing literature profiling the expression of both receptors on primary NK cells derived from healthy donors. Siglec-7 expression was also observed on primary expanded NK cells (92.8 ± 2.4%), but Siglec-9 expression was not observed (**Figure 3.2C**). Strong Siglec-7 expression was also recorded on NK cells isolated from BMAs supplied by MM patients, although Siglec-9 expression was not recorded (85.2 ± 17.5%, **Figure 3.2D**).

When NK cell lines KHYG-1 and NK-92 were analysed, partial Siglec-7 expression was observed on both KHYG-1 and NK-92, while Siglec-9 expression was not observed on either cell line (**Figure 3.3B**).

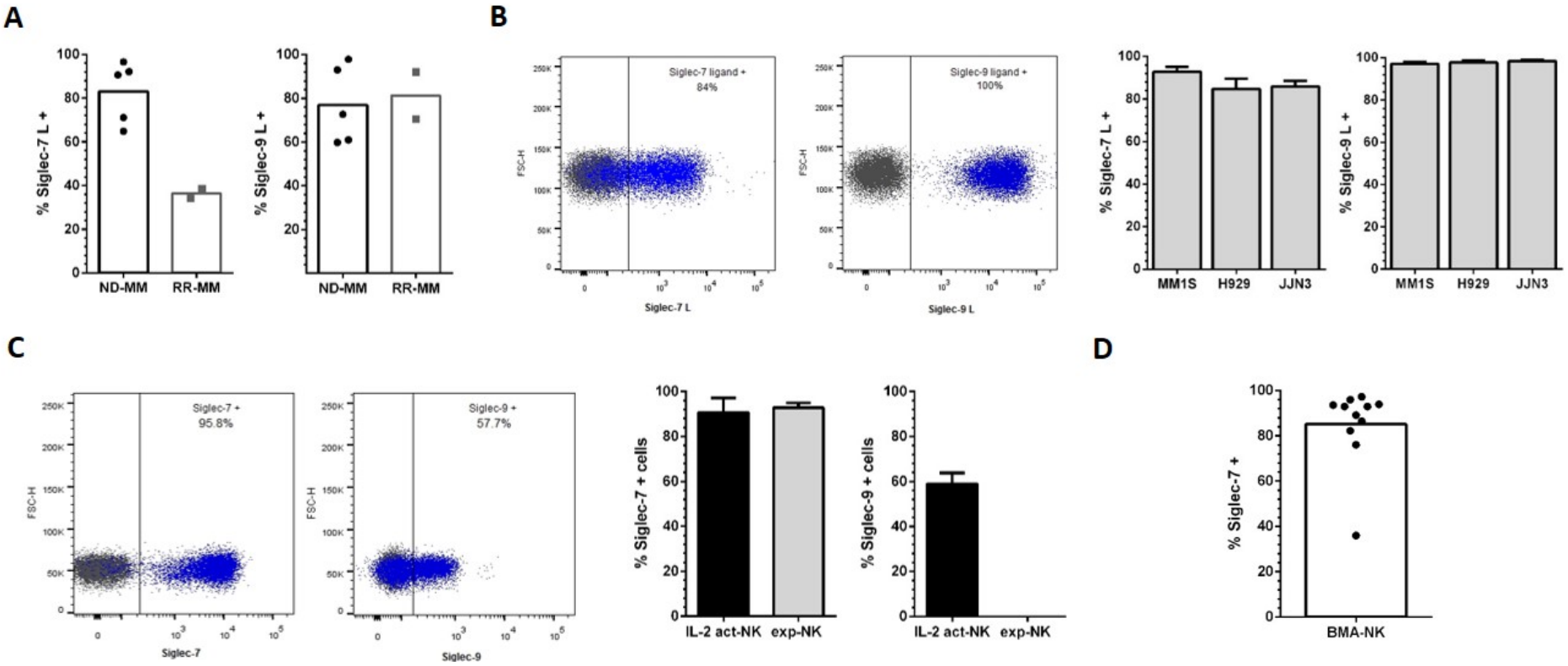


Figure 3.2 Siglec-7L, Siglec-9L and their cognate receptors are expressed by primary MM and NK cells and cell lines, respectively.

Phenotyping of MM and NK cells was carried out using recombinant Siglec-7 and Siglec-9 Fc chimeras to determine Siglec ligand expression on MM cells, whereas NK cells were stained with anti-Siglec-7 and anti-Siglec-9 antibodies. Cells were analysed for expression using flow cytometry. **(A)** Siglec-7L and Siglec-9L expression was recorded on primary MM cells isolated from fresh BMAs from n=5 ND MM and n=2 RR MM patient samples. Graphs in **A** represents mean Siglec-7L and Siglec-9L expression and individual as well as individual values from each sample analysed. **(B)** MM cell lines MM1S, H929 and JJN3 were stained for the expression of Siglec-7L and Siglec-9L using recombinant Siglec-7 and Siglec-9 Fc chimeras. Data in **B** is presented as both an individual dot blot to represent n=1 stain of MM1S for Siglec-7L and Siglec-9L and mean expression from n=3 independent biological repeats for MM1S, H929 and JJN3 + SD. **(C)** Primary IL-2 activated and expanded NK cells, were stained for the expression of Siglec-7 and Siglec-9 using anti-Siglec-7 and anti-Siglec-9 antibodies. Data in **C** represents both n=1 stain of primary IL-2 activated NK cells for Siglec-7 and Siglec-9 and combined n=7 stains from independent donors for IL-2 activated and expanded NK cells + SD. **(D)** NK cells isolated from BMAs provided by MM patients were stained for the expression of Siglec-7. Data in **D** represents mean Siglec-7 positive NK cells as well as individual data points from n=11 patient samples.

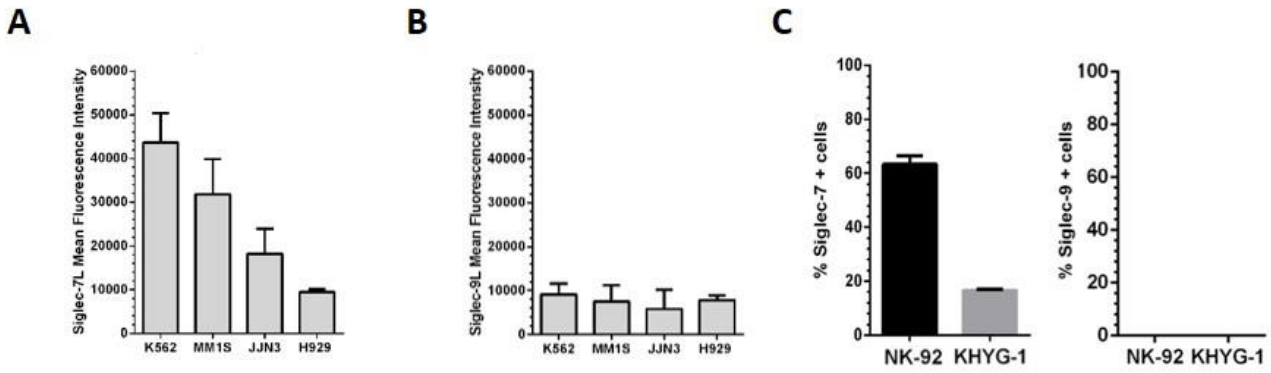


Figure 3.3 Expression of Siglec-7L and Siglec-9L, and their cognate receptors varies across MM and NK cell lines, respectively.

Further analysis of the expression of Siglec-7L, Siglec-9L and their cognate receptors was carried out in target cancer cell lines and NK cell lines. **(A,B)** MFI of Siglec-7L$^+$ and Siglec-9L$^+$ MM cell lines MM1S, H929 and JJN3 and the AML cell line K562 was determined. Data in **A** and **B** represents mean MFI of Siglec-7L$^+$ and Siglec-9L$^+$ cells from n=3 independent biological stains of each cell line + SD. **(C)** Siglec-7 and Siglec-9 expression on NK cell lines KHYG-1 and NK-92 was determined using anti-Siglec-7 and Siglec-9 antibodies. Data in **C** represents mean expression of Siglec-7 and Siglec-9 expression + SD from n=3 independent biological stains of KHYG-1 and NK-92.

3.4.2 PSGL-1 is a predominant ligand for Siglec-7

Cell lysates from MM1S, H929 and JJN3 cell lines were either untreated or desialylated using NEURA before being incubated with magnetic bead-Siglec-7 Fc chimera complexes. Siglec-7 Fc chimeras-binding proteins were isolated and analysed using mass spectrometry.

Mass spectrometry analysis of Siglec-7 Fc chimera-binding proteins revealed P-selectin glycoprotein ligand 1 (PSGL-1) to be the prominent Siglec-7L on all MM cell lines analysed. CD43 was also identified as a Siglec-7L, however its expression was restricted to JJN3 cells, while no pulldown of CD43 from MM1S and H929 cell lysates was observed (**Figure 3.4)**. Binding of PSGL-1 to Siglec-7 Fc chimeras was near completely abolished in H929 lysates pre-treated with NEURA, and abolished completely in MM1S and JJN3 cell lysates pre-treated with NEURA. Similarly, a significant reduction of binding of CD43 was also observed when JJN3 cell lysates were pre-treated with NEURA. PSGL-1 expression was not confirmed on these cell lines using flow cytometry, however previous studies have observed expression on H929 and MM1S cell lines [319].

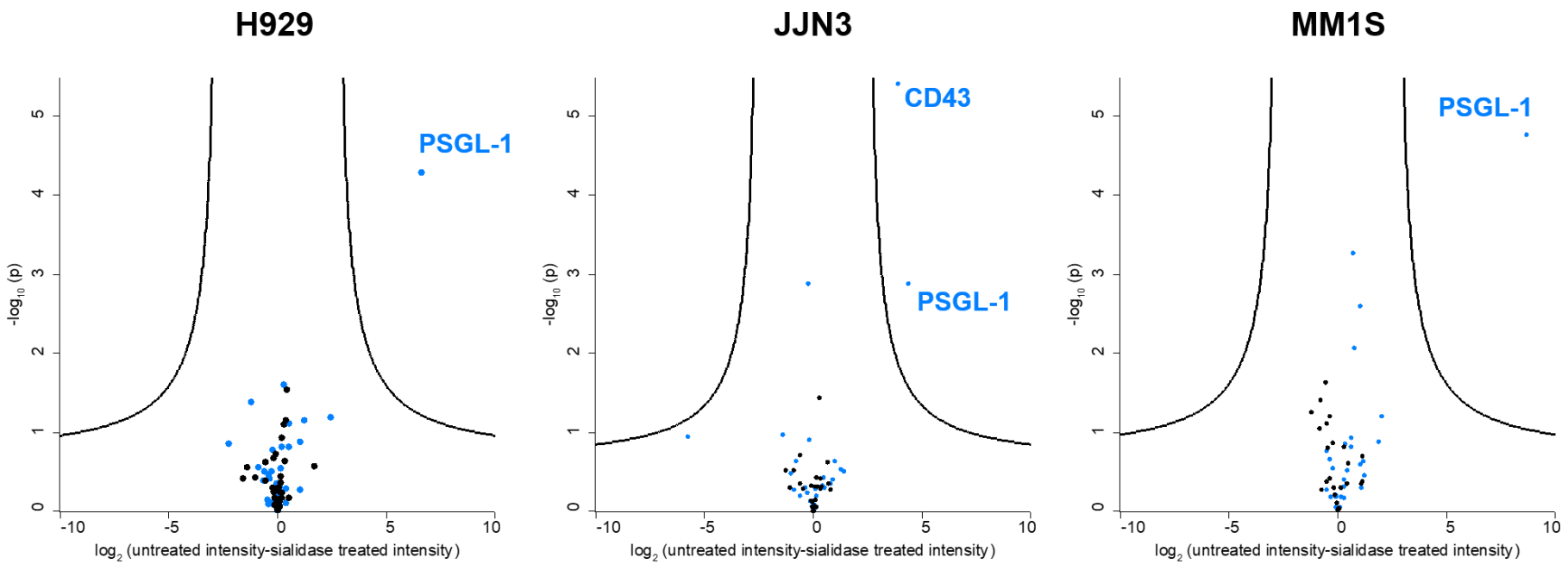


Fig. 3.4 PSGL-1 is a prominent Siglec-7L expressed by MM cell lines, while CD43 also acts as a Siglec-7L but its expression is restricted to JJN3 cells.

MM cell lysates from H929, JJN3 and MM1S MM cell lines were untreated or desialylated using NEURA and subsequently incubated with magnetic bead-Siglec-7 Fc chimera complexes after which bound proteins were isolated and analysed using mass spectrometry. Data is presented as a volcano plot where the X-axis represents log fold changes of intensity of specific protein pulldown between NEURA-treated and untreated lysates and Y-axis represents statistical significance. Proteins outside black lines were considered Siglec-7L with statistical significance ($p<0.05$). Dots represent proteins which were detected in the proteomics runs and known to be expressed on the cell surface. Blue dots represent proteins which are known to contain at least one or more N or O-linked glyco-binding site (as determined by UniProt). n=3 biological repeats for each cell line.

3.4.3 Aberrant expression of Siglec-7 and Siglec-9 is observed on NK cells, $CD4^+$ and $CD8^+$ T cells and monocytes/macrophages from MM patient BMAs compared to the same subsets from the PB of healthy donors

$CD138^-$ fractions from MM patient BMAs were kindly provided by the Blood Cancer Biobank Ireland. PBMCs were isolated from peripheral blood provided by healthy donors. Only viable, single cells were analysed in this assay. n=6 $CD138^-$ fractions from different MM patients were analysed, while n=5 independent PBMC fractions were isolated from healthy donor-derived PB to act as a control group. The most ideal control for this assay would have been healthy donor-derived BMAs, however this was not available during the course of this project.

Siglec-7 and Siglec-9 expression, as well as the MFI of Siglec-7 and Siglec-9 positive cells, was recorded on $CD56^+/CD3^-$ NK cells from MM BMAs. Overall, there was no statistically significant change in the number of Siglec-7^+ NK cells in MM patient BMAs compared to PBMC-derived NK cells. However, a strong increase in the MFI of Siglec-7^+ NK cells from MM patient BMAs was observed (**Figure 3.5A**). A statistically significant decrease in Siglec-9^+ NK cells from MM patient BMAs was observed compared to PBMC-derived NK cells. Additionally, a strong decrease in the MFI of Siglec-9^+ NK cells from MM patient BMAs was also observed (**Figure 3.5B**).

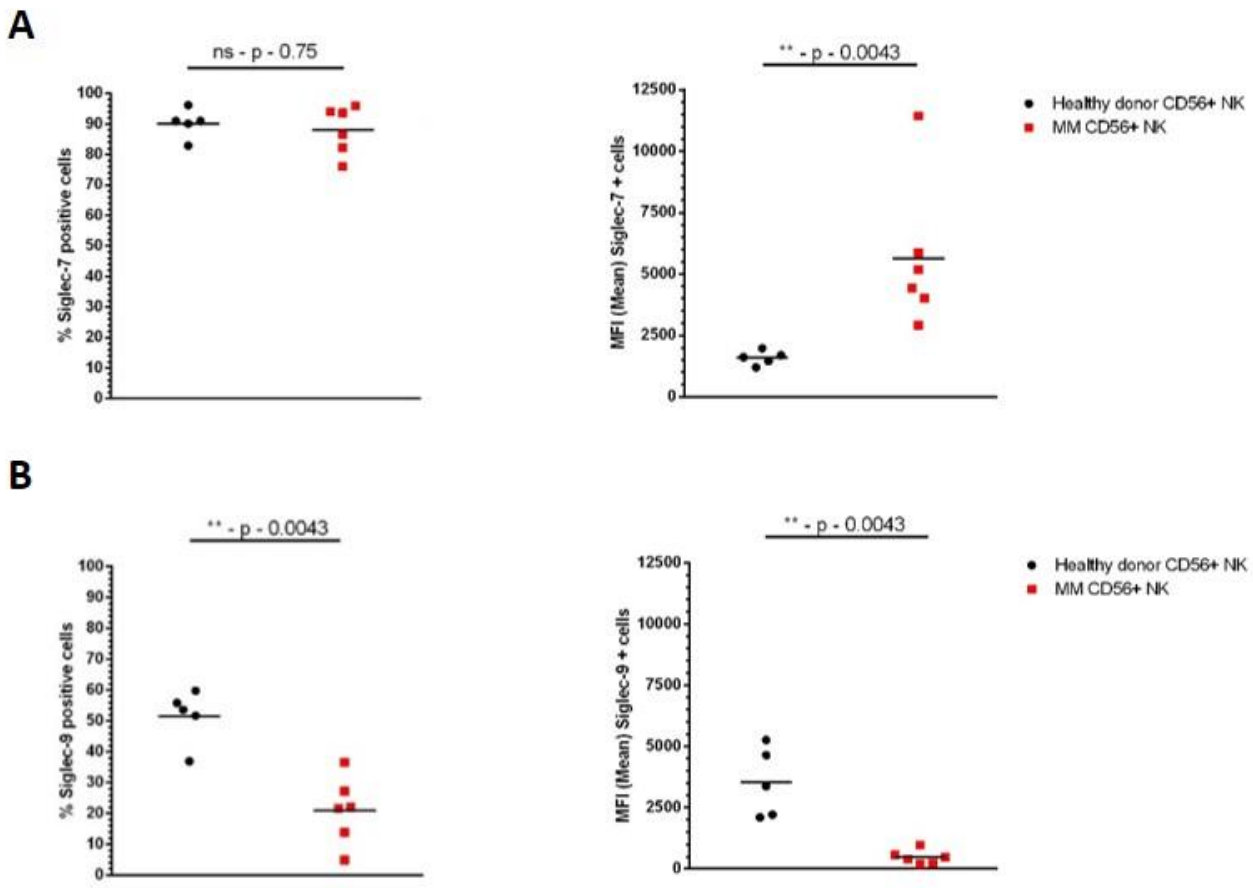


Figure 3.5 Abnormal Siglec-7 and Siglec-9 expression was recorded on NK cells from MM patient BMAs when compared to NK cells from healthy donor-provided PB.

Viable $CD56^+/CD3^-$ NK cells from $CD138^-$ fractions isolated from MM patient BMAs were analysed using flow cytometry for the expression of Siglec-7 and Siglec-9 and expression was compared to that on $CD56^+/CD3^-$ NK cells from the PB of healthy donors. **(A)** Expression of Siglec-7 in the form of overall Siglec-7^+ positive NK cells as well as MFI of Siglec-7^+ NK cells were recorded in NK cells from MM patient BMAs (n=6) and compared to healthy donor PB-derived NK cells (n=5). Data in **A** represents mean Siglec-7^+ cells and MFI of Siglec-7^+ cells as well as individual values from each sample analysed. **(B)** Expression of Siglec-9 in the form of overall Siglec-9^+ NK cells, and the MFI of Siglec-9^+ NK cells were recorded in NK cells from MM patient BMAs (n=6) and compared to healthy donor PB-derived NK cells (n=5). Data in **B** represents mean Siglec-9^+ cells and MFI of Siglec-9^+ cells as well as individual values from each sample analysed. Data analysed using Mann-Whitney unpaired t-test, ** - $p < 0.01$.

No significant changes in Siglec-7 and Siglec-9 expression were recorded on $CD56^+/CD3^-/CD4^+$ and $CD56^+/CD3^-/CD8^+$ T cells from MM patient BMAs compared to healthy donor controls (**Figure 3.6A,B**).

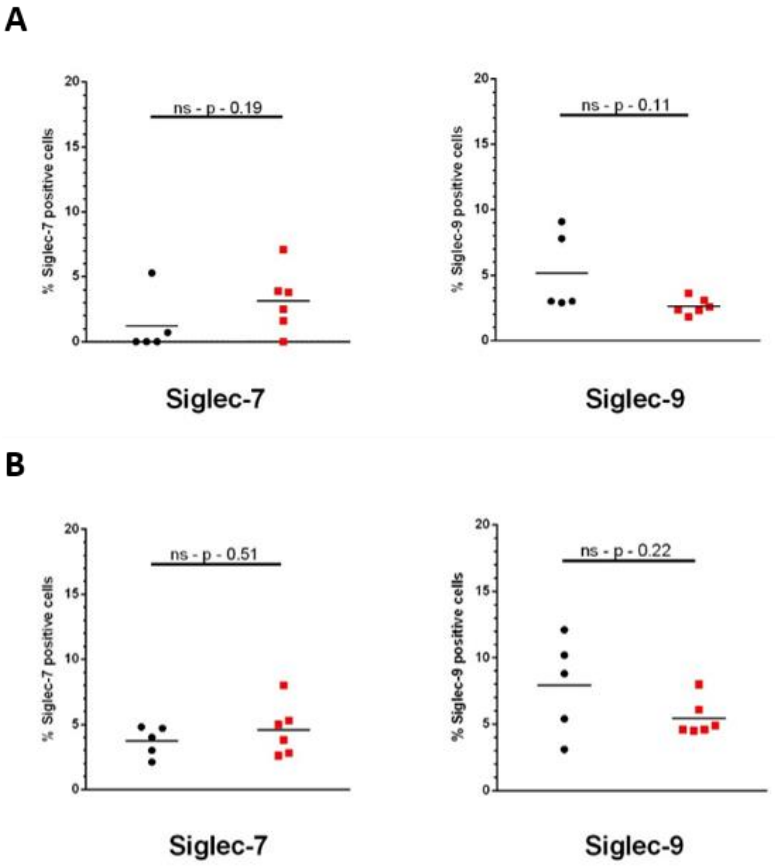


Figure 3.6 Minor changes in expression of Siglec-7 and Siglec-9 were observed on $CD4^+$ and $CD8^+$ T cells from MM patient BMAs compared to healthy donor PBMC controls.

Viable $CD56^-/CD3^+/CD4^+$ and $CD56^-/CD3^+/CD8^+$ T cells from $CD138^-$ fractions of MM patient BMAs were stained for the expression of Siglec-7 and Siglec-9 and expression was compared to viable $CD56^-/CD3^+/CD4^+$ and $CD56^-/CD3^+/CD8^+$ T cells from the PB of healthy donors. **(A)** Expression of Siglec-7 and Siglec-9 in the form of overall Siglec-7^+ and Siglec-9^+ $CD4^+$ T cells from MM patient BMAs (n=6) was recorded and compared to healthy donor PB-derived $CD4^+$ T cells (n=5). Data in **A** represents mean Siglec-7^+ and Siglec-9^+ cells as well as individual values from each sample analysed **(B)** Expression of Siglec-7 and Siglec-9 in the form of overall Siglec-7^+ and Siglec-9^+ $CD8^+$ T cells was recorded in $CD8^+$ T cells from MM patient BMAs (n=6) and compared to healthy donor PB-derived NK cells (n=5). Data in **B** represents mean Siglec-7^+ and Siglec-9^+ $CD8^+$ T cells as well as individual values from each sample analysed. Data analysed using Mann-Whitney unpaired t-test.

No significant changes in the percentage of Siglec-7^+ and Siglec-9^+ $CD56^-/CD3^-/CD14^+/CD11b^+$ monocytes/macrophages was observed between macrophages/monocytes from MM patient BMAs and healthy donor-derived PB. Siglec-7 and Siglec-9 were both strongly expressed by monocytes/macrophages from both healthy donor PB and MM patient-derived BMAs (**Figure 3.7A**). However, the MFI of Siglec-7^+ monocytes/macrophages was

notably higher in macrophages/monocytes from MM patient BMAs compared to monocytes/macrophages from healthy donor-derived PB (**Figure 3.7B**). Conversely, the MFI of Siglec-9^{+} monocytes/macrophages was strongly decreased in monocytes/macrophages from MM patient BMAs compared to healthy donor-derived PB (**Figure 3.7B**).

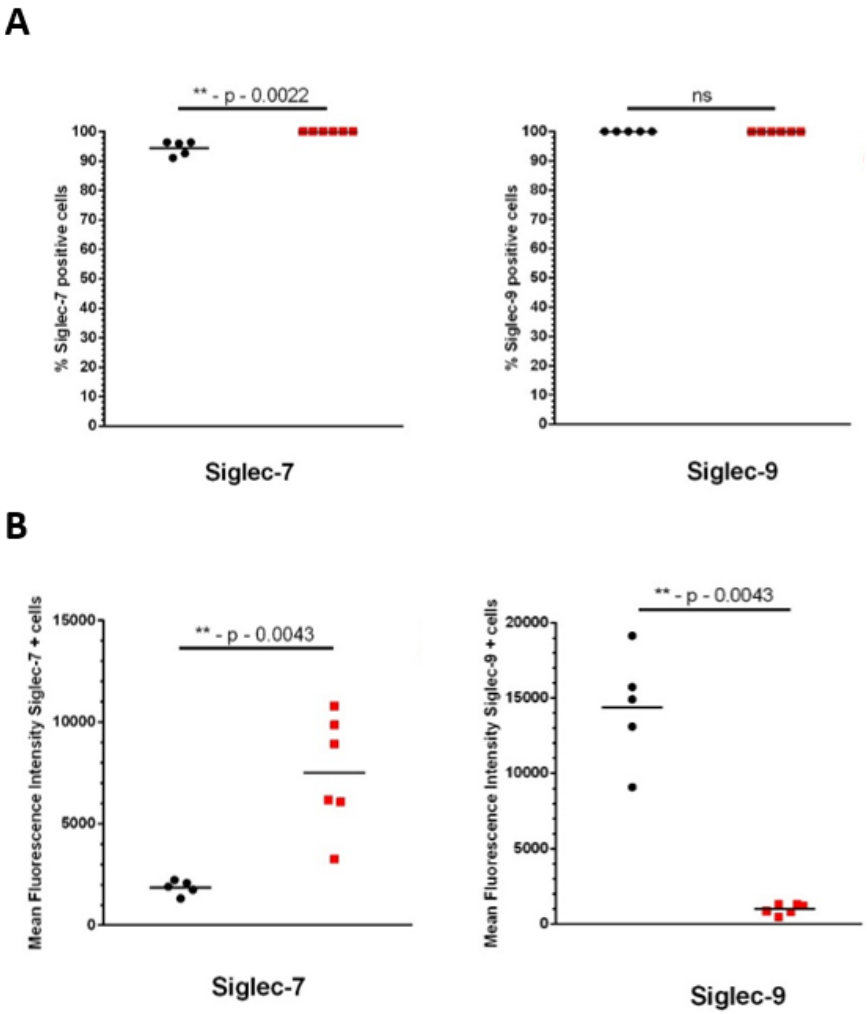


Figure 3.7 Expression of Siglec-7 and Siglec-9 in monocytes/macrophages from MM patient BMAs is significantly different from healthy donor PBMCs controls.

Viable $CD56^-/CD3^-/CD14^+/CD11b^+$ monocytes/macrophages from MM patient BMAs were stained for the expression of Siglec-7 and Siglec-9 which was subsequently compared to the expression of Siglec-7 and Siglec-9 on monocytes/macrophages isolated from the PB of healthy donors. **(A)** Expression of Siglec-7 and Siglec-9 was measured in the form of overall $Siglec\text{-}7^+$ and $Siglec\text{-}9^+$ monocytes/macrophages from MM patient BMAs (n=6) and was compared to monocytes/macrophages from healthy donor-supplied PB (n=5). Data in **A** represents mean $Siglec\text{-}7^+$ and $Siglec\text{-}9^+$ monocytes/macrophages as well as individual values from each sample analysed. **(B)** MFI of $Siglec\text{-}7^+$ and $Siglec\text{-}9^+$ monocytes/macrophages from MM patient BMAs was (n=6) recorded and compared to the MFI of $Siglec\text{-}7^+$ and $Siglec\text{-}9^+$ monocytes/macrophages from healthy donor-supplied PBMCs (n=5). Data in **B** represents mean MFI of $Siglec\text{-}7^+$ and $Siglec\text{-}9^+$ monocytes/macrophages as well as individual values from each sample analysed. Data analysed using Mann-Whitney unpaired t-test. ** - $p < 0.01$.

3.4.4 Hypoxia does not conclusively dysregulate the expression of Siglec-7L or Siglec-9L, or their cognate receptors, on MM and NK cells respectively

Expanded primary NK cells, NK cell lines NK-92 and KHYG-1 and MM cell lines MM1S, JJN3 and H929 were cultured either under normoxia (21% O_2, cultured in a regular tissue culture incubator) or hypoxia (1% O_2) for 72 hours after which Siglec-7L and Siglec-9L expression was recorded on MM cell lines MM1S, H929 and JJN3. Siglec-7 and Siglec-9 expression was also measured on expanded primary NK cells, while Siglec-7 expression was measured on NK cell lines NK-92 and KHYG-1. No toxicity was caused by culturing expanded NK cells, NK cell lines or MM cell lines under hypoxia (data not shown).

No change in the expression of Siglec-7 or Siglec-9 was observed on primary expanded NK cells cultured under hypoxia compared to expanded NK cells from the same donor cultured under normoxia (**Figure 3.8A**). Additionally, no observable change in Siglec-7 was observed on NK-92 NK cells (**Figure 3.8B**). Interestingly, a trend towards increased Siglec-7 expression was observed on KHYG-1 NK cells cultured under hypoxia compared to normoxia, but was not statistically significant from n=5 independent biological repeats (**Figure 3.8C**).

No observable change in overall Siglec-7L$^+$ or Siglec-9L$^+$ positive MM1S cells was observed between MM1S cultured under either hypoxia or normoxia (**Figure 3.8D**). Furthermore, when the MFI of Siglec-7L$^+$ or Siglec-9L$^+$ MM1S cells was analysed, no observable change between MM1S cultured under hypoxia or normoxia was found. In H929, increased Siglec-7L expression was observed on cells cultured under hypoxia compared to cells cultured under normoxia. No observable change in the overall Siglec-9L$^+$ H929 was observed. When the MFI of Siglec-7L$^+$ and Siglec-9L$^+$ was analysed, a trend towards increased MFI of Siglec-7L$^+$ and Siglec-9L$^+$ in H929 was observed (**Figure 3.8E**). In JJN3, no change in the overall Siglec-7$^+$ cells was observed, while a statistically significant minor increase in Siglec-9L$^+$ cells was observed in JJN3 cultured under hypoxia. When the MFI of Siglec-7L$^+$ cells was analysed, similar values were obtained for JJN3 cultured under normoxia and hypoxia. A trend towards increased MFI of Siglec-9L$^+$ cells was observed on JJN3 cultured under hypoxia (**Figure 3.8F**).

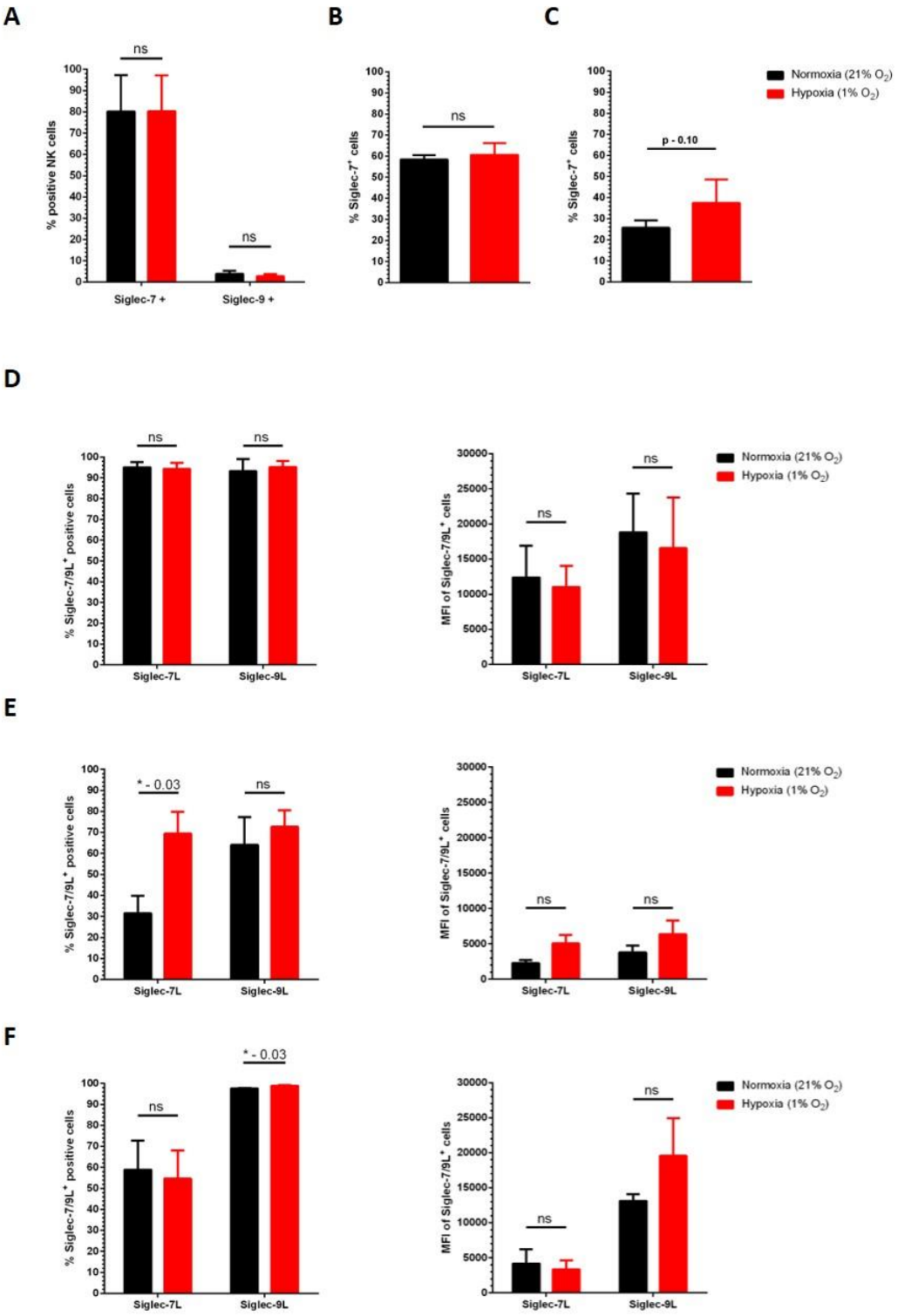

A
B
C
ns
% positive NK cells
Siglec-7 +
Siglec-9 +
% Siglec-7+ cells
p - 0.10
Normoxia (21% O2)
Hypoxia (1% O2)
D
% Siglec-7/9L+ positive cells
MFI of Siglec-7/9L+ cells
Siglec-7L
Siglec-9L
E
* - 0.03
F

Figure 3.8 Expression of Siglec-7L, Siglec-9L and their cognate receptors can potentially be dysregulated by hypoxic conditions.

(A) Siglec-7 and Siglec-9 expression was measured on expanded primary NK cell lines cultured under normoxia or hypoxia. **(B,C)** Siglec-7 expression was measured on **(B)** NK-92 and **(C)** KHYG-1 NK cell lines cultured under either normoxia or hypoxia. **(D,E,F)** Siglec-7L and Siglec-9L expression was measured on MM cell lines **(D)** MM1S, **(E)** H929 and **(F)** JJN3 cultured under normoxia or hypoxia. Graphs represent mean overall Siglec-7^+, Siglec-9^+ or Siglec-$7L^+$ and Siglec-$9L^+$ NK or MM cells, respectively, + SEM, or MFI of Siglec-7^+, Siglec-9^+, Siglec-$7L^+$ and Siglec-$9L^+$ NK or MM cells, respectively. N=5 for **A** and **C,** n=4 for **B**, n=4 for **D**, **E** and **F**. Data analysed using Student's unpaired *t*-test using Welch's correction.

3.4.5 NEURA treatment results in near complete removal of α2,3-linked sialic acid, Siglec-7L and Siglec-9L expression on MM cells

K562, JJN3 and H929 cell lines were treated with either NEURA or a GLYCO control, after which cells were stained with MAA or SNA lectins recognising α2,3-linked and α2,6-linked sialic acids, respectively. Siglec-7L and Siglec-9L expression was also recorded. NEURA treatment strongly reduced α2,3-linked sialic acids, in particular in JJN3 and H929 MM cell lines, but did not completely abolish expression (**Figure 3.9A**). NEURA treatment seemed to have little effect on α2,6-linked sialic acid expression, the basal expression of which was quite low in comparison to α2,3-linked sialic acids (**Figure 3.9B**). Meanwhile, NEURA treatment completely abrogated Siglec-7L and Siglec-9L on all cell lines (**Figure 3.9C** and **Figure 3.9D**, respectively).

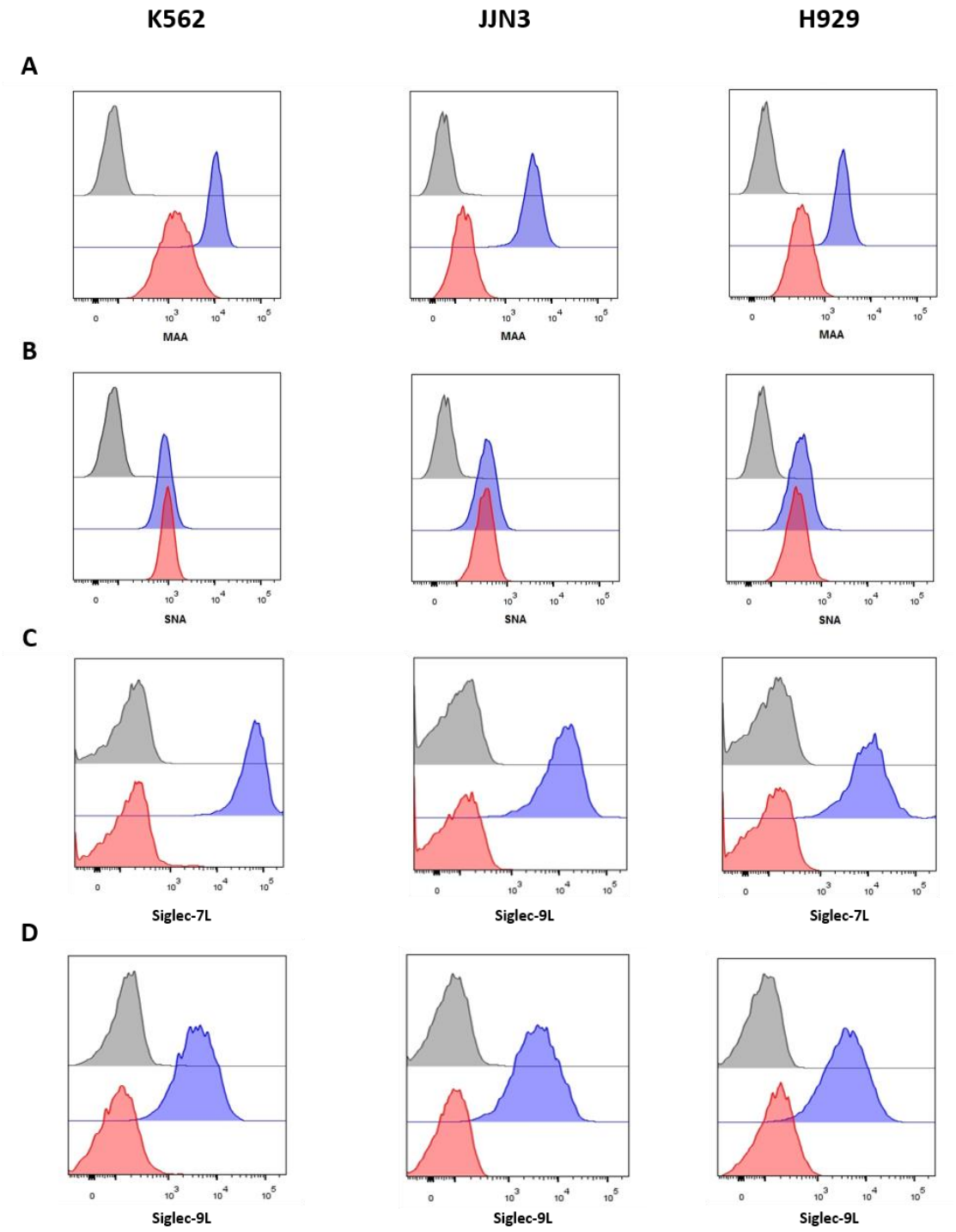


Figure 3.9 Neuraminidase treatment results in reduced sialic acid expression and completely abolishes Siglec-7L and Siglec-9L expression.

Target cell lines K562, JJN3 and H929 were treated with either NEURA (red) or a GLYCO control (blue) and subsequently stained for the expression of **(A)** α2,3-linked and **(B)** α2,6-linked sialic acids as well as **(C)** Siglec-7L and **(D)** Siglec-9L expression. Cells were also stained with a relevant Fc chain control (grey). Graph represents an individual histogram representative of n=3 repeats for (**A**, **B**, **C**, **D**).

3.4.6 NEURA-mediated desialylation of JJN3 and H929 MM cells strongly enhances primary NK cell-mediated cytotoxicity and increases NK cell activation

Desialylation of JJN3 and H929 resulted in strongly enhanced cytotoxicity mediated by primary NK cell types, including naïve, IL-2 activated and expanded NK cells. Strong increases in cytotoxicity were observed at all E:T ratios used for each type of primary NK cell used and were statistically significant (**Figure 3.10A,B,C**). An example of the gating strategy used to analyse data from cytotoxicity assays in this project can be found at (**Figure 3.11**).

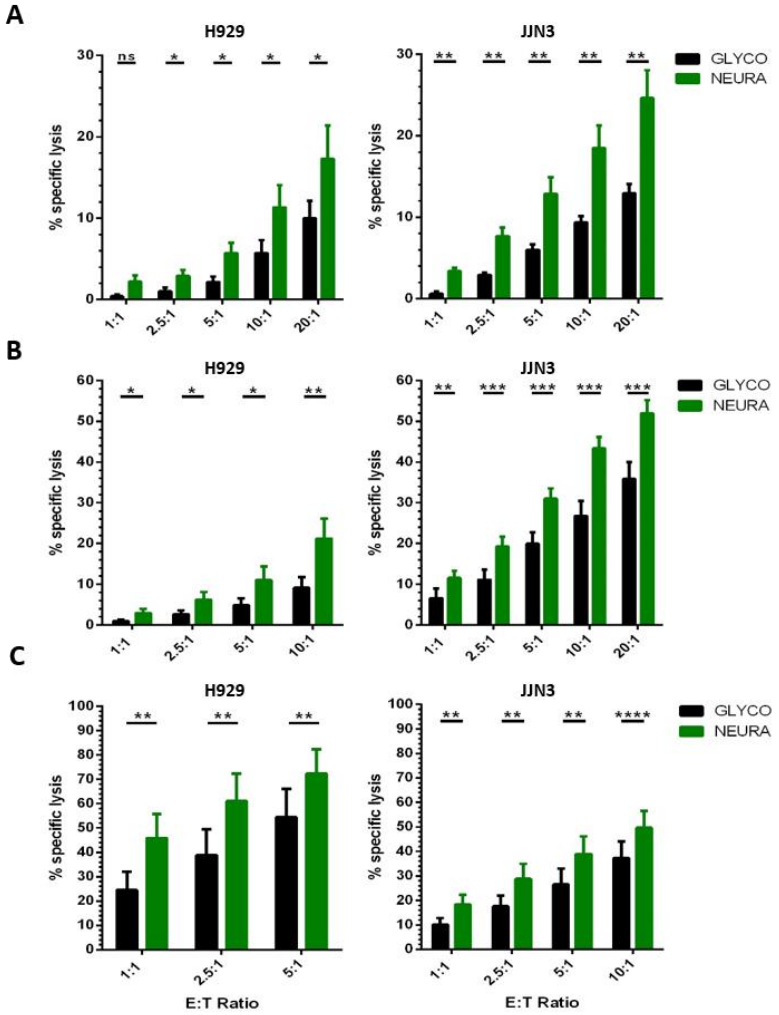


Figure 3.10 NEURA-mediated cell surface desialylation of H929 and JJN3 MM cells results in strongly enhanced NK cell cytotoxicity in co-cultures compared to glycobuffer-treated controls.

MM cell lines H929 and JJN3 were desialylated using NEURA or treated with a GLYCO control, and subsequently co-cultured with **(A)** naïve, **(B)** IL-2 activated and **(C)** expanded primary NK cells at indicated E:T ratios. All cytotoxicity assays were carried out for 4 hours. Graph represents mean specific lysis of either H929 or JJN3 + SEM. n=7 for (**A**, **B**, **C**). Data was analysed using Student's paired *t*-test. * - $p < 0,05$, ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$.

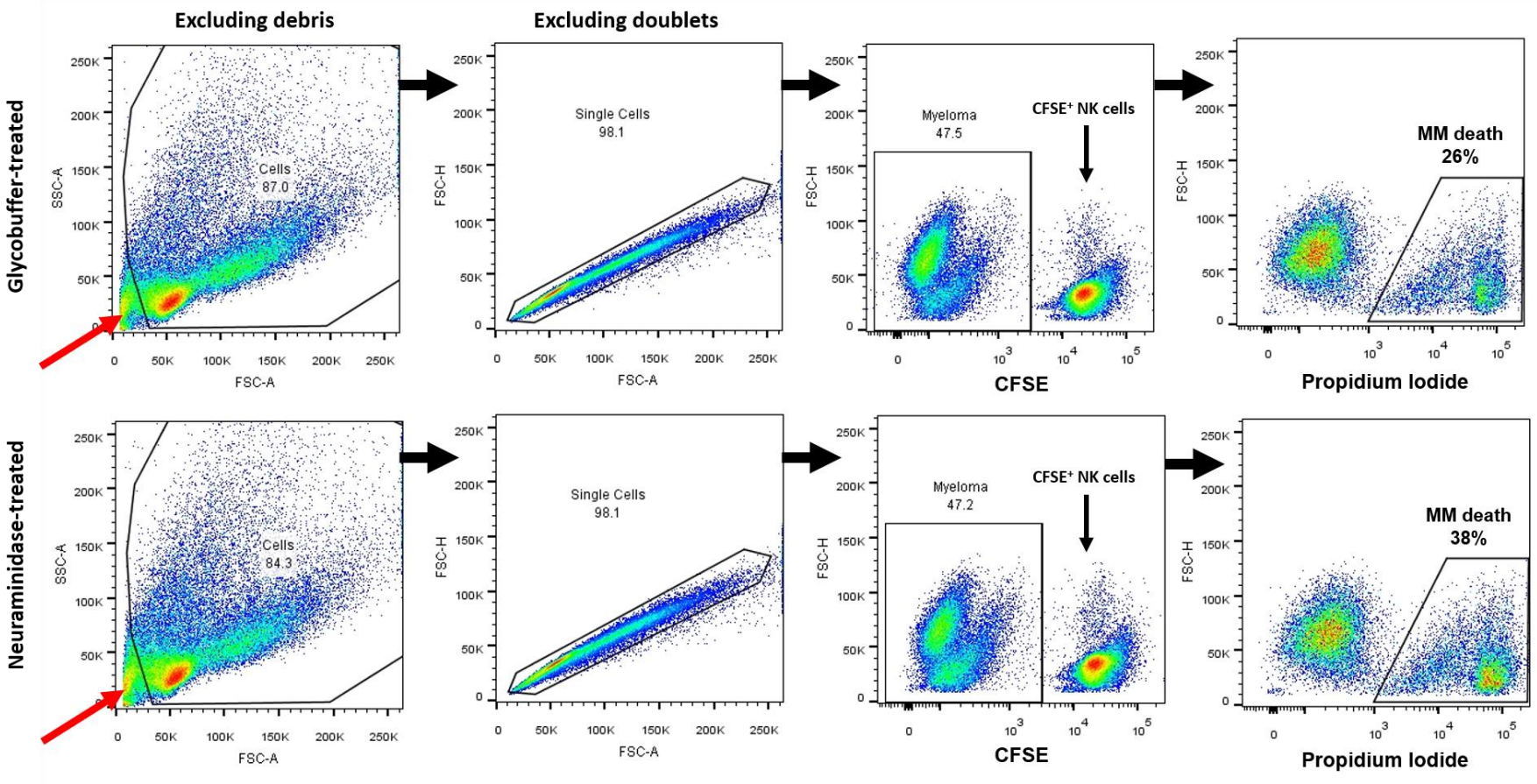


Figure 3.11 An example of the flow-based cytotoxicity assay gating strategy used in this project.

The aim of the gating strategy in this scenario is to measure the viability of $CFSE^-$ single MM cells. Cellular debris is removed from the analysis by appropriate gating in FSC-A vs SSC-A plots. Doublets are then excluded by gating in FSC-A vs FSC-H plots. Single cells were then determined as being MM ($CFSE^-$) or NK cells ($CFSE^+$). Cell death of either MM or NK cells was then analysed using propidium iodide (P. Iodide). Graph represents an n=1 example of a 1:1 E:T ratio cytotoxicity assay using expanded primary NK cells and NEURA or GLYCO-treated H929 MM cells.

During the course of these cytotoxicity assays, it was observed that NEURA-treatment was toxic to MM1S cells (**Figure 3.12A**). MM1S cells were considered as one of the more sensitive cell lines used in this project, in particular when compared to H929 and JJN3. Thus, an alternative approach to desialylate MM cells which may be more tolerable was needed. To do this the cell permeable sialic acid analogue which acts as a sialyltransferase inhibitor (SIA) was employed. After treatment with SIA, no toxicity was observed towards MM1S cells. Furthermore, enhanced primary expanded NK cell cytotoxicity was observed against desialylated MM1S, compared to DMSO-treated controls (**Figure 3.12B**).

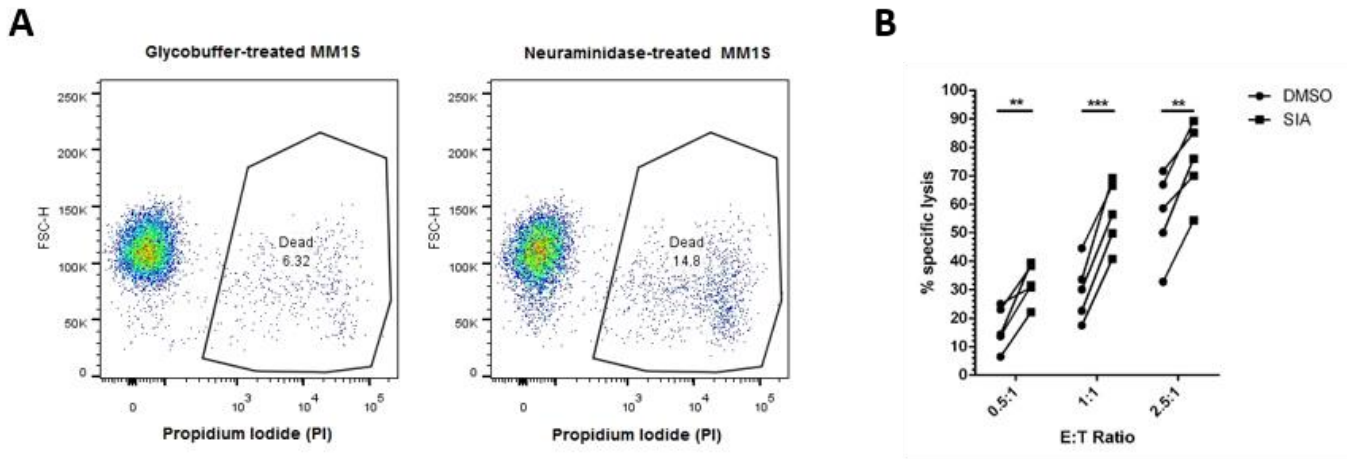


Figure 3.12 Toxicity of neuraminidase treatment to MM1S can be overcome by using the cell permeable sialyltransferase inhibitor SIA, while still obtaining a strong cytotoxic NK cell response.

Due to the toxicity observed to MM1S from NEURA treatment, SIA was used as an alternative desialylating approach to enhance NK cell cytotoxicity. **(A)** An example raw dot plot file demonstrating the toxicity of NEURA treatment to MM1S cells. **(B)** MM1S treated with SIA were strongly sensitized to primary expanded NK cells. Graph in **A** represents a single biological repeat of n=5, while **B** represents data from n=5 biological repeats using primary NK cells expanded from the PB of healthy donors. Data in **B** was analysed using Student's paired *t*-test, ** - $p < 0.01$, *** - $p < 0.001$.

Having observed enhanced NK cell cytotoxicity in cytotoxicity assays with desialylated MM cells, expression of CD107a on the NK cell surface was measured to confirm increased NK cell activation. When primary IL-2 activated NK cells were co-cultured with NEURA-treated K562, H929 or JJN3 for one hour and then examined, a consistent and reproducible increase in CD107a was observed on bulk NK cells co-cultured with desialylated target cells compared to GLYCO-treated controls (**Figure 3.13A,B**). In order to further examine this, another

degranulation assay was carried out, but this time NK cells were divided into subsets based on Siglec-7 and Siglec-9 expression, rather than analysing the activation of bulk NK cells in response to desialylated target cells. The subsets analysed were Siglec-7^+/Siglec-9^+, Siglec-7^+/Siglec-9^-, Siglec-7^-/Siglec-9^+ and Siglec-7^-/Siglec-9^- NK cells. Statistically significant increases in CD107a expression were observed on Siglec-7^+/Siglec-9^+ and Siglec-7^+/Siglec-9^- subsets, while a trend for increased CD107a expression was observed on Siglec-7^-/Siglec-9^+, but was not statistically significant. Interestingly, a statistically significant increase in CD107a expression was observed on Siglec-7^-/Siglec-9^- NK cells (**Figure 3.13C**).

Finally, the expression of cytokines TNF-α and IFN-γ within expanded primary NK cells when co-cultured with desialylated K562, JJN3 and H929 target cells were measured. As discussed in **3.1**, NK cells secrete pro-inflammatory cytokines as well as being cytotoxic effectors of the innate immune system. Having observed increased primary expanded NK cell cytotoxicity upon co-culture with desialylated JJN3 and H929 in **Figure 3.10C**, it was of interest to determine the effect of this on TNF-α and IFN-γ secretion. Firstly, CD107a expression on expanded primary NK cells co-cultured with NEURA or GLYCO-treated K562, JJN3 and H929 was recorded. Increased CD107a expression on NK cells cultured desialylated K562, JJN3 and H929 was observed, although only K562 was statistically significant while non-significant trends were observed in JJN3 and H929 (**Figure 3.13D**).

When intracellular expression of TNF-α and IFN-γ was measured statistically significant increases in the number of TNF-α^+ and IFN-γ^+ NK cells was observed in expanded NK cells co-cultured with NEURA-treated K562 cells compared to GLYCO-treated controls. A statistically significant increase in the number of IFN-γ^+ NK cells was also observed in expanded NK cells co-cultured with desialylated H929 compared to GLYCO-treated H929. No significantly increased numbers of TNF-α^+ and IFN-γ^+ NK cells were observed after co-culture with desialylated H929 and JJN3 (**Figure 3.13E,F**).

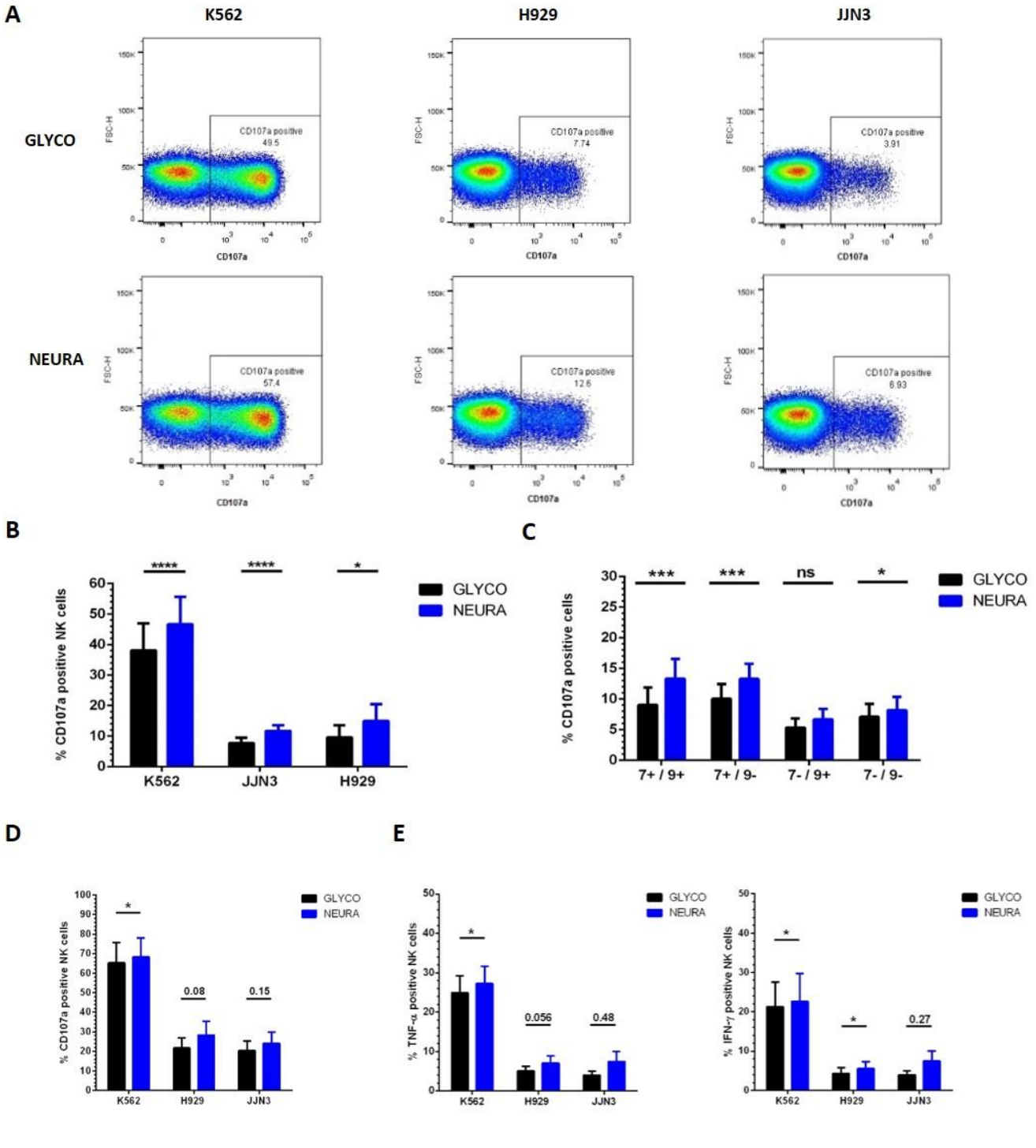


Figure 3.13 NK cells co-cultured with desialylated K562, JJN3 and H929 are more highly activated and have higher intracellular expression of TNF-α and IFN-γ compared to NK cells co-cultured with GLYCO-treated control target cells.

Primary NK cells (IL-2 activated and expanded) were co-cultured with target cell lines K562, JJN3 and H929 which were either pre-treated with NEURA or GLYCO, after which CD107a expression was measured on NK cells or TNF-α and IFN-γ expression was measured within NK cells. **(A)** Individual dot blot representatives of CD107a expression on IL-2 activated NK cells co-cultured with NEURA-treated or GLYCO-treated K562, JJN3 and H929. **(B)** Combined results of n=7 independent biological repeats of **A**. **(C)** IL-2 activated NK cells were divided into subsets based on Siglec-7 and Siglec-9 expression after which CD107a expression on each subset. **(D)** Expanded primary NK cells were co-cultured with K562, JJN3 and H929 pre-treated with NEURA or GLYCO after which CD107a expression was measured.

(E) TNF-α and IFN-γ expression within expanded NK cells was measured after co-culture with K562, JJN3 and H929 pre-treated with NEURA or GLYCO. n=7 for **B**, **C**, D, n=5 for E. Graphs in **B**, **C**, and **D** represent mean CD107a$^+$ NK cells + SEM. Graphs in **E** represent mean TNF-α$^+$ and IFN-γ$^+$ NK cells + SEM. Data in **B**, **C**, **D** and **E** were analysed using Student's paired *t*-test. * - $p < 0.05$, *** - $p < 0.001$, **** - $p < 0.0001$.

3.4.7 Desialylation of MM cells does not unmask NKG2D ligands

Hypersialylation of the tumour cell surface has previously been hypothesized to mask ligands for the activating NKG2D receptor expressed by NK cells. To investigate whether this could contribute to enhanced NK cell cytotoxicity upon desialylation, MM cell lines were pre-treated with NEURA or GLYCO, after which expression of MIC A/B and ULBP1-6 was recorded and compared.

Treatment with NEURA resulted in abolishment of sialic acid expression on MM cell lines (data not shown). However, no observable difference in levels of MIC A/B and ULBP1-6 expression were observed on desialylated MM1S, H929 and JJN3 MM cell lines compared to GLYCO-treated controls (**Figure 3.14**). Interestingly, in the case of the erythroleukemia cell line K562, increased detection of ULBP-2/5/6 and ULBP-3 was observed on desialylated K562 compared to GLYCO-treated controls (**Figure 3.14**).

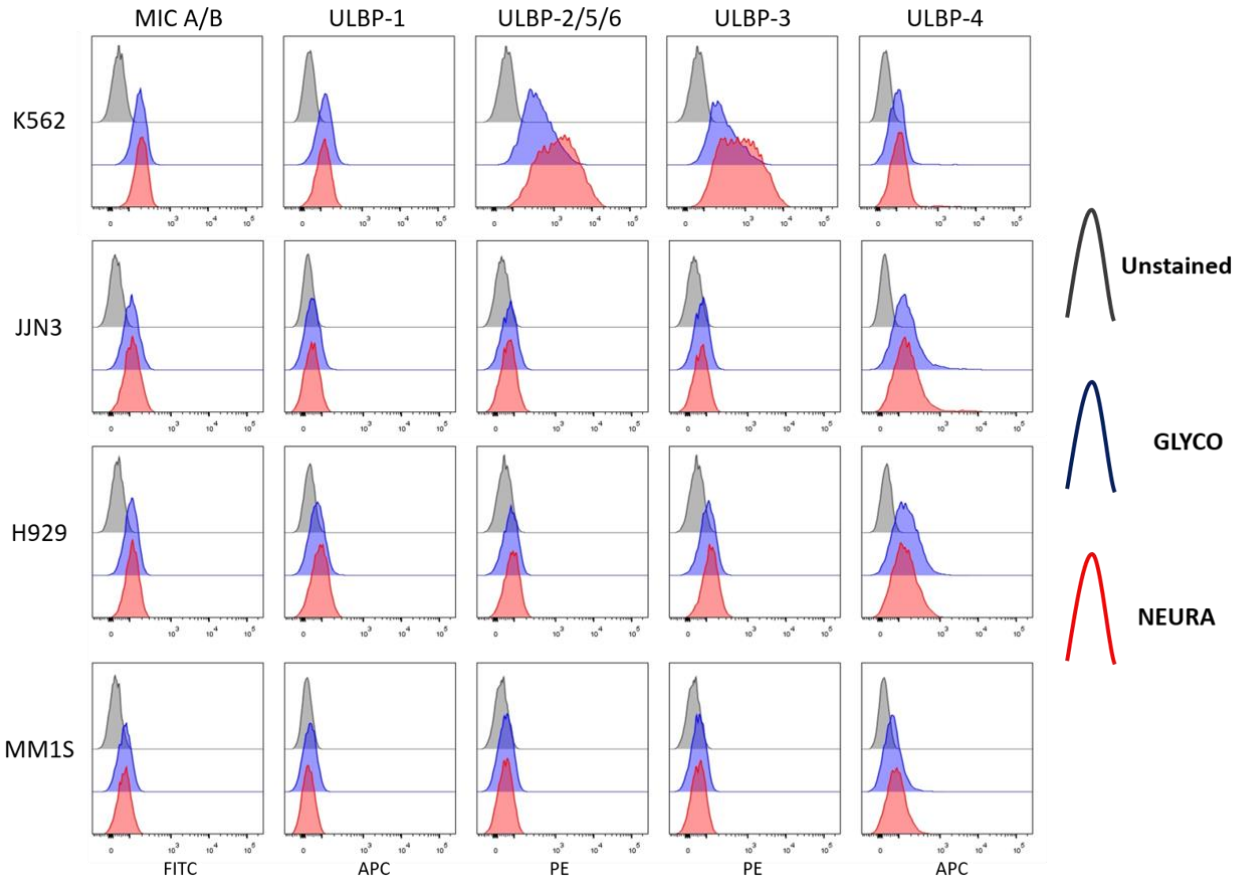


Fig 3.14 NKG2D ligand expression is not increased on MM cells after desialylation, but is increased in the CML cell line K562.

MM cell lines MM1S, H929 and JJN3 and erythroleukemia cell line K562 were pre-treated with NEURA or GLYCO, after which MIC A/B and ULBP-1-6 expression was recorded. Graph represents individual histogram representatives of n=3 independent biological repeats. Grey histograms represent unstained control cells, red histograms represent NKG2D ligand expression on NEURA-treated target cells while blue histograms represent the expression of NKG2D ligands on GLYCO-treated controls.

3.5 Discussion

While previous studies have addressed the role of hypersialylation and Siglecs in regulating NK cell cytotoxicity against cancer, this had never been addressed in the setting of MM prior to this project. In order to examine this thoroughly, it was necessary to start with preliminary phenotyping assays to measure Siglec ligand expression on MM cells, and to determine the effect of MM cell surface desialylation on NK-cell mediated cytotoxicity. The data from these initial assays could then be used to form the basis of future work, including targeting specific Siglec receptors of relevance in MM, should cell surface hypersialylation facilitate evasion of NK cells.

3.5.1 Siglec-7L and Siglec-9L expression on primary MM and MM cell lines confirms the potential for sialic acid-derived Siglec ligands to inhibit NK cell cytotoxicity against MM

It was necessary to carry out analysis of MM cells and NK cells to determine the expression of Siglec ligands and their cognate receptors respectively, to determine whether Siglec-Siglec ligand interactions are possible in MM. As mentioned previously, the most common method of determining Siglec-7L and Siglec-9L expression is to use recombinant Siglec-7 and Siglec-9 chimeras in a flow cytometry-based assay [190-193]. Primary MM cells stained with Siglec-7 and Siglec-9 Fc chimeras were highly decorated with ligands for Siglec-7 and Siglec-9. A trend towards decreased expression of Siglec-7L was observed on primary MM cells from RR MM patients. However, with a sample size of n=2 it is difficult to say whether decreased expression of Siglec-7L in RR MM is due to previous treatment regimens, or whether this is simply variance within this population of patients. Strong expression of Siglec-7L and Siglec-9L was also observed on a panel of routinely used MM cell lines. By analysing the MFI of Siglec-7L$^+$ and Siglec-9L$^+$ MM cells the cell lines with the highest expression of Siglec-7L and Siglec-9L were identified. Combined, these data demonstrate conclusively that MM cells are highly decorated with both Siglec-7L and Siglec-9L.

To confirm that interactions between Siglec-7L and Siglec-9L and their cognate receptors is possible in MM the expression of Siglec-7 and Siglec-9 on NK cells and NK cell

lines was measured, again using flow cytometry-based analysis. Analysis of IL-2 activated NK cells confirmed strong expression of Siglec-7 and partial expression of Siglec-9. As IL-2 activated NK cells were isolated from freshly sourced peripheral blood and cultured overnight before expression of Siglec-7 and Siglec-9 was measured, they remain phenotypically similar to NK cells in the circulation. Additionally, by observing expression of both Siglec-7 and Siglec-9 on IL-2 activated NK cells at levels comparable to published literature, the antibody titrations and flow cytometry techniques used were confirmed to be accurate at an early stage in the project. Furthermore, this ensured confidence in the data obtained when staining NK cell lines or expanded primary NK cells to determine the expression of Siglec-7 and Siglec-9 in subsequent follow-up assays.

Interestingly, strong expression of Siglec-7 was observed on expanded primary NK cells and was maintained throughout the course of the expansion while Siglec-9 expression was completely lost within the first week. Additionally, while partial expression of Siglec-7 was observed on the NK cell lines KHYG-1 and NK-92, no Siglec-9 expression was observed on either cell line. The prevalence of Siglec-7 expression across NK cell lines and primary expanded NK cells was influential, as it provided suitable models to use when investigating the role of the receptor in follow-up assays. Conversely, the lack of Siglec-9 expression across NK cell lines and expanded NK cells prevented a thorough analysis of its effect on NK cell anti-MM functions in this project. It is worth noting that interrupting Siglec-9-Siglec-9L interactions using a blocking antibody has been shown to enhance NK cell cytotoxicity against Burkitt's lymphoma [320]. Considering the presence of both Siglec-9 on a subset of NK cells and Siglec-9L on MM cells, Siglec-9 is therefore likely involved in NK cell regulation against MM and should be considered as a promising checkpoint inhibitor to target in future studies.

3.5.2 PSGL-1 and CD43 act as Siglec-7L in MM, with PSGL-1 appearing to be more prominent than CD43

The identification of P-selectin glycoprotein ligand-1 (PSGL-1), and CD43 as Siglec-7L in MM is important in the context of research into hypersialylation and its role in regulating NK cell activity in this disease. PSGL-1, observed to be highly expressed in MM

biopsies as well as on MM cell lines, can bind to E, P and L-selectins – cell surface lectin-like adhesion molecules. P-selectin is expressed heavily by endothelial cells during inflammation while E-selectin expression can also be induced following exposure to cytokines such as IL-1 and TNF, ultimately allowing the egress of white blood cells such as neutrophils and monocytes from the circulation to areas of infection [321]. Interestingly, interactions between PSGL-1 and P/E-selectin facilitate MM cell proliferation, homing and contribute to resistance to therapies[188, 322, 323]. Antibody blockade of PSGL-1 in-vivo resulted in retention of MM cells in the circulation, sensitizing them to the PI bortezomib, leading to increased survival of mice along with reduced tumour burden [322]. Coupled with the elucidation of its role as a ligand for Siglec-7, PSGL-1 represents an important tumor antigen in MM which could be targeted to potentiate the benefit of future NK cell-based adoptive therapies. It was not surprising to observe that CD43 also acts as a Siglec-7L in MM, considering a previous study utilising the same Siglec-7L pulldown and analysis methodology identified CD43 as a Siglec-7L in AML [309]. Furthermore, in MM CD43 was a strong candidate to act as a Siglec-7L as its expression has been recorded in hematopoietic cells and it is known that CD43 is aberrantly glycosylated in cancer [324]. However, it is likely that PSGL-1 represents the most prominent Siglec-7L on MM cells due to its successful pulldown in three separate MM cell lines whereas CD43 was expressed solely by JJN3.

The identification of PSGL-1 and CD43 as prominent Siglec-7L in MM is important, as individual patients can be examined for their expression and variable expression may potentially identify patient subsets which might obtain the highest therapeutic response from Siglec-7-targeting immunotherapeutic approaches. Furthermore, as PSGL-1 expression is known to increase throughout MM disease progression, a novel NK cell-based therapy targeting PSGL-1 would likely be of benefit to patients all stages of the disease and may be particularly valuable to patients with advanced disease [323].

Finally, the near complete lack of pulldown of both PSGL-1 and CD43 from MM cell lysates pre-treated with NEURA prior to incubation with Siglec-7 Fc chimera-magnetic bead complexes indicates that the sialylation of both of these proteins is critical for efficient binding to Siglec-7. Thus, targeted strategies to desialylate MM cells would likely prevent PSGL-1/CD43 binding to Siglec-7, and would abrogate any inhibition of NK cell functions.

3.5.3 Comparison of Siglec-7 and Siglec-9 expression on immune cell subsets from the MM TME to healthy donor-derived PBMCs

As mentioned previously, the MM TME is known to be immunosuppressive, with NK cells isolated from the BM of MM patients demonstrated to be hypofunctional [306]. Furthermore hypoxia, a common feature within the TME, has been shown to downregulate the expression of activating NK-cell receptors NKp46, NKp30, and NKG2D [325]. Therefore, it was of interest to examine the expression of Siglec-7 and Siglec-9 on NK cells from BMAs provided by MM patients and compare to expression on NK cells from the PB of healthy donors. However, previous studies have shown Siglec-9 expression to be elevated in peripheral blood-circulating $CD4^+$ and $CD8^+$ T cells in a multitude of cancers, including Burkitt's lymphoma, hepatocellular carcinoma, non-small cell lung cancer and melanoma [320]. Siglec-7 and Siglec-9 have been documented to impair the upregulation of gene transcription following T cell activation by reducing phosphorylation of ZAP-70, suggesting that Siglec-7 and Siglec-9 can dampen T cell functions as well as NK cells [294]. Siglec-7 and Siglec-9 have also been shown to drive monocyte to macrophage differentiation, with monocyte-derived macrophages being demonstrated to contribute to poor clinical outcome in pancreatic cancer [326]. The clear potential for Siglec-7 and Siglec-9 to be influential in several immune cell types in cancer coupled with the desire to utilise precious primary MM samples to the full meant it was also of interest to profile the expression of Siglec-7 and Siglec-9 on not only NK cells but $CD4^+$ and $CD8^+$ T cells as well as monocytes/macrophages.

It is worth noting that matched PB as a source of NK cells, T cells and monocytes/macrophages from the same MM patients that supplied the BMAs would have served as the ideal control for this experiment, however these were not available from the Blood Cancer Ireland Biobank. Alternatively, another suitable control would be to compare the expression of Siglec-7 and Siglec-9 on immune cell subsets from BMAs of healthy donors in order to assess the role of the abnormal MM TME in dysregulating Siglec-7 or Siglec-9 expression on immune cells. However, this is challenging due to the difficulty in obtaining healthy donors willing to undergo BMA. Therefore, it is currently difficult to ascertain specifically the mechanisms responsible for increased Siglec-7 expression observed on MM BMA-derived NK cells compared to NK cells from the periphery of healthy donors. It is possible

that a healthy BME could also dysregulate the expression of Siglec-7 or Siglec-9 and that the results observed in this assay were due to this and not specifically the MM TME itself. Regardless, this finding is extremely promising and was the most exciting result from this assay. These data emphasize the potential for Siglec-7 to be an important regulator of NK cell anti-cancer , particularly in an already immunosuppressive TME. This is further amplified by the decreased expression of Siglec-9 observed on MM BMA-derived NK cells, presenting Siglec-7 as the most influential NK cell-expressed Siglec receptor within the MM TME niche. As with Siglec-7, it is difficult to provide a specific mechanism responsible for the decreased expression of Siglec-9 on NK cells from the MM TME. In an attempt to understand the role of hypoxia on Siglec and Siglec ligand expression on NK cells and MM cells respectively, NK cell lines and expanded NK cells were cultured under hypoxia or normoxia after which Siglec-7 and Siglec-9 expression was recorded. However, no conclusive increase in Siglec-7 or Siglec-9 expression was observed on primary expanded NK cells or NK cell lines. There are several alternative mechanisms by which Siglec-7 expression could be increased such as interactions with TME-residing BMSCs. Previously, NK cells treated with supernatant from activated BMSCs displayed a more senescent phenotype, with aberrant expression of CD16 and CXCR4 observed [327]. Additionally, exposure to immunosuppressive factors from local cell types such as TGF-β could also influence Siglec-7 or Siglec-9 expression, as TGF-β has previously been shown to downregulate activating NK cell receptors NKG2D and NKp30. Studies to address these potential contributors to aberrant Siglec-7 and Siglec-9 are discussed in **6**.

Expression of Siglec-7 and Siglec-9 on $CD4^+$ and $CD8^+$ T cells from the TME was comparable to T cells from the PB with only minor subsets of T cells being Siglec-7^+ and Siglec-9^+. Meanwhile, upregulated Siglec-7 expression (as determined by MFI of Siglec-7^+ cells) on monocytes/macrophages could potentially result in a more pro-inflammatory state within the TME, as Siglec-7 stimulation induces the secretion of pro-inflammatory cytokines and chemokines such as IL-6, IL-8 and TNF-α in monocytes [328]. The strong decrease in Siglec-9, determined by comparing MFI of Siglec-9^+ cells, on monocytes/macrophages from the TME was observed compared to PB may suggest that macrophages are more likely to be polarized into an M2 state, which has been shown to be pro-cancerous through supporting angiogenesis and secreting immunosuppressive molecules [329]. While the MFI of Siglec-9^+ on monocytes/macrophages from the TME was significantly decreased, all cells remained

positive overall for Siglec-9. This suggests that while Siglec-9 may still prevent polarization of macrophages in the MM TME, the signalling pathway is likely significantly reduced.

Collectively, these data demonstrate the potential importance of the MM TME in creating an immunosuppressive and pro-cancerous environment, which may facilitate the dampening of NK cell responses by creating a more inhibitory receptor NK cell phenotype including the strong upregulation of Siglec-7 expression.

3.5.4 Hypoxia may result in abnormal Siglec-7L and Siglec-9L expression on MM cells

Conflicting and inconclusive data was observed in MM cell lines cultured under hypoxia with both Siglec-7L and Siglec-9L expression being both upregulated or downregulated depending on the cell line. It is possible that culturing cell lines in-vitro for 72 hours might not be sufficient to observe a true effect on Siglec ligand expression. Long-term culture within a murine model capable of providing a more realistic TME with a hypoxic nature might result in more definitive results. This is also true when considering the effects of the other TME factors on Siglec or Siglec ligand expression on NK or MM cells, respectively. Recreating the BM is extremely difficult considering the multitude of factors needed for a realistic model. Furthermore, the effects on Siglec or Siglec ligand expression when cells are simply incubated under hypoxia might not be a valid representation of the true biological effect unless the TME is fully mimicked. Nevertheless, the potential for the TME to alter Siglec or Siglec ligand expression cannot be discounted and potentially plays a role in enhancing the regulatory effect of Siglec-7 on NK cell anti-cancer capabilities.

Recent research proposes utilising a hydrogel-based layered co-culture system in order to replicate the AML BME. This system was used to co-culture AML cells with BMSCs while simultaneously exposing them to common immunosuppressive cytokines such as TGF-β or microenvironmental factors such as hypoxia [330]. Replicating this assay but within MM might reflect clearer the effects of the BME on Siglec ligand or cognate receptor expression on MM and NK cells, respectively. Furthermore, this assay could be modified to reflect the MM TME by including TME-residing cells or conditioned media from MM cell cultures in order

to investigate the role of the MM TME in dysregulating Siglec ligand or receptor expression, compared to the normal healthy BME.

3.5.5 Desialylation of the MM cell surface strongly enhanced primary NK cell-mediated cytotoxicity

Having concluded that interactions between sialic acid-derived Siglec-7L and Siglec-9L on MM cells and inhibitory Siglec-7 and Siglec-9 receptors on NK cells are possible, the role of hypersialylation in facilitating evasion of NK cell-mediated cytotoxicity was next addressed. Several studies have shown that desialylation of the tumour cell surface results enhances NK cell-mediated cytotoxicity in leukaemia, cervical and breast cancer cell lines [190, 305]. As this was the first study investigating the role of hypersialylation in regulating NK cell activity in MM, these experiments were carried out using a panel of MM cell lines instead of a single MM cell line. MM1S, H929 and JJN3 were chosen due to the strong expression of both Siglec-7L and Siglec-9L observed on each cell line.

The strong enhancement of primary NK cell cytotoxicity against desialylated target cells compliments the results observed by previous groups in different cancers, but also demonstrated the importance of this novel therapeutic strategy in MM. As well as increased tumour cell lysis upon desialylation, increased NK cell activation was also observed on NK cells co-cultured with desialylated MM cells as determined by CD107a expression. Additionally, when subsets of NK cells (generated based on Siglec-7 and Siglec-9 expression) were analysed, the strongest increase in activation was in Siglec-7^+ subsets (Siglec-7^+/Siglec-9^+ and Siglec-7^+/Siglec-9^-). These data suggest that Siglec-7 appears to have a more influential role in regulating NK cell cytotoxicity against MM cells than Siglec-9. Finally, minor increases in TNF-α^+ and IFN-γ^+ NK cells were observed after co-culture with both desialylated MM cells and the CML cell line K562, indicating that targeted desialylation of tumor cells might not only result in enhanced NK cell-mediated cytotoxicity but potentiate the activation of macrophages as well as controlling cell growth or even stimulate apoptosis within tumour cells [243, 331, 332].

Previously, hypersialylation has been theorised to mask ligands for the activating NK cell receptor NKG2D. This may contribute to stronger enhancements in NK cell-mediated

cytotoxicity due to abolishment of inhibitory signalling mediated by Siglecs while simultaneously providing an activating signal through binding of unmasked MIC A/B or ULBP-1-6 to NKG2D [173]. Indeed, desialylation of breast cancer cells resulted in enhanced binding to recombinant NKG2D Fc chimeras [305]. However, while no increase in NKG2D ligand expression was observed on desialylated MM cells, an increase in ULBP-3 and ULBP-4 was observed in the erythroleukemia cell line K562, indicating that this axis may be relevant in leukaemia. This is particularly important as NKG2D CAR-T cells have demonstrated promise in treating leukaemia in a patient trial [333, 334]. Potentially, desialylation of leukaemia cells may result in enhanced NKG2D ligand availability, which could in turn enhance the efficacy of NKG2D CAR-based immunotherapies. However, this needs to be investigated further. An immediate follow up assay would be to determine the expression of NKG2D ligands measured on both desialylated and control-treated primary leukaemia cells, as opposed to cell lines. This would also be relevant in MM, where NKG2D ligand expression was measured only on MM cell lines in this project and not on primary MM cells. However, this data suggests that increased NKG2D signalling is not a mechanism by which NK cell cytotoxicity is potentiated upon encountering desialylated MM cells in this project, further indicating that Siglec receptors expressed by NK cells, and Siglec-7 in particular, may be heavily responsible for regulating NK cell functions against MM.

3.5.6 Interpretation

The findings of this chapter demonstrate the relevance of the hypersialylated MM cell surface in facilitating evasion of NK cell-mediated immunosurveillance. With the more recent rise in interest in cellular therapies for both solid and haematological malignancies, the role of NK cells has never been of more importance [335-338]. In particular, CAR-NK cell and adoptive NK cell transfer-based therapies have recently been utilised in clinical trials [282]. While CAR T-cells are currently in the spotlight, NK cells offer a number of potential advantages to CAR-T cells. Notably, NK cells do not cause graft versus host disease and HLA downregulation by tumour cells to escape T-cell immunosurveillance strongly induces NK cell cytotoxicity as NK cells recognize HLA Class I molecules as a marker of self [273]. However, adoptive NK cell

transfer-based therapies could be limited by the hypersialylated tumour cell surface regulating NK cell activities such as cytotoxicity and cytokine secretion.

Elucidation of the ligands for Siglec-7 expressed by MM cells is of benefit to the field. Knowing the structure of the underlying glycoprotein(s) to which sialic acid-derived Siglec-7L and Siglec-9L are attached may lead to an understanding of the affinity with which they bind to Siglec-7 and Siglec-9, thereby allowing a predominant ligand to be identified and targeted in future studies. Furthermore, with the presence of more than one Siglec-7L on MM cells, patients could be screened for the expression of both PSGL-1 and CD43, and patients with high expression of one, or both, of these ligands could be identified. Screening patient MM cells in this manner could help to identify patients who may be most suitable for future NK cell-based therapies or trials targeting Siglec-7 or the hypersialylated cell surface. The near abolishment of these ligands binding to Siglec-7 Fc chimeras upon treatment of cell lysates with NEURA confirms that targeted desialylation could weaken, or abolish completely, the inhibitory Siglec-7-Siglec-7L signalling pathway. While this data is important, it is also imperative to elucidate the identity of Siglec-9L in MM, as screening of both healthy donor-derived and patient BMA-derived NK cells as well primary MM cells and MM cell lines reveals the expression of Siglec-9 and Siglec-9L on NK cells and MM cells, respectively. These data indicate that the Siglec-9-Siglec-9L axis can also lead to inhibition of NK cell functions against MM, although perhaps not to the extent of Siglec-7.

Considering the increases in NK cell-mediated cytotoxicity, as well as activation and cytokine secretion, observed upon encountering desialylated MM cells, targeted desialylation represents a promising future therapeutic strategy to enhance NK cell-based anti-MM, or cancer in general, therapies. Indeed, targeted desialylation of the tumour cell surface has been attempted and has produced promising results. Antibody-decorated nanoparticles loaded with SIA have been used to specifically desialylate melanoma cells, and demonstrated long term SIA release and prolonged sialic acid blockade. Furthermore, in an in-vivo model treatment of mice with these SIA-containing nanoparticles prevented lung cancer metastasis in a murine model [(314)]. Additionally, antibody-sialidase conjugates have also been created, with the antibody directing the sialidase to the tumour cell, allowing targeted desialylation. This approach achieved promising results when targeting $HER2^{+}$ breast cancer cells in-vitro and has also been shown to inhibit tumour growth in mice [(339)]. The toxicity observed to MM1S

MM cells by NEURA treatment should also be discussed at this point. Untargeted treatment of the TME with a naked sialidase could result in toxicity towards all BM-residing cell types and may induce negative side effects in patients. However SIA, as a cell permeable sialyltransferase inhibitor, represents a more passive alternative to desialylate tumour cells and no toxicity was observed in MM cells treated with SIA. However, it is important to consider the potential toxicity of desialylating agents to non-MM cells in the BM, and the health issues that may arise because of this, which emphasizes the need to provide desialylating agents specifically targeting MM cells for future studies, especially in in-vivo work.

The promise of targeting the hypersialylated tumour cell surface to enhance NK cell anti-cancer functions is therefore very strong. Future studies that could be carried out using this data as a rationale are discussed in **6**. However, as discussed in **1.1.7**, targeting immune checkpoints can lead to disappointing results when used as a monotherapy, as observed in PD-1 targeting studies [(139)]. Therefore, it was of interest to determine whether desialylation could be combined with an existing clinical therapy for MM to further enhance NK cell anti-MM functions. This work is described in **Chapter 4.**

Chapter 4:
Desialylation can be combined with anti-CD38 moAbs to elicit a potent anti-MM response

4.1 Introduction

Based on the expression of two markers: CD16 and CD56, NK cells can be subdivided into two main populations. $CD56^{bright}$ $CD16^{dim}$ NK cells, which represent a maximum of 10% of NK cells within PB, are considered weakly cytotoxic compared to $CD56^{dim}$ $CD16^{bright}$ NK cells, which constitute up to 90% of NK cells within PB [340, 341]. This is due to lower expression of perforin, granzymes and lytic granules within $CD56^{bright}$ $CD16^{dim}$ NK cells [342]. However, while weakly cytotoxic, $CD56^{bright}$ $CD16^{dim}$ NK cells are potent secretors of cytokines such as TNF-α and IFN-γ [340]. Conversely, due to decreased expression of CD16, $CD56^{bright}$ $CD16^{dim}$ NK cells perform weaker ADCC compared to $CD16^{bright}$ NK cells. NK cells are critical effectors of ADCC within the immune system and therapeutic moAbs, which bind to antigens ideally expressed solely by cancer cells and engage CD16 on NK cells via the Fc chain of the antibody, have been introduced in cancer therapies. As well as inducing ADCC mediated by NK cells, moAbs can also stimulate CDC. CDC is initiated by the binding of the complement protein C1q to the Fc portion of moAbs, after which the complement cascade is initiated eventually forming the membrane attack complex (MAC). The MAC forms pores in the MM cells surface, leading to target cell lysis [343]. Finally, opsonized cancer cells can also be phagocytosed by macrophages demonstrating the potential of therapeutic moAbs to eliminate cancer cells using a range of mechanisms [344]. In MM, two important immunotherapeutic moAbs are approved for use in the clinic: Elotuzumab and Daratumumab.

4.1.1 Elotuzumab

Elotuzumab is a humanized moAb which recognizes cell-surface glycoprotein CD2 subset 1 (CS1, also known as signalling lymphocyte activation molecule F7 (SLAMF7), and was the first monoclonal antibody approved for MM treatment [345]. SLAMF7 mediates cell-cell binding between MM cells and BMSCs, enhancing MM proliferation and survival [346]. Attractively, SLAMF7 is expressed by over 90% of BM-residing MM cells, but is not expressed by other hematopoietic cell types. Several pre-clinical in-vivo studies have demonstrated that Elotuzumab induces strong NK cell responses against MM, with co-cultures between PBMCs (containing NK cells) and MM cells treated with Elotuzumab and the IMiD lenalidomide

strongly enhancing NK cell activation, cytokine release and expression of adhesion markers [347]. In a murine MM model, Elotuzumab treatment resulted in decreased tumour volume, which was near completely reversed if NK cells were depleted from the mice, or if the mice were treated with a variant of Elotuzumab mutated to prevent binding between the Fc chain of the moAb and CD16 [348]. Interestingly, in the same study antibody blockade of PD-1 strongly enhanced the effects of Elotuzumab, leading to enhanced antitumour efficacy mediated by NK cells [348]. In 2015, Elotuzumab was approved to treat MM in combination with lenalidomide and dexamethasone after promising clinical trial results in the ELOQUENT Phase III study [349]. Regarding NK cells, Elotuzumab treatment can potentially trigger NK cells to target each other in a process known as fratricide, due to their own expression of SLAMF7. However, several studies in vitro and in vivo have demonstrated that Elotuzumab treatment induces minimal NK cell fratricide [279, 350, 351].

4.1.2 Daratumumab

Dara is an IgG1κ human antibody that recognizes the type-II transmembrane glycoprotein CD38 and was first used in the clinic to treat MM in 2015 [352]. Initially, Dara was approved for use in patients who had undergone ≥ 3 previous lines of therapy which included IMiDs and PIs, or patients who were double refractory to both IMiDs and PIs [353]. Further studies demonstrated the potential for Dara in MM therapy. In the CASTOR trial Dara combined with bortezomib and dexamethasone (DVd) was superior than bortezomib and dexamethasone alone (Vd), with DVd-treated patients experiencing prolonged PFS and more impressive overall responses, including minimal residual disease (MRD) [354]. Furthermore, in the phase III POLLUX trial, Dara, in combination with lenalidomide and dexamethasone (DRd) gave a greater therapeutic response than lenalidomide and dexamethasone alone (Rd) with patients also exhibiting longer progression-free survival and deeper responses [355]. Due to this Dara was approved for use in combination with Vd or Rd in RR-MM. Dara has also received FDA approval for use in combination with pomalidomide and dexamethasone in the treatment of RR-MM patients.

Currently, studies are also being conducted into the potential of Dara in ND-MM patients, with trials reporting promising results. In a Phase II trial carried out by Mayo Clinic,

Dara in combination with ixazomib, lenalidomide and dexamethasone showed promising results with all ND-MM patients who underwent four courses of treatment experiencing ≥ partial response (PR). Of these patients, 50% expressed a very good PR [356]. The phase II Lyra trial studied the efficacy of Dara combined with CYBORD (cyclophosphamide, bortezomib and dexamethasone) in a population of ND-MM patients including transplant eligible and ineligible patients, along with patients who had relapsed after one prior treatment line. In ND-MM patients, 44% observed ≥ very good PR with 5% experiencing CR. Responses were further improved in patients who underwent longer treatment [357]. Further studies investigating Dara efficacy in ND-MM are currently ongoing.

Dara binds to CD38 with high affinity and can induce CDC, ADCP and ADCC. In freshly isolated MM cells from both ND-MM and RR-MM patients Dara treatment induced CDC of MM cells [358]. ADCC is a process which requires expression of the target antigen on the cancer cell surface, a moAb targeting the antigen with an Fc chain and an effector cell expressing the FcγR (CD16) receptor. More recently, several studies have utilised a variant of CD16 with a single nucleotide polymorphism of F158V. This mutation results in enhanced affinity for Fc chain presented by moAbs, and studies have shown that this F158V-containing variant of CD16 is more efficient at inducing ADCC than the wildtype version [359]. One of the drawbacks of utilising Dara in MM treatment in regards to NK cells is their own expression of CD38. As with Elotuzumab, the self-expression of CD38 on NK cells results in Dara-induced fratricide of NK cells [360]. In order to overcome fratricide, the $CD38^{low}$ NK cell line KHYG-1 has been used as an effector of ADCC and demonstrated no fratricide when co-cultured with Dara-treated MM cells. Furthermore, these $CD38^{low}$ KHYG-1 NK cells were transfected with F158V CD16 messenger RNA (mRNA) which, when compared to wild-type KHYG-1, elicited significantly superior ADCC against CD38-expressing primary MM cells and MM cell lines [233].

In an attempt to evade Dara-induced ADCC MM cells can rapidly lose CD38 expression after initial treatment with Dara, to an extent where 90% of non-cleared MM cells after Dara treatment were observed to lack CD38 expression [163, 361]. Countering depletion of CD38 is key to maximising the efficacy of Dara. The CD38 upregulating agent all-*trans* retinoic acid (ATRA) has been demonstrated to robustly increase the expression of CD38 on MM cells. ATRA interacts with the retinoic acid-α receptor (RAR-α) to induce CD38 expression [362]. Furthermore, ATRA treatment in combination with Dara enhanced the efficacy of Dara in both

in vitro assays and in a humanized mouse model [162]. Interestingly, ATRA treatment of MM cells was also observed to decrease the expression of the complement-inhibitory proteins CD55 and CD59 in MM cell lines, which are typically complement-resistant, and in primary MM cells [162]. Thus, there is strong pre-clinical evidence to suggest that CD38 upregulation in combination with Dara treatment can enhance NK cell anti-MM functions.

4.1.3 The potential for desialylating strategies to be combined with ADCC to enhance NK cell anti-cancer capabilities

More recently, ADCC has been combined with desialylation to enhance NK cell anti-cancer cytotoxicity. The potential for targeting the hypersialylated tumour cell surface in combination with therapeutic moAbs to simultaneously reduce inhibitory signals and enhance activating signals is a promising novel immunotherapeutic strategy. This was initially proposed by Xiao et al., who conjugated a sialidase enzyme to Trastuzumab (Tras); a therapeutic moAb targeting Herceptin-expressing breast cancer cells. Treatment of breast cancer cell lines (HER-2$^+$ and HER-2$^-$) with this antibody-drug conjugate (ADC) demonstrated selective desialylation of HER2$^+$ cells, while HER2$^-$ cells were not desialylated. Furthermore, the ADC conjugate performed well in cytotoxicity assays where Tras, the Tras-sialidase ADC and sialidase were used individually. Treatment with the Tras-sialidase ADC resulted in strongly potentiated NK cell cytotoxicity compared to Tras and sialidase treatment alone. When NK cells were removed from the assay, no cytotoxicity was observed, implicating NK cells as the sole effectors of cytotoxicity and ADCC in this experimental setup. Interestingly, using a panel of HER2$^+$ cells lines, it was observed that the HER2^{low} target cells were sensitized by Tras-sialidase compared to HER2^{moderate} and HER2^{high} target cells [305]. This indicates that cancer cells which respond poorly to moAb therapies might benefit most from a combinational approach of moAb therapy and tumor cell desialylation. This research was then followed up where an in vivo study using the Tras-sialidase conjugate demonstrated that HER2$^+$ breast cancer cells were desialylated in a murine model where Tras-resistant cancers were established. Tras-sialidase treatment resulted in inhibited tumour growth and prolonged survival due to enhanced immune cell infiltration and activation, including NK cells

[339]. Therefore, targeted interruption of inhibition through the sialic acid-Siglec axis represents a promising method of enhancing moAb therapies where NK cells are key effectors.

Furthermore, the role of hypersialylation in masking expression of antigens on the tumour cell surface cannot be underestimated. Previous reviews have suggested that hypersialylation can mask ligands expressed by genetically damaged or malignantly transformed cells which are typically recognized by the activating NK cell receptor NKG2D, contributing to an underwhelming NK cell response [173, 300]. Indeed, NKG2D Fc chimera-binding to breast cancer cells was enhanced after desialylation, indicating the heightened presence of NKG2D ligands after cancer cell surface desialylation [305]. Furthermore, in MM desialylation of the cell surface resulted in increased expression of BCMA [363]. BCMA also represents a promising target for novel immunotherapies, in particular as the target for ADC, moAbs and CAR-T cells [364]. Considering that BCMA is a glycoprotein and that other promising target antigens in MM such as MUC-1 (a reported ligand for Siglec-9), CD38 and SLAMF7 are also glycoproteins, targeting the hypersialylated MM cell surface therefore has potential as a novel means of stimulating a heightened moAb-induced NK cell anti-cancer response [365].

In this chapter the expression of moAb target antigens BCMA, CD38, MUC-1 and SLAMF7 was measured on desialylated MM cell lines and compared to expression of the same antigens on GLYCO-treated control cells. The potential for anti-CD38 moAb treatment in combination with MM cell desialylation was investigated by carrying out co-cultures of expanded NK cells and Dara-treated, desialylated MM cells. To combat Dara-resistant CD38^{low}MM cells, desialylation and Dara treatment of MM cells was combined with ATRA to upregulate CD38 expression and enhance Dara-induced NK cell-mediated ADCC.

4.2 Hypothesis and Objectives

4.2.1 Hypothesis

I. Desialylation of the MM cells surface can unmask therapeutic target antigens targeted by moAb-based immunotherapies

II. Desialylation can be combined with immunotherapeutic moAbs to enhance NK cell cytotoxicity and ADCC

4.2.2 Objectives

I. Desialylate the MM cell surface, using either a sialidase or sialyltransferase inhibitor, and compare the expression of several target antigens on desialylated and sialylated MM cell lines

II. Combine desialylation with immunotherapeutic moAb treatment of MM cells and subsequently co-culture with primary NK cells to measure MM cell death

4.3 Study design

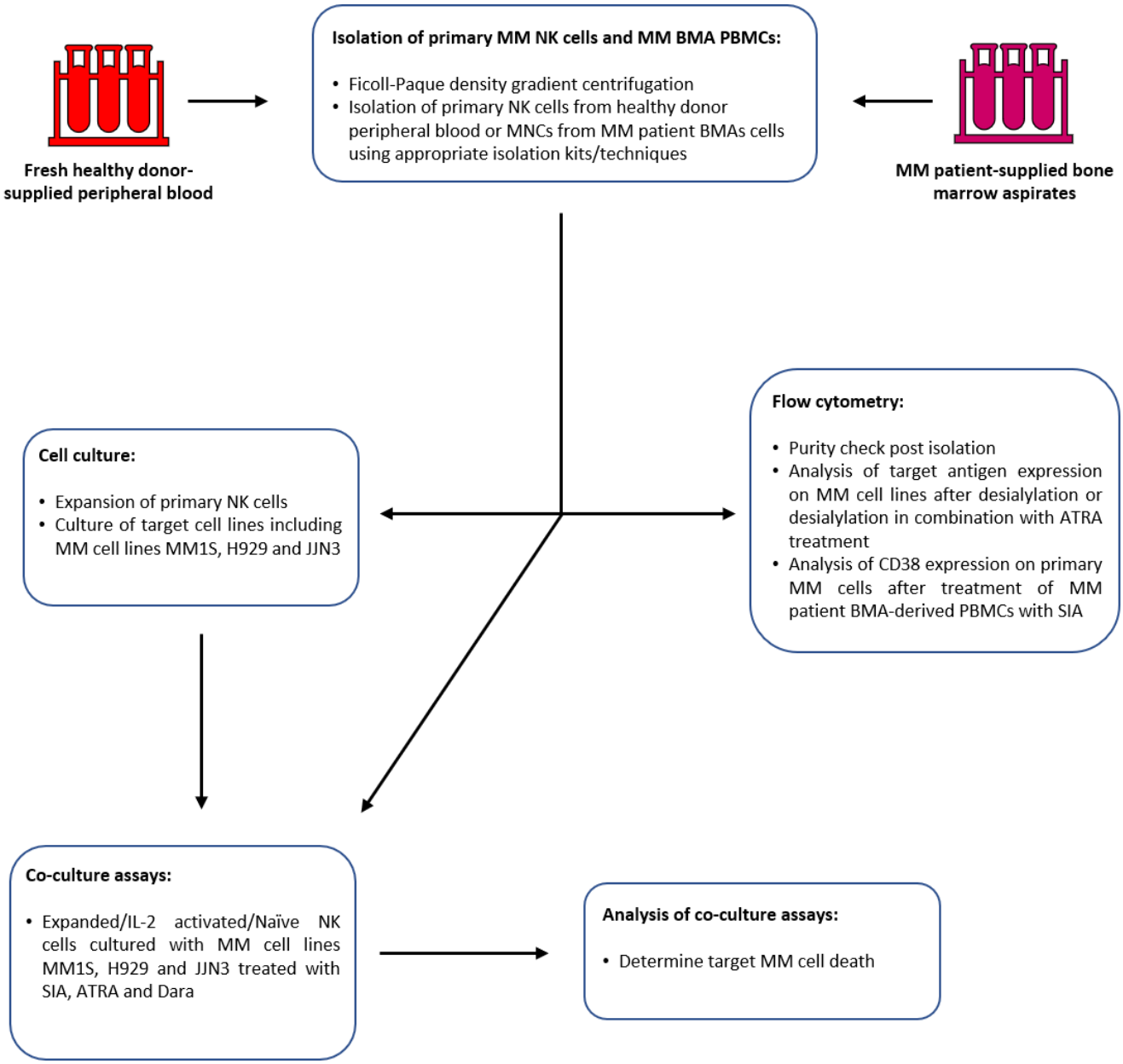


Figure 4.1 Schematic representation of the experimental work carried out in this chapter

An overview of the workflow carried out in this chapter. The expression of several potential or existing therapeutic antigens was measured after desialylation and compared to controls. Having observed increased CD38 detection upon desialylation, further studies were carried out to investigate the potential of combining desialylation with Dara treatment to induce a potent NK cell anti-MM response. Additionally, the expression of CD38 on JJN3 treated with SIA and ATRA was measured and compared to ATRA or SIA treatments alone. Primary expanded NK cells were co-cultured with MM cell lines treated with SIA, Dara, ATRA or combinations of these to determine the effect on NK cell-mediated ADCC.

4.4 Results

4.4.1 Increased detection of CD38 is observed on desialylated primary MM cells and MM cell lines

MM cell lines H929 and MM1S were desialylated using SIA, resulting in a significant reduction of sialic acids on the cell surface as determined using MAA staining to measure a2,3-linked sialic acid expression (**Figure 4.2**). Expression of BCMA, SLAMF7, MUC1 and CD38 was measured on DMSO-treated and SIA-treated MM1S and H929. A minor but consistent increase in the detection of BCMA was observed on both MM1S and H929, however no reproducible increase in the detection of SLAMF7 or MUC1 was observed. However, a reproducibly consistent increase in the detection of CD38 was observed on desialylated H929 and MM1S (**Figure 4.2**).

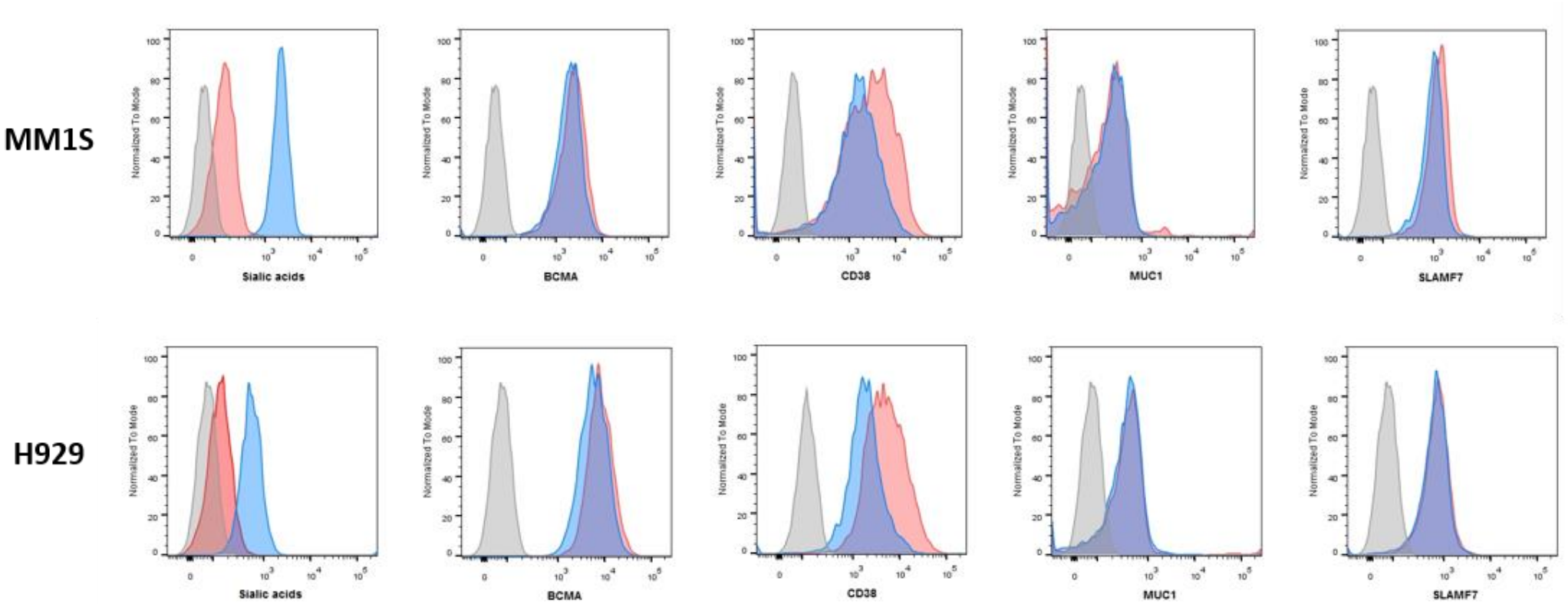


Figure 4.2 Increased detection of BCMA and CD38 is observed on desialylated MM cell lines, but no observable change in MUC1 and SLAMF7 was recorded.

MM1S and H929 MM cell lines were treated with SIA (red) or DMSO (blue) for 72 hours, after which the expression of a2,3-linked sialic acids and promising potential immunotherapeutic antigens was recorded. Unstained cells were used to optimise initial gating and laser voltages (grey). A significant reduction, but not complete abolishment, of sialic acids was observed in SIA-treated MM1S and H929. Minor increases in the detection of BCMA were observed in desialylated MM1S and H929, and strong increases in the detection of CD38 were observed in both cell lines. No increase in the expression of MUC1 or SLAMF7 were observed in either cell line. Figure represents n=1 individual histogram representative of n=3 total independent biological repeats.

To expand on these initial observations MM1S, H929 and JJN3 were treated with either NEURA, SIA or relevant GLYCO/DMSO controls, after which CD38 expression was recorded. Reproducible increases in the detection of CD38 were observed on MM1S and H929 MM cell lines desialylated using both NEURA and SIA (**Figure 4.3A,B**). Interestingly, no increase in the detection of CD38 was observed on JJN3 MM cells treated with either NEURA or SIA (**Figure 4.3A,B)**.

MNCs were isolated from MM patient-derived BMAs and treated with SIA for 40 and 64 hours, after which CD38 expression was measured on $CD38^+/CD138^+$ MM cells. a2,3-linked sialic acid expression was also measured on SIA-treated and DMSO-treated MNCs and a decrease in the MFI of MAA-stained MM cells was observed in SIA-treated cells. Simultaneously, the MFI of CD38 was increased on $CD38^+/CD138^+$ MM cells treated with SIA compared to DMSO-treated $CD38^+/CD138^+$ MM cells (**Figure 4.3C**).

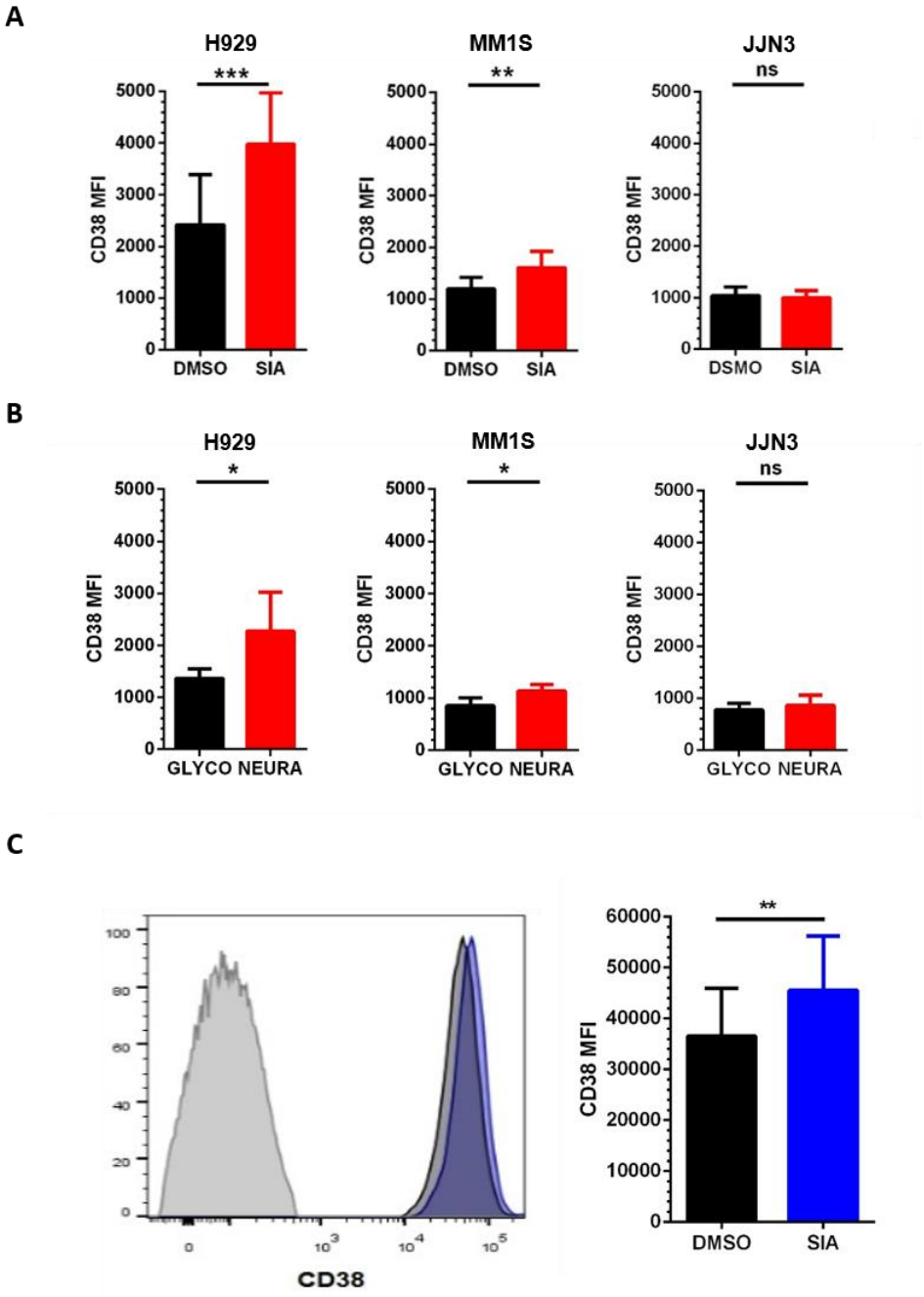


Figure 4.3 Desialylation of MM cells results in increased CD38 detection in both MM cell lines and primary MM cells.

MM cell lines MM1S, H929 and JJN3 were desialylated using either **(A)** NEURA, **(B)** SIA, or relevant controls after which CD38 expression was measured using flow cytometry. **(C)** MNCs isolated from MM patient-derived BMAs were treated with either SIA or DMSO for 64 hours after which CD38 expression was determined on $CD38^+/CD138^+$ primary MM cells. Graphs in **A** and **B** represent MFI of $CD38^+$ MM cell lines and graphs in **C** represent $CD38^+/CD138^+$ primary MM cells + SEM. n=4 for **A**, **B**, n=6 for **C**. Data analysed using Student's unpaired *t*-test (**A**, **B**) and Student's paired *t*-test (**C**). * - $p < 0.05$, ** - $p < 0.01$.

4.4.2 Desialylation in combination with Daratumumab treatment results in increased NK cell-mediated target cell lysis compared to either treatment alone

H929 and JJN3 MM cells were treated with either SIA or DMSO for 72 hours after which they were treated with either Dara, or PBS, before being co-cultured with expanded $CD16^+$ NK cells at a 1:1 E:T ratio. Partial CD16 expression was confirmed on expanded primary NK cells used in these co-cultures using flow cytometry (**Figure 4.4**).

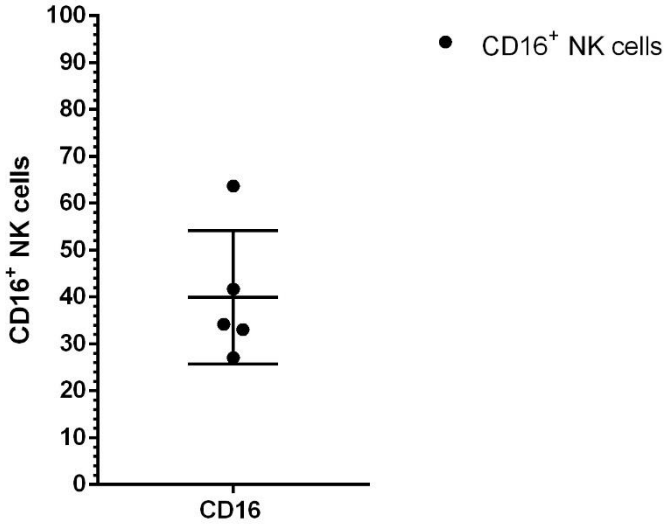


Figure 4.4 CD16 is expressed by expanded primary NK cells

CD16 expression was measured on expanded primary NK cells using an anti-CD16 antibody and flow cytometry analysis. Graph represents CD16 expression on n=5 individual NK cell expansions, mean overall CD16 expression ± SD. n=5.

DMSO or PBS-treated H929 and JJN3 (untreated control to establish basal NK cell-mediated cytotoxicity) were most resistant to NK cell-mediated cytotoxicity, as expected. Strong ADCC of DMSO, Dara-treated H929 was observed, but this was not replicated in DMSO, Dara-treated JJN3. SIA, PBS-treated H929 and JJN3 were more readily lysed by NK cells, echoing the findings of **Chapter 3**. In H929, Dara treatment induced higher levels of NK cell-mediated specific lysis than SIA alone (**Figure 4.5**).

Finally, combined treatment of SIA and Dara in H929 resulted in enhanced NK cell-mediated cytotoxicity compared to SIA or Dara alone. However, no observable change in NK cell-mediated lysis of JJN3 was observed in JJN3 treated with both SIA and Dara compared to JJN3 treated with SIA alone (**Figure 4.5**). No NK cell fratricide was observed in any of the cytotoxicity assays carried out for this experiment.

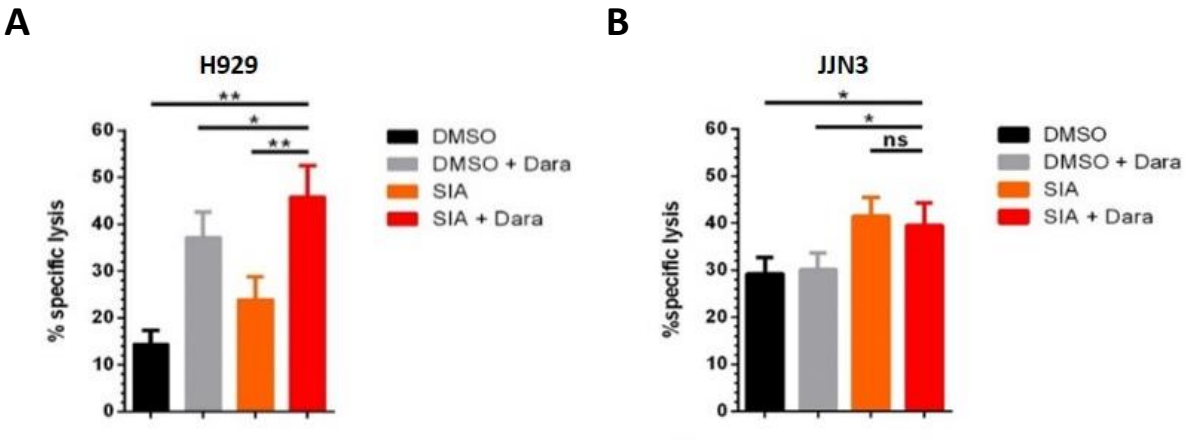


Figure 4.5 Treatment with both SIA and Dara resulted in increased NK cell-mediated lysis of H929, but not JJN3, compared to SIA or Dara alone

(A) H929 and **(B)** JJN3 were treated with SIA or DMSO for 72 hours prior to being treated with PBS or Dara and subsequently co-cultured with $CD16^+$ expanded primary NK cells at a 1:1 E:T ratio. Graph represents mean specific lysis + SEM, data was analysed using one-way ANOVA, n=5 for **A**, **B**. * - $p < 0.05$, ** - $p < 0.01$.

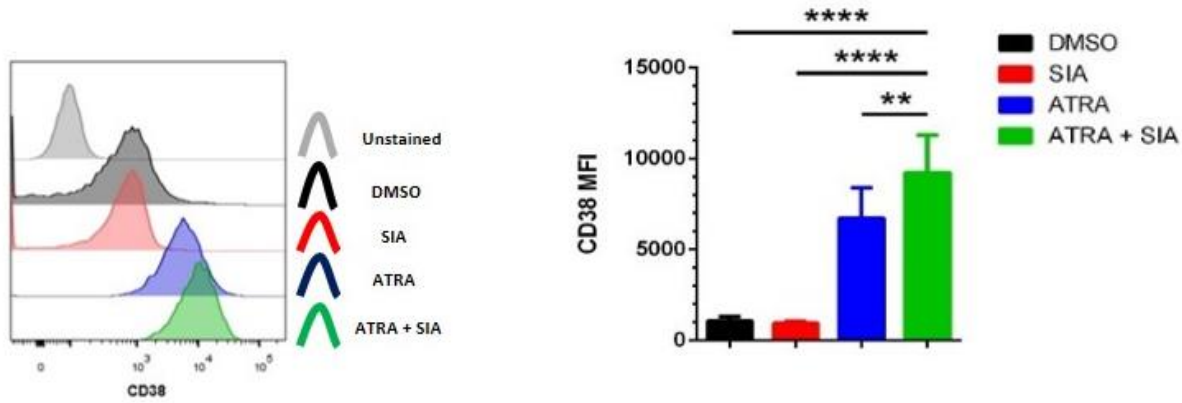
4.4.3 ATRA treatment to upregulate CD38 expression can be combined with desialylation and Daratumumab treatment to enhance NK cell cytotoxicity against $CD38^{low}$ MM cells

As observed in **4.4.2**, JJN3 MM cells were not sensitized to NK cell-mediated ADCC following Dara treatment. Furthermore, JJN3 appeared to have the lowest expression of CD38 of the three MM cell lines used, as shown in **Figure 4.3 A,B**. Therefore, JJN3 were treated with ATRA in order to upregulate CD38 expression and to sensitize them to Dara-induced NK cell-mediated ADCC.

ATRA treatment of JJN3 resulted in a > 6-fold increase in CD38 expression, as determined by MFI of $CD38^{+}$ JJN3 cells, compared to JJN3 treated with DMSO (**Figure 4.6A**). Additionally, JJN3 were treated with SIA alone or a combination of SIA and ATRA. As previously observed in **4.4.1**, analysis of SIA-treated JJN3 revealed no increase in detectable CD38. However, JJN3 treated with both SIA and ATRA had a > 8- fold increase in CD38 expression, as determined by MFI of $CD38^{+}$ JJN3 cells, compared to JJN3 treated with DMSO alone (**Figure 4.6A**)

JJN3 treated with either DMSO alone, SIA alone, ATRA alone and ATRA in combination with SIA were then treated with Dara and co-cultured with $CD16^{+}$ expanded primary NK cells. As can be observed in **Figure 4.6B**, JJN3 treated with DMSO and Dara were not sensitized to NK cell-mediated ADCC, replicating the results displayed in **Figure 4.5B**. JJN3 treated with ATRA and Dara were sensitized to NK cell-mediated ADCC. However, JJN3 treated with SIA, ATRA and Dara were further sensitized and were significantly more readily lysed by NK cells compared to JJN3 solely treated with Dara (**Figure 4.6B**). No fratricide of NK cells was observed in any of the cytotoxicity assays carried out for this experiment.

A



B

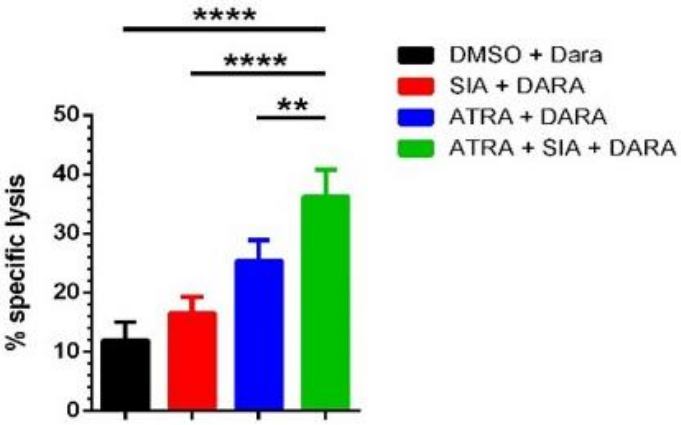


Figure 4.6 ATRA treatment of CD38^{low} JJN3 MM cells can be combined with SIA and Dara to potentiate NK cell-mediated ADCC

JJN3 were treated with DMSO, SIA, ATRA or ATRA and SIA after which **(A)** CD38 expression was measured or **(B)** JJN3 were co-cultured with CD16^{+} expanded NK cells at a 1:1 E:T ratio. Histogram in **A** is a single representative of n=5 independent biological repeats of the described treatment while combined results of n=5 are presented as a bar graph depicting MFI of CD38^{+} JJN3 + SEM. Graph in **B** represent mean specific lysis of n=5 independent cytotoxicity assays + SEM. Data analysed using one-way ANOVA. ** - $p < 0.01$, **** - $p < 0.0001$.

4.4.4 CD38 knockout on expanded NK cells could maintain NK cell-mediated cytotoxicity whilst reducing the risk of fratricide

To minimize the risk posed to NK cells through Dara-induced fratricide, genetic interruption of *CD38* was carried out to abolish CD38 expression on NK cells. CD38 knockout ($CD38^{KO}$) resulted in near complete loss of CD38 expression on NK cells as determined by flow cytometry (**Figure 4.7A**). Using these $CD38^{KO}$ NK cells a repeat of the experiment described in **4.4.2** was carried out, using both mock and $CD38^{KO}$ NK cells in cytotoxicity assays with Dara-treated H929 MM cells. Whilst minor fratricide was observed in both mock and $CD38^{KO}$ NK cells when co-cultured with Dara-treated H929 target cells, there appeared to be less fratricide in $CD38^{KO}$ NK cells (**Figure 4.7B**). Furthermore, in a comparison of the ability of $CD38^{KO}$ NK cells in carrying out lysis of target cells, similar levels of specific cytotoxicity were observed between mock and $CD38^{KO}$ NK cells (**Figure 4.7C**).

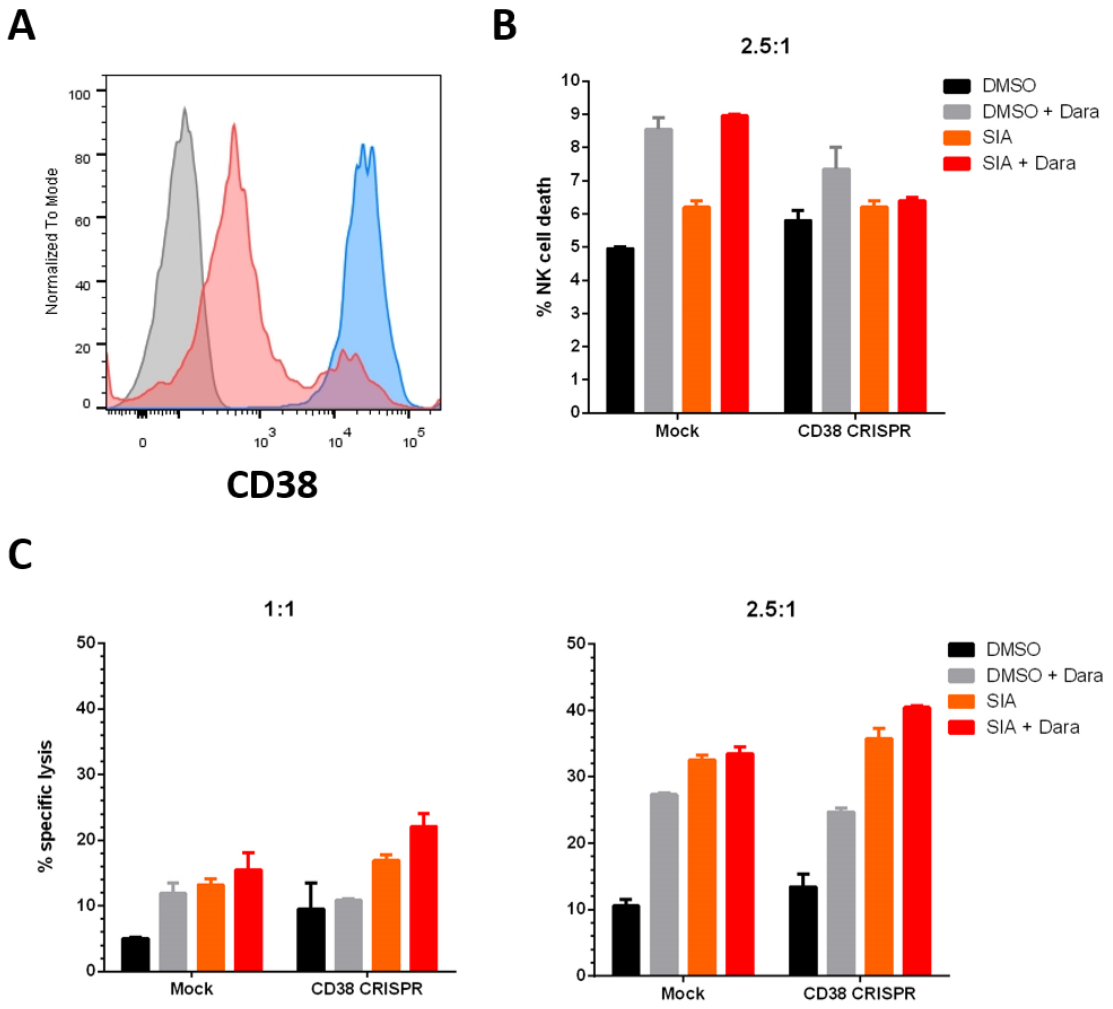


Figure 4.7 CD38^{KO} NK cells can carry out effective ADCC whilst reducing the risk of Daratumumab-induced fratricide

CD38 was targeted using CRISPR/Cas9 to generate CD38^{KO} NK cells. **(A)** CD38 expression levels were recorded in mock-electroporated (blue) and CRISPR/Cas9-targeted NK cells (red) to measure KO efficiency. **(B)** Fratricide in mock and CD38^{KO} NK cells was measured after co-culture with H929 pre-treated with SIA, Dara or both. **(C)** Efficacy of CD38^{KO} NK cells was measured against pre-treated H929 in co-culture assays and compared to mock-electroporated NK cells. n=1 for (**A**, **B**, **C**), graphs in **B**, **C** represent mean specific lysis of H929 + SEM from n=1 cytotoxicity assay where each condition was assessed in duplicate.

4.5 Discussion

Whilst hypersialylation has been demonstrated to inhibit NK cell function in different cancer types through Siglec-Siglec ligand interactions, the impact of a dense layer of sialic acids on the tumour cell surface cannot be discounted when considering alternate NK cell-mediated functions, such as ADCC. Previous studies have shown that, upon desialylation, increased BCMA expression was detected on the MM cell surface [363]. Additionally, it has been shown that NKG2D Fc chimera binding to breast cancer cells was increased upon desialylation, indicating an increased presence of ligands MIC A/B and ULBP-1-6 on the cancer cells [305]. This conceived the hypothesis that hypersialylation may also mask ligands for other potential therapeutic target antigens, particularly glycoproteins such as CD38 and SLAMF7 which could themselves be sialylated.

In work carried out for this chapter, desialylation of MM cells resulted in the increased detection of CD38 on primary MM cells and MM cell lines. Additionally, a combination of desialylation with Dara treatment combined the benefit of desialylation in enhancing NK cell cytotoxicity with ADCC to elicit a potent anti-MM response by expanded $CD16^{+}$ NK cells. Results also showed that CD38 can be upregulated on MM cells using ATRA which, combined with desialylation and Dara, resulted in strongly enhanced NK cell-mediated lysis of $CD38^{low}$ MM cell lines. Taken together, these data demonstrate that desialylation can be combined with a frontline existing therapy for both ND and RR-MM patients to enhance the therapeutic effect. In this discussion the results of these findings and their novelty and implications are discussed.

4.5.1 Desialylation of the MM cell surface increases detection of CD38, as determined by flow cytometry

Upon desialylation of both MM cell lines and primary MM cells, a consistent and reproducible increase in the detection of CD38 was observed by flow cytometry compared to control MM cells treated with GLYCO or DMSO. The word detection is used here instead of expression, to prevent the interpretation from this sentence that desialylation actively upregulates CD38 expression. CD38 is a glycoprotein and may itself be terminated with sialic

acids which physically prevent CD38 antibodies bind to their recognized epitope. However, it is also possible that the density of sialic acids expressed on the MM cell surface may prevent CD38-CD38 antibody binding, and that CD38 may not be itself sialylated. It is unlikely that CD38 expression is upregulated upon desialylation, as the immediate nature of NEURA treatment (45 minutes) likely doesn't allow sufficient time for upregulated CD38 expression to occur on the cell surface. Therefore, it is more likely that CD38 is not fully exposed to anti-CD38 antibodies due to physical hinderance caused by sialic acid expression, or possibly by the sialylation of CD38 itself. While it was previously observed that NEURA treatment can be toxic to MM1S MM cells, in this chapter SIA was also used to desialylate MM cells, resulting in enhanced CD38 detection. Considering work done by other groups in combing sialidase enzymes to antibodies, or loading nanoparticles with sialyltransferase inhibitor, promising potential strategies to desialylate MM cells and expose CD38 to therapeutic anti-CD38 moAbs such as Dara do exist and warrant further investigation in order to develop such a strategy for the desialylation of MM cells in the TME [305, 314, 366]. Finally, it is worth noting that upon desialylation with either NEURA or SIA a minor, yet consistent and reproducible increase, in detected BCMA by flow cytometry was observed, similar to the results shown by Huang et al. (**Figure 4.2**) [363]. Observing the same results in this project allowed confident interpretation of the results obtained when the expression of antigens targeted by moAb-based immunotherapies such as CD38, SLAMF7 and MUC-1 were analysed on sialylated and desialylated MM cells.

The discovery that detection of CD38 upon desialylation is increased is extremely significant due to the use of anti-CD38 therapeutic moAbs, such as Dara, in the clinic. A novel strategy combining targeted desialylation of the MM cell surface with anti-CD38 moAbs could be used to stimulate enhanced ADCC and cytotoxicity mediated by NK cells, delivering a potent anti-MM response. This is discussed in detail in **4.5.2**

4.5.2 Desialylation can be combined with the anti-CD38 therapeutic moAb Daratumumab to enhance NK cell cytotoxicity

Previous studies have shown that desialylation can be combined with Trastuzumab to enhance ADCC-mediated by NK cells against breast cancer cells [305]. Therefore, desialylation of the MM cells in combination with Dara treatment was carried out in an attempt to enhance NK cell-mediated ADCC against MM cell lines. As observed in **Figure 4.5**, combining SIA with Dara resulted in strongly increased $CD16^+$ NK cell-mediated ADCC of $CD38^+$ H929 MM cells compared to Dara or SIA treatments alone. Expanded primary NK cells used in these assays were observed to express CD16, and thus the potential for ADCC was confirmed. However, the levels of CD16 expression varied across each expanded NK cell donor. Expansion of NK cells has previously been shown to resulted in downregulation of CD16 expression on NK cells expanded ex vivo for adoptive transfer in a clinically approved manner [367]. This represents a potential issue if desialylation were to be combined with an moAb such as Dara in the setting of adoptive NK cell transfer, potentially dampening the therapeutic response of the moAb. Despite downregulation of CD16, $CD16^+$ NK cells were still able to carry out potent ADCC against H929 MM cells, suggesting that minimal CD16 downregulation might only have a minor impact NK cell-mediated ADCC. As discussed previously, KHYG-1 NK cells have been genetically modified to express haCD16 using mRNA, resulting in potent ADCC of Dara-coated MM cells [233]. Should CD16 expression be lost on NK cells primed for adoptive transfer, mRNA-induced expression of CD16 could maximise ADCC mediated by NK cells. Alternatively, ADAM17 – a disintegrin and metalloprotease has been implicated in dampening NK cell-mediated ADCC by inducing CD16 shedding from NK cells. KO of ADAM17 using CRISPR/Cas9 has been observed to prevent shedding of CD16 and enhance NK cell-mediated ADCC against Burkitt's lymphoma [368]. This is discussed further in **5.1.2**. Therefore, should CD16 expression be reduced on NK cells expanded for adoptive transfer to cancer patients, there are mechanisms to restore or upregulate CD16 expression, thus allowing retained NK cell-mediated ADCC capacity.

The potential of creating an anti-CD38 antibody/sialidase conjugate to treat MM is promising, considering the success of the Trastuzumab-sialidase conjugate in treating breast cancer both in-vitro and in-vivo [305, 339]. With such a successful model to replicate, this

strategy could be rapidly reproduced and therefore could quickly provide a novel therapeutic treatment for in vivo studies, and potentially clinical trials, in MM.

4.5.3 ATRA treatment to upregulate CD38 expression can be combined with Daratumumab and desialylation to further enhance NK cell cytotoxicity

In order to escape Dara-mediated destruction, MM cells lose expression of CD38 as a mechanism of resistance [163, 361, 369]. To overcome this, ATRA has been used to upregulate CD38 expression on MM cells, in the process sensitizing them to Dara-mediated ADCC and CDC [162]. Furthermore, ATRA and Dara have been shown to display synergistic anti-MM activity [162]. ATRA upregulates CD38 on myeloid cells by interacting with retinoic acid-alpha receptor, and is used clinically to treat acute promyelocytic leukaemia [370, 371]. As no increased detection of CD38 on desialylated JJN3 MM cells or NK cell-mediated ADCC when JJN3 were treated with Daratumumab was observed, JJN3 MM cells were classified as JJN3^{low} and treated with ATRA to upregulate CD38 expression. As expected, ATRA treatment led to a significant upregulation of CD38 expression, as determined by flow cytometry. However, the exciting result was that JJN3 treated with both SIA and ATRA had significantly higher CD38 expression compared JJN3 treated with ATRA alone (**Figure 4.6A**). Despite the strong upregulation of CD38 induced by ATRA, it is therefore likely that the therapeutic potential of ATRA treatment would not be met in MM, due to the hypersialylated MM cell surface preventing maximal ADCC by NK cells. However, combining ATRA with a desialylating agent could expose more CD38 to anti-CD38 moAbs such as Dara, leading to enhanced NK cell-mediated ADCC.

4.5.4 CD38^{KO} NK cells retain the capacity to mediate ADCC of Daratumumab-treated MM cells

One of the drawbacks of using Dara as a treatment in MM is the uniform expression of CD38 on NK cells, leading to fratricide where NK cells target other NK cells decorated with Dara [372]. However, several studies have shown that NK cells lacking CD38, either through the use of CD38^{low} NK cell lines or CRISPR/Cas9 mediated knockout of CD38 in expanded NK cells

can minimize the risk of fratricide, elicit a more potent ADCC-mediated response against MM cells and MM cell lines treated with Dara, as well as resulting in a longer persistence of NK cells in a humanized murine model [233, 360, 373]. A trend for this was observed when *CD38* was targeted using CRISPR/Cas9 in expanded NK cells in this project. Efficient KO of CD38 on NK cells appeared to elicit a minor reduction in fratricide when mock and $CD38^{KO}$ NK cells were co-cultured with desialylated H929 treated with Dara. Furthermore, $CD38^{KO}$ NK cells appeared to have retained capacity to elicit Dara-induced ADCC, with comparable amounts of H929 cell death observed to be caused by both mock and $CD38^{KO}$ cells. It is worth noting that this assay was carried out n=1 times, however the data appearing to match previous observations suggests that $CD38^{KO}$ NK cells might elicit the most potent anti-MM response if desialylation were to be combined with anti-CD38 moAb treatment.

4.5.5 Interpretation

The potential for combining desialylation strategies with an existing frontline MM therapy is very promising. The impact of proposing an approach which, in-vitro at least, enhances Dara-induced NK cell-mediated ADCC of MM cells warrants further and more detailed research into this potential combinational treatment. Therapies combining ATRA treatment, anti-CD38 antibodies and desialylating agents also all represent avenues of approach to enhance NK cell anti-MM cellular therapy. Combining desialylation with Dara could represent a suitable approach in patients who have basally higher MM CD38 expression, whereas including ATRA might be most beneficial to patients who have previously been treated with Dara and who may therefore lack CD38 expressing MM cells. These therapies would all need to be established as safe in animal models before transition to trials or the clinic.

The work carried out in this chapter provides the rationale for many future studies, with the aim of enhancing NK cell anti-MM functions, specifically either cytotoxicity or ADCC. Additionally, in combination with the work carried out by Bertozzi et. al in establishing the premise of using desialylation to enhance NK cell-mediated ADCC, this work also serves to demonstrate the immunotherapeutic potential of using targeted desialylation of the MM cell surface. Future studies that could stem from this research are discussed in **6. Discussion**.

CHAPTER 5: CRISPR/Cas9-mediated interruption of checkpoint inhibitor expression enhances NK cell anti-MM functions

5.1 Introduction

5.1.1 Targeting immune checkpoints to enhance NK cell anti-cancer functions

As discussed in **1.2.6**, targeted interruption of interactions between NK cell immune checkpoints and their cognate ligands has been shown to enhance NK cell functionality against several cancer types. While this approach was initially carried out in T cells, NK cells have recently been attracting attention as potential benefactors of checkpoint inhibitor disruption to induce cancer therapy. moAbs such as IPH2101 and lirilumab, targeting the classic inhibitory KIR receptor KIR2DL1/2/3, are tolerable by MM patients as well as being highly efficient at binding KIR2D, however the use of IPH2101 as a monotherapy did not yield impressive results in MM patients [374]. Furthermore, results of this trial demonstrated that IPH2101 treatment resulted in decreased expression of KIR2D receptor on NK cells, reduced responsiveness of NK cells was observed [375]. However, in combination with the IMiD lenalidomide, IPH2101 reported promising results, with treated MM patients demonstrating survival-free progression of up to 24 months [376]. Additionally, lirilumab was also shown to enhance the efficacy of Elotuzumab, where a combination of the two drugs gave promising results in mouse models and enhanced Elotuzumab-mediated ADCC towards MM cells [377, 378]. Furthermore, IPH2101 has shown promising potential against AML, emphasising the potential of targeting NK cell checkpoints in multiple cancer types [379].

Targeting leukocyte Ig-like receptors (LIRs) on NK cells has also shown promise. In particular, LIRB1 (also known as ILT2) has been observed to inhibit NK cell cytotoxicity against target cancer cells in both NK cell lines and primary NK cells [380, 381]. Blockade of LIRB1, enhanced clearance of CLL cells in patients when combined with lenalidomide [382]. Furthermore, studies have shown promising results when the inhibitory receptor NKG2A was targeted for blockade. Poor prognosis has been observed upon upregulation of the NKG2A ligand, which has been reported in several cancer types including CLL, colon, lung, head and neck cancer [383]. The use of the blocking antibody Monalizumab - targeting NKG2A - was reported to be safe and tolerable in haematological malignancies and displayed promising results by improving NK cell cytotoxicity against CLL cells in vitro [382].

More recently, attention has focused on alternative novel immune checkpoints such as PD-1, CD96 and Siglec-7/9. PD-1 is an inhibitory receptor binding PD-L1, normally expressed by macrophages, dendritic cells and activated T and B cells [168]. However, cancer cells have been documented to express PD-L1 as a mechanism of resistance to T and NK cell-mediated immunosurveillance [384]. Studies have also shown that IFN-γ secreted by NK cells and T cells can upregulate PD-L1 on cancer cells, promoting cancer cell survival [385]. Furthermore, combining Elotuzumab and anti-PD-1 blocking antibodies promoted activation of tumour infiltrating NK and T cells as well as enhancing intra-tumoral cytokine and chemokine release [348]. Additionally, approaches combining anti-CD38 and anti-PD-1 antibodies have also shown promise, with the combination proving effective in a MM murine model as well as demonstrating success in regulating lung cancer growth and breast cancer metastasis in murine models [386, 387]. However, conflicting data around the success of PD-1 immune checkpoints in clinical MM treatment and the inconsistent expression of PD-L1 on MM cells suggests that PD-1 blockade might not be an ideal immunotherapeutic approach against MM [388]. Therefore, there is a necessity to investigate other promising immune checkpoints which may be hijacked by MM to evade NK cell-mediated immunosurveillance. Recently, focus has turned to immune checkpoints which have not been previously fully investigated. For example, with the recent implication of Siglec-7 and Siglec-9 in regulating NK cell functions against several cancer types, including AML and breast cancer, inhibitory Siglec receptors represent a novel target in MM [190, 304, 320, 389]. However, while blocking antibodies have been used to inhibit Siglec-Siglec ligand interactions, this is the extent of the work carried out in targeting Siglec-7 to enhance NK cell anti-cancer activity using an approach that could be translatable to the clinic to treat MM.

Interest in the disruption of immune checkpoints in MM is not solely focused on Siglecs. Alternate promising immune checkpoints exist, such as CD96 and TIGIT [172]. CD96 and TIGIT are inhibitory receptors, expressed by both T cells and NK cells. CD96 and TIGIT compete for the same preferred ligand: CD155 and to a lesser extent CD111, CD112, CD113 and Nectin-4, with CD96 binding to CD155 with less affinity than TIGIT [390, 391]. The expression of CD96 and TIGIT on NK and T cells has also been demonstrated to be upregulated in cancer resulting in this axis becoming highly promising as a novel target of cellular therapies [172]. Currently. TIGIT has been the subject of more research than CD96. In MM, the function of TIGIT has

predominantly been examined in T cells, notably $CD8^+$ T cells where TIGIT blockade prevented T cell exhaustion, utilised by MM cells to facilitate immune-escape [392]. Furthermore, blockade of TIGIT reversed exhaustion of NK cells in murine models and in colonic cancer patients, resulting in a potent anti-tumour response [218]. While the role of TIGIT as an inhibitory receptor on NK and T cells is well understood, conflicting data surrounds the role of CD96 in MM. Studies have shown CD96 to be both an activating and inhibiting NK cell receptor, although of late more evidence has been published presenting CD96 as an inhibitory receptor [221, 222, 393]. However, while the role of CD96 has predominantly been studied in relation to its impact in regulating cytokine release, the role of CD96 has not been studied with regards to NK cell cytotoxicity.

There are therefore, a multitude of promising immune checkpoints expressed by NK cells which could be targeted to generate novel NK cell-based immunotherapies to treat MM. However, the most suitable means of targeting these immune checkpoints remains to be fully explored. While the vast majority of the research into immune checkpoints has been carried out using blocking antibodies, there has been little investigation into generating genetically modified NK cells lacking inhibitory receptors hijacked by cancer cells to promote immune-escape. Adoptive transfer of such genetically modified NK cells represents a promising novel immunotherapeutic strategy, which could offer significant responses for cancer patients which may have hypofunctional NK cells, such as MM BM-residing NK cells which have elevated Siglec-7 expression as observed in this project. Several strategies have previously been used to modify NK cells including retroviral transduction where IL-2 complementary DNA (cDNA) was used to generate NK cells capable of endogenous secretion of IL-2, increasing their persistence in vivo [394]. More recently, strategies using viral vectors have been used to generate CAR-expressing NK cells as well as NK cells with cytokine-encoding genes, such as IL-2 and IL-15 [395]. However, the efficacy of viral vectors can be an issue in NK cells. Furthermore, viral vectors are used primarily for the delivery of genetic material to be expressed by NK cells (such as IL-2 and IL-15), while there has been little research done into using viral-based delivery systems to prevent or disrupt gene expression. However, thanks to recent advancements in science, targeted disruption of genes in NK cells is now possible using the clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system.

5.1.2 CRISPR/Cas9 and its use in NK cells

CRISPR represents one of the most significant advancements in science in recent times. CRISPR originates as part of the prokaryotic adaptive immune system, working to eliminate foreign nucleic acids from within host bacteria [(396)]. Using a spacer sequence, CRISPR RNA (crRNAs) are transcribed by ribonucleic acid (RNA) polymerase. These crRNAs then recognise, and bind with high affinity, to complementary sequences expressed on the foreign DNA. When bound, the crRNA acts as a guide, recruiting Cas9 enzymes which induce a double stranded break in the DNA, cleaving and degrading the foreign DNA and abrogating the threat to the bacteria [(397)]. The genetic blueprint for Cas enzymes is expressed generally adjacent to the CRISPR, however while several Cas genes have been observed, the first Cas observed to have nuclease activity was Cas9, discovered in *Streptococcus thermophilus* by Alexander Bolotin in 2005 [(398)]. Cas9 contains two endonuclease domains: RuvC and HNH. The HNH domain cleaves the target strand of DNA, while RuvC cleaves the non-target sequence [(399)]. Once the potential of CRISPR was realised, scientists immediately began to experiment with this technique to try and combat disease, generate more productive crop yields and eliminate pathogens.

In an attempt to treat disease, the CRISPR/Cas9 system was developed to target genes of interest for KO. Using pre-designed sgRNAs, a gRNA:Cas9 complex is formed between the gRNA and Cas9. After transfection to the target cell using electroporation or lipofection, the complex can recognize and bind the complementary sequence coded by the gRNA. Once bound to the target gene, a double stranded break is created. Following the generation of the double stranded break, one of two main genetic repair mechanisms are activated: the non-homologous end joining (NHEJ) pathway and the homology directed repair (HDR) pathway. While the HDR pathway is highly efficient, NHEJ is the more prominent pathway within human cells and despite being highly prevalent, the process is highly error-prone [(400)]. This can result in small single nucleotide insertions or deletions, resulting in the way the triplet code of the gene is read, often leading to the generation of a malfunctional protein. With this, genetic interruption of gene expression became a possibility.

Increased success in using CRISPR/Cas9 to enhance NK cell activities has recently been reported. The publishing and availability of detailed protocols have allowed

researchers to access and edit NK cells using CRISPR/Cas9 with relative ease, producing exciting data in a short period of time [401, 402]. PB-derived NK cells were targeted with CRISPR/Cas9 to KO a disintegrin and metalloprotease 17 (*ADAM17)* and programmed cell death protein 1 (*PDCD1)* genes. ADAM17 rapidly cleaves CD16 from the NK cell surface upon NK cell activation, and can limit the efficacy of immunotherapeutic approaches centralised around NK cell-mediated ADCC. KO of ADAM17 in NK cells resulted in significantly higher levels of CD16 on NK cells, comparable with the levels obtained when an ADAM17 inhibitor was used. Furthermore, ADAM17 KO NK cells were capable of ADCC in the presence of rituximab, with degranulation and IFN-γ secretion also strongly enhanced compared to NK cells transfected with just Cas9 alone [368]. In the same study, PD-1 was targeted for KO and PD-1 KO NK cells displayed increased cytotoxic capacity against a range of cancer cell lines (including prostate, AML, CLL and ovarian cancer), again compared to NK cells transfected with Cas9 alone [368]. In the case of both ADAM17 and PD-1, high KO efficiency was observed. Excitingly, these KO cells were cultured to clinically relevant numbers without losing efficacy of KO, highlighting the potential of this novel strategy to generate potent NK cells suitable in cancer therapies [368]. Additionally, it has been demonstrated that CD16 expression in NK-92 cells can be re-activated by Cas9-mediated promoter insertion, leading to the expression of CD16 on NK-92 cells, which then were able to carry out Herceptin-induced ADCC against breast cancer cell lines. Interestingly, not only was CD16 expression re-activated in NK-92 cells lines, but the same process was applied to allow the re-expression of the activating DNAM-1 receptor. Expression of DNAM-1 resulted in highly increased NK cell-mediated lysis of breast and cervical cancer cell lines [403]. CRISPR/Cas9 therefore, represents a promising mechanism of generating genetically engineered potent NK cells lacking checkpoint inhibitory receptors hijacked by cancer cells.

In this chapter Siglec-7 and CD96 were targeted using the CRISPR/Cas9 system to generate Siglec-7^{KO} and CD96^{KO} NK cells, and the impact of abolished Siglec-7 and CD96 expression on NK cell responses against MM cell lines expressing ligands for both receptors was recorded. Furthermore, the impact of CD96^{KO} on cytokine release as well as cytotoxicity was assessed by quantifying the presence of both IFN-γ and TNF-α in NK cells co-cultured with target MM cells after CD96^{KO}. As a suitable control for studies into CD96, TIGIT^{KO} NK cells were generated using CRISPR/Cas9 and used in cytotoxicity assays in parallel with CD96^{KO} NK cells.

5.2 Hypothesis and Objectives

5.2.1 Hypothesis

I. Disruption of Siglec-7 expression using CRISPR/Cas9 on NK cells will enhance NK cell-mediated cytotoxicity against Siglec-7L^{+} MM target cells

II. Disruption of CD96 expression using CRISPR/Cas9 on NK cells will enhance NK cell-mediated cytotoxicity against CD155^{+} MM target cells

5.2.2 Objectives

I. Generate Siglec-7^{KO} and CD96^{KO} NK cells with a high level of KO and minimal toxicity using a multi-guide-based approach

II. Carry out cytotoxicity assays with Siglec-7^{KO} and CD96^{KO} NK cells and cytokine release assays with CD96^{KO} NK cells to determine the effect of Siglec-7^{KO} or CD96^{KO} on NK cell-mediated cytotoxicity or TNF-α and IFN-γ secretion

5.3 Study Design

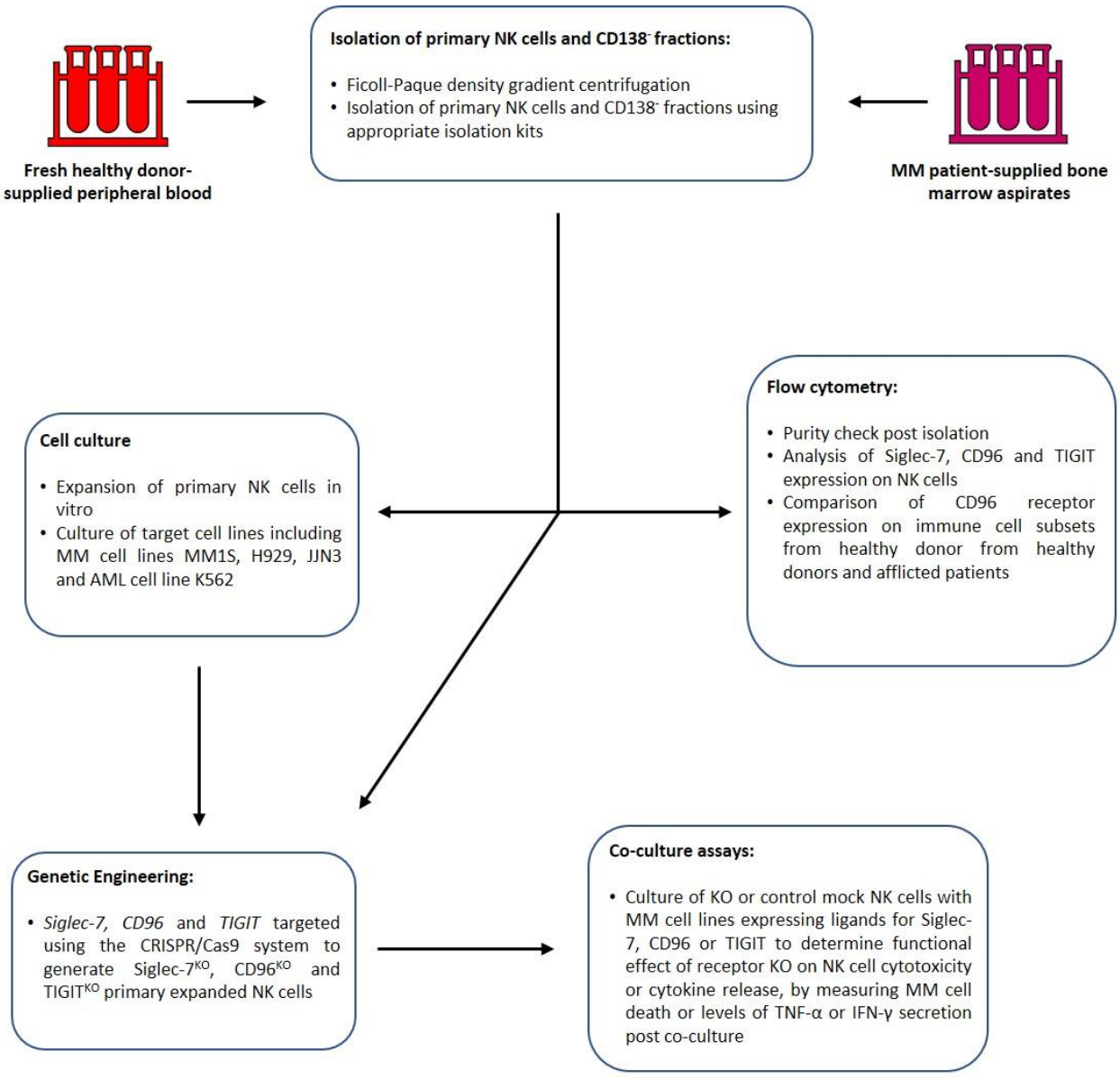


Figure 5.1 Schematic representation of the experimental work carried out in this chapter

An overview of the workflow carried out in this chapter. The expression of several promising NK cell checkpoint receptors was measured after isolation of primary NK cells and throughout NK cell expansion. Siglec-7, CD96 and TIGIT were targeted for KO using the CRISPR/Cas9 system and single/multi guide approaches. Efficacy of KO was measured by flow cytometry, comparing KO NK cells to mock electroporated controls. The functional effect of Siglec-7, CD96 and TIGIT KO was measured using co-culture assays with MM cell lines expressing ligands for Siglec-7, CD96 and TIGIT and analysing the effect of KO on expanded primary NK cell cytotoxicity and cytokine release post co-culture

5.4 Results

5.4.1 Genetic disruption of *Siglec-7* resulting in cell surface KO of Siglec-7 enhances NK cell-mediated cytotoxicity against Siglec-7L^{+} MM cell lines

Three separate sgRNAs were chosen for initial experiments and were designed to target an early region of the first exon of *Siglec-7*. The gRNA sequences, protospacer adjacent motif (PAM) sites, strand targeted as well as off-target and on-target scores are detailed in **Table 5.1**.

Table 5.1 Specific details of three designed gRNAs used for initial investigations into CRISPR/Cas9-mediated KO of Siglec-7

Guide #	Sequence	Strand	PAM	On-target score	Off-target score
1	GAGTTCCGTGACCGTGCAAG	+	AGG	65	90
2	AGTCAGTCTGGCTGTCCACT	-	GGG	53	25
3	CGGAACCAGTAGCCATGAAC	-	TGG	69	62

As an exploratory assay, the sgRNAs detailed above were used to target *Siglec-7* in IL-2 activated NK cells isolated from the PB of n=1 healthy donor. All sgRNAs resulted in decreased the number of Siglec-7^{+} NK cells overall, however Guide #2 was observed to have the highest KO efficiency and was chosen to be used in follow-up assays to target *Siglec-7* in expanded NK cells (**Figure 5.2)**.

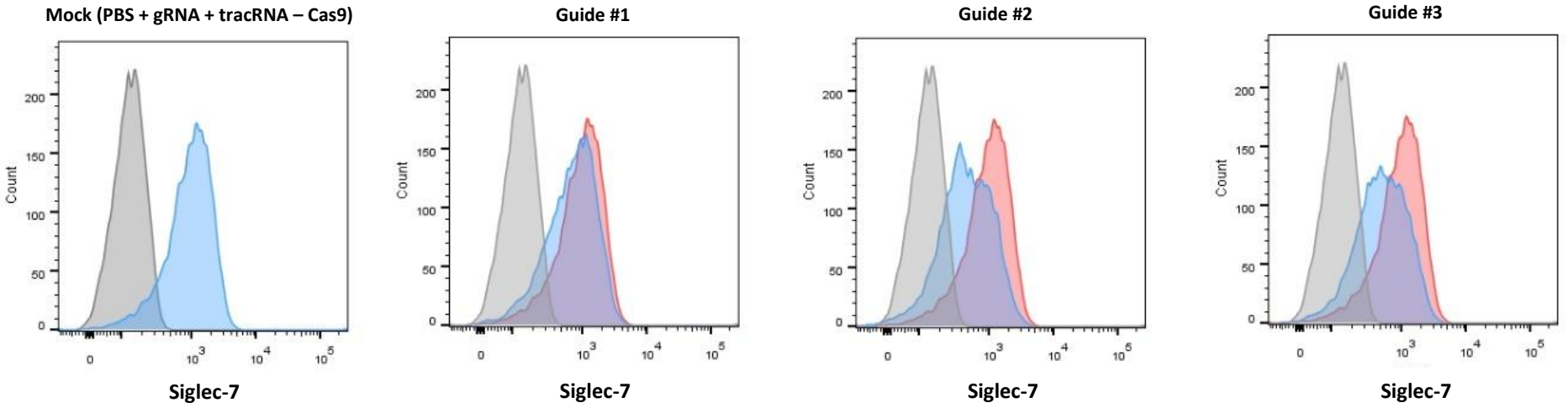


Figure 5.2 Analysis of Siglec-7 expression after genetic interruption of *Siglec-7* using the CRISPR/Cas9 system and an sgRNA. *Siglec-7* was targeted in IL-2 activated primary NK cells using the CRISPR/Cas9 system and three sgRNAs or sgRNA without Cas9 as a mock control. Expression of Siglec-7 was measured on viable $CD56^{+}/CD3^{-}$ NK cells consistently after electroporation. Graph represents the data from n=1 preliminary donor, where Siglec-7 expression was recorded six days after transfection. In "Mock", wild-type Siglec-7 expression for this donor is indicated in a blue histogram, whereas for each sgRNA basal Siglec-7 expression from "Mock" is shown in red while expression of Siglec-7 in NK cells targeted by the respective guides is shown in blue histograms. Unstained NK cells are displayed as grey histograms. n=1.

Guide #2 was used to target *Siglec-7* in primary expanded NK cells and resulted in approximately 50% complete KO, as determined by flow cytometry (**Figure 5.3A,B**). Minimal toxicity was observed towards NK cells from electroporation, facilitated by the clinically approved MaxCyte GT transfection system (data not shown). Siglec-7 expression was recorded daily from day 0 post-transfection until day 7 post-transfection. Analysis of Siglec-7 expression across this time period demonstrated that the highest levels of KO were observed between day 6 and day 8 post-transfection. However, when co-cultured with Siglec-7L^{+} MM cell lines H929 and JJN3 there was no observable increase in lysis of H929 or JJN3 by Siglec-7^{KO} NK cells was observed when compared to Mock controls (**Figure 5.3C**).

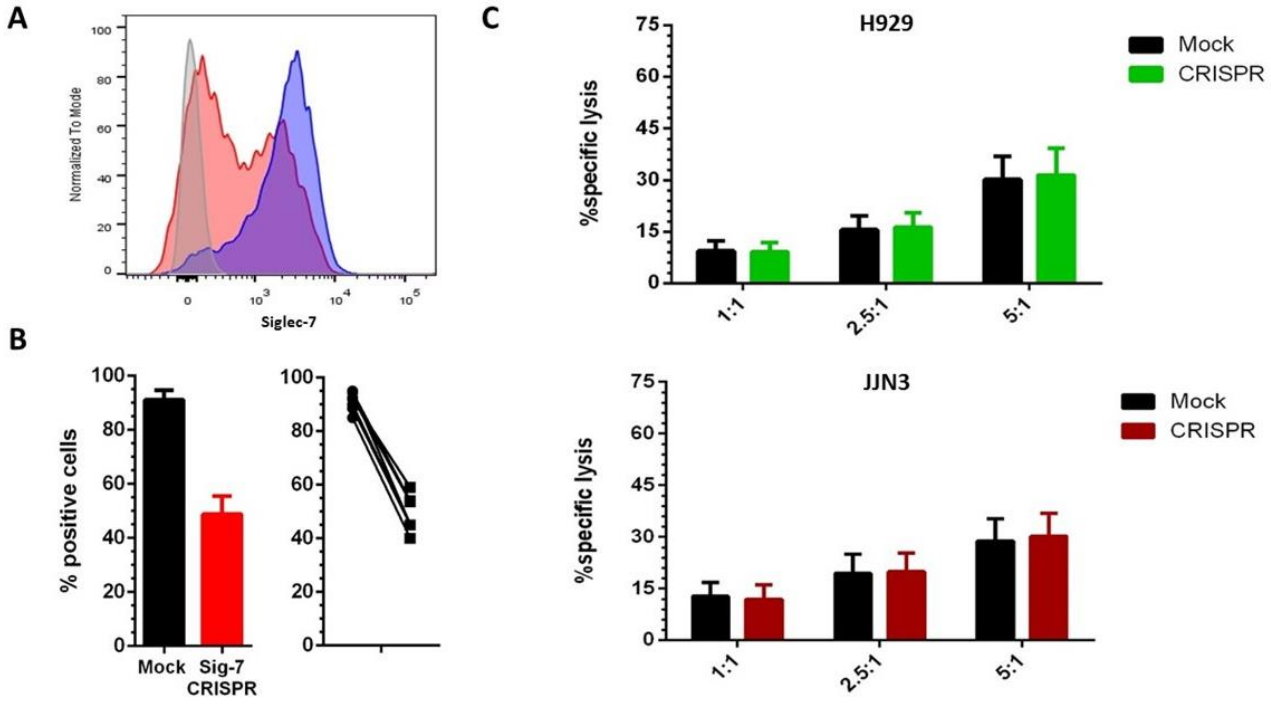


Figure 5.3 Partial KO of Siglec-7 on expanded primary NK cells does not enhance cytotoxicity against Siglec-7L^{+} MM cell lines. *Siglec-7* was targeted in expanded primary NK cells using sgRNA Guide #2 **(A)** KO of Siglec-7 was determined by flow cytometry and presented as a comparison in histogram format, where *Siglec-7* was targeted using CRISPR/Cas9 (red), or a PBS Mock control (blue). **(B)** KO of Siglec-7 across n=7 independent healthy donor-derived expanded NK cells was measured and combined. **(C)** Siglec-7^{KO} or Mock NK cells were co-cultured with Siglec-7L^{+} MM cell lines H929 and JJN3 and mean specific lysis of MM cells + SEM was determined and compared. Graph in **A** represents n=1 repeat of Siglec-7 expression measurement on Mock or CRISPR-targeted NK cells used subsequent cytotoxicity assays. Graph in **B** represents combined and individual data demonstrating Siglec-7 KO across n=7 independent donors. Graph in **C** represents mean specific lysis of both JJN3 and H929 MM cells when co-cultured with Mock or Siglec-7^{KO} NK cells in cytotoxicity assays at several indicated E:T ratios. n=1 for **A**, n=7 for **B** and **C**. Data in **C** analysed using Student's paired *t*-test.

In an attempt to enhance the efficacy of Siglec-7 KO in expanded by NK cells *Siglec*-7-targeting pooled sgRNAs were purchased from Synthego as part of the Gene KO KIT V2. Instead of using a single sgRNA approach, the Gene KO KIT V2 uses three pooled sgRNAs targeting a similar region within the initial coding exon of *Siglec-7*. The sgRNA sequences used in this kit to target *Siglec-7* in this project are detailed in **Table 5.2**. Double-stranded breaks induced by the three separate gRNAs within a close vicinity result in a fragment deletion of the exon, thus securing a higher level of genetic interruption than a single nucleotide polymorphism induced by a sgRNA approach would yield. Using the Gene KO KIT V2 resulted in a considerably higher level of complete Siglec-7 KO, as determined by flow cytometry, than observed using the sgRNAs detailed in **Table 5.1** (**Figure 5.4A,B**).

When Mock and Siglec-7^{KO} NK cells were co-cultured with Siglec-7L^{+} MM cell lines H929 and JJN3, consistent increases in the specific lysis of Siglec-7L^{+} MM cells by Siglec-7^{KO} NK cells were observed, compared to Mock NK cells. The increases in cytotoxicity mediated by Siglec-7^{KO} NK cells varied across the n=7 donors used, and ranged from increases of 5% specific lysis to 25% specific lysis (**Figure 5.4B**).

Table 5.2 The sequences of the three pooled sgRNAs used to target *Siglec-7* for genetic disruption using the CRISPR/Cas9 system. * - indicate 2'-O-methyl analogues and 3'-phosphorothiate internucleotide linkages. No PAM site, strand to be targeted or on and off-target scores were provided for these gRNAs.

Guide #	Sequence
1	A*G*G*ACAGAAGAGUAACCGGA
2	G*A*C*ACACAUGCCCUCUUGCA
3	G*G*G*CAGGGAAUGAUAUAAGC

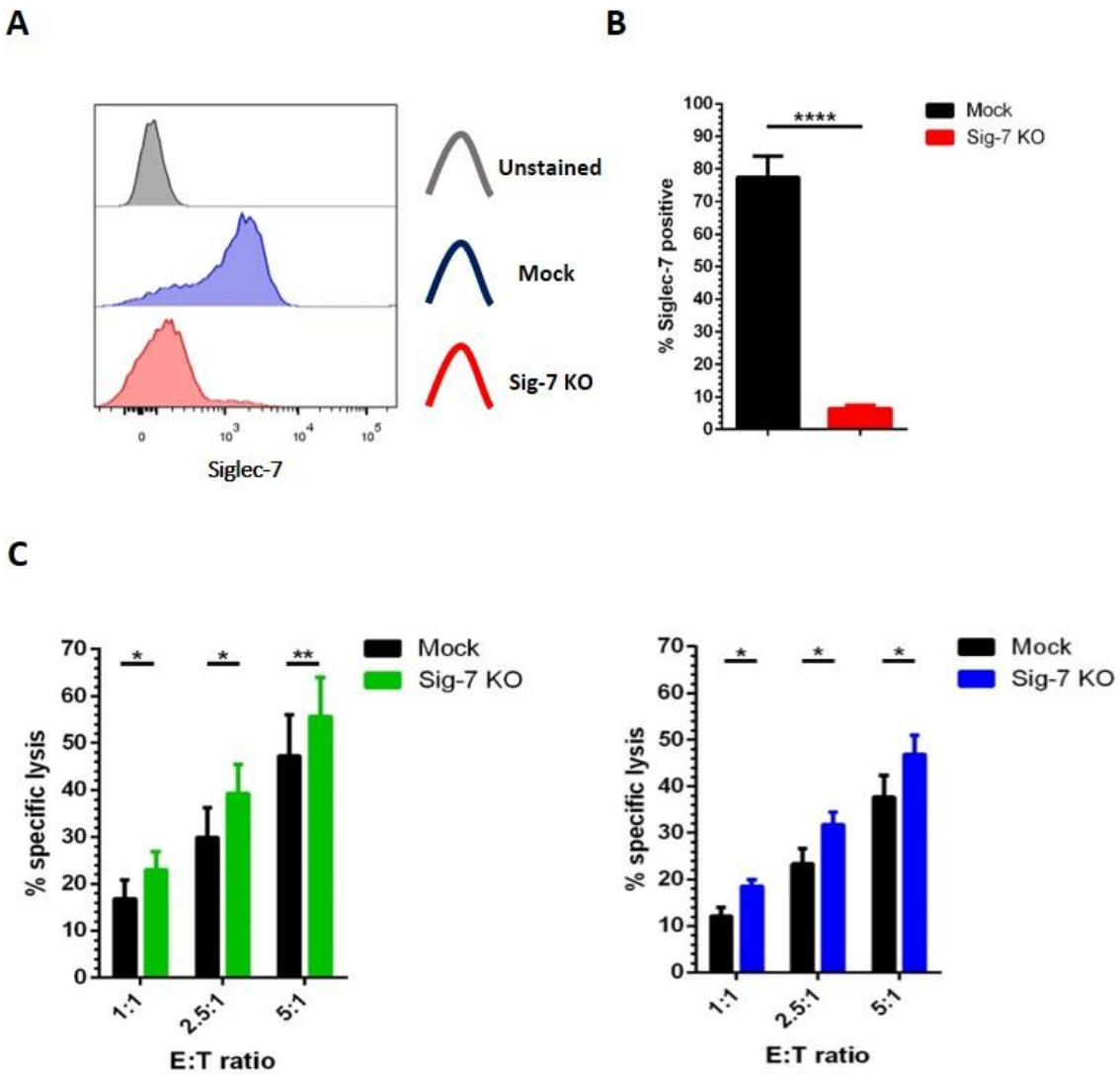


Figure 5.4 Efficient KO of Siglec-7 on primary expanded NK cells results in enhanced cytotoxicity. against Siglec-7L^{+} MM cell lines. (A,B) Expression of Siglec-7, as determined by flow cytometry, was measured on mock electroporated NK cells and NK cells transfected with three pooled sgRNAs targeting *Siglec-7* and Cas9 enzyme and displayed as (**A**) a n=1 representative histogram demonstrating Siglec-7 KO readout in flow cytometry and (**B**) combined levels of Siglec-7 KO across n=7 independent healthy donor-derived expanded NK cells. **(C)** Mock and Siglec-7^{KO} NK cells were co-cultured with Siglec-7L^{+} MM cell lines H929 and JJN3 in cytotoxicity assays for 4 hours after which specific lysis of MM was calculated and compared. Data in **C** represents mean specific lysis calculated after n=7 independent repeats of the assay + SEM. Data in **B** and **C** was analysed using Student's paired *t*-test, * - $p < 0.05$, ** - $p < 0.01$, **** - $p < 0.0001$.

5.4.2 Siglec-7^{KO} NK cells retain the capacity to mediate ADCC in the presence of Daratumumab, and can be combined to further enhance cytotoxicity of CD38^{+}/Siglec-7L^{+} MM cells

In **Chapter 4**, it was demonstrated that MM cell desialylation (including removal of sialic acid-derived Siglec-7L), can be combined with Dara treatment to enhance NK cell-mediated lysis of CD38^{+}/Siglec-7L^{+} H929 MM cells. Having observed consistent and reproducible increases in NK cell-mediated lysis of Siglec-7L^{+} MM cells when co-cultured with Siglec-7^{KO} NK cells (**Figure 5.4B**), Dara treatment was subsequently combined with Siglec-7^{KO} NK cells to observe the effect of Siglec-7 KO on NK cell capacity to mediate ADCC. CD16 expression was measured on Siglec-7^{KO} NK cells, and was comparable to the expression of CD16 on mock electroporated NK cells (**Figure 5.5A**). H929 were chosen as an appropriate target MM cell line for this assay due to the high expression of both CD38 and Siglec-7L.

Siglec-7^{KO} NK cells in combination with Dara treatment of H929 resulted in statistically significant increased lysis of H929 MM cells compared to Dara treatment in combination with mock electroporated NK cells, or Siglec-7^{KO} NK cells alone (**Figure 5.5B**). In line with the observations in **Figure 5.4C**, Siglec-7^{KO} cells demonstrated increased cytotoxicity against CD38^{+}/Siglec-7L^{+} H929 compared to mock electroporated NK cells (**Figure 5.5B**).

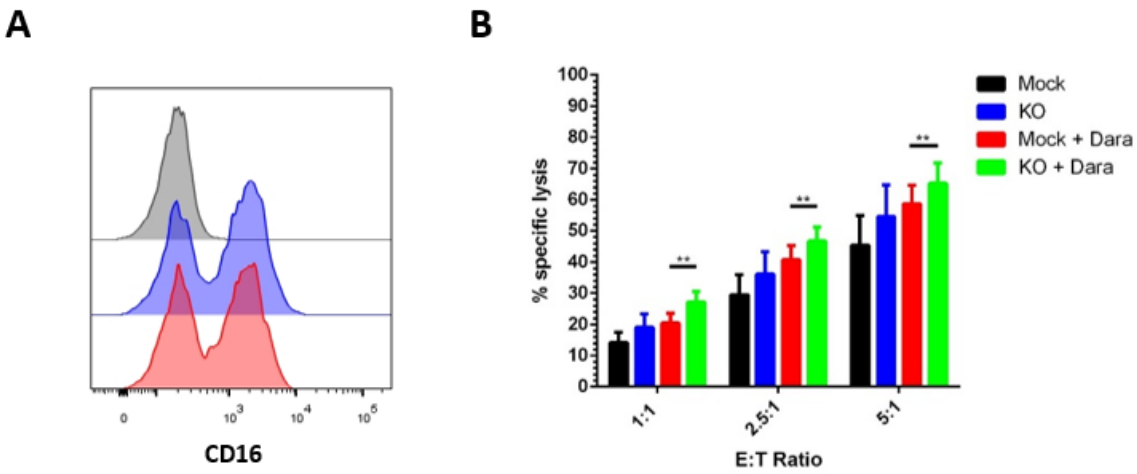


Figure 5.5 Siglec-7^{KO} NK cells in combination with Daratumumab elicit more potent cytotoxicity of CD38^{+}/Siglec-7L^{+} MM cells than Siglec-7^{KO} NK cells or Daratumumab treatment alone. (A) CD16 expression was measured on mock electroporated and Siglec-7^{KO} NK cells to confirm capacity for ADCC and that Siglec-7^{KO} did not impact CD16 expression. **(B)** Mock electroporated or Siglec-7^{KO} NK cells were generated and co-cultured with CD38^{+}/Siglec-7L^{+} H929 MM cells pre-treated with either PBS or Dara after which specific lysis of H929 was recorded and compared. Histogram in **A** is a single representative of n=6 independent biological repeats. Data in **B** represents mean specific lysis of n=6 independent biological repeats of the cytotoxicity assays + SEM. Data in **B** analysed using one-way ANOVA, ** - $p < 0.01$.

5.4.3 CD96 is an inhibitory NK cell receptor, and KO of CD96 enhances cytotoxicity and cytokine release against CD155^{+} MM cell lines

As discussed in **5.1**, while attention has focused on the role of lesser-known checkpoint receptors in inhibiting NK cell cytotoxicity against cancer cells, this interest is not limited to Siglec-7 and Siglec-9. CD96 has been reported to be both an activating and inhibitory NK cell receptor. To elucidate fully its role in regulating NK cell functions against MM, *CD96* was targeted using CRISPR/Cas9 to generate CD96 KO NK cells. Having observed efficient KO of Siglec-7 using the pooled sgRNA approach, three pooled gRNAs targeting *CD96* were purchased from Synthego, again as part of the Gene KO KIT V2. The sequence of these gRNAs is detailed in **Table 5.3**.

Table 5.3 The sequences of the three pooled sgRNAs used to target *CD96* for genetic disruption using the CRISPR/Cas9 system. * - indicate 2'-O-methyl analogues and 3'-phosphorothiate internucleotide linkages. No PAM site, strand to be targeted or on and off-target scores were provided for these gRNAs.

Guide #	Sequence
1	A*G*G*CACAGUAGAAGCCGUAU
2	C*G*U*GCAGAUGCAAUGGUCCA
3	G*A*A*AAUGUUUAUGCUACACU

At the same time as targeting CD96, a relevant control to confirm the results obtained in these assays was to target the known inhibitory NK cell receptor TIGIT for KO using CRISPR/Cas9. TIGIT and CD96 bind to the same common ligand – CD155, expressed by MM cells. By targeting TIGIT for KO using CRISPR/Cas9, a suitable comparable set of results was obtained and could be compared to the data generated using CD96^{KO} NK cells in functional assays to confirm the role of CD96 in regulating NK cell functions against CD155^{+} MM cells. As with Siglec-7 and CD96, three pooled sgRNAs targeting *TIGIT* were purchased from Synthego as part of the Gene KO KIT V2. The sequence of these gRNAs is detailed in **Table 5.4**.

Table 5.4 The sequences of the three pooled gRNAs used to target *TIGIT* for genetic disruption using the CRISPR/Cas9 system. * - indicate 2'-O-methyl analogues and 3'-phosphorothiate internucleotide linkages. No PAM site, strand to be targeted or on and off-target scores were provided for these gRNAs.

Guide #	Sequence
1	G*G*C*CAUUUGUAAUGCUGACU
2	A*U*G*UCACCUCUCCUCCACCA
3	U*C*U*UCCCUAGGAAUGAUGAC

Strong expression of CD96 was observed on expanded primary NK cells, while only a subset of expanded primary NK cells expressed TIGIT (**Figure 5.7**). In **Chapter 3**, increased Siglec-7 and decreased Siglec-9 expression was observed on NK cells from MM patient-derived BMAs. Within the same immunophenotyping assay, CD96 expression was also recorded on BMA-derived NK cells compared to NK cells from the PB of healthy donors (**Figure 5.6**). Analysis revealed that CD96 expression is elevated on NK cells from the TME compared to NK cells from PB supplied by healthy donors (**Figure 5.6**). Using CRISPR/Cas9, genetic disruption of *CD96 and TIGIT* resulted in strong KO of CD96 and TIGIT, as determined by flow cytometry (**Figure 5.7**). Comparable with Siglec-7, maximum KO of both CD96 and TIGIT was recorded 6-7 days post transfection. No toxicity was observed to NK cells from the electroporation process, as observed when *Siglec-7* was targeted.

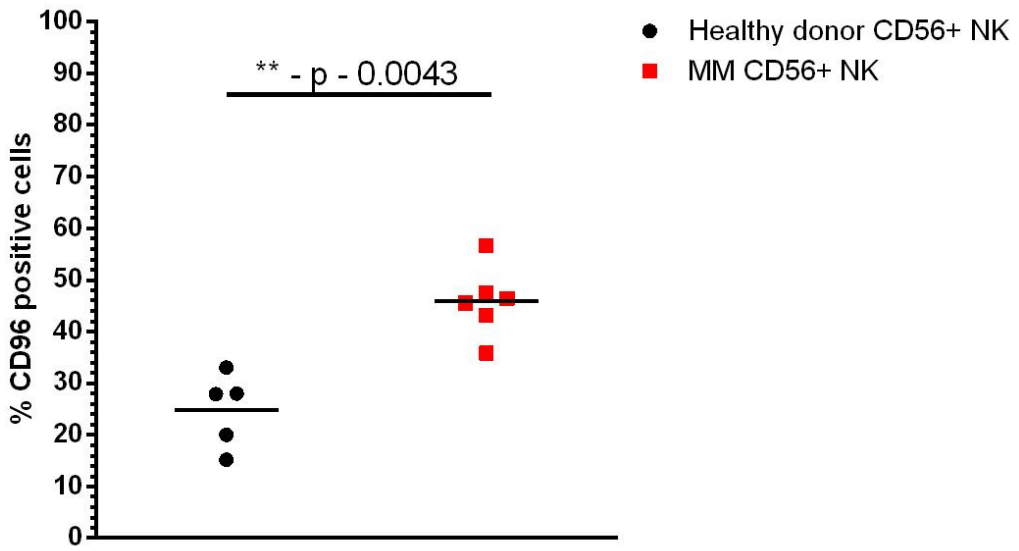


Figure 5.6 CD96 expression on MM patient BMA-derived NK cells is elevated compared to NK cells from the PB of healthy donors. Viable primary $CD56^{+}/CD3^{+}$ NK cells from either peripheral blood of healthy donors (black) or BMAs supplied by healthy donors (red) were screened for the expression of CD96. Graph represents mean CD96 expression on healthy donor or MM BMA-derived NK cells as well as individual values for each donor. n=5 for black, n=6 for red. Data analysed using Mann-Whitney unpaired t-test, ** - $p < 0.01$.

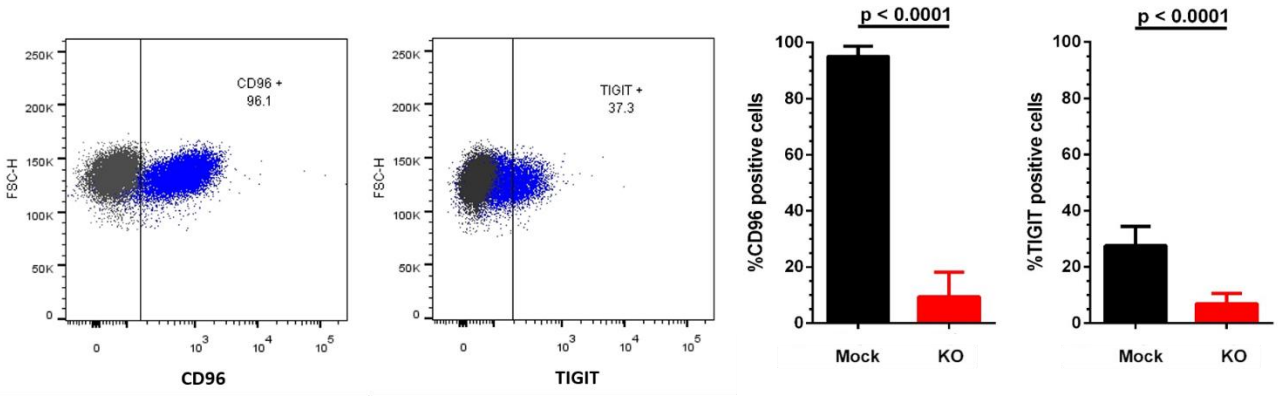


Figure 5.7 Disruption of *CD96* and *TIGIT* using CRISPR/Cas9 results in strong KO of CD96 and TIGIT, as determine by flow cytometry. (A) Basal expression of CD96 and TIGIT was first determined using flow cytometry-based phenotyping. **(B)** Using CRISPR/Cas9, $CD96^{KO}$ and $TIGIT^{KO}$ NK cells were generated and used in subsequent co-culture-based assays. Graphs represent mean $CD96^{+}$ and $TIGIT^{+}$ on NK cells targeted with CRISPR/Cas9 or mock electroporated as a control. n=6 for both CD96 and TIGIT. Data analysed using Student's paired *t*-test.

A panel of potential suitable target MM cell lines were previously screened for the expression CD155, CD111 and CD112, recognized ligands of both CD96 and TIGIT (**Figure 5.8**) [404].

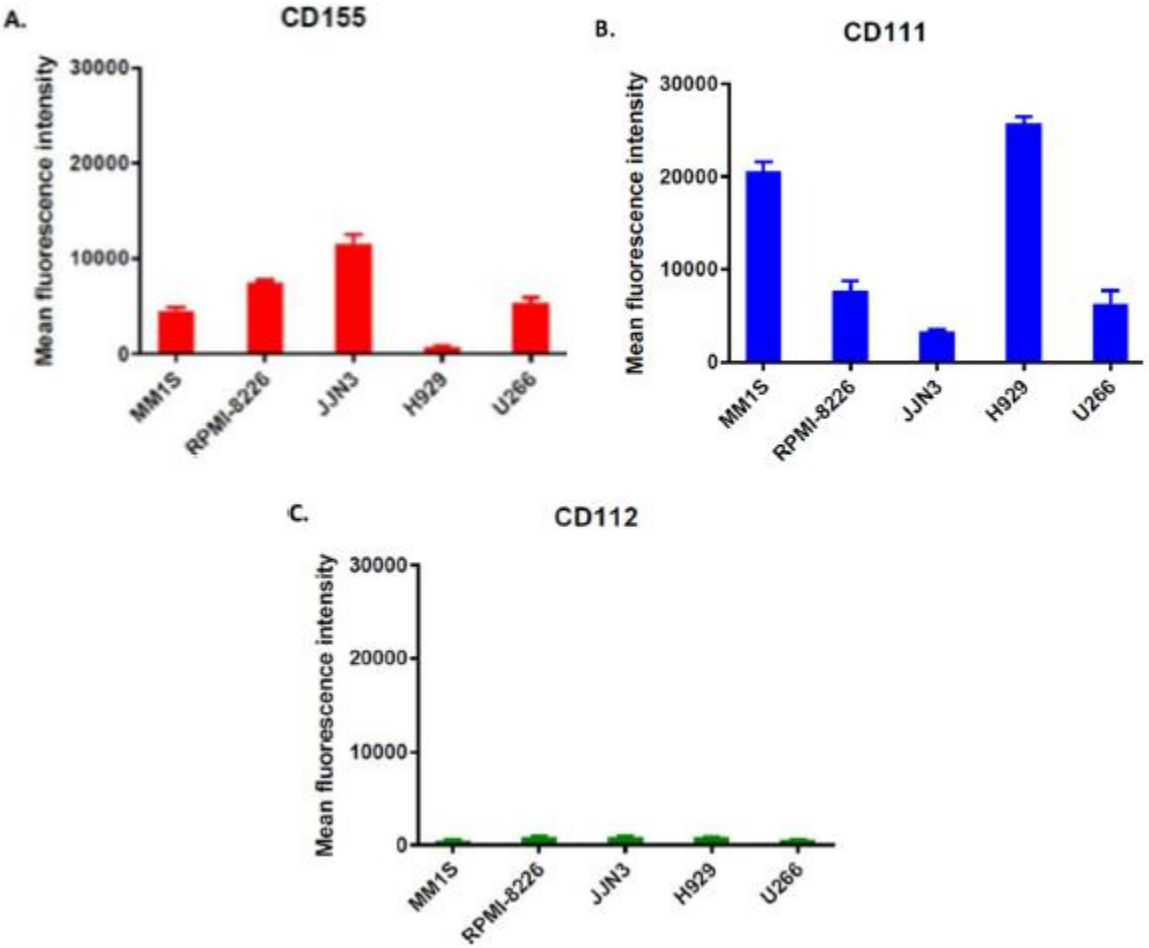


Figure 5.8 CD155, CD111 and CD112 expression was recorded on a panel of MM cell lines. Using flow cytometry and anti-CD155, anti-CD111 and anti-CD112 antibodies, a panel of MM cell lines were screened for the expression of ligands for CD96 and TIGIT. Data represents MFI expression of **(A)** $CD155^+$, **(B)** $CD111^+$, **(C)** $CD112^+$ MM cells + SEM, n=3 for each cell line. Figure adapted from Duggan, 2018.

MM cell lines JJN3 and U266 were chosen as target MM cell lines for co-culture assays with $CD96^{KO}$ or $TIGIT^{KO}$ NK cells, based on data previously gathered within the group confirming the expression of CD155 on these cell lines [404]. JJN3 were observed to have the highest expression of CD155, the preferred ligand for both CD96 and TIGIT while U266 were

observed to have lower expression of CD155 (**Figure 5.8A**). JJN3 were considered to be $CD155^{high}$ while U266 were considered to be $CD155^{low}$.

When $CD96^{KO}$ NK cells were co-cultured with $CD155^{+}$ JJN3 and U266, moderate increases in cytotoxicity were observed, compared to mock NK cells. The strongest increases were observed against JJN3 where $CD96^{KO}$ resulted in statistically significant increases in cytotoxicity mediated by $CD96^{KO}$ NK cells were observed at all E:T ratios used (**Figure 5.9A**). Similar results were observed against U266 MM cells, with statistically significant increases in cytotoxicity mediated by $CD96^{KO}$ NK cells compared to mock electroporated NK cells at 1:1 and 5:1 E:T ratios, while a non-significant trend was observed at 2.5:1 E:T ratio (**Figure 5.9A**).

Comparable results were also observed when $TIGIT^{KO}$ NK cells were co-cultured with JJN3 and U266, with $TIGIT^{KO}$ NK cells-mediating increased lysis of both JJN3 and U266, compared to mock electroporated NK cells alone. Similarly, the strongest increases were observed against JJN3 and were statistically significant at all E:T ratios used, while non-significant trends for increased lysis mediated by $TIGIT^{KO}$ NK cells were observed against U266 (**Figure 5.9B**).

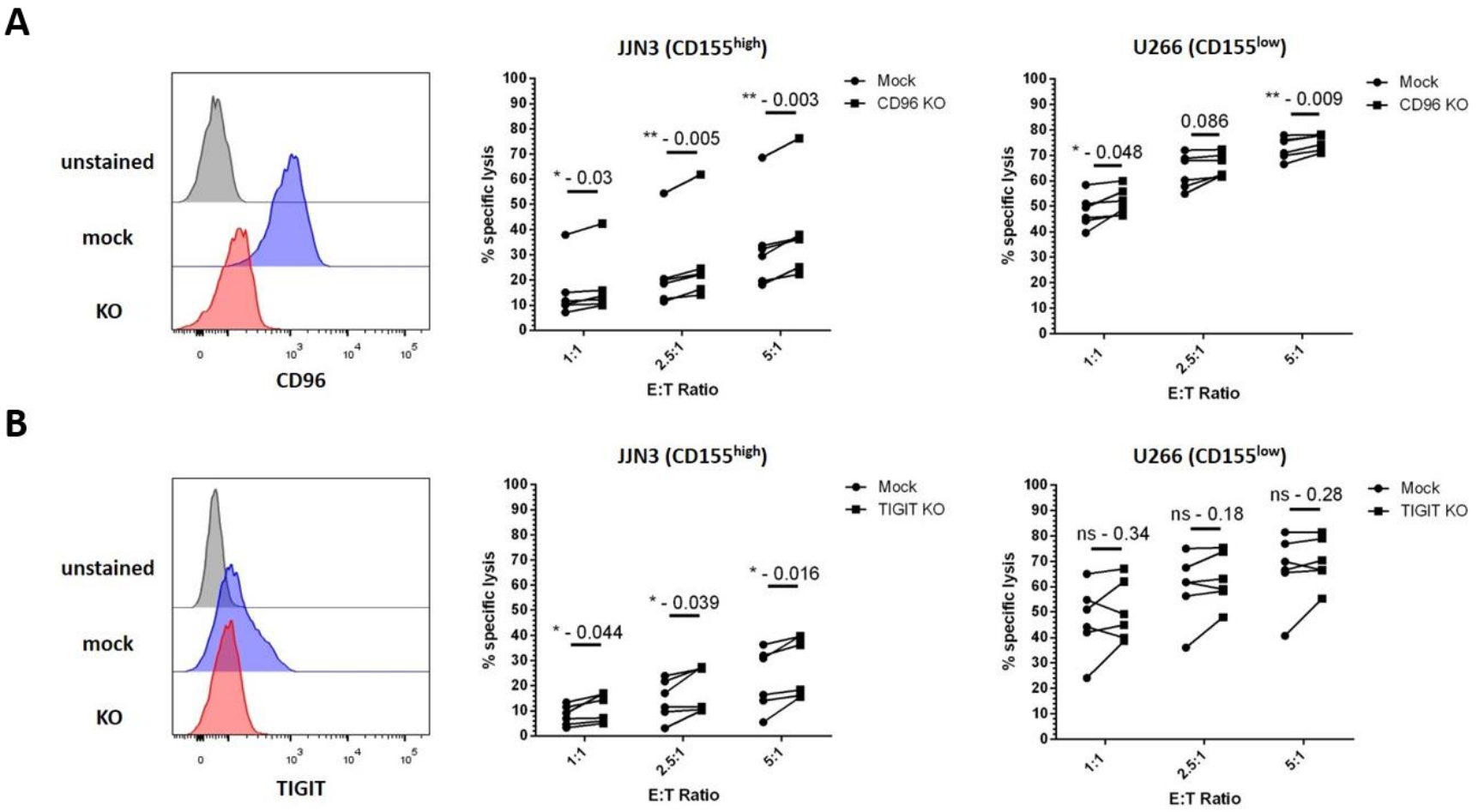


Figure 5.9 KO of CD96 and TIGIT in expanded primary NK cells results in enhanced cytotoxicity of CD155^{+} MM cell lines. (A) CD96^{KO} and **(B)** TIGIT^{KO} NK cells (or mock NK cells as a control) were generated using CRISPR/Cas9 and co-cultured with CD155^{high} and CD155^{low} JJN3 and U266 MM cell lines in cytotoxicity assays. Data in **A** represents one individual readout of CD96 KO using CRISPR/Cas9, as determined by flow cytometry and the subsequent cytotoxicity assays using n=6 individual healthy donor-derived NK cells displaying individual results for each donor. Data in **B** represents one individual readout of TIGIT KO using CRISPR/Cas9, as determined by flow cytometry and the subsequent cytotoxicity assays using n=6 individual healthy-donor derived NK cells displaying individual result for each donor. Data in **A** and **B** was analysed using Student's paired *t*-test, * - $p < 0.05$, ** - $p < 0.01$

Next, secretion of cytokines IFN-γ and TNF-α was measured by mock, CD96^{KO} and TIGIT^{KO} NK cells when co-cultured with CD155^{+} JJN3 and U266 MM cell lines. CD96^{KO} NK cells secreted significantly higher levels of IFN-γ when co-cultured with both JJN3 and U266 compared to mock NK cells. While the increases were statistically significant in U266, it was not possible to run statistical analysis on the results of IFN-γ secretion after co-culture with JJN3 due to levels of IFN-γ secreted by mock electroporated NK cells being below the lower limit of quantitation (LLQ). A trend towards enhanced TNF-α secretion by CD96^{KO} when co-cultured with U266 was observed, but no enhanced TNF-α secretion was apparent when after co-cultures with JJN3 (**Figure 5.10A**).

Similar to CD96^{KO} NK cells, TIGIT^{KO} NK cells secreted significantly higher levels of IFN-γ than mock NK cells which was observed and was statistically significant when co-cultured with both JJN3 and U266. TIGIT^{KO} did not appear to enhance TNF-α secretion by KO NK cells compared to mock NK cells (**Figure 5.10B**).

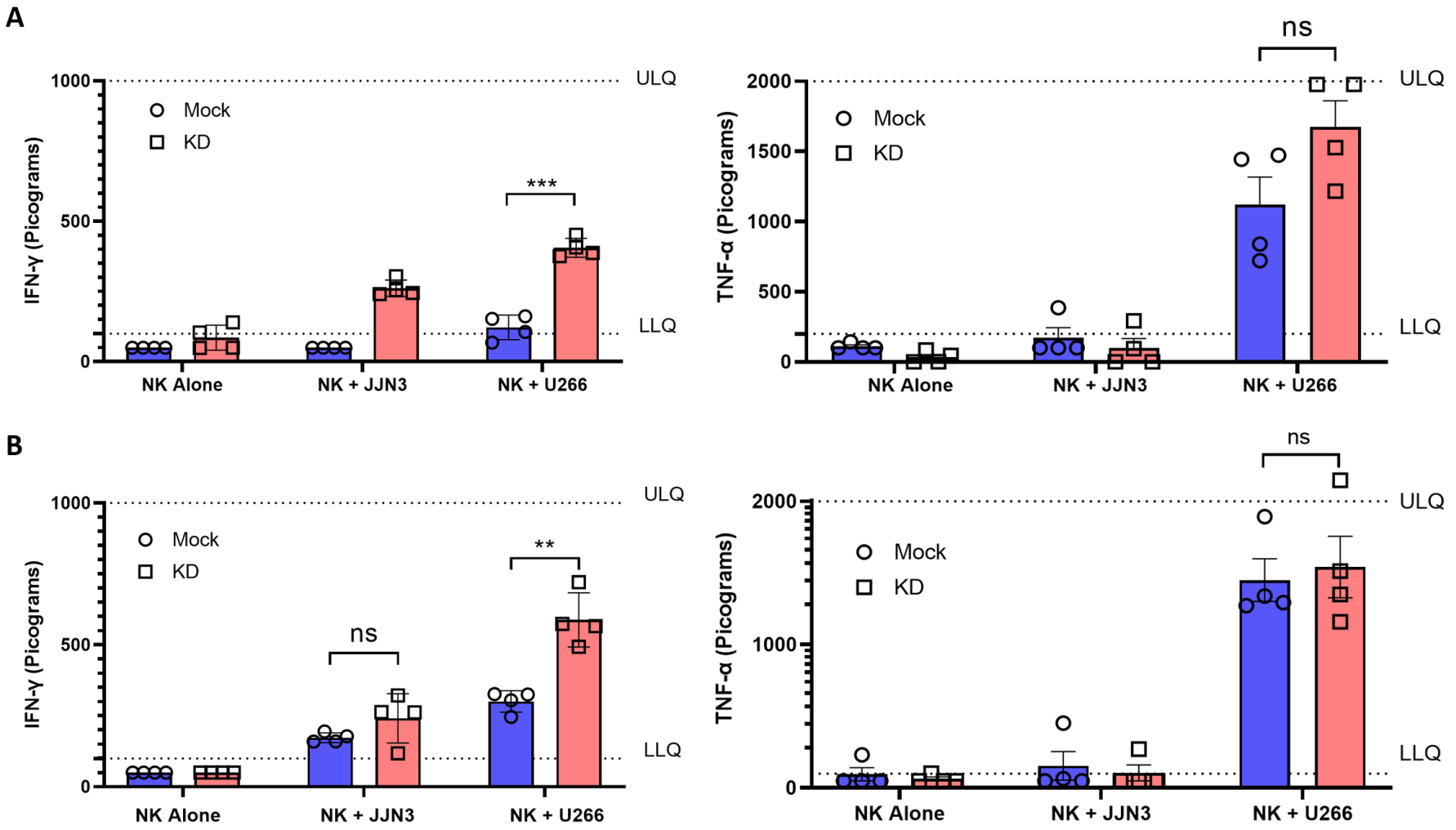


Figure 5.10 CD96^{KO} and TIGIT^{KO} results in significant enhancement of IFN-γ when co-cultured with CD155^{+} MM cell lines, while only minor increases in TNF-α secretion was observed, compared levels of secretion observed by mock electroporated NK cells. (A) CD96^{KO} and **(B)** TIGIT^{KO} NK cells (or mock NK cells as a control) were generated using CRISPR/Cas9, and co-cultured with CD155^{+} MM cell lines JJN3 and U266, after which supernatant from the cytotoxicity assays were collected and analysed for levels of IFN-γ and TNF-α in ELISAs. Data in **A** and **B** represents mean levels of IFN-γ and TNF-α as well as individual values from n=4 independent biological repeats. Upper limit of quantification (ULQ) and lower limit of quantification (LLQ) were included in each graph. Data was analysed using Student's paired *t*-test, ** - $p < 0.01$, *** - $p < 0.0001$

5.5 Discussion

5.5.1 Siglec-7 regulates NK cell cytotoxicity against MM cells, which can be overcome by genetic disruption of *Siglec-7* using CRISPR/Cas9

Siglec-7 has previously been implicated in regulating NK cell cytotoxicity against ovarian, breast and leukaemia cancers by employing blocking antibodies to abolish interactions between Siglec-7 and Siglec-7L, preventing the generation of an inhibitory stimulus to the NK cell [190]. Additionally, retrovirally-transduced Siglec-7 expression on the NK cell line NK-92 resulted in decreased cytotoxicity against sialic-acid polymer-coated ALL Jurkat cells. Overwhelming evidence has been presented implicating sialic acids, and hypersialylation in facilitating immune-evasion. Thus, attention has focused on targeting this axis of NK cell inhibition, with therapies such as sialidase-antibody conjugates, blocking antibodies and small molecule inhibitors targeting Siglec-7 all shown to enhance NK cell cytotoxicity [190, 305, 405]. However, no previous studies have addressed the role of Siglec-7 in regulating NK cell activity against MM, where MM cells are known to be highly decorated with sialic acids capable of acting as Siglec-7L [190, 304, 405]. However, as discussed in **5.1**, CRISPR/Cas9 represents a promising novel approach to generate genetically modified NK cells for adoptive transfer, lacking inhibitory receptors which maybe hijacked by malignant cells. In this chapter, investigations into utilising CRISPR/Cas9 to genetically disrupt *Siglec-7*, and indeed *CD96* and *TIGIT*, revealed no observable toxicity to expanded primary NK cells generated by both the electroporation and genetic disruption processes.

Initially, partial KO of Siglec-7 was observed using a sgRNA approach with approximately 50% of NK cells completely losing Siglec-7, as determined by flow cytometry. However, no observable increases in cytotoxicity were observed by this population of NK cells compared to mock electroporated NK cells when co-cultured with Siglec-7L^{+} MM cell lines. In an attempt to enhance Siglec-7 KO, a multi-sgRNA approach was used, using pooled three sgRNAs instead of the previous single sgRNA approach. Markedly enhanced KO of Siglec-7 was obtained using the multi-gRNA strategy, with >90% complete KO being recorded on expanded NK cells. Enhancements in cytotoxicity by Siglec-7^{KO} NK cells compared to mock NK

cells conclusively implicate Siglec-7 as an inhibitory receptor which regulates NK cell activity against MM cells.

While consistent enhancements in cytotoxicity were observed, the range of these enhancements varied from 5%-25% in specific lysis of either JJN3 or H929 MM cells. This may be attributed to the complex expression profile of both activating and inhibitory receptors expressed by NK cells. As reported by Angelo et al., variation in expression of common NK cell receptors such as NKG2D, DNAM-1 and NKp30 was observed across healthy donor PB-derived NK cells [406]. In addition to this, the overall percentage of NK cells within PBMCs in PB was also observed to be varied, ranging from 2-12% [406]. Therefore, it is likely that certain patients may have an increased therapeutic response to Siglec-7^{KO} NK cell-based immunotherapies. Siglec-7 has been shown to define a particularly active NK cell subset, where Siglec-7^{+} NK cells had higher levels of activating receptors such as CD16, DNAM1 and CD38 while also being observed to have lower levels of inhibitory receptors such as NKG2A and CD158b, when compared to Siglec-7^{-} NK cells [297]. Given the high levels of Siglec-7 expression on NK cells, targeting Siglec-7 using the CRISPR/Cas9 system represents a novel approach to enhance NK cell-mediated anti-MM functions.

When comparing the efficacy of desialylating agents such as NEURA or SIA against Siglec-7 KO using CRISPR/Cas9, it was observed that Siglec-7^{KO} on NK cells resulted in more moderate increases in cytotoxicity, while desialylating agents elicited a more potent response (**Figure 3.10** and **Figure 5.4**). This could be for a number of reasons. Firstly, desialylation may result in the increased exposure of ligands for activating receptors expressed by NK cells. As observed by increased CD38 detection following desialylation using both NEURA or SIA, variation in the expression of markers is to be expected upon desialylation of the MM cell surface (**Figure 4.3**). Whilst there were no increases in the detection of NKG2D ligands on MM cells following desialylation (**Figure 3.14**), the potential for increased availability of ligands for the plethora of other activating and inhibitory receptors expressed by NK cells cannot be ruled out. Increased presentation of activating ligands mediated by MM cell-surface desialylation could lead to enhanced NK cell-mediated cytotoxicity which could in turn be wrongly accredited to the abolishment of Siglec-7-Siglec-7L interactions. Secondly, the role of Siglec-9 must also be considered when analysing the strong enhancements in IL-2 activated and naïve NK cell cytotoxicity against desialylated MM cells (**Figure 3.10**). Previous

studies have shown that blockade of Siglec-9 using blocking antibodies results in enhanced primary NK cell-mediated cytotoxicity [190, 320]. However, while naïve and IL-2 activated NK cells express Siglec-9 partially, no expression of Siglec-9 was detected on expanded NK cells, which were used for CRISPR/Cas9 studies. Therefore, when trying to account for the reason as to why desialylating agents can offer enhanced NK cell cytotoxicity compared to Siglec-7 KO using CRISPR/Cas9 Siglec-9 cannot be implicated in this scenario.

Finally, the effects of CRISPR/Cas9 on NK cell functions such as cytotoxicity must be considered. Electroporation uses a short electrical pulse to form holes in the NK cell membrane, through which genetic material can travel into the cell. Electroporation has been demonstrated to result in up to 90% transfection of mRNA in NK cells, including within resting NK cells as well as expanded or activated NK cells, while demonstrating little toxicity to NK cells. However the effects of electroporation on the phenotype or function capabilities of NK cells has yet to be fully detailed [407]. While both mock and Siglec-7^{KO} NK cells underwent the same electroporation process, it must be considered that electroporation and the subsequent genetic interruption of *Siglec-7* may potentially limit the therapeutic effect seen when Siglec-7^{KO} NK cells encounter MM cells. However, it must be said that there is no existing evidence yet to suggest this and it is merely a suggestion. Furthermore, the MaxCyte GT transfection system used to electroporate NK cells in this study is GMP-compliant and represents a valuable tool which can be upscaled to produce genetically engineered NK cells at a clinically relevant number. Therefore, it is likely that there is little impact of the transfection process on NK cell cytotoxicity.

Nevertheless, the data gathered in this project confirm Siglec-7 as an important NK cell receptor in the context of MM which should be considered for future investigations, particularly when considering adoptive NK cell-based therapies. Considering the high expression of Siglec-7L on a myriad of different cancer types, targeting Siglec-7 (and Siglec-9) represents an immunotherapeutic strategy which could be used in numerous different settings. Within MM, the high expression of Siglec-7 on NK cells from the TME must also be noted (**Figure 3.5A**), suggesting that the Siglec-7-Siglec-7L axis could be even more exaggerated in this setting. Interestingly, Siglec-9 expression appears to be decreased on NK cells from within the TME and targeting Siglec-7 may induce a more therapeutic response (**Figure 3.5B**). Nevertheless, Siglec-7 and Siglec-9 have both been implicated in regulating NK

cell cytotoxicity, and dual targeting of both receptors would likely offer an overall improved NK cell response than targeting either Siglec-7 or Siglec-9 alone.

5.5.2 CD96 regulates NK cell functions against cancer, and targeted KO of CD96 using CRISPR/Cas9 can enhance NK anti-MM functions

Conflicting studies have suggested CD96 to be both an activating and inhibitory receptor in NK cells [221, 222, 408]. In the context of MM little is known about the role of CD96 in impacting NK cell functions. Having observed increased cytotoxicity by Siglec-7^{KO} NK cells against Siglec-$7L^{+}$ MM cells, and generating KO NK cells with ease and precision, CRISPR/Cas9 was used to elucidate the role of CD96 in influencing NK cell activity against MM. Furthermore, CD96 on NK cells has been documented to increase on expanded NK cells, and therefore could represent a potential regulator of adoptive NK cell cell-based therapies when encountering $CD155^{+}$, $CD112^{+}$ or $CD111^{+}$ MM cells in-vivo [409]. Adoptive transfer of NK cells requires expansion prior to infusion. Indeed, when NK cells were expanded in this chapter, high CD96 expression was observed on expanded NK cells, compared to the same NK cells upon isolation and prior to expansion. Furthermore, CD96 on NK cells from MM patient-derived BMAs have higher expression of CD96 compared to NK cells from the PB of healthy donors, indicating that CD96 may be more influential in regulating NK cells in the MM TME. Indeed CD96, as well as the inhibitory NK cell receptor TIGIT are attracting attention as potential targets in immunotherapy of late [172]. KO of TIGIT was carried out as a control for this experiment, with TIGIT being a widely recognised inhibitory NK cell receptor also binding CD155; the preferred ligand for CD96 [219]. Targeting of TIGIT using antagonistic moAbs or siRNA in in-vitro studies and in murine models respectively have demonstrated success in preventing tumour growth and enhancing NK cell functions such as cytokine secretion, degranulation and cytotoxicity [172, 410] . By targeting TIGIT in parallel with CD96 and observing results similar to previously published literature it allowed accurate interpretation of results of CD96 KO studies using the CRISPR/Cas9 system, which has not been previously frequently used to abrogate immune checkpoint signalling.

As with Siglec-7, almost complete KO of CD96 and TIGIT was obtained using the multi-gRNA CRISPR/Cas9 approach (**Figure 5.7**). Enhancements in $CD96^{KO}$ NK cell-mediated cytotoxicity upon co-culture with $CD155^{+}$ MM cell lines compared to mock electroporated NK cells, confirming that CD96 is an inhibitory NK cell receptor. Similar increases in cytotoxicity were observed when $TIGIT^{KO}$ NK cells were co-cultured with the same MM cell lines, validating the finding of the cytotoxicity assays with $CD96^{KO}$ NK cells (**Figure 5.9**). $CD96^{KO}$ NK cells displayed greater enhancements in cytotoxicity when co-cultured with $CD155^{high}$ JJN3 MM cells compared to $CD155^{low}$ U266, indicating that MM patients with high tumour cell surface expression of CD155 might be most sensitized to $CD96^{KO}$ NK cells. Other studies have found that CD96 has no impact on NK cell cytotoxicity against different cancer cell types and is more involved in regulating NK cell cytokine production [222]. However, the data collected in this chapter conclusively shows that CD96 is an inhibitory receptor within the context of MM which regulates NK cell anti-MM activities.

As CD96 has previously been implicated in regulating cytokine production by NK cells, supernatants from co-cultures with $CD96^{KO}$ or mock electroporated NK cells and $CD155^{+}$ MM cell lines JJN3 and U266 were collected and analysed for IFN-γ and TNF-α levels. $CD96^{KO}$ NK and $TIGIT^{KO}$ cells robustly produced increased levels of IFN-γ compared to mock electroporated NK cells, while no conclusive increase in secretion of TNF-α was observed by $CD96^{KO}$ and $TIGIT^{KO}$ NK cells (**Figure 5.10**). These data reiterate that CD96 is an inhibitory NK cell receptor in the context of MM, but demonstrate that CD96 regulates NK cell cytotoxicity as well as potentially regulating cytokine secretion. Despite studies suggesting that CD96 does not have a role in regulating NK cell cytotoxicity, but regulates cytokine release instead, the work in this chapter demonstrates that CD96 regulates NK cell cytotoxicity against MM. This work presents CD96 as having a more prominent role in regulating NK cell cytotoxicity than cytokine secretion as several trends towards increased IFN-γ and TNF-α were observed but were not statistically significant. However, this may have been a result of too few biological replicates of the experiment and statistical significance would likely be reached if the sample size was increased in this assay.

With the prevalent expression of CD96 on expanded NK cells, on which several novel NK cell-based adoptive therapies have been demonstrated, CD96 represents an important immunotherapeutic target, which could result in enhanced NK cell anti-MM functions.

Indeed, combining CD96 KO with targeted disruption of other inhibitory receptors, such as Siglec-7, Siglec-9 and TIGIT could generate potent NK cells with enhanced cytotoxicity and cytokine producing potential in the setting of MM.

5.5.3 Interpretation

While the data gathered in this chapter clearly demonstrate the role of both Siglec-7 and CD96 in regulating NK cell cytotoxicity, and cytokine release in the case of CD96, the full therapeutic effect of this strategy would likely be best revealed within an in-vivo setting. Considering the complexity of the TME in MM, with many environmental factors such as hypoxia, cytokine secretion and tumour-residing cells all interacting with NK cells, a study within this setting would likely elucidate fully the potential of targeting checkpoint receptors such as Siglec-7, Siglec-9 and CD96 as monotherapies or indeed as combinatory treatments.

With electroporation systems, such as the MaxCyte GT used in this chapter, capable of producing clinically relevant numbers of genetically modified NK cells the potential for producing NK cells for use in adoptive transfer therapies is very promising [282, 411]. Targeting inhibitory receptors in expanded NK cells using CRISPR/Cas9 could also conceivably be combined with the myriad of recently documented promising potential NK cell-based therapies. Genetic modification using CRISPR/Cas9 could be combined with CAR expression, mRNA-mediated expression of a high-affinity CD16 receptor or blocking antibodies to generate highly potent NK cells, for use in the treatment of a multitude of cancer types and not just MM. With the implication of Siglec-7, or at least hypersialylation, in facilitating regulation of NK cell cytotoxicity, the importance of targeting this axis is apparent. Furthermore, an approach to successfully overcome this axis of inhibition could not only be applied to MM, but to the myriad of cancer types where Siglec-7L has been recorded on malignant cells [190]. Similar to Siglec-7, the expression of the preferred ligand for CD96 - CD155 - is often overexpressed in cancer [412-414]. Therefore, an approach targeting CD96 could also conceivably be used against a wide range of cancer types.

It is evident therefore, that there is significant potential in targeting novel checkpoint receptors in NK cells to elicit a more potent-anti tumour response. Several strategies utilising

NK cells to combat cancer have been demonstrated and show promise, however genetic modification to generate modified NK cells lacking inhibitory receptors which may be hijacked by MM, and cancer in general had until now not been attempted. The work performed for this chapter present genetic modification of NK cells as a tool that can be used to potentiate both cytotoxicity and cytokine production when in co-culture with MM cells. Furthermore, the relative ease of generating genetically modified expanded NK cells present adoptive NK cell therapy as a viable option for treating a multitude of cancer types, and warrants significant continues research and investment. Excitingly, the enhancements in NK cell engineering indicate that targeted checkpoint receptors could also be combined with other strategies, to deliver a significantly enhanced NK cell population for use against cancer.

Chapter 6:
Discussion and Future Studies

6.1 Discussion

Aberrant glycosylation of the tumour cell surface is considered a hallmark of cancer [173, 300, 415]. The impact of hypersialylation on cancer progression is significant, as hypersialylation has been shown to lead to abnormal cell trafficking and drug resistance [179, 185, 186, 415]. However, attention has only recently focused on the role of the hypersialylated tumour cell surface in regulating the anti-cancer activity of innate immune cell types, such as NK cells. As discussed in **1.2.6**, NK cells are critical components of the innate response, patrolling both the circulation and bone marrow milieus with the aim of eradicating virally infected, genetically damaged and malignantly transformed cells. Hypersialylation has been implicated in facilitating evasion of NK cell-mediated immunosurveillance in several cancer types, such as breast cancer and leukaemia [190, 305]. However, the impact of the hypersialylation in regulating NK cell anti-MM functions had not, until this project, been addressed. This axis of NK cell regulation in MM demanded research due to the difficulty in treating in MM, as the disease still remains incurable despite recent advancements in medicine enhancing patient outlook in recent decades. Furthermore, MM cells are known to be hypersialylated with a rise in sialyltransferase activity being recorded in PBMCs and MNCs from MM patients, demonstrated also to increase throughout disease progression [302]. Coupled with the necessity of further research into developing novel therapies to target MM, hypersialylation and its role in regulating NK cell anti-cancer functions represented a promising avenue to enhance NK cell anti-cancer activity against MM. Additionally, NK cells have recently been attracting attention as promising effectors of novel adoptive cellular therapies [272, 302, 416]. Coupled with the necessity of further research into developing novel therapies to target MM, hypersialylation and its role in regulating NK cell anti-cancer functions represented a promising avenue to enhance NK cell anti-cancer activity against MM. Additionally, NK cells have recently been attracting attention as promising effectors of novel adoptive cellular therapies [272, 302, 416]. Coupled with the necessity of further research into developing novel therapies to target MM, hypersialylation and its role in regulating NK cell anti-cancer functions represented a promising avenue to enhance NK cell anti-cancer activity against MM. Additionally, NK cells have recently been attracting attention as promising effectors of novel adoptive cellular therapies [272, 416]. While CAR-T cells have been prominent

within the cellular therapy field of late, NK cells represent a viable alternative, capable of being utilised to develop cheaper CAR-based therapies, and providing an alternative to T cells which can be rejected by the patient's immune system upon infusion [282]. However, the possibility for MM to hijack inhibitory NK cell signalling pathways mediated by receptors such as Siglec-7 to facilitate immune-evasion could dampen the therapeutic response generated by these NK cell-based immunotherapies. As there had been no prior research into the role of hypersialylation in regulating NK cell anti-MM functions, several exploratory experiments were needed to understand whether this axis of NK cell inhibition was possible in this disease before further detailed research could be carried out. In this chapter, the key findings from this project are discussed, and future studies are proposed with the intention of further developing NK cell-based therapies that hold promise in treating MM, as highlighted by the findings of this project.

6.2 MM cells strongly express Siglec-7L and Siglec-9L

Studies have previously demonstrated that a myriad of cancer types are highly decorated with Siglec-7L, Siglec-9L or in some cases, both Siglec-7L and Siglec-9L [190, 191, 417, 418]. The detection of Siglec-7L and Siglec-9L on both primary MM cells from both ND-MM and RR-MM patients confirmed that inhibition of NK cell anti-cancer functions via Siglec receptor-Siglec ligands interactions is possible within MM. Thus, both Siglec-7 and Siglec-9 represent novel target immune checkpoints that could be targeted to enhance the functions of NK cells within MM patients. Understanding when these therapies could be used within the course of the disease is important. Expression of both Siglec-7L and Siglec-9L on MM cells from RR-MM patients appears to be lower than in ND patients. However, with a very small sample size it is impossible to say that this is biologically true. In future, studies could profile MM cells isolated from patients who have received prior treatments, in an attempt to establish whether Siglec-7L or Siglec-9L expression can be altered by a certain treatment regimen. Should Siglec-7L or Siglec-9L expression be aberrantly expressed after treatment, MM patients may become more or less suitable for future Siglec-7/9-targeting NK cell-based immunotherapies. Alternatively, should Siglec-7L or Siglec-9L expression remain consistent or increase throughout the course of the disease in spite of treatments, then patients may receive benefit

from such an NK cell-based immunotherapy targeting Siglec-7/9 at all stages of MM. Therefore, a thorough understanding of the profile of Siglec-7L or Siglec-9L expression throughout the course of MM progression is necessary to provide rationale for further in-vivo studies or identify potential patients for clinical trials targeting this axis of NK cell inhibition.

While Siglec-7L and Siglec-9L expression was recorded on MM cell lines, the culture of these cells in an incubator is not representative of the MM TME. As discussed in **1.1.6**, the MM TME is extremely complex, with MM cells influenced by the hypoxic nature of the TME, TME-residing cells and a wide range of cytokines and chemokines present in the TME. It is possible therefore, that the TME could result in aberrant expression of Siglec-7L and Siglec-9L. Although the effect of hypoxia on Siglec-7/9L expression on MM cells displayed trends for both increased and decreased Siglec-7L and Siglec-9L expression, there was no statistically significant changes observed. This assay could be repeated with MM cells cultured for longer times under hypoxia as a suitable follow-up assay. Additionally, the expression of individual sialyltransferases in MM cells cultured under hypoxia could be quantified using qPCR, to determine whether hypoxia specifically dysregulates sialyltransferase expression. However, MM cells could also be cultured with BM-residing cells such as stromal cells to observe the effects of interactions between the cell types on Siglec-7/9L expression. Finally, MM cells could be cultured with cytokines implicated with MM survival and proliferation within the TME, such as IL-6 or IGF-1, to observe the effects of exposure on expression of Siglec-7/9L. Understanding the expression of Siglec-7L and Siglec-9L throughout the course of MM, as well as the microenvironmental factors responsible for dysregulated Siglec ligand expression, if any, will help to identify at what stage of the disease a Siglec-7/9-targeting therapy may be of most benefit to patients and the mechanisms by which this axis of inhibition is exaggerated to promote NK cell-mediated immune evasion by MM cells.

6.3 PSGL-1 is a prominent Siglec-7L in MM, with CD43 also acting as a ligand

As discussed in **3.5**, the elucidation of the identity of the underlying glycans or proteins terminated with sialic acids moieties acting as ligands for Siglec-7 and Siglec-9 is important in MM. The confirmation of PSGL-1 as a highly expressed Siglec-7L is exciting, as previous studies have implicated PSGL-1 in contributing to MM proliferation, survival and drug resistance [(323)].

Antibody blockade of PSGL-1 has been shown to cause MM cell retention in the circulation, thereby sensitizing them to the PI bortezomib [322]. Furthermore, PSGL-1 blockade increased survival and decreased tumour burden in a murine MM model [323]. As well as acting for a ligand for cell adhesion molecules P and E-selectin, the data gathered in this project reveals that PSGL-1 also acts as a Siglec-7L, confirming that PSGL-1 contributes to MM disease progression and survival using several different mechanisms. Additionally, studies into the role of PSGL-1 in other immune cell types have revealed an inhibitory effect on both DCs and T cells [419]. Strategies used to-date to target PSGL-1 in an attempt to treat clinical diseases include blocking antibodies and recombinant immunoglobulins which compete for PSGL-1 and molecular therapies [420]. As a negative regulator of several immune cell subsets, studies into targeting PSGL-1 should be emphasized due to the negative influence on several immune cell subsets, including DCs and T cells as well as NK cells [419].

As discussed in **3.5**, the elucidation of the identity of the underlying glycans or proteins terminated with sialic acids moieties acting as ligands for Siglec-7 and Siglec-9 is important in MM. The confirmation of PSGL-1 as a highly expressed Siglec-7L is exciting, as previous studies have implicated PSGL-1 in contributing to MM proliferation, survival and drug resistance [323]. Antibody blockade of PSGL-1 has been shown to cause MM cell retention in the circulation, thereby sensitizing them to the PI bortezomib [322]. Furthermore, PSGL-1 blockade increased survival and decreased tumour burden in a murine MM model [323]. As well as acting for a ligand for cell adhesion molecules P and E-selectin, the data gathered in this project reveals that PSGL-1 also acts as a Siglec-7L, confirming that PSGL-1 contributes to MM disease progression and survival using several different mechanisms. Additionally, studies into the role of PSGL-1 in other immune cell types have revealed an inhibitory effect on both DCs and T cells [419]. Strategies used to-date to target PSGL-1 in an attempt to treat clinical diseases include blocking antibodies and recombinant immunoglobulins which compete for PSGL-1 and molecular therapies [420]. As a negative regulator of several immune cell subsets, studies into targeting PSGL-1 should be emphasized due to the negative influence on several immune cell subsets, including DCs and T cells as well as NK cells [419].

Specifically, future studies around the role of PSGL-1 in regulating NK cell activities should start with PSGL-1 blockade on MM cells in-vitro, followed by co-cultures with Siglec-7^+ NK cells in cytotoxicity assays to determine whether interference of interactions between

Siglec-7 and PSGL-1 could enhance NK cell anti-MM functions. Both NK cell cytotoxicity and cytokine secretion should be measured to determine the effect of abolished PSGL-1-Siglec-7 interactions on NK cell functions. This could be followed up by murine model studies where Siglec-7$^+$ NK cells could be administered to MM-afflicted mice in conjunction with PSGL-1 blocking antibodies, for example, to determine the effect of blockade of Siglec-7-PSGL-1 interactions on survival of mice and MM tumour burden and proliferation. As CD43 was also observed to act as a Siglec-7L in the JJN3 MM cell line, these studies could also be simply replicated, again using blocking antibodies, to understand the role of CD43 in regulating NK cell cytotoxicity, thereby allowing a comparison between the benefits of targeting both PSGL-1 and CD43 individually. Although the identification of PSGL-1 and CD43 as Siglec-7L was carried out using MM cell lines, confirmation that PSGL-1 or CD43 could be pulled down from primary MM cell lysates incubated with Siglec-7 Fc chimeras-magnetic bead complexes would complement this data. However, this is unlikely due to the difficulty in isolating large amounts of primary MM cells from patient samples. However, PSGL-1 is known to be expressed by MM cells, and has been observed in MM biopsies, therefore confirming the potential for inhibition of NK cells in a Siglec-7-dependent manner in vivo [323]. As mentioned in **6.2**, Siglec-7L and Siglec-9L expression should be recorded in MM cells from patient BMAs to track their expression across disease progression or after treatment. As CD43 was also confirmed as a Siglec-7L in MM, the expression of both PSGL-1 and CD43 could be measured using specific antibodies in a flow cytometry-based assay. Utilising a Siglec-7 Fc chimera which would only confirm the expression of Siglec-7L and not the percentage makeup of PSGL-1 or CD43 from the total of Siglec-7L$^+$ MM cells. This would allow the generation of a full profile of Siglec-7L expression in MM. These experiments also be carried out upon the elucidation of the identity of Siglec-9L in MM as inhibition of NK cell anti-cancer functions in a Siglec-9-dependent manner is still possible in MM.

Finally, while targeting Siglec-7L represents a promising novel therapeutic approach in MM, it is important to consider the potential regulatory role of Siglec-9 in NK cells. While expressed at a lower level in NK cells in comparison to Siglec-7, blockade of Siglec-9 in primary NK cells has resulted in enhanced NK cell cytotoxicity against leukaemia and cervical cancer cells [190, 320]. Thus, elucidation of Siglec-9L in pulldown and mass spectrometry assays as carried out in this project could also reveal novel targets for NK cell-based immunotherapies

specifically targeting Siglec-9, but may also highlight the importance of other glycans or proteins in driving MM progression, as is the case with PSGL-1. Upon elucidation of the identity, Siglec-9L expression could also be measured using specific antibodies, again to profile the expression of individual ligands throughout the course of the disease as opposed to merely detecting the presence or absence of Siglec-9L.

6.4 The hypersialylated MM cell surface facilitates evasion of NK cell-mediated immunosurveillance which can be overcome by targeted desialylation

A simple experiment to understand the role of hypersialylation in facilitating evasion of NK cells is to desialylate MM cells, conduct cytotoxicity assays with Siglec-7^{+} NK cells and subsequently compare the levels of specific NK cell-mediated lysis of desialylated and control-treated MM cells. Comprehensive increases in NK cell-mediated cytotoxicity were observed by several different type of primary NK cell, including activated, expanded and naïve NK cells against desialylated MM cell lines. This data is in line with previously published literature, where desialylated breast cancer, cervical cancer and leukaemia cells were demonstrated to be more susceptible to NK cells [190, 305]. Both a sialidase enzyme and sialyltransferase inhibitor were used to effectively desialylate MM cells and enhance NK cell-mediated cytotoxicity, providing more than one strategy that can be utilised to achieve desialylation. Toxicity observed by sialidase treatment of MM1S suggests that sialidase in a naked form may be toxic to not only MM but also surrounding BM-residing cell types. However, SIA was used to achieve desialylation without causing adverse toxicity to MM cells, including both primary and MM cell lines. Primary MM cells could therefore conceivably be desialylated with SIA prior to co-culture with NK cells in cytotoxicity assays without a toxic side effect, providing accurate results. It is likely that freshly isolated MM cells would be needed for this assay, which could be hampered by the difficulty in culturing primary MM cells in vitro post-isolation. SIA treatment of primary MM cells and MM cell lines in this project was between 2-3 days, however it is possible that treatment for a shorter period of time might still abolish sufficient quantities of sialic acids to sensitize MM cells to NK cells. After initial treatment with SIA, MM cell lines could be collected at regular time intervals during the treatment to observe how quickly a potentiation of NK cell-mediated cytotoxicity was observed. This time length

could then be applied to freshly isolated primary MM cells to avoid high levels of apoptosis, which occurs rapidly post-isolation.

The success of using both a sialidase and sialyltransferase inhibitor to enhance NK cell cytotoxicity is promising, as several groups have developed methods of targeted desialylation using both of these agents. An ADC of sialidase and moAb has shown promise in enhancing NK cell cytotoxicity, by both desialylation and moAb-induced ADCC [304, 305, 339]. Furthermore, animal model studies have successfully used SIA encapsulated within bisphosphate-coated nanoparticles to specifically deliver the inhibitor to the BM, decreasing melanoma metastasis and providing long term sialic acid blockade [314]. It is possible that both of these approaches could be adapted to treat MM, where MM cells are known to reside in the BM. A sialidase-moAb conjugate could conceivably be created, with several moAbs being used clinically to treat MM, such as Dara and Elotuzumab. Future studies could focus on the development of these strategies to develop a method of selectively desialylating MM cells within a BM-microenvironment. It is likely that this would need to take place in a murine model with established MM, due to the difficulty in successfully replicating a MM TME in vitro. The establishment of MM tumours within murine models from MM cells pre-treated with a sialidase/sialyltransferase inhibitor would be helpful in understanding how beneficial MM cell surface desialylation would be within such a model by tracking proliferation and survival of MM cells as well as their effect on overall mouse survival.

6.5 Desialylation of MM cells results in increased detection of CD38 and can be combined with anti-CD38 antibody moAbs to enhance NK cell cytotoxicity

As mentioned previously, the potential for hypersialylation to conceal ligands for activating or inhibitory NK cell receptors as well as mask important antigens targeted by therapeutic moAbs must be considered. Increased BCMA expression has been recorded on desialylated MM cells [363]. Due to its selective expression on malignantly transformed plasma cells, BCMA represents a promising target antigen for therapies including ADCs, CAR-T cells and bispecific T cell engagers (BiTEs) [364]. To examine whether desialylation of MM cells could represent a method of enhancing the efficacy of such treatments, as well as antibody-based

therapies such as moAbs, the expression of a panel of target antigens was determined upon desialylation and compared to sialylated MM cells as a control. While a minor increase in BCMA expression was recorded upon desialylation, the consistent and reproducible increase in the detection of the glycoprotein CD38 was the most interesting finding from this assay. CD38 is targeted in MM clinically by anti-CD38 moAbs such as Dara and increased availability of the antigen upon desialylation represented a method of enhancing the efficacy of Dara-mediated therapy, such as ADCC, CDC and ADCP. Indeed, combining tumour cell surface desialylation with Dara enhanced NK cell cytotoxicity against $CD38^{+}$ MM cell lines, and subsequent assays revealed increased detection of CD38 on primary MM cells desialylated using SIA. As a mechanism of resistance described in **1.1.8**, MM cells may lose CD38 expression after Dara treatment. However, treatment of the $CD38^{low}$ MM cell line JJN3 with ATRA strongly upregulated CD38 expression, which was further potentiated upon desialylation combined with ATRA treatment. Follow up cytotoxicity assays revealed that JJN3 treated with ATRA, SIA and Dara were more readily lysed by NK cells than either SIA or ATRA combined with Daratumumab individually. This work demonstrates that the efficacy of a clinically used frontline therapy for MM - anti-CD38 moAbs such as Dara and Isatuximab – can potentially be combined with targeted tumour cell surface desialylation to enhance NK cell-mediated cytotoxicity against MM.

While the data in these assays was obtained by using MM cell lines H929 and JJN3, the repetition of these experiments using primary MM cells would strongly enhance the impact of this finding. During the course of this study, it was difficult to obtain sufficient MM BMA samples or a high enough volume of BMA in the samples obtained, to isolate the required number of primary MM cells for these experiments. However, this assay could be carried out if sufficient fresh samples were available. As mentioned in **6.4**, it is worth noting that primary MM culture post-isolation is extremely difficult and it may be too difficult to maintain a high level of viability throughout the time needed for ATRA or SIA treatment to be efficient. One of the most appropriate approaches to counteract this would be to develop an anti-CD38-sialidase ADC, similar to the Trastuzumab/sialidase antibody-sialidase conjugate utilised to enhance the efficacy of Trastuzumab/NK cell-mediated ADCC against breast cancer [305]. This conjugate could be utilised in both in vitro and in vivo studies to assess efficacy as well as determine toxicity of the conjugate, if any, to MM cells in vitro or murine models in vivo. This

approach would prevent the need to design and develop a desialylating strategy separate from Dara treatment. It is worth noting, that at the time of writing, ATRA has not been approved to treat MM thus indicating that patients with low CD38 expression, such as patients previously treated with Daratumumab, would perhaps not benefit from an anti-CD38 antibody-sialidase conjugate. However, Dara has been combined with ATRA in a clinical trial for RR-MM and was well tolerated, confirming the potential of ATRA for future approval and use clinically [421]. Finally, as BCMA expression was also recorded to increase on MM cells upon desialylation, the assays carried out in this chapter of the project could be repeated, but targeting BCMA instead of CD38 in order to potentiate NK cell cytotoxicity. Furthermore, an antibody-sialidase conjugate targeting BCMA could also be created, thus providing multiple targets of such a strategy, allowing the comparison in efficacy of both approaches and the selection of the most efficient conjugate. These data confirm the potential of combining desialylation with an anti-CD38 moAb to enhance the efficacy of treatment.

It is clear therefore, that desialylation can be combined with moAb therapy to enhance Dara/NK cell mediated-ADCC against MM cells, however there is potential for desialylation to be potentiate other moAb-induced anti-MM mechanisms of action. While this study addresses the role of desialylation in enhancing ADCC, moAbs such as Dara can also target tumour cells by stimulating CDC and ADCP [163, 353, 358, 369, 422]. The effects of MM cell surface desialylation on CDC and ADCP were not addressed in this project, but should be investigated to determine the full potential of desialylation in enhancing Dara-mediated lysis of MM cells. MM cell lines are known to be extremely resistant to CDC due to high expression of anti-complement-inhibitory proteins such as CD55 and CD59 [163]. Therefore, future CDC studies should be carried out against primary MM cells, which have been demonstrated to be sensitive to CDC, in order to fully investigate the effects of desialylation on Dara-induced CDC [423]. Finally, Dara has been shown to induce phagocytosis of both MM cell lines and primary MM cells by macrophages [358]. Simple co-cultures of MM cells pre-treated with a desialylating agent or relevant control, followed by treatment with Dara and co-culture with macrophages would be a simple introductory assay to determine the effect of MM cell surface desialylation on Dara-mediated ADCP. [423]. Finally, Dara has been shown to induce phagocytosis of both MM cell lines and primary MM cells by macrophages [358]. Simple co-cultures of MM cells pre-treated with a desialylating agent or relevant control, followed by treatment with Dara and

co-culture with macrophages would be a simple introductory assay to determine the effect of MM cell surface desialylation on Dara-mediated ADCP. [423]. Finally, Dara has been shown to induce phagocytosis of both MM cell lines and primary MM cells by macrophages [358]. Simple co-cultures of MM cells pre-treated with a desialylating agent or relevant control, followed by treatment with Dara and co-culture with macrophages would be a simple introductory assay to determine the effect of MM cell surface desialylation on Dara-mediated ADCP [423]. Finally, Dara has been shown to induce phagocytosis of both MM cell lines and primary MM cells by macrophages [358]. Simple co-cultures of MM cells pre-treated with a desialylating agent or relevant control, followed by treatment with Dara and co-culture with macrophages would be a simple introductory assay to determine the effect of MM cell surface desialylation on Dara-mediated ADCP.

6.6 CRISPR/Cas9 can be used to target immune checkpoints such as Siglec-7, CD96 and TIGIT to enhance NK cell cytotoxicity against cancer

Targeting immune checkpoints is not a novel concept with several inhibitory NK cell receptors already targeted and demonstrating successful enhancements in NK cell anti-tumour responses [264, 306, 335, 375, 383]. To-date, this has been achieved by incorporating blocking antibodies, as described in **1.2.6**. However, while concerns of the efficacy of these treatments as monotherapies exist, combinational approaches of blocking antibodies with other relevant drugs have elicited a more potent therapeutic response in several cancers, with ongoing trials including several more blocking antibodies [262, 264, 265, 374, 424]. In MM the only checkpoint inhibitor of NK cells targeted in clinical trial studies is PD-1, which did not demonstrate an impressive response in patients as a monotherapy, but has shown promising results in combination with the IMiDs lenalidomide and pomalidomide [388, 425, 426].

As NK cells possess a range of inhibitory receptors which can be hijacked by cancer cells to promote immune escape including Siglec-7, Siglec-9, TIGIT and CD96, it is unlikely that MM patients will be able to receive treatments targeting each checkpoint inhibitor as a combinational treatment regimen in the near future. However, CRISPR/Cas9 represents a promising avenue for the generation of genetically engineered NK cells lacking these

inhibitory receptors, which can subsequently be used in adoptive transfer to cancer patients. Inhibition of Siglec-7 and Siglec-9 signalling using blocking antibodies has previously been demonstrated to enhance NK cell cytotoxicity against several cancer cell types, but not in MM [190, 427]. To propose a novel method of enhancing NK cell cytotoxicity against MM, and cancer in general, *Siglec-7* was targeted using CRISPR/Cas9 to abolish Siglec-7 on the NK cell surface, thereby preventing inhibitory signalling. CRISPR/Cas9-mediated KO of Siglec-7 enhanced NK cell cytotoxicity against Siglec-7L$^+$ MM cell lines in every donor, although variation between donors was observed. The variation between donors could be due to the varying expression profile of both activating and inhibitory receptors on the NK cell surface between individuals, with the influence of specific checkpoint receptor varying as a result of abnormal signalling by variation in receptor expression [406].

When comparing the increases in NK cell cytotoxicity as a result of Siglec-7 KO and desialylation induced by NEURA or SIA, it is apparent that desialylation induces a more potent NK cell response than Siglec-7 KO using CRISPR/Cas9. The effect of desialylating the MM cell surface on antigen expression must not be ignored when considering this. Removal of sialic acids may reveal ligands for activating NK cell receptors such as NKG2D thereby contributing to an overall enhancement in cytotoxicity compared to abolished Siglec-7-Siglec-7L interactions. Indeed, enhanced binding to NKG2D Fc chimeras was observed upon desialylation of breast cancer cells, and increased ULBP-3 and ULBP-4 expression was recorded on the CML cell line K562 in this project [305]. However, no increase in expression of MIC-A/B or ULBP-1-6 was observed on desialylated MM cell lines, confirming that increases in NK cell cytotoxicity upon MM cell surface desialylation were not as a result of increased stimulation of NK cells through enhanced NKG2D receptor-ligand signalling. However, it is not possible to rule out the possibility of desialylation revealing ligands for other activating NK cell receptors. To identify the mechanisms contributing to potentiated NK cell cytotoxicity, ligands for activating and inhibitory NK cell receptors expressed by target cancer cells should be analysed and compared upon desialylation to identify whether the expression of any are increased/decreased by tumour cell surface desialylation. This knowledge will help to determine whether the increases in NK cell-mediated cytotoxicity upon tumour cell surface desialylation are entirely in a Siglec-7/9-dependent manner. Finally, it would be of interest to examine the expression of Siglec-7 and Siglec-9 on expanded NK cells from expansion

protocols which are used to generate NK cells suitable for adoptive transfer to cancer patients. As no expression of Siglec-9 on expanded NK cells expanded using the Miltenyi expansion protocol was recorded, NK cell expansion possibly results in loss of Siglec-9 expression. Knowing this data will allow researchers to determine whether the benefit of adoptive NK cell-based therapies is likely to be dampened by Siglec-9 signalling. If so, it would be possible to target Siglec-7 and Siglec-9 simultaneously using CRISPR/Cas9 to abolish negative signalling to NK cells through Siglec receptors.

Variable, yet consistent, increases in NK cell-mediated cytotoxicity were also observed when CD96 and TIGIT expression was individually abolished using CRISPR/Cas9. While enhancements in cytotoxicity by expanded NK cells from each healthy donor used was observed, enhancements varied between donors, similar to Siglec-7 KO cytotoxicity assays. Subsequent assays investigating the role of CD96 revealed that CRISPR/Cas9-mediated CD96 KO enhanced not just NK cell cytotoxicity, but also the secretion of the influential cytokine IFN-γ. While conflicting data has presented CD96 as both an activating and inhibitory receptor, this data confirms conclusively that CD96 is an inhibitory NK cell receptor in MM. Comparisons between single-guide and multi-guide approaches to generate KO NK cells revealed that multi-guide strategies generated higher levels of KO, as determined by flow cytometry, whereas the single-guide approach was unable to generate a high level of KO. Future studies targeting immune checkpoints in NK cells might therefore benefit best from utilising a multi-guide approach to generate KO NK cells. The availability of clinically approved electroporation systems, such as the MaxCyte GT used in this project, is important to the advancement of this therapeutic approach being able to facilitate high levels of KO with minimal effects on NK cell viability while also being able to successfully electroporate clinically relevant numbers of NK cells at the same time.

As observed in this project, NK cells from patient-derived BMAs have elevated Siglec-7 and CD96 expression, which could enhance the regulatory role they play in-vivo. It is therefore possible that the role of these receptors could be even more enhanced within the MM TME. A murine model where MM has been established could conceivably be treated with adoptive NK cell transfer, including both mock electroporated and genetically modified NK cells lacking Siglec-7 or CD96 after which disease progression, tumour size and survival of mice could be measured to determine any therapeutic effect. Indeed, NK cells where multiple

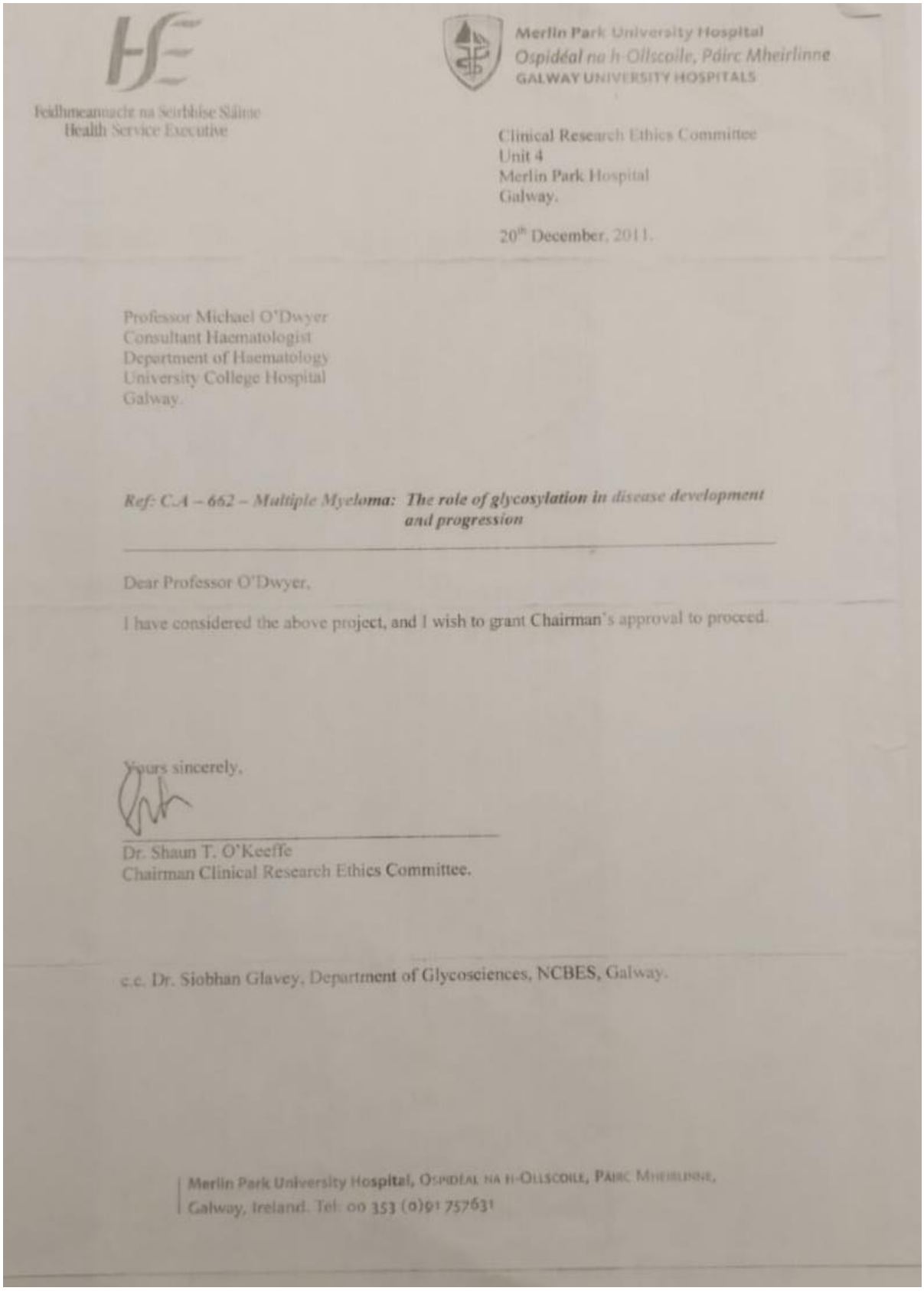
checkpoint receptors have been targeted could also be used and compared to NK cells where only one receptor has been targeted, to examine the potential of targeting multiple NK cell inhibitory receptors at once. Tagging the NK cells with a tracking dye such as green fluorescent protein (GFP) would also allow the tracking of the genetically modified NK cells, and examine their persistence in-vivo compared to a control group of NK cells. Generating a protocol for the consistent and reproducible KO of multiple NK cell receptors at the same time would be needed to ensure high efficiency of KO on NK cells, and should be optimised prior to an in-vivo study being carried out.

Of particular interest would be whether NK cells from MM patient-derived BMAs could be expanded and used in functional assays against MM cells. Due to the high expression of Siglec-7 on NK cells from the BM TME compared to NK cells from healthy donor-derived PB, these NK cells represent a good model for further studies into the role of hypersialylation and Siglec-7 in particular. However, whether sufficient NK cells to start expansions or perform functional assays could be gathered from MM patient-derived BMAs is a potential hinderance for this work. However, NK cell expansions can be initiated using a low number of starting cells and require only viable NK cells which could be easily isolated from a fresh MM patient-derived BMA . As an example, in this project, expansions were routinely started using less than 10^6 NK cells. Therefore, it is possible that sufficient NK cells could be gathered from BMAs which can be used in expansions and further functional studies to investigate the potential of enhancing the anti-cancer capabilities of hypofunctional NK cells.

Finally, while this approach generated positive results with enhanced NK cell cytotoxicity upon Siglec-7 KO, CD96 KO and TIGIT KO, it would be interesting to see if these results are similar when cytotoxicity assays between KO or mock NK cells were co-cultured with primary MM cells. In Chapter 3, high expression of Siglec-7L was observed on MM cells isolated from ND patient-derived BMAs. However, primary MM cells are considered highly resistant to NK cells in comparison to MM cell lines. Another approach to investigate the true therapeutic potential of targeting these immune checkpoints would be to investigate the effect of genetically engineered NK cells administered to murine models where MM has been established. As discussed in **1.1.6**, the bone marrow microenvironment contributes to MM cell survival and is considered strongly immunosuppressive. It would be of interest to see the benefits of genetically engineered NK cells in models where the MM TME can be replicative

of the human BME. CRISPR/Cas9 therefore represents a promising approach to generate hyper functional NK cells suitable for cancer therapy.

Appendix A: Ethical Approval Documents



Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Merlin Park University Hospital
Ospidéal na h-Ollscoile, Páirc Mheirlinne
GALWAY UNIVERSITY HOSPITALS

Clinical Research Ethics Committee
Unit 4
Merlin Park Hospital
Galway.

20th December, 2011.

Professor Michael O'Dwyer
Consultant Haematologist
Department of Haematology
University College Hospital
Galway.

Ref: CA – 662 – Multiple Myeloma: ***The role of glycosylation in disease development and progression***

Dear Professor O'Dwyer,

I have considered the above project, and I wish to grant Chairman's approval to proceed.

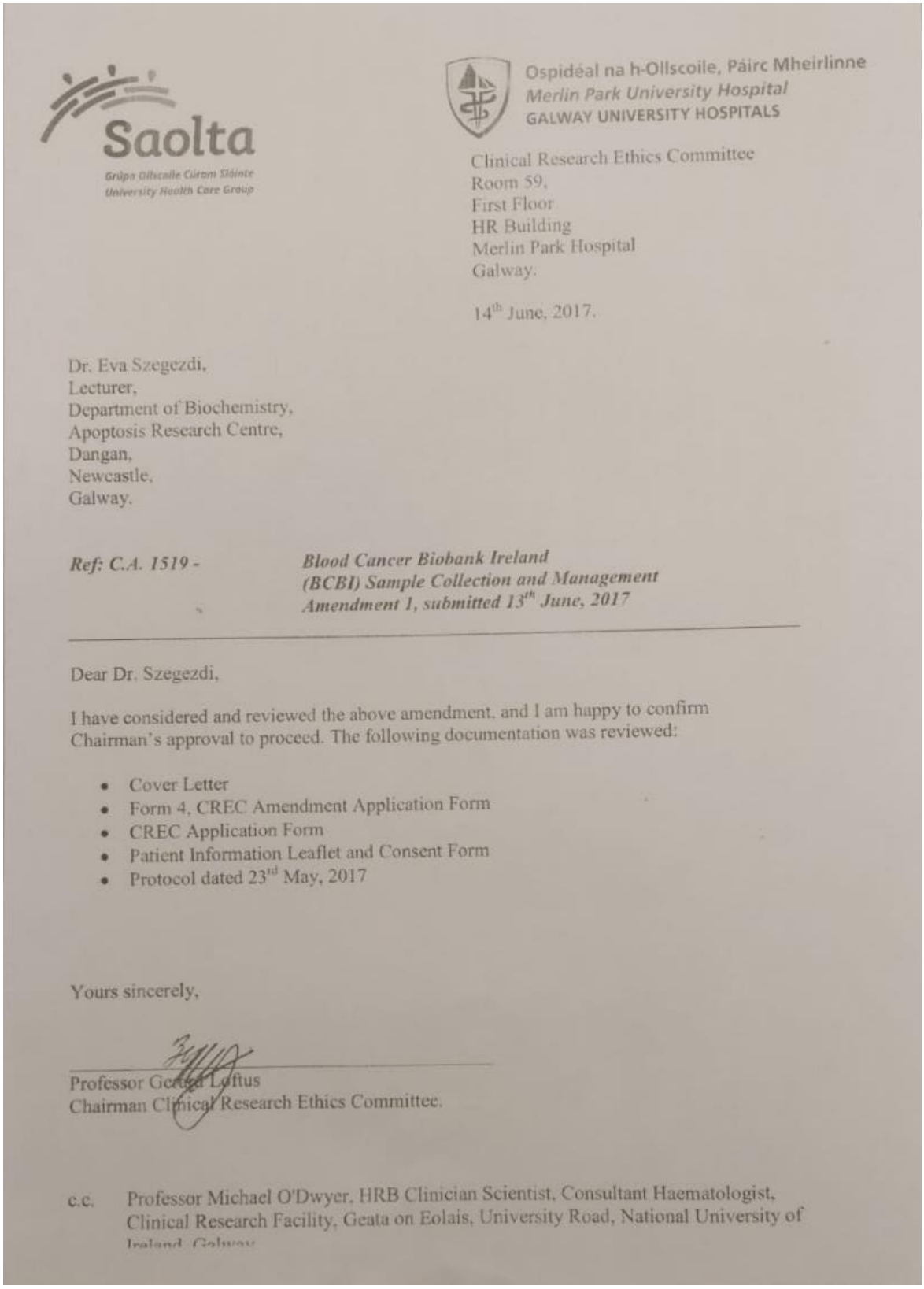
Yours sincerely,

Dr. Shaun T. O'Keeffe
Chairman Clinical Research Ethics Committee.

c.c. Dr. Siobhan Glavey, Department of Glycosciences, NCBES, Galway.

Merlin Park University Hospital, Ospidéal na h-Ollscoile, Páirc Mheirlinne,
Galway, Ireland. Tel: 00 353 (0)91 757631

Figure A.1 Ethical approval for collection of bone marrow aspirates from MM patients for this project



Saolta
Grúpa Ollscoile Cúram Sláinte
University Health Care Group

Ospidéal na h-Ollscoile, Páirc Mheirlinne
Merlin Park University Hospital
GALWAY UNIVERSITY HOSPITALS

Clinical Research Ethics Committee
Room 59,
First Floor
HR Building
Merlin Park Hospital
Galway.

14th June, 2017.

Dr. Eva Szegezdi,
Lecturer,
Department of Biochemistry,
Apoptosis Research Centre,
Dangan,
Newcastle,
Galway.

Ref: C.A. 1519 - ***Blood Cancer Biobank Ireland (BCBI) Sample Collection and Management Amendment 1, submitted 13th June, 2017***

Dear Dr. Szegezdi,

I have considered and reviewed the above amendment, and I am happy to confirm Chairman's approval to proceed. The following documentation was reviewed:

- Cover Letter
- Form 4, CREC Amendment Application Form
- CREC Application Form
- Patient Information Leaflet and Consent Form
- Protocol dated 23rd May, 2017

Yours sincerely,

Professor Gerard Loftus
Chairman Clinical Research Ethics Committee.

c.c. Professor Michael O'Dwyer, HRB Clinician Scientist, Consultant Haematologist, Clinical Research Facility, Geata on Eolais, University Road, National University of Ireland, Galway

Fig A.2 Ethical approval for the collection of MM samples by the Blood Cancer Biobank Ireland

Saolta
Grúpa Ollscoile Cúram Sláinte
University Health Care Group

Ospidéal na h-Ollscoile, Páirc Mheirlinne
Merlin Park University Hospital
GALWAY UNIVERSITY HOSPITALS

Clinical Research Ethics Committee
Room 59
First Floor
HR Building
Merlin Park Hospital
Galway.

4th August, 2017.

Professor Michael O'Dwyer
HRB Clinician Scientist
Consultant Haematologist
Clinical Research Facility
Geata on Eolais
University Road
National University of Ireland
Galway.

Ref: C.A. 1805 - ***An in-vitro investigation into platelet aggregation & interaction with myeloma cells***

Dear Professor O'Dwyer,

I have considered and reviewed the above submission, and I wish to confirm that I am happy to confirm Chairman's approval to proceed.

Yours sincerely,

Professor B. Gerard Loftus
Chairman Clinical Research Ethics Committee.

c.c. Dr. Robert Henderson, Research Fellow to Professor Michael O'Dwyer, Clinical Research Facility, Geata on Eolais, University Road, National University of Ireland Galway.

Ospidéal na h-Ollscoile, Páirc Mheirlinne, Merlin Park University Hospital,
Galway, Ireland. Tel: 00 353 (0)91 757631

Fig A.3 Ethical approval for the collection of peripheral blood from healthy donors for NK cell isolation

Appendix B: Healthy Blood Consent Forms and Information Leaflet

HRB Clinical Research Facility, Galway
Áis Taighde Chliniciúil HRB, Gaillimh

Study Consent Form

Collection of Healthy Donor Blood Samples

Study Principle investigators: Dr Janusz Krawczyk, Professor Michael O'Dwyer
Institution: University Hospital Galway and NUI Galway, Ireland

Please read each sentence and think about your choice. After reading each sentence, initial each box to confirm your consent. If you have any questions, please talk to the nurse before you sign and date this form.

		Initials
1	I am an adult and of my own free will and not under duress I am taking part in this study.	
2	I confirm that I have read and understood the information sheet for the above research and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.	
3	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason and without my rights being affected in any way	
4	I agree to provide peripheral blood samples for the purpose of the study.	
5	I give up all rights to any samples donated and processed as part of this study, as well as rights to any research using these samples, or their products, and to any discovery or product arising from the use of these donated samples or any part, derivative or product of the samples.	

Print Subject Name	Subject Signature	Date
Print Witness Name (if Applicable)	Witness Signature	Date
Print Researcher Name	Researcher Signature	Date

Figure A. Consent form used for collection of healthy donor-derived peripheral blood throughout this project

NUI Galway
OÉ Gaillimh

NCBES
National Centre for
Biomedical Engineering Science
National University of Ireland, Galway

Irish
Cancer
Society

ICAT

Dear Donor,

You are most likely reading this as you have been asked to supply peripheral blood for academic research purposes here at NUI Galway. Allow us to explain why we look for peripheral blood from healthy donors for our research!

Natural Killer (NK) cells are a type of white blood cell who's job it is to recognize and destroy cancerous cells. They are one of the body's frontline defences against our own cells when they become malignantly transformed. They are also highly stimulated by monoclonal antibody (Daratumumab in Multiple Myeloma, for example) treatments and are key effectors of the therapeutic response.

Unfortunately, cancer cells have evolved methods to mask themselves from the surveillance carried out by NK cells, allowing them to migrate freely throughout the circulation and establish new tumour sites. This is particularly true in the case of Multiple Myeloma, where Myeloma cells have developed a thick layer of sugars known as sialic acids on the cell surface. This essentially acts as a cloaking mechanism, preventing the NK cells from recognizing them as cancer cells in need of destruction. Our work investigates methods to enhance the potency of NK cells by removing the sialic acids from the MM cell surface, or by genetically engineering the NK cells to eliminate the inhibitory receptors they possess which are stimulated by sialic acids (these are known as Siglecs). We are also researching other methods to enhance NK cells, for example by creating 'CAR-NK' cells which are modified to recognise specific markers present on the cancer cell surface in multiple myeloma, and acute myeloid leukaemia.

Why do we want fresh blood? We take fresh blood from consenting healthy donors and isolate the NK cells. These NK cells are known as "primary NK" cells, and data gathered using primary NK cells is considered much more impactful than data using NK cell lines which have been "immortalized" and are designed for long term culture in a laboratory incubator. Undergoing immortalization results in the NK cell lines losing some of the characteristics of fresh primary NK cells, and the data gathered may not be truly representative of what is happening within the body. Thus, data gathered using primary NK cells is preferable for higher impact publications and presentations, as well as being a more accurate indicator of the potential therapeutic advantages of our studies! Primary NK cells are also likely to be the basis of future NK cell-based therapies, and this research can also directly contribute to their development.

We hope this helps you to understand what exactly your blood is being used for! Thank you for supporting our work!

Kind Regards,

O'Dwyer lab

Figure B. Information leaflet provided to healthy donors

Appendix C:

Publications, presentations and achievements

Publications:

- ***Daly J,*** Sarkar S, Nation A, Stark J, Riley N, Bertozzi C, Carlsten M, O'Dwyer M: Targeting the hypersialylated Multiple Myeloma cell surface represents a novel therapeutic strategy to enhance NK cell-mediated tumor responses (In preparation)
- Sarkar S, Chauhan SKS, ***Daly J***, Natoni A, Fairfield H, Henderson R, Nolan E, Swan D, Hu J, Reagan MR, et al.: The CD38(low) natural killer cell line KHYG1 transiently expressing CD16(F158V) in combination with daratumumab targets multiple myeloma cells with minimal effector NK cell fratricide. Cancer Immunol. Immunother. 2020, 69(3):421-434. doi: 10.1007/s00262-019-02477-8.
- ***Daly J***, Carlsten M, O'Dwyer M: **Sugar Free: Novel Immunotherapeutic Approaches Targeting Siglecs and Sialic Acids to Enhance Natural Killer Cell Cytotoxicity Against Cancer**. *Frontiers in immunology* 2019, **10**:1047-1047. doi: 10.3389/fimmu.2019.01047

Presentations (Oral):

- ***Daly J,*** Sarkar S, Natoni A, Carlsten M, O'Dwyer M. Immunotherapeutic targeting of Siglec-7 as a novel approach to potentiate the cytotoxic functions of Natural Killer cells against Multiple Myeloma. Haematology Association of Ireland Conference 2018.
- ***Daly J,*** Sarkar S, Natoni A, Carlsten M, O'Dwyer M. Hypersialylation Protects Multiple Myeloma Cells from NK Cell-Mediated Immunosurveillance and This Can be Overcome By Targeted Desialylation Using a Sialyltransferase Inhibitor. ASH 61st Annual Meeting and Exposition (December 7-10, 2019), 652. Myeloma: Pathophysiology and Pre-Clinical Studies, excluding Therapy: Modeling Cellular Immunity and Tumor Microenvironment in Multiple Myeloma.
- ***Daly J***, Sarkar S, Natoni A, Carlsten M, O'Dwyer M. Hypersialylation protects Multiple Myeloma cells from NK cell-mediated immunosurveillance and this can be overcome by targeted desialylation or genetic modification of NK cells. Virtual Cancer Conference: CRISPR in Cancer Research (May 2020), (https://www.youtube.com/watch?v=kvltXoMa4CQ&t=75s)

Presentations (Poster):

- ***Daly J,*** Sarkar S, Duggan T, Hu J, Natoni A, McEllistrim C, Henderson R, McCarthy L, O'Dwyer M. Targeting Siglec-7 – A novel therapeutic approach to potentiate the cytotoxic functions of Natural Killer cells against Multiple Myeloma. ASH 59th Annual Meeting and Exposition (December 7-10th, 2017), 652. Myeloma: Pathophysiology and Pre-Clinical Studies, excluding Therapy: Poster I.
- ***Daly J,*** Sarkar S, Natoni A, Hu J, Henderson R, Duggan T, McCarthy P.L, McEllistrim C, O'Dwyer M. Targeting Siglec-7: A novel immunotherapeutic approach to potentiate the cytotoxic functions of Natural Killer cells against MM cells. Biology and preclinical, European Myeloma Network 1st Meeting 2018.
- ***Daly J,*** Sarkar S, Natoni A, Carlsten M, O'Dwyer M. Hypersialylation protects Myeloma cells from NK cell mediated killing and this can be overcome by targeted desialylation using a sialyltransferase inhibitor. 17th International Myeloma Workshop, Poster Session I FP-181.
- ***Daly J,*** Sarkar S, Natoni A, Carlsten M, O'Dwyer M. Hypersialylation protects Multiple Myeloma cells from NK cell-mediated immunosurveillance and this can be overcome by targeted desialylation using a sialyltransferase inhibitor. 56th IACR Annual Conference 2020, Tumour Immunology, Poster Session 1.
- ***Daly J,*** Gurney M, O'Dwyer M. Knockout of CD96 or TIGIT using CRISPR/Cas9 enhances NK cell-induced cytotoxicity and cytokine production in the presence of CD155 expressing MM cells. European Haematology Association Virtual Conference 2021.

Achievements:

- Irish Cancer Society PhD Student of the year 2020
- Best Student Poster, Irish Association of Cancer Research Conference 2020
- JCI Galway TOYP award 2020 for Medical Innovation

7. References:

1. Solly S: **Remarks on the pathology of mollities ossium; with cases**. *Medico-chirurgical transactions* 1844, **27**:435-498.438.
2. Macintyre W: **Case of Mollities and Fragilitas Ossium, accompanied with urine strongly charged with animal matter**. *Medico-chirurgical transactions* 1850, **33**:211-232.
3. Garcia-Lopez P, Garcia-Marin V, Freire M: **The histological slides and drawings of cajal**. *Frontiers in neuroanatomy* 2010, **4**:9-9.
4. Marschalkó Tv: **Ueber die sogenannten Plasmazellen, ein Beitrag zur Kenntniss der Herkunft der entzündlichen Infiltrationszellen**. *Archiv für Dermatologie und Syphilis* 1895, **30**:3-52.
5. Wright JH: **A case of multiple myeloma**. *Journal of the Boston Society of Medical Sciences* 1900, **4**:195.
6. Geschickter CF: **Multiple myeloma as a single lesion**. *Annals of surgery* 1930, **92**:425.
7. Arinkin M: **Die intravitale untersuchungsmethodik des knochenmarks**. *Folia haematol* 1929, **38**:192.199.
8. Rosenthal N: **Value of the sternal puncture in the diagnosis of multiple myeloma**. *J. Mt Sinai Hosp.* 1938, **4**:1001-1019.
9. Waldenstrom J: **Studies on conditions associated with disturbed gamma globulin formation (gammopathies)**.
10. Kyle RA: **Multiple myeloma: an odyssey of discovery**.
11. Alwall N: **Urethane and stilbamidine in multiple myeloma report on two cases**.
12. Holland Jr Fau - Hosley H, Hosley H Fau - Scharlau C, Scharlau C Fau - Carbone PP, Carbone Pp Fau - Frei E, 3rd, Frei E 3rd Fau - Brindley CO, Brindley Co Fau - Hall TC, Hall Tc Fau - Shnider BI, Shnider Bi Fau - Gold GL, Gold Gl Fau - Lasagna L, Lasagna L Fau - Owens AH, Jr., et al.: **A controlled trial of urethane treatment in multiple myeloma**.
13. Blokhin N Fau - Larionov L, Larionov L Fau - Perevodchikova N, Perevodchikova N Fau - Chebotareva L, Chebotareva L Fau - Merkulova N, Merkulova N: **[Clinical experiences with sarcolysin in neoplastic diseases]**.
14. Bergsagel De Fau - Sprague CC, Sprague Cc Fau - Austin C, Austin C Fau - Griffith KM, Griffith KM: **Evaluation of new chemotherapeutic agents in the treatment of multiple myeloma. IV. L-Phenylalanine mustard (NSC-8806)**.

15. Hoogstraten B Fau - Sheehe PR, Sheehe Pr Fau - Cuttner J, Cuttner J Fau - Cooper T, Cooper T Fau - Kyle RA, Kyle Ra Fau - Oberfield RA, Oberfield Ra Fau - Townsend SR, Townsend Sr Fau - Harley JB, Harley Jb Fau - Hayes DM, Hayes Dm Fau - Costa G, Costa G Fau - Holland JF, et al.: **Melphalan in multiple myeloma**.
16. Alexanian R Fau - Haut A, Haut A Fau - Khan AU, Khan Au Fau - Lane M, Lane M Fau - McKelvey EM, McKelvey Em Fau - Migliore PJ, Migliore Pj Fau - Stuckey WJ, Jr., Stuckey Wj Jr Fau - Wilson HE, Wilson HE: **Treatment for multiple myeloma. Combination chemotherapy with different melphalan dose regimens**.
17. Lee Bj Fau - Sahakian G, Sahakian G Fau - Clarkson BD, Clarkson Bd Fau - Krakoff IH, Krakoff IH: **Proceedings: Combination chemotherapy of multiple myeloma with alkeran, cytoxan, vincristine, prednisone, and BCNU**.
18. Case Dc Jr Fau - Lee DJ, 3rd, Lee Dj 3rd Fau - Clarkson BD, Clarkson BD: **Improved survival times in multiple myeloma treated with melphalan, prednisone, cyclophosphamide, vincristine and BCNU: M-2 protocol**.
19. **Combination chemotherapy versus melphalan plus prednisone as treatment for multiple myeloma: an overview of 6,633 patients from 27 randomized trials. Myeloma Trialists' Collaborative Group**.
20. Thomas Ed Fau - Lochte HL, Jr., Lochte Hl Jr Fau - Lu WC, Lu Wc Fau - Ferrebee JW, Ferrebee JW: **Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy**.
21. Osserman Ef Fau - DiRe LB, DiRe Lb Fau - DiRe J, DiRe J Fau - Sherman WH, Sherman Wh Fau - Hersman JA, Hersman Ja Fau - Storb R, Storb R: **Identical twin marrow transplantation in multiple myeloma**.
22. Barlogie B Fau - Alexanian R, Alexanian R Fau - Dicke KA, Dicke Ka Fau - Zagars G, Zagars G Fau - Spitzer G, Spitzer G Fau - Jagannath S, Jagannath S Fau - Horwitz L, Horwitz L: **High-dose chemoradiotherapy and autologous bone marrow transplantation for resistant multiple myeloma**.
23. Hemminki K, Försti A, Hansson M: **Incidence, mortality and survival in multiple myeloma compared to other hematopoietic neoplasms in Sweden up to year 2016**. *Scientific Reports* 2021, **11**:17272.
24. Palumbo A, Anderson K: **Multiple Myeloma**. *New England Journal of Medicine* 2011, **364**:1046-1060.

25. Kyle RA, Gertz Ma Fau - Witzig TE, Witzig Te Fau - Lust JA, Lust Ja Fau - Lacy MQ, Lacy Mq Fau - Dispenzieri A, Dispenzieri A Fau - Fonseca R, Fonseca R Fau - Rajkumar SV, Rajkumar Sv Fau - Offord JR, Offord Jr Fau - Larson DR, Larson Dr Fau - Plevak ME, et al.: **Review of 1027 patients with newly diagnosed multiple myeloma**.
26. Bladé J, Kyle Ra Fau - Greipp PR, Greipp PR: **Multiple myeloma in patients younger than 30 years. Report of 10 cases and review of the literature**.
27. Lynch HT, Sanger Wg Fau - Pirruccello S, Pirruccello S Fau - Quinn-Laquer B, Quinn-Laquer B Fau - Weisenburger DD, Weisenburger DD: **Familial multiple myeloma: a family study and review of the literature**.
28. Landgren O, Gridley G Fau - Turesson I, Turesson I Fau - Caporaso NE, Caporaso Ne Fau - Goldin LR, Goldin Lr Fau - Baris D, Baris D Fau - Fears TR, Fears Tr Fau - Hoover RN, Hoover Rn Fau - Linet MS, Linet MS: **Risk of monoclonal gammopathy of undetermined significance (MGUS) and subsequent multiple myeloma among African American and white veterans in the United States**.
29. Waxman AJ, Mink Pj Fau - Devesa SS, Devesa Ss Fau - Anderson WF, Anderson Wf Fau - Weiss BM, Weiss Bm Fau - Kristinsson SY, Kristinsson Sy Fau - McGlynn KA, McGlynn Ka Fau - Landgren O, Landgren O: **Racial disparities in incidence and outcome in multiple myeloma: a population-based study**.
30. Cowan AJ, Allen C, Barac A, Basaleem H, Bensenor I, Curado MP, Foreman K, Gupta R, Harvey J, Hosgood HD, et al.: **Global Burden of Multiple Myeloma: A Systematic Analysis for the Global Burden of Disease Study 2016**. *JAMA oncology* 2018, **4**:1221-1227.
31. van de Donk NWCJ, Pawlyn C, Yong KL: **Multiple myeloma**. *The Lancet* 2021, **397**:410-427.
32. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK: **Improved survival in multiple myeloma and the impact of novel therapies**. *Blood* 2008, **111**.
33. Schaapveld M, Visser O Fau - Siesling S, Siesling S Fau - Schaar CG, Schaar Cg Fau - Zweegman S, Zweegman S Fau - Vellenga E, Vellenga E: **Improved survival among younger but not among older patients with Multiple Myeloma in the Netherlands, a population-based study since 1989**.
34. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2016**.

35. Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, et al.: **Improved survival in multiple myeloma and the impact of novel therapies**. *Blood* 2008, **111**:2516-2520.

36. Kristinsson SY, Landgren O Fau - Dickman PW, Dickman Pw Fau - Derolf AR, Derolf Ar Fau - Björkholm M, Björkholm M: **Patterns of survival in multiple myeloma: a population-based study of patients diagnosed in Sweden from 1973 to 2003**.

37. Turesson I, Velez R, Kristinsson SY, Landgren O: **Patterns of improved survival in patients with multiple myeloma in the twenty-first century: a population-based study**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010, **28**:830-834.

38. Brenner H, Gondos A Fau - Pulte D, Pulte D: **Recent major improvement in long-term survival of younger patients with multiple myeloma**.

39. Hillengass J, Usmani S, Rajkumar SV, Durie BGM, Mateos M-V, Lonial S, Joao C, Anderson KC, García-Sanz R, Riva E, et al.: **International myeloma working group consensus recommendations on imaging in monoclonal plasma cell disorders**. *The Lancet Oncology* 2019, **20**:e302-e312.

40. Sonneveld P, Avet-Loiseau H, Lonial S, Usmani S, Siegel D, Anderson KC, Chng W-J, Moreau P, Attal M, Kyle RA, et al.: **Treatment of multiple myeloma with high-risk cytogenetics: a consensus of the International Myeloma Working Group**. *Blood* 2016, **127**:2955-2962.

41. Katzmann JA, Dispenzieri A Fau - Kyle RA, Kyle Ra Fau - Snyder MR, Snyder Mr Fau - Plevak MF, Plevak Mf Fau - Larson DR, Larson Dr Fau - Abraham RS, Abraham Rs Fau - Lust JA, Lust Ja Fau - Melton LJ, 3rd, Melton Lj 3rd Fau - Rajkumar SV, Rajkumar SV: **Elimination of the need for urine studies in the screening algorithm for monoclonal gammopathies by using serum immunofixation and free light chain assays**.

42. Rajkumar SV, Dimopoulos MA, Palumbo A, Blade J, Merlini G, Mateos MV, Kumar S, Hillengass J, Kastritis E, Richardson P, et al.: **International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma**.

43. Nakaya A, Fujita S, Satake A, Nakanishi T, Azuma Y, Tsubokura Y, Hotta M, Yoshimura H, Ishii K, Ito T, et al.: **Impact of CRAB Symptoms in Survival of Patients with Symptomatic Myeloma in Novel Agent Era**. *Hematology reports* 2017, **9**:6887-6887.

44. Kyle RA, Therneau Tm Fau - Rajkumar SV, Rajkumar Sv Fau - Larson DR, Larson Dr Fau - Plevak MF, Plevak Mf Fau - Offord JR, Offord Jr Fau - Dispenzieri A, Dispenzieri A Fau - Katzmann JA, Katzmann Ja Fau - Melton LJ, 3rd, Melton LJ, 3rd: **Prevalence of monoclonal gammopathy of undetermined significance**.

45. Kyle R, Durie B, Rajkumar SV, Landgren O, Bladé J, Merlini G, Kröger N, Einsele H, Vesole D, Dimopoulos M: **Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management**. *Leukemia* 2010, **24**:1121-1127.

46. Landgren O, Graubard B, Kumar S, Kyle R, Katzmann J, Murata K, Costello R, Dispenzieri A, Caporaso N, Mailankody S: **Prevalence of myeloma precursor state monoclonal gammopathy of undetermined significance in 12372 individuals 10–49 years old: a population-based study from the National Health and Nutrition Examination Survey**. *Blood cancer journal* 2017, **7**:e618-e618.

47. Bladé J, Rosiñol L Fau - Cibeira MT, Cibeira Mt Fau - de Larrea CF, de Larrea CF: **Pathogenesis and progression of monoclonal gammopathy of undetermined significance**.

48. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, Melton III LJ: **A long-term study of prognosis in monoclonal gammopathy of undetermined significance**. *New England Journal of Medicine* 2002, **346**:564-569.

49. Rosiñol L, Cibeira MT, Montoto S, Rozman M, Esteve J, Filella X, Bladé J: **Monoclonal gammopathy of undetermined significance: predictors of malignant transformation and recognition of an evolving type characterized by a progressive increase in M protein size**. In *Mayo Clinic Proceedings*: Elsevier: 2007:428-434.

50. Blade J, Lopez-Guillermo A, Rozman C, Cervantes F, Salgado C, Aguilar JL, Vives-Corrons JL, Montserrat E: **Malignant transformation and life expectancy in monoclonal gammopathy of undetermined significance**. *British journal of haematology* 1992, **81**:391-394.

51. Kyle RA, Remstein Ed Fau - Therneau TM, Therneau Tm Fau - Dispenzieri A, Dispenzieri A Fau - Kurtin PJ, Kurtin Pj Fau - Hodnefield JM, Hodnefield Jm Fau - Larson DR, Larson Dr Fau - Plevak MF, Plevak Mf Fau - Jelinek DF, Jelinek Df Fau - Fonseca R, Fonseca R

Fau - Melton LJ, 3rd, et al.: **Clinical course and prognosis of smoldering (asymptomatic) multiple myeloma**.

52. Rosiñol L, Bladé J Fau - Esteve J, Esteve J Fau - Aymerich M, Aymerich M Fau - Rozman M, Rozman M Fau - Montoto S, Montoto S Fau - Giné E, Giné E Fau - Nadal E, Nadal E Fau - Filella X, Filella X Fau - Queralt R, Queralt R Fau - Carrió A, et al.: **Smoldering multiple myeloma: natural history and recognition of an evolving type**.
53. Pérez-Persona E, Vidriales Mb Fau - Mateo G, Mateo G Fau - García-Sanz R, García-Sanz R Fau - Mateos M-V, Mateos Mv Fau - de Coca AG, de Coca Ag Fau - Galende J, Galende J Fau - Martín-Nuñez G, Martín-Nuñez G Fau - Alonso JM, Alonso Jm Fau - de Las Heras N, de Las Heras N Fau - Hernández JM, et al.: **New criteria to identify risk of progression in monoclonal gammopathy of uncertain significance and smoldering multiple myeloma based on multiparameter flow cytometry analysis of bone marrow plasma cells**.
54. Pérez-Persona E, Mateo G Fau - García-Sanz R, García-Sanz R Fau - Mateos M-V, Mateos Mv Fau - de Las Heras N, de Las Heras N Fau - de Coca AG, de Coca Ag Fau - Hernández JM, Hernández Jm Fau - Galende J, Galende J Fau - Martín-Nuñez G, Martín-Nuñez G Fau - Bárez A, Bárez A Fau - Alonso JM, et al.: **Risk of progression in smouldering myeloma and monoclonal gammopathies of unknown significance: comparative analysis of the evolution of monoclonal component and multiparameter flow cytometry of bone marrow plasma cells**.
55. Rawstron AC, Orfao A Fau - Beksac M, Beksac M Fau - Bezdickova L, Bezdickova L Fau - Brooimans RA, Brooimans Ra Fau - Bumbea H, Bumbea H Fau - Dalva K, Dalva K Fau - Fuhler G, Fuhler G Fau - Gratama J, Gratama J Fau - Hose D, Hose D Fau - Kovarova L, et al.: **Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders**.
56. Minges Wols HA: **Plasma Cells**. *eLS* 2015:1-11.
57. Lu LL, Suscovich TJ, Fortune SM, Alter G: **Beyond binding: antibody effector functions in infectious diseases**. *Nature Reviews Immunology* 2018, **18**:46-61.
58. Corre J, Munshi N, Avet-Loiseau H: **Genetics of multiple myeloma: another heterogeneity level?** *Blood* 2015, **125**:1870-1876.
59. Prideaux SM, Conway O'Brien E, Chevassut TJ: **The Genetic Architecture of Multiple Myeloma**. *Advances in Hematology* 2014, **2014**:16.

60. Smadja N, Fruchart C, Isnard F, Louvet C, Dutel J, Cheron N, Grange M, Monconduit M, Bastard C: **Chromosomal analysis in multiple myeloma: cytogenetic evidence of two different diseases**. *Leukemia* 1998, **12**:960-969.

61. Smadja NVr, Bastard C, Brigaudeau C, Leroux D, Fruchart C, on behalf of the Groupe Français de Cytogénétique Hm: **Hypodiploidy is a major prognostic factor in multiple myeloma**. *Blood* 2001, **98**:2229-2238.

62. Chesi M, Bergsagel PL: **Advances in the pathogenesis and diagnosis of multiple myeloma**. *International Journal of Laboratory Hematology* 2015, **37**:108-114.

63. Chng WJ, Glebov O, Bergsagel PL, Kuehl WM: **Genetic events in the pathogenesis of multiple myeloma**. *Best practice & research. Clinical haematology* 2007, **20**:571-596.

64. Chesi M, Bergsagel Pl Fau - Brents LA, Brents La Fau - Smith CM, Smith Cm Fau - Gerhard DS, Gerhard Ds Fau - Kuehl WM, Kuehl WM: **Dysregulation of cyclin D1 by translocation into an IgH gamma switch region in two multiple myeloma cell lines**.

65. Martinez-Garcia E, Popovic R Fau - Min D-J, Min Dj Fau - Sweet SMM, Sweet Sm Fau - Thomas PM, Thomas Pm Fau - Zamdborg L, Zamdborg L Fau - Heffner A, Heffner A Fau - Will C, Will C Fau - Lamy L, Lamy L Fau - Staudt LM, Staudt Lm Fau - Levens DL, et al.: **The MMSET histone methyl transferase switches global histone methylation and alters gene expression in t(4;14) multiple myeloma cells**.

66. Chesi M, Bergsagel Pl Fau - Shonukan OO, Shonukan Oo Fau - Martelli ML, Martelli Ml Fau - Brents LA, Brents La Fau - Chen T, Chen T Fau - Schröck E, Schröck E Fau - Ried T, Ried T Fau - Kuehl WM, Kuehl WM: **Frequent dysregulation of the c-maf proto-oncogene at 16q23 by translocation to an Ig locus in multiple myeloma**.

67. Hanamura I, Iida S Fau - Akano Y, Akano Y Fau - Hayami Y, Hayami Y Fau - Kato M, Kato M Fau - Miura K, Miura K Fau - Harada S, Harada S Fau - Banno S, Banno S Fau - Wakita A, Wakita A Fau - Kiyoi H, Kiyoi H Fau - Naoe T, et al.: **Ectopic expression of MAFB gene in human myeloma cells carrying (14;20)(q32;q11) chromosomal translocations**.

68. Bergsagel PL, Kuehl Wm Fau - Zhan F, Zhan F Fau - Sawyer J, Sawyer J Fau - Barlogie B, Barlogie B Fau - Shaughnessy J, Jr., Shaughnessy J, Jr.: **Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma**.

69. Prideaux SM, Conway O'Brien E, Chevassut TJ: **The Genetic Architecture of Multiple Myeloma**. *Advances in Hematology* 2014, **2014**:864058.

70. Onodera N, McCabe NR, Rubin CM: **Formation of a hyperdiploid karyotype in childhood acute lymphoblastic leukemia [see comments]**. 1992.

71. Avet-Loiseau H, Gerson F, Magrangeas F, Minvielle S, Harousseau J-L, Bataille Rg: **Rearrangements of the c-myc oncogene are present in 15% of primary human multiple myeloma tumors**. *Blood, The Journal of the American Society of Hematology* 2001, **98**:3082-3086.

72. Abdallah NA-O, Baughn LB, Rajkumar SV, Kapoor P, Gertz MA-O, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Dingli D, et al.: **Implications of MYC Rearrangements in Newly Diagnosed Multiple Myeloma**.

73. Shirazi F, Jones RJ, Singh RK, Zou J, Kuiatse I, Berkova Z, Wang H, Lee HC, Hong S, Dick L, et al.: **Activating KRAS, NRAS, and BRAF mutants enhance proteasome capacity and reduce endoplasmic reticulum stress in multiple myeloma**. *Proceedings of the National Academy of Sciences* 2020, **117**:20004.

74. Annunziata CM, Davis RE, Demchenko Y, Bellamy W, Gabrea A, Zhan F, Lenz G, Hanamura I, Wright G, Xiao W: **Frequent engagement of the classical and alternative NF-κB pathways by diverse genetic abnormalities in multiple myeloma**. *Cancer cell* 2007, **12**:115-130.

75. Keats JJ, Fonseca R, Chesi M, Schop R, Baker A, Chng W-J, Van Wier S, Tiedemann R, Shi C-X, Sebag M: **Promiscuous mutations activate the noncanonical NF-κB pathway in multiple myeloma**. *Cancer cell* 2007, **12**:131-144.

76. Chauhan D, Uchiyama H, Akbarali Y, Urashima M, Yamamoto K-l, Libermann TA, Anderson KC: **Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B**. 1996.

77. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H: **Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas**. *Nature* 1988, **332**:83-85.

78. Tu Y, Renner S, Xu F-h, Fleishman A, Taylor J, Weisz J, Vescio R, Rettig M, Berenson J, Krajewski S: **BCL-X expression in multiple myeloma: possible indicator of chemoresistance**. *Cancer research* 1998, **58**:256-262.

79. Aronson LI, Davenport EL, Giuntoli SG, Srikanth M, Smith E, Morgan GJ, Davies F: **Autophagy is a key myeloma survival pathway that can be manipulated therapeutically to enhance apoptosis**. *Blood* 2010, **116**:4083.

80. Jagannathan-Bogdan M, Zon LI: **Hematopoiesis**. *Development (Cambridge, England)* 2013, **140**:2463-2467.

81. Manier S, Sacco A, Leleu X, Ghobrial IM, Roccaro AM: **Bone Marrow Microenvironment in Multiple Myeloma Progression**. *Journal of Biomedicine and Biotechnology* 2012, **2012**:5.

82. Hou J, Wei R, Qian J, Wang R, Fan Z, Gu C, Yang Y: **The impact of the bone marrow microenvironment on multiple myeloma (Review)**. *Oncol Rep* 2019, **42**:1272-1282.

83. Kawano Y, Moschetta M, Manier S, Glavey S, Görgün GT, Roccaro AM, Anderson KC, Ghobrial IM: **Targeting the bone marrow microenvironment in multiple myeloma**. *Immunological Reviews* 2015, **263**:160-172.

84. Ribatti D, Nico B, Vacca A: **Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma**. *Oncogene* 2006, **25**:4257-4266.

85. Menu E, Asosingh K, Indraccolo S, De Raeve H, Van Riet I, Van Valckenborgh E, Van de Broek I, Fujii N, Tamamura H, Van Camp B: **The involvement of stromal derived factor 1alpha in homing and progression of multiple myeloma in the 5TMM model**. *haematologica* 2006, **91**:605-612.

86. Moser-Katz T, Joseph NS, Dhodapkar MV, Lee KP, Boise LH: **Game of Bones: How Myeloma Manipulates Its Microenvironment**. *Frontiers in Oncology* 2021, **10**.

87. Maiso P, Mogollón P, Ocio EM, Garayoa M: **Bone Marrow Mesenchymal Stromal Cells in Multiple Myeloma: Their Role as Active Contributors to Myeloma Progression**. *Cancers* 2021, **13**.

88. Kumar S, Witzig Te Fau - Timm M, Timm M Fau - Haug J, Haug J Fau - Wellik L, Wellik L Fau - Fonseca R, Fonseca R Fau - Greipp PR, Greipp Pr Fau - Rajkumar SV, Rajkumar SV: **Expression of VEGF and its receptors by myeloma cells**.

89. Tanno T, Lim Y Fau - Wang Q, Wang Q Fau - Chesi M, Chesi M Fau - Bergsagel PL, Bergsagel Pl Fau - Matthews G, Matthews G Fau - Johnstone RW, Johnstone Rw Fau - Ghosh N, Ghosh N Fau - Borrello I, Borrello I Fau - Huff CA, Huff Ca Fau - Matsui W, et al.: **Growth differentiating factor 15 enhances the tumor-initiating and self-renewal potential of multiple myeloma cells**.

90. Giuliani N, Storti P, Bolzoni M, Palma BD, Bonomini S: **Angiogenesis and multiple myeloma**. *Cancer microenvironment : official journal of the International Cancer Microenvironment Society* 2011, **4**:325-337.

91. Roccaro AM, Sacco A, Maiso P, Azab AK, Tai Y-T, Reagan M, Azab F, Flores LM, Campigotto F, Weller E: **BM mesenchymal stromal cell–derived exosomes facilitate multiple myeloma progression**. *The Journal of clinical investigation* 2013, **123**:1542-1555.

92. Wang J, Hendrix A, Hernot S, Lemaire M, De Bruyne E, Van Valckenborgh E, Lahoutte T, De Wever O, Vanderkerken K, Menu E: **Bone marrow stromal cell–derived exosomes as communicators in drug resistance in multiple myeloma cells**. *Blood, The Journal of the American Society of Hematology* 2014, **124**:555-566.

93. Gabrilovich DI, Nagaraj S: **Myeloid-derived suppressor cells as regulators of the immune system**. *Nature Reviews Immunology* 2009, **9**:162-174.

94. Diaz-Montero CM, Salem Ml Fau - Nishimura MI, Nishimura Mi Fau - Garrett-Mayer E, Garrett-Mayer E Fau - Cole DJ, Cole Dj Fau - Montero AJ, Montero AJ: **Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy**.

95. Ramachandran IR, Martner A Fau - Pisklakova A, Pisklakova A Fau - Condamine T, Condamine T Fau - Chase T, Chase T Fau - Vogl T, Vogl T Fau - Roth J, Roth J Fau - Gabrilovich D, Gabrilovich D Fau - Nefedova Y, Nefedova Y: **Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow**.

96. Huang B, Pan Py Fau - Li Q, Li Q Fau - Sato AI, Sato Ai Fau - Levy DE, Levy De Fau - Bromberg J, Bromberg J Fau - Divino CM, Divino Cm Fau - Chen S-H, Chen SH: **Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host**.

97. Movahedi K, Guilliams M Fau - Van den Bossche J, Van den Bossche J Fau - Van den Bergh R, Van den Bergh R Fau - Gysemans C, Gysemans C Fau - Beschin A, Beschin A Fau - De Baetselier P, De Baetselier P Fau - Van Ginderachter JA, Van Ginderachter JA: **Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity**.

98. Curiel TJ, Coukos G Fau - Zou L, Zou L Fau - Alvarez X, Alvarez X Fau - Cheng P, Cheng P Fau - Mottram P, Mottram P Fau - Evdemon-Hogan M, Evdemon-Hogan M Fau - Conejo-

Garcia JR, Conejo-Garcia Jr Fau - Zhang L, Zhang L Fau - Burow M, Burow M Fau - Zhu Y, et al.: **Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival**.

99. Podar K, Anderson KC: **The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications**. *Blood* 2005, **105**:1383-1395.

100. Bhutani M, Turkbey B, Tan E, Kemp TJ, Pinto LA, Berg AR, Korde N, Minter AR, Weiss BM, Mena E, et al.: **Bone marrow angiogenesis in myeloma and its precursor disease: a prospective clinical trial**. *Leukemia* 2014, **28**:413-416.

101. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC: **Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets**. *Nat Rev Cancer* 2007, **7**:585-598.

102. Oranger A, Carbone C, Izzo M, Grano M: **Cellular Mechanisms of Multiple Myeloma Bone Disease**. *Clinical and Developmental Immunology* 2013, **2013**:289458.

103. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, Shaughnessy JD: **The Role of the Wnt-Signaling Antagonist DKK1 in the Development of Osteolytic Lesions in Multiple Myeloma**. *New England Journal of Medicine* 2003, **349**:2483-2494.

104. Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Akiyama M, Hideshima T, Chauhan D, Joseph M, Libermann TA: **Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors**. *Cancer cell* 2004, **5**:221-230.

105. Georgii-Hemming P, Wiklund Hj Fau - Ljunggren O, Ljunggren O Fau - Nilsson K, Nilsson K: **Insulin-like growth factor I is a growth and survival factor in human multiple myeloma cell lines**.

106. Vanderkerken K, Asosingh K Fau - Braet F, Braet F Fau - Van Riet I, Van Riet I Fau - Van Camp B, Van Camp B: **Insulin-like growth factor-1 acts as a chemoattractant factor for 5T2 multiple myeloma cells**.

107. Ge NL, Rudikoff S: **Insulin-like growth factor I is a dual effector of multiple myeloma cell growth**.

108. Ogata A, Chauhan D Fau - Urashima M, Urashima M Fau - Teoh G, Teoh G Fau - Treon SP, Treon Sp Fau - Anderson KC, Anderson KC: **Blockade of mitogen-activated protein kinase cascade signaling in interleukin 6-independent multiple myeloma cells**.

109. Harrison Js Fau - Rameshwar P, Rameshwar P Fau - Chang V, Chang V Fau - Bandari P, Bandari P: **Oxygen saturation in the bone marrow of healthy volunteers**.

110. Azab AK, Hu J, Quang P, Azab F, Pitsillides C, Awwad R, Thompson B, Maiso P, Sun JD, Hart CP, et al.: **Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features**. *Blood* 2012, **119**:5782-5794.

111. Storti P, Bolzoni M, Donofrio G, Airoldi I, Guasco D, Toscani D, Martella E, Lazzaretti M, Mancini C, Agnelli L, et al.: **Hypoxia-inducible factor (HIF)-1α suppression in myeloma cells blocks tumoral growth in vivo inhibiting angiogenesis and bone destruction**. *Leukemia* 2013, **27**:1697-1706.

112. Muz B, De La Puente P, Azab F, Luderer M, Azab A: **Hypoxia promotes stem cell-like phenotype in multiple myeloma cells**. *Blood cancer journal* 2014, **4**:e262-e262.

113. Hu J, Handisides Dr Fau - Van Valckenborgh E, Van Valckenborgh E Fau - De Raeve H, De Raeve H Fau - Menu E, Menu E Fau - Vande Broek I, Vande Broek I Fau - Liu Q, Liu Q Fau - Sun JD, Sun Jd Fau - Van Camp B, Van Camp B Fau - Hart CP, Hart Cp Fau - Vanderkerken K, et al.: **Targeting the multiple myeloma hypoxic niche with TH-302, a hypoxia-activated prodrug**.

114. Hu J, Van Valckenborgh E Fau - Xu D, Xu D Fau - Menu E, Menu E Fau - De Raeve H, De Raeve H Fau - De Bruyne E, De Bruyne E Fau - Xu S, Xu S Fau - Van Camp B, Van Camp B Fau - Handisides D, Handisides D Fau - Hart CP, Hart Cp Fau - Vanderkerken K, et al.: **Synergistic induction of apoptosis in multiple myeloma cells by bortezomib and hypoxia-activated prodrug TH-302, in vivo and in vitro**.

115. Hideshima T, Chauhan D Fau - Podar K, Podar K Fau - Schlossman RL, Schlossman Rl Fau - Richardson P, Richardson P Fau - Anderson KC, Anderson KC: **Novel therapies targeting the myeloma cell and its bone marrow microenvironment**.

116. Azab AK, Runnels Jm Fau - Pitsillides C, Pitsillides C Fau - Moreau A-S, Moreau As Fau - Azab F, Azab F Fau - Leleu X, Leleu X Fau - Jia X, Jia X Fau - Wright R, Wright R Fau - Ospina B, Ospina B Fau - Carlson AL, Carlson Al Fau - Alt C, et al.: **CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy**.

117. Ghiringhelli F, Larmonier N Fau - Schmitt E, Schmitt E Fau - Parcellier A, Parcellier A Fau - Cathelin D, Cathelin D Fau - Garrido C, Garrido C Fau - Chauffert B, Chauffert B Fau -

Solary E, Solary E Fau - Bonnotte B, Bonnotte B Fau - Martin F, Martin F: **CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative**.

118. Rajkumar SV, Harousseau J-L, Durie B, Anderson KC, Dimopoulos M, Kyle R, Blade J, Richardson P, Orlowski R, Siegel D: **Consensus recommendations for the uniform reporting of clinical trials: report of the International Myeloma Workshop Consensus Panel 1**. *Blood, The Journal of the American Society of Hematology* 2011, **117**:4691-4695.
119. van de Velde H, Londhe A, Ataman O, Johns HL, Hill S, Landers E, Berlin JA: **Association between complete response and outcomes in transplant-eligible myeloma patients in the era of novel agents**. *European journal of haematology* 2017, **98**:269-279.
120. Rajkumar SV: **Thalidomide: tragic past and promising future**.
121. Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, Munshi N, Anaissie E, Wilson C, Dhodapkar M: **Antitumor activity of thalidomide in refractory multiple myeloma**. *New England Journal of Medicine* 1999, **341**:1565-1571.
122. Dimopoulos MA, Anagnostopoulos A Fau - Weber D, Weber D: **Treatment of plasma cell dyscrasias with thalidomide and its derivatives**.
123. Lacy MQ, Allred JB, Gertz MA, Hayman SR, Short KD, Buadi F, Dispenzieri A, Kumar S, Greipp PR, Lust JA: **Pomalidomide plus low-dose dexamethasone in myeloma refractory to both bortezomib and lenalidomide: comparison of 2 dosing strategies in dual-refractory disease**. *Blood, The Journal of the American Society of Hematology* 2011, **118**:2970-2975.
124. Schey S, Fields P, Bartlett J, Clarke I, Ashan G, Knight R, Streetly M, Dalgleish A: **Phase I study of an immunomodulatory thalidomide analog, CC-4047, in relapsed or refractory multiple myeloma**. *Journal of Clinical Oncology* 2004, **22**:3269-3276.
125. Ribatti D: **A historical perspective on milestones in multiple myeloma research**. *European Journal of Haematology* 2018, **100**:221-228.
126. Richardson PG, Blood E, Mitsiades CS, Jagannath S, Zeldenrust SR, Alsina M, Schlossman RL, Rajkumar SV, Desikan KR, Hideshima T: **A randomized phase 2 study of lenalidomide therapy for patients with relapsed or relapsed and refractory multiple myeloma**. *Blood* 2006, **108**:3458-3464.

127. Dimopoulos M, Spencer A Fau - Attal M, Attal M Fau - Prince HM, Prince Hm Fau - Harousseau J-L, Harousseau Jl Fau - Dmoszynska A, Dmoszynska A Fau - San Miguel J, San Miguel J Fau - Hellmann A, Hellmann A Fau - Facon T, Facon T Fau - Foà R, Foà R Fau - Corso A, et al.: **Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma**.

128. Mateos M-V, Hernández M-T, Giraldo P, de la Rubia J, de Arriba F, Corral LL, Rosiñol L, Paiva B, Palomera L, Bargay J, et al.: **Lenalidomide plus Dexamethasone for High-Risk Smoldering Multiple Myeloma**. *New England Journal of Medicine* 2013, **369**:438-447.

129. Adams J, Palombella Vj Fau - Sausville EA, Sausville Ea Fau - Johnson J, Johnson J Fau - Destree A, Destree A Fau - Lazarus DD, Lazarus Dd Fau - Maas J, Maas J Fau - Pien CS, Pien Cs Fau - Prakash S, Prakash S Fau - Elliott PJ, Elliott PJ: **Proteasome inhibitors: a novel class of potent and effective antitumor agents**.

130. Orlowski RZ, Stinchcombe Te Fau - Mitchell BS, Mitchell Bs Fau - Shea TC, Shea Tc Fau - Baldwin AS, Baldwin As Fau - Stahl S, Stahl S Fau - Adams J, Adams J Fau - Esseltine D-L, Esseltine Dl Fau - Elliott PJ, Elliott Pj Fau - Pien CS, Pien Cs Fau - Guerciolini R, et al.: **Phase I trial of the proteasome inhibitor PS-341 in patients with refractory hematologic malignancies**.

131. Richardson PG, Barlogie B Fau - Berenson J, Berenson J Fau - Singhal S, Singhal S Fau - Jagannath S, Jagannath S Fau - Irwin D, Irwin D Fau - Rajkumar SV, Rajkumar Sv Fau - Srkalovic G, Srkalovic G Fau - Alsina M, Alsina M Fau - Alexanian R, Alexanian R Fau - Siegel D, et al.: **A phase 2 study of bortezomib in relapsed, refractory myeloma**.

132. van de Donk NWCJ, Kamps S, Mutis T, Lokhorst HM: **Monoclonal antibody-based therapy as a new treatment strategy in multiple myeloma**. *Leukemia* 2012, **26**:199-213.

133. Rosenblatt J, Avigan D: **Targeting the PD-1/PD-L1 axis in multiple myeloma: a dream or a reality?**

134. Gajewski TF, Schreiber H Fau - Fu Y-X, Fu YX: **Innate and adaptive immune cells in the tumor microenvironment**.

135. Mahoney KM, Freeman GJ, McDermott DF: **The Next Immune-Checkpoint Inhibitors: PD-1/PD-L1 Blockade in Melanoma**.

136. Jing W, Li M, Zhang Y, Teng F, Han A, Kong L, Zhu H: **PD-1/PD-L1 blockades in non-small-cell lung cancer therapy**. *OncoTargets and therapy* 2016, **9**:489-502.

137. Rosenblatt J, Glotzbecker B Fau - Mills H, Mills H Fau - Vasir B, Vasir B Fau - Tzachanis D, Tzachanis D Fau - Levine JD, Levine Jd Fau - Joyce RM, Joyce Rm Fau - Wellenstein K, Wellenstein K Fau - Keefe W, Keefe W Fau - Schickler M, Schickler M Fau - Rotem-Yehudar R, et al.: **PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine**.

138. Liu J, Hamrouni A Fau - Wolowiec D, Wolowiec D Fau - Coiteux V, Coiteux V Fau - Kuliczkowski K, Kuliczkowski K Fau - Hetuin D, Hetuin D Fau - Saudemont A, Saudemont A Fau - Quesnel B, Quesnel B: **Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway**.

139. Lesokhin AM, Ansell SM, Armand P, Scott EC, Halwani A, Gutierrez M, Millenson MM, Cohen AD, Schuster SJ, Lebovic D, et al.: **Nivolumab in Patients With Relapsed or Refractory Hematologic Malignancy: Preliminary Results of a Phase Ib Study**.

140. Luptakova K, Rosenblatt J Fau - Glotzbecker B, Glotzbecker B Fau - Mills H, Mills H Fau - Stroopinsky D, Stroopinsky D Fau - Kufe T, Kufe T Fau - Vasir B, Vasir B Fau - Arnason J, Arnason J Fau - Tzachanis D, Tzachanis D Fau - Zwicker JI, Zwicker Ji Fau - Joyce RM, et al.: **Lenalidomide enhances anti-myeloma cellular immunity**.

141. Borrello I, Noonan KA: **Marrow-Infiltrating Lymphocytes - Role in Biology and Cancer Therapy**.

142. Noonan K, Matsui W Fau - Serafini P, Serafini P Fau - Carbley R, Carbley R Fau - Tan G, Tan G Fau - Khalili J, Khalili J Fau - Bonyhadi M, Bonyhadi M Fau - Levitsky H, Levitsky H Fau - Whartenby K, Whartenby K Fau - Borrello I, Borrello I: **Activated marrow-infiltrating lymphocytes effectively target plasma cells and their clonogenic precursors**.

143. Noonan KA, Huff CA, Davis J, Lemas MV, Fiorino S, Bitzan J, Ferguson A, Emerling A, Luznik L, Matsui W, et al.: **Adoptive transfer of activated marrow-infiltrating lymphocytes induces measurable antitumor immunity in the bone marrow in multiple myeloma**.

144. Garfall AL, Stadtmauer EA, Maus MV, Hwang W-T, Vogl DT, Cohen AD, Weiss BM, Porter DL, Frey N, Milone M, et al.: **Pilot Study of Anti-CD19 Chimeric Antigen Receptor T Cells (CTL019) in Conjunction with Salvage Autologous Stem Cell Transplantation for Advanced Multiple Myeloma**. *Blood* 2016, **128**:974-974.

145. Ali SA, Shi V, Maric I, Wang M, Stroncek DF, Rose JJ, Brudno JN, Stetler-Stevenson M, Feldman SA, Hansen BG, et al.: **T cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma**.

146. Boussi L, Niesvizky R: **Advances in immunotherapy in multiple myeloma**.

147. Berenson JR, Lichtenstein A, Porter L, Dimopoulos MA, Bordoni R, George S, Lipton A, Keller A, Ballester O, Kovacs MJ: **Efficacy of pamidronate in reducing skeletal events in patients with advanced multiple myeloma**. *New England Journal of Medicine* 1996, **334**:488-493.

148. Morgan GJ, Child JA, Gregory WM, Szubert AJ, Cocks K, Bell SE, Navarro-Coy N, Drayson MT, Owen RG, Feyler S: **Effects of zoledronic acid versus clodronic acid on skeletal morbidity in patients with newly diagnosed multiple myeloma (MRC Myeloma IX): secondary outcomes from a randomised controlled trial**. *The lancet oncology* 2011, **12**:743-752.

149. Kyle RA, Yee GC, Somerfield MR, Flynn PJ, Halabi S, Jagannath S, Orlowski RZ, Roodman DG, Twilde P, Anderson K: **American Society of Clinical Oncology 2007 clinical practice guideline update on the role of bisphosphonates in multiple myeloma**. *Journal of Clinical Oncology* 2007, **25**:2464-2472.

150. Papadas A, Asimakopoulos F: **Mechanisms of Resistance in Multiple Myeloma**. In *Mechanisms of Drug Resistance in Cancer Therapy*. Edited by Mandalà M, Romano E: Springer International Publishing; 2018:251-288.

151. Turner JG, Gump JL, Zhang C, Cook JM, Marchion D, Hazlehurst L, Munster P, Schell MJ, Dalton WS, Sullivan DM: **ABCG2 expression, function, and promoter methylation in human multiple myeloma**. *Blood* 2006, **108**:3881-3889.

152. Grogan TM, Spier CM, Salmon SE, Matzner M, Rybski J, Weinstein RS, Scheper RJ, Dalton WS: **P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy**. 1993.

153. Chen Q, Van der Sluis PC, Boulware D, Hazlehurst LA, Dalton WS: **The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells**. *Blood* 2005, **106**:698-705.

154. Cho HJ, Mei A, Fukui J, Tung K, Leshchenko VV, Lagana A, Patel M, Kim-Schulze S, Perumal D, Chari A: **MAGE-a mediate resistance to chemotherapy in multiple myeloma through regulation of Bcl-2 proteins**. *Blood* 2016, **128**:3277.

155. Fischer ES, Böhm K, Lydeard JR, Yang H, Stadler MB, Cavadini S, Nagel J, Serluca F, Acker V, Lingaraju GM, et al.: **Structure of the DDB1–CRBN E3 ubiquitin ligase in complex with thalidomide**. *Nature* 2014, **512**:49-53.

156. Kortüm KM, Mai EK, Hanafiah NH, Shi C-X, Zhu Y-X, Bruins L, Barrio S, Jedlowski P, Merz M, Xu J, et al.: **Targeted sequencing of refractory myeloma reveals a high incidence of mutations in CRBN and Ras pathway genes**. *Blood* 2016, **128**:1226-1233.

157. Oerlemans R, Franke NE, Assaraf YG, Cloos J, van Zantwijk I, Berkers CR, Scheffer GL, Debipersad K, Vojtekova K, Lemos C: **Molecular basis of bortezomib resistance: proteasome subunit β5 (PSMB5) gene mutation and overexpression of PSMB5 protein**. *Blood, The Journal of the American Society of Hematology* 2008, **112**:2489-2499.

158. Ri M, Iida S, Nakashima T, Miyazaki H, Mori F, Ito A, Inagaki A, Kusumoto S, Ishida T, Komatsu H: **Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress**. *Leukemia* 2010, **24**:1506-1512.

159. Zhou W, Yang Y, Xia J, Wang H, Salama ME, Xiong W, Xu H, Shetty S, Chen T, Zeng Z: **NEK2 induces drug resistance mainly through activation of efflux drug pumps and is associated with poor prognosis in myeloma and other cancers**. *Cancer cell* 2013, **23**:48-62.

160. Xu D, Hu J, De Bruyne E, Menu E, Schots R, Vanderkerken K, Van Valckenborgh E: **Dll1/Notch activation contributes to bortezomib resistance by upregulating CYP1A1 in multiple myeloma**. *Biochemical and biophysical research communications* 2012, **428**:518-524.

161. Chauhan D, Tian Z, Nicholson B, Kumar KS, Zhou B, Carrasco R, McDermott JL, Leach CA, Fulcinniti M, Kodrasov MP: **A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance**. *Cancer cell* 2012, **22**:345-358.

162. Nijhof IS, Groen RWJ, Lokhorst HM, van Kessel B, Bloem AC, van Velzen J, de Jong-Korlaar R, Yuan H, Noort WA, Klein SK, et al.: **Upregulation of CD38 expression on multiple myeloma cells by all-trans retinoic acid improves the efficacy of daratumumab**. 2015, **29**:2039-2049.

163. Nijhof IS, Casneuf T, van Velzen J, van Kessel B, Axel AE, Syed K, Groen RWJ, van Duin M, Sonneveld P, Minnema MC, et al.: **CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma**. *Blood* 2016, **128**:959.

164. de Haart SJ, Holthof L, Noort WA, Minnema MC, Emmelot ME, Aarts-Riemens T, Doshi P, Sasser K, Yuan H, de Bruijn J, et al.: **Sepantronium bromide (YM155) improves daratumumab-mediated cellular lysis of multiple myeloma cells by abrogation of bone marrow stromal cell-induced resistance**.

165. Uluçkan O, Becker Sn Fau - Deng H, Deng H Fau - Zou W, Zou W Fau - Prior JL, Prior Jl Fau - Piwnica-Worms D, Piwnica-Worms D Fau - Frazier WA, Frazier Wa Fau - Weilbaecher KN, Weilbaecher KN: **CD47 regulates bone mass and tumor metastasis to bone**.

166. Russ A, Hua AB, Montfort WR, Rahman B, Riaz IB, Khalid MU, Carew JS, Nawrocki ST, Persky D, Anwer F: **Blocking "don't eat me" signal of CD47-SIRPα in hematological malignancies, an in-depth review**.

167. Pardoll DM: **The blockade of immune checkpoints in cancer immunotherapy**. *Nature Reviews Cancer* 2012, **12**:252-264.

168. Sharpe AH, Wherry Ej Fau - Ahmed R, Ahmed R Fau - Freeman GJ, Freeman GJ: **The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection**.

169. Okamoto M, Inaba T Fau - Yamada N, Yamada N Fau - Uchida R, Uchida R Fau - Fuchida SI, Fuchida Si Fau - Okano A, Okano A Fau - Shimazaki C, Shimazaki C Fau - Taniwaki M, Taniwaki M: **Expression and role of MHC class I-related chain in myeloma cells**.

170. Dhatchinamoorthy K, Colbert JD, Rock KL: **Cancer Immune Evasion Through Loss of MHC Class I Antigen Presentation**. *Frontiers in Immunology* 2021, **12**.

171. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, Groh V, Spies T, Pollio G, Cosman D: **HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells**. *Blood* 2005, **105**:251-258.

172. Dougall WC, Kurtulus S, Smyth MJ, Anderson AC: **TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy**.

173. Büll C, den Brok MH, Adema GJ: **Sweet escape: Sialic acids in tumor immune evasion**. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 2014, **1846**:238-246.

174. Kooner AS, Yu H, Chen X: **Synthesis of N-Glycolylneuraminic Acid (Neu5Gc) and Its Glycosides**. *Frontiers in Immunology* 2019, **10**.

175. Gottschalk A: *The chemistry and biology of sialic acids and related substances*: CUP Archive; 1960.

176. Li Y, Chen X: **Sialic acid metabolism and sialyltransferases: natural functions and applications**. *Applied microbiology and biotechnology* 2012, **94**:887-905.

177. Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi M-A, Samyn-Petit B, Julien S, Delannoy P: **The human sialyltransferase family**. *Biochimie* 2001, **83**:727-737.

178. Costa AF, Campos D, Reis CA, Gomes C: **Targeting Glycosylation: A New Road for Cancer Drug Discovery**. *Trends in Cancer* 2020, **6**:757-766.

179. Rodrigues E, Macauley MS: **Hypersialylation in Cancer: Modulation of Inflammation and Therapeutic Opportunities**. *Cancers* 2018, **10**.

180. Miyagi T: **Aberrant expression of sialidase and cancer progression**. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences* 2008, **84**:407-418.

181. Almaraz RT, Tian Y, Bhattarcharya R, Tan E, Chen S-H, Dallas MR, Chen L, Zhang Z, Zhang H, Konstantopoulos K, et al.: **Metabolic Flux Increases Glycoprotein Sialylation: Implications for Cell Adhesion and Cancer Metastasis**. *Molecular & Cellular Proteomics* 2012, **11**.

182. Swindall AF, Bellis SL: **Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells**. *Journal of Biological Chemistry* 2011, **286**:22982-22990.

183. Guadamillas MC, Cerezo A, Del Pozo MA: **Overcoming anoikis–pathways to anchorage-independent growth in cancer**. *Journal of cell science* 2011, **124**:3189-3197.

184. Julien S, Ivetic A, Grigoriadis A, QiZe D, Burford B, Sproviero D, Picco G, Gillett C, Papp SL, Schaffer L: **Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers**. *Cancer research* 2011, **71**:7683-7693.

185. Schultz MJ, Swindall AF, Wright JW, Sztul ES, Landen CN, Bellis SL: **ST6Gal-I sialyltransferase confers cisplatin resistance in ovarian tumor cells**. *Journal of ovarian research* 2013, **6**:1-9.

186. Punch PR, Irons EE, Manhardt CT, Marathe H, Lau JTY: **The sialyltransferase ST6GAL1 protects against radiation-induced gastrointestinal damage**. *Glycobiology* 2020, **30**:446-453.

187. Glavey SV, Manier S, Natoni A, Sacco A, Moschetta M, Reagan MR, Murillo LS, Sahin I, Wu P, Mishima Y, et al.: **The sialyltransferase ST3GAL6 influences homing and survival in multiple myeloma**. *Blood* 2014.

188. Natoni A, Smith TAG, Keane N, McEllistrim C, Connolly C, Jha A, Andrulis M, Ellert E, Raab MS, Glavey SV, et al.: **E-selectin ligands recognised by HECA452 induce drug resistance in myeloma, which is overcome by the E-selectin antagonist, GMI-1271**. *Leukemia* 2017, **31**:2642-2651.

189. Varki A: **Since there are PAMPs and DAMPs, there must be SAMPs? Glycan "self-associated molecular patterns" dampen innate immunity, but pathogens can mimic them**. *Glycobiology* 2011, **21**:1121-1124.

190. Jandus C, Boligan KF, Chijioke O, Liu H, Dahlhaus M, Démoulins T, Schneider C, Wehrli M, Hunger RE, Baerlocher GM, et al.: **Interactions between Siglec-7/9 receptors and ligands influence NK cell–dependent tumor immunosurveillance**. *The Journal of Clinical Investigation* 2014, **124**:1810-1820.

191. Kawasaki Y, Ito A, Withers DA, Taima T, Kakoi N, Saito S, Arai Y: **Ganglioside DSGb5, preferred ligand for Siglec-7, inhibits NK cell cytotoxicity against renal cell carcinoma cells**. *Glycobiology* 2010, **20**:1373-1379.

192. Läubli H, Pearce OMT, Schwarz F, Siddiqui SS, Deng L, Stanczak MA, Deng L, Verhagen A, Secrest P, Lusk C, et al.: **Engagement of myelomonocytic Siglecs by tumor-associated ligands modulates the innate immune response to cancer**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**:14211-14216.

193. Läubli H, Alisson-Silva F, Stanczak MA, Siddiqui SS, Deng L, Verhagen A, Varki N, Varki A: **Lectin Galactoside-binding Soluble 3 Binding Protein (LGALS3BP) Is a Tumor-associated Immunomodulatory Ligand for CD33-related Siglecs**. *The Journal of Biological Chemistry* 2014, **289**:33481-33491.

194. Herberman Rb Fau - Nunn ME, Nunn Me Fau - Holden HT, Holden Ht Fau - Lavrin DH, Lavrin DH: **Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells**.

195. Kiessling R Fau - Klein E, Klein E Fau - Wigzell H, Wigzell H: **"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype**.

196. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S: **Innate or adaptive immunity? The example of natural killer cells**. *Science (New York, N.Y.)* 2011, **331**:44-49.

197. Paul S, Lal G: **The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy**. *Frontiers in Immunology* 2017, **8**.

198. Ferlazzo G, Carrega P: **Natural killer cell distribution and trafficking in human tissues**. *Frontiers in immunology* 2012, **3**:347.

199. Sun JC: **Transcriptional Control of NK Cells**.

200. De Obaldia ME, Bhandoola A: **Transcriptional regulation of innate and adaptive lymphocyte lineages**.

201. Kim S, Iizuka K, Kang H-SP, Dokun A, French AR, Greco S, Yokoyama WM: **In vivo developmental stages in murine natural killer cell maturation**. *Nature immunology* 2002, **3**:523-528.

202. Abel AM, Yang C, Thakar MS, Malarkannan S: **Natural Killer Cells: Development, Maturation, and Clinical Utilization**. *Frontiers in Immunology* 2018, **9**.

203. Lugthart G, Melsen JE, Vervat C, van Ostaijen-Ten Dam MM, Corver WE, Roelen DL, van Bergen J, van Tol MJ, Lankester AC, Schilham MW: **Human lymphoid tissues harbor a distinct CD69+ CXCR6+ NK cell population**. *The Journal of Immunology* 2016, **197**:78-84.

204. Stegmann KA, Robertson F, Hansi N, Gill U, Pallant C, Christophides T, Pallett LJ, Peppa D, Dunn C, Fusai G: **CXCR6 marks a novel subset of T-bet lo Eomes hi natural killer cells residing in human liver**. *Scientific reports* 2016, **6**:1-10.

205. Harmon C, Robinson MW, Fahey R, Whelan S, Houlihan DD, Geoghegan J, O'Farrelly C: **Tissue-resident Eomeshi T-betlo CD56bright NK cells with reduced proinflammatory potential are enriched in the adult human liver**. *European journal of immunology* 2016, **46**:2111-2120.

206. Stabile H, Fionda C, Gismondi A, Santoni A: **Role of Distinct Natural Killer Cell Subsets in Anticancer Response**. *Frontiers in Immunology* 2017, **8**.

207. Barrow AD, Trowsdale J: **You say ITAM and I say ITIM, let's call the whole thing off: the ambiguity of immunoreceptor signalling**. *European Journal of Immunology* 2006, **36**:1646-1653.

208. Topham NJ, Hewitt EW: **Natural killer cell cytotoxicity: how do they pull the trigger?** *Immunology* 2009, **128**:7-15.

209. Alter G, Malenfant JM, Altfeld M: **CD107a as a functional marker for the identification of natural killer cell activity**. 2004, **294**:15-22.

210. Aktas E, Kucuksezer UC, Bilgic S, Erten G, Deniz G: **Relationship between CD107a expression and cytotoxic activity**. *Cellular Immunology* 2009, **254**:149-154.

211. Veillette A, Latour S, Davidson D: **Negative regulation of immunoreceptor signaling**. *Annual review of immunology* 2002, **20**:669-707.

212. Rajalingam R: **Overview of the killer cell immunoglobulin-like receptor system**. *Immunogenetics* 2012:391-414.

213. Rodríguez JA: **HLA-mediated tumor escape mechanisms that may impair immunotherapy clinical outcomes via T-cell activation**. *Oncology letters* 2017, **14**:4415-4427.

214. Ljunggren H-G, Kärre K: **In search of the 'missing self': MHC molecules and NK cell recognition**. 1990, **11**:237-244.

215. Kärre K: **NK Cells, MHC Class I Molecules and the Missing Self**. *Scandinavian Journal of Immunology* 2002, **55**:221-228.

216. Braud VM, Allan DS, O'Callaghan CA, Söderström K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH: **HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C**. *Nature* 1998, **391**:795-799.

217. Sun H, Sun C: **The Rise of NK Cell Checkpoints as Promising Therapeutic Targets in Cancer Immunotherapy**. *Frontiers in Immunology* 2019, **10**.

218. Zhang Q, Bi J, Zheng X, Chen Y, Wang H, Wu W, Wang Z, Wu Q, Peng H, Wei H, et al.: **Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity**. *Nature Immunology* 2018, **19**:723-732.

219. Chauvin J-M, Zarour HM: **TIGIT in cancer immunotherapy**. *Journal for ImmunoTherapy of Cancer* 2020, **8**:e000957.

220. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, Levine Z, Beiman M, Dassa L, Achdout H: **The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity**. *Proceedings of the National Academy of Sciences* 2009, **106**:17858-17863.

221. Georgiev H, Ravens I, Papadogianni G, Bernhardt G: **Coming of Age: CD96 Emerges as Modulator of Immune Responses**. *Frontiers in Immunology* 2018, **9**.

222. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M: **Cutting Edge: CD96 (Tactile) Promotes NK Cell-Target Cell Adhesion by Interacting with the Poliovirus Receptor (CD155)**. *The Journal of Immunology* 2004, **172**:3994.

223. Humphrey MB, Lanier LL, Nakamura MC: **Role of ITAM-containing adapter proteins and their receptors in the immune system and bone**. *Immunological reviews* 2005, **208**:50-65.

224. Pende D, Rivera P, Marcenaro S, Chang C-C, Biassoni R, Conte R, Kubin M, Cosman D, Ferrone S, Moretta L: **Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity**. *Cancer research* 2002, **62**:6178-6186.

225. Skov S, Pedersen MT, Andresen L, Straten PT, Woetmann A, Ødum N: **Cancer cells become susceptible to natural killer cell killing after exposure to histone deacetylase inhibitors due to glycogen synthase kinase-3–dependent expression of MHC class I–related chain A and B**. *Cancer research* 2005, **65**:11136-11145.

226. Valés-Gómez M, Chisholm SE, Cassady-Cain RL, Roda-Navarro P, Reyburn HT: **Selective induction of expression of a ligand for the NKG2D receptor by proteasome inhibitors**. *Cancer research* 2008, **68**:1546-1554.

227. Kruse PH, Matta J, Ugolini S, Vivier E: **Natural cytotoxicity receptors and their ligands**. *Immunology & Cell Biology* 2013, **92**:221-229.

228. Vaidya SV, Stepp SE, McNerney ME, Lee J-K, Bennett M, Lee K-M, Stewart CL, Kumar V, Mathew PA: **Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells**. *The Journal of Immunology* 2005, **174**:800-807.

229. Tangye SG, Lazetic S, Woollatt E, Sutherland GR, Lanier LL, Phillips JH: **Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP**. *The Journal of Immunology* 1999, **162**:6981-6985.

230. Tangye SG, Cherwinski H, Lanier LL, Phillips JH: **2B4-mediated activation of human natural killer cells**. 2000, **37**:493-501.

231. Parolini S, Bottino C, Falco M, Augugliaro R, Giliani S, Franceschini R, Ochs HD, Wolf H, Bonnefoy JY, Biassoni R, et al.: **X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells**. *The Journal of experimental medicine* 2000, **192**:337-346.

232. Hatjiharissi E, Xu L, Santos DD, Hunter ZR, Ciccarelli BT, Verselis S, Modica M, Cao Y, Manning RJ, Leleu X, et al.: **Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the Fc{gamma}RIIIa-158 V/V and V/F polymorphism**. *Blood* 2007, **110**:2561-2564.

233. Sarkar S, Chauhan SKS, Daly J, Natoni A, Fairfield H, Henderson R, Nolan E, Swan D, Hu J, Reagan MR, et al.: **The CD38(low) natural killer cell line KHYG1 transiently expressing CD16(F158V) in combination with daratumumab targets multiple myeloma cells with minimal effector NK cell fratricide**.

234. Sonar S, Lal G: **Role of tumor necrosis factor superfamily in neuroinflammation and autoimmunity**. *Frontiers in immunology* 2015, **6**:364.

235. Screpanti V, Wallin RP, Ljunggren H-G, Grandien A: **A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells**. *The Journal of Immunology* 2001, **167**:2068-2073.

236. Batista IA, Quintas SA-O, Melo SA: **The Interplay of Exosomes and NK Cells in Cancer Biology. LID - 10.3390/cancers13030473 [doi] LID - 473**.

237. Zhu L, Kalimuthu S, Gangadaran P, Oh JM, Lee HW, Baek SH, Jeong SY, Lee SW, Lee J, Ahn BC: **Exosomes Derived From Natural Killer Cells Exert Therapeutic Effect in Melanoma**.

238. Fauriat C, Long EO, Ljunggren H-G, Bryceson YT: **Regulation of human NK-cell cytokine and chemokine production by target cell recognition**. *Blood, The Journal of the American Society of Hematology* 2010, **115**:2167-2176.

239. Roda JM, Parihar R, Magro C, Nuovo GJ, Tridandapani S, Carson WE: **Natural killer cells produce T cell–recruiting chemokines in response to antibody-coated tumor cells**. *Cancer research* 2006, **66**:517-526.

240. Bluman EM, Bartynski KJ, Avalos BR, Caligiuri MA: **Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines**. *Journal of Clinical Investigation* 1996, **97**:2722-2727.

241. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, Yagita H, Okumura K: **Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis**. *The Journal of experimental medicine* 2001, **193**:661-670.

242. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD: **Demonstration of an interferon γ-dependent tumor surveillance system in immunocompetent mice**. *Proceedings of the National Academy of Sciences* 1998, **95**:7556.

243. Wang R, Jaw JJ, Stutzman NC, Zou Z, Sun PD: **Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1**. *Journal of leukocyte biology* 2012, **91**:299-309.

244. Ortaldo JR, Winkler-Pickett R, Wigginton J, Horner M, Bere EW, Mason AT, Bhat N, Cherry J, Sanford M, Hodge DL: **Regulation of ITAM-positive receptors: role of IL-12 and IL-18**. *Blood* 2006, **107**:1468-1475.

245. Chaix J, Tessmer MS, Hoebe K, Fuséri N, Ryffel B, Dalod M, Alexopoulou L, Beutler B, Brossay L, Vivier E: **Cutting edge: Priming of NK cells by IL-18**. *The Journal of Immunology* 2008, **181**:1627-1631.

246. Cai G, Kastelein RA, Hunter CA: **IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-γ when combined with IL-18**. *European journal of immunology* 1999, **29**:2658-2665.

247. Smyth MJ, Swann J, Kelly JM, Cretney E, Yokoyama WM, Diefenbach A, Sayers TJ, Hayakawa Y: **NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer**. *Journal of Experimental Medicine* 2004, **200**:1325-1335.

248. Takaki R, Hayakawa Y, Nelson A, Sivakumar PV, Hughes S, Smyth MJ, Lanier LL: **IL-21 enhances tumor rejection through a NKG2D-dependent mechanism**. *The Journal of Immunology* 2005, **175**:2167-2173.

249. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G: **Reciprocal activating interaction between natural killer cells and dendritic cells**. *The Journal of experimental medicine* 2002, **195**:327-333.

250. Mocikat R, Braumüller H, Gumy A, Egeter O, Ziegler H, Reusch U, Bubeck A, Louis J, Mailhammer R, Riethmüller G, et al.: **Natural Killer Cells Activated by MHC Class ILow**

Targets Prime Dendritic Cells to Induce Protective CD8 T Cell Responses. *Immunity* 2003, **19**:561-569.

251. Ferlazzo G, Moretta L: **Dendritic cell editing by natural killer cells**. *Critical Reviews™ in Oncogenesis* 2014, **19**.

252. Bélanger S, Tu MM, Rahim MMA, Mahmoud AB, Patel R, Tai L-H, Troke AD, Wilhelm BT, Landry J-R, Zhu Q: **Impaired natural killer cell self-education and "missing-self" responses in Ly49-deficient mice**. *Blood, The Journal of the American Society of Hematology* 2012, **120**:592-602.

253. Ebihara T, Jonsson AH, Yokoyama WM: **Natural killer cell licensing in mice with inducible expression of MHC class I**. *Proceedings of the National Academy of Sciences* 2013, **110**:E4232-E4237.

254. Choi T, Ferris ST, Matsumoto N, Poursine-Laurent J, Yokoyama WM: **Ly49-dependent NK cell licensing and effector inhibition involve the same interaction site on MHC ligands**. *The Journal of Immunology* 2011, **186**:3911-3917.

255. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song Y-J, Yang L, French AR, Sunwoo JB, Lemieux S, Hansen TH: **Licensing of natural killer cells by host major histocompatibility complex class I molecules**. *Nature* 2005, **436**:709-713.

256. Qu C-K, Nguyen S, Chen J, Feng G-S: **Requirement of Shp-2 tyrosine phosphatase in lymphoid and hematopoietic cell development**. *Blood, The Journal of the American Society of Hematology* 2001, **97**:911-914.

257. Yusa S-i, Campbell KS: **Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) can play a direct role in the inhibitory function of killer cell Ig-like receptors in human NK cells**. *The Journal of Immunology* 2003, **170**:4539-4547.

258. Lisovsky I, Isitman G, Song R, DaFonseca S, Tremblay-McLean A, Lebouché B, Routy J-P, Bruneau J, Bernard NF: **A higher frequency of NKG2A+ than of NKG2A− NK cells responds to autologous HIV-infected CD4 cells irrespective of whether or not they coexpress KIR3DL1**. *Journal of virology* 2015, **89**:9909-9919.

259. Ivarsson MA, Loh L, Marquardt N, Kekäläinen E, Berglin L, Björkström NK, Westgren M, Nixon DF, Michaëlsson J: **Differentiation and functional regulation of human fetal NK cells**. *The Journal of clinical investigation* 2013, **123**:3889-3901.

260. Dorfman JR, Raulet DH: **Acquisition of Ly49 receptor expression by developing natural killer cells**. *The Journal of experimental medicine* 1998, **187**:609-618.

261. Elliott JM, Wahle Ja Fau - Yokoyama WM, Yokoyama WM: **MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment**.

262. Tinker AV, Hirte HW, Provencher D, Butler M, Ritter H, Tu D, Azim HA, Paralejas P, Grenier N, Hahn S-A: **Dose-ranging and cohort-expansion study of monalizumab (IPH2201) in patients with advanced gynecologic malignancies: a trial of the Canadian Cancer Trials Group (CCTG): IND221**. *Clinical Cancer Research* 2019, **25**:6052-6060.

263. Marín R, Ruiz-Cabello F Fau - Pedrinaci S, Pedrinaci S Fau - Méndez R, Méndez R Fau - Jiménez P, Jiménez P Fau - Geraghty DE, Geraghty De Fau - Garrido F, Garrido F: **Analysis of HLA-E expression in human tumors**.

264. Minetto P, Guolo F, Pesce S, Greppi M, Obino V, Ferretti E, Sivori S, Genova C, Lemoli RM, Marcenaro E: **Harnessing NK Cells for Cancer Treatment**. *Frontiers in Immunology* 2019, **10**.

265. Vey N, Bourhis J-H, Boissel N, Bordessoule D, Prebet T, Charbonnier A, Etienne A, Andre P, Romagne F, Benson D: **A phase 1 trial of the anti-inhibitory KIR mAb IPH2101 for AML in complete remission**. *Blood, The Journal of the American Society of Hematology* 2012, **120**:4317-4323.

266. Korde N, Carlsten M, Lee M-J, Minter A, Tan E, Kwok M, Manasanch E, Bhutani M, Tageja N, Roschewski M: **A phase II trial of pan-KIR2D blockade with IPH2101 in smoldering multiple myeloma**. *Haematologica* 2014, **99**:e81.

267. Nayyar G, Chu Y, Cairo MS: **Overcoming Resistance to Natural Killer Cell Based Immunotherapies for Solid Tumors**. *Frontiers in Oncology* 2019, **9**.

268. Kantarjian H, Stein A, Gökbuget N, Fielding AK, Schuh AC, Ribera J-M, Wei A, Dombret H, Foà R, Bassan R, et al.: **Blinatumomab versus Chemotherapy for Advanced Acute Lymphoblastic Leukemia**. *New England Journal of Medicine* 2017, **376**:836-847.

269. Kantarjian HM, DeAngelo DJ, Stelljes M, Martinelli G, Liedtke M, Stock W, Gökbuget N, O'Brien S, Wang K, Wang T, et al.: **Inotuzumab Ozogamicin versus Standard Therapy for Acute Lymphoblastic Leukemia**. *New England Journal of Medicine* 2016, **375**:740-753.

270. Lancman G, Sastow DL, Cho HJ, Jagannath S, Madduri D, Parekh SS, Richard S, Richter J, Sanchez L, Chari A: **Bispecific Antibodies in Multiple Myeloma: Present and Future**. *Blood cancer discovery* 2021, **2**:423-433.

271. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F: **Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants**. *Science* 2002, **295**:2097-2100.

272. Fang F, Wang W, Chen M, Tian Z, Xiao W: **Technical advances in NK cell-based cellular immunotherapy**. *Cancer biology & medicine* 2019, **16**:647-654.

273. Rezvani K: **Adoptive cell therapy using engineered natural killer cells**. *Bone marrow transplantation* 2019, **54**:785-788.

274. Imai C, Iwamoto S, Campana D: **Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells**. *Blood* 2005, **106**:376-383.

275. Ni Z, Knorr DA, Bendzick L, Allred J, Kaufman DS: **Expression of chimeric receptor CD4ζ by natural killer cells derived from human pluripotent stem cells improves in vitro activity but does not enhance suppression of HIV infection in vivo**. *Stem cells* 2014, **32**:1021-1031.

276. Li Y, Hermanson DL, Moriarity BS, Kaufman DS: **Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity**. *Cell stem cell* 2018, **23**:181-192. e185.

277. Chang Y-H, Connolly J, Shimasaki N, Mimura K, Kono K, Campana D: **A chimeric receptor with NKG2D specificity enhances natural killer cell activation and killing of tumor cells**. *Cancer research* 2013, **73**:1777-1786.

278. Romanski A, Uherek C, Bug G, Seifried E, Klingemann H, Wels WS, Ottmann OG, Tonn T: **CD 19-CAR engineered NK-92 cells are sufficient to overcome NK cell resistance in B-cell malignancies**. *Journal of cellular and molecular medicine* 2016, **20**:1287-1294.

279. Hsi ED, Steinle R Fau - Balasa B, Balasa B Fau - Szmania S, Szmania S Fau - Draksharapu A, Draksharapu A Fau - Shum BP, Shum Bp Fau - Huseni M, Huseni M Fau - Powers D, Powers D Fau - Nanisetti A, Nanisetti A Fau - Zhang Y, Zhang Y Fau - Rice AG, et al.: **CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma**.

280. Jiang H, Zhang W, Shang P, Zhang H, Fu W, Ye F, Zeng T, Huang H, Zhang X, Sun W, et al.: **Transfection of chimeric anti-CD138 gene enhances natural killer cell activation and killing of multiple myeloma cells**. *Molecular Oncology* 2014, **8**:297-310.

281. Maroto-Martín E, Encinas J, García-Ortiz A, Alonso R, Leivas A, Paciello M, Garrido V, Cedena T, Ugalde L, Powell Jr D: **PS1209 NKG2D and bcma-car NK cells efficiently eliminate multiple myeloma cells. A comprehensive comparison between two clinically relevant CARS**. *HemaSphere* 2019, **3**:550-551.

282. Schmidt P, Raftery MJ, Pecher G: **Engineering NK Cells for CAR Therapy—Recent Advances in Gene Transfer Methodology**. *Frontiers in Immunology* 2021, **11**.

283. Shah UA, Mailankody S: **CAR T and CAR NK cells in multiple myeloma: Expanding the targets**. *Best Practice & Research Clinical Haematology* 2020, **33**:101141.

284. Felices M, Lenvik TR, Davis ZB, Miller JS, Vallera DA: **Generation of BiKEs and TriKEs to Improve NK Cell-Mediated Targeting of Tumor Cells**. *Methods in molecular biology (Clifton, N.J.)* 2016, **1441**:333-346.

285. Tay SS, Carol H, Biro M: **TriKEs and BiKEs join CARs on the cancer immunotherapy highway**. *Human Vaccines & Immunotherapeutics* 2016, **12**:2790-2796.

286. Baluna R, Vitetta ES: **Vascular leak syndrome: a side effect of immunotherapy**. *Immunopharmacology* 1997, **37**:117-132.

287. Bachanova V, Cooley S, Defor TE, Verneris MR, Zhang B, McKenna DH, Curtsinger J, Panoskaltsis-Mortari A, Lewis D, Hippen K: **Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein**. *Blood, The Journal of the American Society of Hematology* 2014, **123**:3855-3863.

288. Vallera DA, Felices M, McElmurry R, McCullar V, Zhou X, Schmohl JU, Zhang B, Lenvik AJ, Panoskaltsis-Mortari A, Verneris MR: **IL15 trispecific killer engagers (TriKE) make natural killer cells specific to CD33+ targets while also inducing persistence, in vivo expansion, and enhanced function**. *Clinical Cancer Research* 2016, **22**:3440-3450.

289. Nicoll G, Ni J, Liu D, Klenerman P, Munday J, Dubock S, Mattei M-G, Crocker PR: **Identification and Characterization of a Novel Siglec, Siglec-7, Expressed by Human Natural Killer Cells and Monocytes**. *Journal of Biological Chemistry* 1999, **274**:34089-34095.

290. von Gunten S, Bochner BS: **Basic and Clinical Immunology of Siglecs**. *Annals of the New York Academy of Sciences* 2008, **1143**:61-82.

291. Crocker PR, Paulson JC, Varki A: **Siglecs and their roles in the immune system**. *Nat Rev Immunol* 2007, **7**:255-266.

292. Angata T, Hayakawa T, Yamanaka M, Varki A, Nakamura M: **Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates**. *The FASEB Journal* 2006, **20**:1964-1973.

293. Falco M, Biassoni R, Bottino C, Vitale M, Sivori S, Augugliaro R, Moretta L, Moretta A: **Identification and Molecular Cloning of P75/Airm1, a Novel Member of the Sialoadhesin Family That Functions as an Inhibitory Receptor in Human Natural Killer Cells**. *The Journal of Experimental Medicine* 1999, **190**:793-802.

294. Ikehara Y, Ikehara Sk Fau - Paulson JC, Paulson JC: **Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9**.

295. Yamaji T, Teranishi T, Alphey MS, Crocker PR, Hashimoto Y: **A Small Region of the Natural Killer Cell Receptor, Siglec-7, Is Responsible for Its Preferred Binding to α2,8-Disialyl and Branched α2,6-Sialyl Residues: A COMPARISON WITH Siglec-9**. *Journal of Biological Chemistry* 2002, **277**:6324-6332.

296. Alphey MS, Attrill H, Crocker PR, van Aalten DMF: **High Resolution Crystal Structures of Siglec-7: INSIGHTS INTO LIGAND SPECIFICITY IN THE SIGLEC FAMILY**. *Journal of Biological Chemistry* 2003, **278**:3372-3377.

297. Shao JY, Yin WW, Zhang QF, Liu Q, Peng ML, Hu HD, Hu P, Ren H, Zhang DZ: **Siglec-7 Defines a Highly Functional Natural Killer Cell Subset and Inhibits Cell-Mediated Activities**. *Scandinavian Journal of Immunology* 2016, **84**:182-190.

298. Zhang JQ, Nicoll G, Jones C, Crocker PR: **Siglec-9, a Novel Sialic Acid Binding Member of the Immunoglobulin Superfamily Expressed Broadly on Human Blood Leukocytes**. *Journal of Biological Chemistry* 2000, **275**:22121-22126.

299. von Gunten S, Yousefi S Fau - Seitz M, Seitz M Fau - Jakob SM, Jakob Sm Fau - Schaffner T, Schaffner T Fau - Seger R, Seger R Fau - Takala J, Takala J Fau - Villiger PM, Villiger Pm Fau - Simon H-U, Simon HU: **Siglec-9 transduces apoptotic and nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment**.

300. Büll C, Stoel MA, den Brok MH, Adema GJ: **Sialic Acids Sweeten a Tumor's Life**. *Cancer Research* 2014, **74**:3199.

301. Varki A: **Letter to the Glyco-Forum: Since there are PAMPs and DAMPs, there must be SAMPs? Glycan "self-associated molecular patterns" dampen innate immunity, but pathogens can mimic them**. *Glycobiology* 2011, **21**:1121-1124.

302. Cohen AM, Allalouf D, Bessler H, Djaldetti M, Malachi T, Levinsky H: **Sialyltransferase activity and sialic acid levels in multiple myeloma and monoclonal gammopathy**. *European Journal of Haematology* 1989, **42**:289-292.

303. Seidenfaden R, Krauter A Fau - Schertzinger F, Schertzinger F Fau - Gerardy-Schahn R, Gerardy-Schahn R Fau - Hildebrandt H, Hildebrandt H: **Polysialic acid directs tumor cell growth by controlling heterophilic neural cell adhesion molecule interactions**.

304. Hudak JE, Canham SM, Bertozzi CR: **Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion**. *Nat Chem Biol* 2014, **10**:69-75.

305. Xiao H, Woods EC, Vukojicic P, Bertozzi CR: **Precision glycocalyx editing as a strategy for cancer immunotherapy**. *Proceedings of the National Academy of Sciences* 2016, **113**:10304-10309.

306. Pittari G, Vago L, Festuccia M, Bonini C, Mudawi D, Giaccone L, Bruno B: **Restoring Natural Killer Cell Immunity against Multiple Myeloma in the Era of New Drugs**. *Frontiers in immunology* 2017, **8**:1444-1444.

307. Sun C, Xu J, Huang Q, Huang M, Wen H, Zhang C, Wang J, Song J, Zheng M, Sun H: **High NKG2A expression contributes to NK cell exhaustion and predicts a poor prognosis of patients with liver cancer**. *Oncoimmunology* 2017, **6**:e1264562.

308. Platonova S, Cherfils-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, André P, Dieu-Nosjean M-C, Alifano M, Régnard J-F: **Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma**. *Cancer research* 2011, **71**:5412-5422.

309. Wisnovsky S, Möckl L, Malaker SA, Pedram K, Hess GT, Riley NM, Gray MA, Smith BAH, Bassik MC, Moerner WE, et al.: **Genome-wide CRISPR screens reveal a specific ligand for the glycan-binding immune checkpoint receptor Siglec-7**. *Proceedings of the National Academy of Sciences* 2021, **118**:e2015024118.

310. Angata T, Varki A: **Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily**. *Glycobiology* 2000, **10**:431-438.

311. Belisle JA, Horibata S, Jennifer GAA, Petrie S, Kapur A, André S, Gabius H-J, Rancourt C, Connor J, Paulson JC, et al.: **Identification of Siglec-9 as the receptor for MUC16 on human NK cells, B cells, and monocytes**. *Molecular Cancer* 2010, **9**:118-118.

312. Sarkar S, Germeraad WTV, Rouschop KMA, Steeghs EMP, van Gelder M, Bos GMJ, Wieten L: **Hypoxia Induced Impairment of NK Cell Cytotoxicity against Multiple Myeloma Can Be Overcome by IL-2 Activation of the NK Cells**. *PLoS ONE* 2013, **8**:e64835.

313. Stanczak MA, Siddiqui SS, Trefny MP, Thommen DS, Boligan KF, von Gunten S, Tzankov A, Tietze L, Lardinois D, Heinzelmann-Schwarz V, et al.: **Self-associated molecular patterns mediate cancer immune evasion by engaging Siglecs on T cells**. *The Journal of clinical investigation* 2018, **128**:4912-4923.

314. Büll C, Boltje TJ, van Dinther EAW, Peters T, de Graaf AMA, Leusen JHW, Kreutz M, Figdor CG, den Brok MH, Adema GJ: **Targeted Delivery of a Sialic Acid-Blocking Glycomimetic to Cancer Cells Inhibits Metastatic Spread**. *ACS Nano* 2015, **9**:733-745.

315. Paolini R, Bernardini G, Molfetta R, Santoni A: **NK cells and interferons**. *Cytokine & Growth Factor Reviews* 2015, **26**:113-120.

316. Pollard KM, Cauvi Dm Fau - Toomey CB, Toomey Cb Fau - Morris KV, Morris Kv Fau - Kono DH, Kono DH: **Interferon-γ and systemic autoimmunity**.

317. Favrot M, Combaret V Fau - Blay JY, Blay Jy Fau - Capdeville R, Capdeville R Fau - Zhou DC, Zhou Dc Fau - Clapisson G, Clapisson G Fau - Chouaib S, Chouaib S Fau - Franks CR, Franks Cr Fau - Philip T, Philip T: **TNF alpha enhancement of NK and LAK cell functions induced by high-dose IL-2 in human peripheral blood mononuclear cells from patients pretreated with alpha IFN + IL-2**.

318. Wensveen FM, Jelenčić V, Polić B: **NKG2D: A Master Regulator of Immune Cell Responsiveness**. *Frontiers in Immunology* 2018, **9**.

319. Azab AK, Quang P, Azab F, Pitsillides C, Thompson B, Chonghaile T, Patton JT, Maiso P, Monrose V, Sacco A, et al.: **P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment**. *Blood* 2012, **119**:1468-1478.

320. Bénac O, Gaudin M, Ors M, Roy AL, Blanc HR, Soulas C, Chanteux S, Rossi B, Gauthier L, Paturel C, et al.: **Abstract 2713: Preclinical development of first-in-class antibodies targeting Siglec-9 immune checkpoint for cancer immunotherapy**. *Cancer Research* 2018, **78**:2713.

321. Springer TA: **Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm**.

322. Muz B, Azab F, de la Puente P, Rollins S, Alvarez R, Kawar Z, Azab AK: **Inhibition of P-Selectin and PSGL-1 Using Humanized Monoclonal Antibodies Increases the Sensitivity of Multiple Myeloma Cells to Bortezomib**. *BioMed Research International* 2015, **2015**:417586.

323. Azab AK, Quang P Fau - Azab F, Azab F Fau - Pitsillides C, Pitsillides C Fau - Thompson B, Thompson B Fau - Chonghaile T, Chonghaile T Fau - Patton JT, Patton Jt Fau - Maiso P, Maiso P Fau - Monrose V, Monrose V Fau - Sacco A, Sacco A Fau - Ngo HT, et al.: **P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment**.

324. Tuccillo FM, de Laurentiis A, Palmieri C, Fiume G, Bonelli P, Borrelli A, Tassone P, Scala I, Buonaguro FM, Quinto I, et al.: **Aberrant glycosylation as biomarker for cancer: focus on CD43**. *BioMed research international* 2014, **2014**:742831-742831.

325. Balsamo M, Manzini C Fau - Pietra G, Pietra G Fau - Raggi F, Raggi F Fau - Blengio F, Blengio F Fau - Mingari MC, Mingari Mc Fau - Varesio L, Varesio L Fau - Moretta L, Moretta L Fau - Bosco MC, Bosco Mc Fau - Vitale M, Vitale M: **Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC**.

326. Rodriguez E, Boelaars K, Brown K, Eveline Li RJ, Kruijssen L, Bruijns SCM, van Ee T, Schetters STT, Crommentuijn MHW, van der Horst JC, et al.: **Sialic acids in pancreatic cancer cells drive tumour-associated macrophage differentiation via the Siglec receptors Siglec-7 and Siglec-9**. *Nature Communications* 2021, **12**:1270.

327. Petri RM, Hackel A, Hahnel K, Dumitru CA, Bruderek K, Flohe SB, Paschen A, Lang S, Brandau S: **Activated Tissue-Resident Mesenchymal Stromal Cells Regulate Natural Killer Cell Immune and Tissue-Regenerative Function**. *Stem cell reports* 2017, **9**:985-998.

328. Varchetta S, Brunetta E, Roberto A, Mikulak J, Hudspeth KL, Mondelli MU, Mavilio D: **Engagement of Siglec-7 Receptor Induces a Pro-Inflammatory Response Selectively in Monocytes**. *PLOS ONE* 2012, **7**:e45821.

329. Jayasingam SD, Citartan M, Thang TH, Mat Zin AA, Ang KC, Ch'ng ES: **Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice**. *Frontiers in oncology* 2020, **9**:1512-1512.

330. O'Reilly E, Zeinabad HA, Nolan C, Sefy J, Williams T, Tarunina M, Hernandez D, Choo Y, Szegezdi E: **Recreating the Bone Marrow Microenvironment to Model Leukemic Stem Cell Quiescence**. *Frontiers in cell and developmental biology* 2021, **9**:662868-662868.

331. Green DS, Young HA, Valencia JC: **Current prospects of type II interferon γ signaling and autoimmunity**. *J Biol Chem* 2017, **292**:13925-13933.

332. Schroder K, Hertzog Pj Fau - Ravasi T, Ravasi T Fau - Hume DA, Hume DA: **Interferon-gamma: an overview of signals, mechanisms and functions**.

333. Sentman CL, Meehan KR: **NKG2D CARs as cell therapy for cancer**. *Cancer journal (Sudbury, Mass.)* 2014, **20**:156-159.

334. Sallman DA, Brayer J, Sagatys EM, Lonez C, Breman E, Agaugué S, Verma B, Gilham DE, Lehmann FF, Davila ML: **NKG2D-based chimeric antigen receptor therapy induced remission in a relapsed/refractory acute myeloid leukemia patient**. *Haematologica* 2018, **103**:e424-e426.

335. Oh S, Lee J-H, Kwack K, Choi S-W: **Natural Killer Cell Therapy: A New Treatment Paradigm for Solid Tumors**. *Cancers* 2019, **11**:1534.

336. Morvan MG, Lanier LL: **NK cells and cancer: you can teach innate cells new tricks**. *Nat Rev Cancer* 2016, **16**:7-19.

337. Shimasaki N, Jain A, Campana D: **NK cells for cancer immunotherapy**. *Nature Reviews Drug Discovery* 2020, **19**:200-218.

338. Leivas A, Rio P, Mateos R, Paciello ML, Garcia-Ortiz A, Fernandez L, Perez-Martinez A, Lee DA, Powell DJ, Jr., Valeri A, et al.: **NKG2D-CAR Transduced Primary Natural Killer Cells Efficiently Target Multiple Myeloma Cells**. *Blood* 2018, **132**:590-590.

339. Gray MA, Stanczak MA, Mantuano NR, Xiao H, Pijnenborg JFA, Malaker SA, Miller CL, Weidenbacher PA, Tanzo JT, Ahn G, et al.: **Targeted glycan degradation potentiates the anticancer immune response in vivo**. *Nature Chemical Biology* 2020, **16**:1376-1384.

340. Cooper MA, Fehniger TA, Caligiuri MA: **The biology of human natural killer-cell subsets**. *Trends in Immunology* 2001, **22**:633-640.

341. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J: **CD56bright natural killer (NK) cells: an important NK cell subset**. *Immunology* 2009, **126**:458-465.

342. Jacobs R, Hintzen G Fau - Kemper A, Kemper A Fau - Beul K, Beul K Fau - Kempf S, Kempf S Fau - Behrens G, Behrens G Fau - Sykora KW, Sykora Kw Fau - Schmidt RE, Schmidt RE: **CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells**.

343. Hendriks D, Choi G, de Bruyn M, Wiersma VR, Bremer E: **Chapter Seven - Antibody-Based Cancer Therapy: Successful Agents and Novel Approaches**. In *International Review of Cell and Molecular Biology*. Edited by Galluzzi L: Academic Press; 2017:289-383. vol 331.]

344. Gül N Fau - Babes L, Babes L Fau - Siegmund K, Siegmund K Fau - Korthouwer R, Korthouwer R Fau - Bögels M, Bögels M Fau - Braster R, Braster R Fau - Vidarsson G, Vidarsson G Fau - ten Hagen TLM, ten Hagen Tl Fau - Kubes P, Kubes P Fau - van Egmond M, van Egmond M: **Macrophages eliminate circulating tumor cells after monoclonal antibody therapy**.

345. Fancher KM, Bunk EJ: **Elotuzumab: The First Monoclonal Antibody for the Treatment of Multiple Myeloma**. *Journal of the advanced practitioner in oncology* 2016, **7**:542-547.

346. Magarotto V, Salvini M, Bonello F, Bringhen S, Palumbo A: **Strategy for the treatment of multiple myeloma utilizing monoclonal antibodies: A new era begins**.

347. Balasa B, Yun R Fau - Belmar NA, Belmar Na Fau - Fox M, Fox M Fau - Chao DT, Chao Dt Fau - Robbins MD, Robbins Md Fau - Starling GC, Starling Gc Fau - Rice AG, Rice AG: **Elotuzumab enhances natural killer cell activation and myeloma cell killing through interleukin-2 and TNF-α pathways**.

348. Bezman NA, Jhatakia A, Kearney AY, Brender T, Maurer M, Henning K, Jenkins MR, Rogers AJ, Neeson PJ, Korman AJ, et al.: **PD-1 blockade enhances elotuzumab efficacy in mouse tumor models**. *Blood advances* 2017, **1**:753-765.

349. Lonial S, Dimopoulos M Fau - Palumbo A, Palumbo A Fau - White D, White D Fau - Grosicki S, Grosicki S Fau - Spicka I, Spicka I Fau - Walter-Croneck A, Walter-Croneck A Fau - Moreau P, Moreau P Fau - Mateos M-V, Mateos Mv Fau - Magen H, Magen H Fau - Belch A, et al.: **Elotuzumab Therapy for Relapsed or Refractory Multiple Myeloma**.

350. Pazina T, James AM, MacFarlane AWt, Bezman NA, Henning KA, Bee C, Graziano RF, Robbins MD, Cohen AD, Campbell KS: **The anti-SLAMF7 antibody elotuzumab mediates NK cell activation through both CD16-dependent and -independent mechanisms**.

351. Collins SM, Bakan CE, Swartzel GD, Hofmeister CC, Efebera YA, Kwon H, Starling GC, Ciarlariello D, Bhaskar S, Briercheck EL, et al.: **Elotuzumab directly enhances NK cell cytotoxicity against myeloma via CS1 ligation: evidence for augmented NK cell function complementing ADCC**. *Cancer immunology, immunotherapy : CII* 2013, **62**:1841-1849.

352. Deaglio S, Mehta K Fau - Malavasi F, Malavasi F: **Human CD38: a (r)evolutionary story of enzymes and receptors**.

353. Abdallah N, Kumar SK: **Daratumumab in untreated newly diagnosed multiple myeloma**. *Therapeutic advances in hematology* 2019, **10**:2040620719894871-2040620719894871.

354. Palumbo A, Chanan-Khan A, Weisel K, Nooka AK, Masszi T, Beksac M, Spicka I, Hungria V, Munder M, Mateos MV, et al.: **Daratumumab, Bortezomib, and Dexamethasone for Multiple Myeloma**. *New England Journal of Medicine* 2016, **375**:754-766.

355. Dimopoulos MA, Oriol A, Nahi H, San-Miguel J, Bahlis NJ, Usmani SZ, Rabin N, Orlowski RZ, Komarnicki M, Suzuki K, et al.: **Daratumumab, Lenalidomide, and Dexamethasone for Multiple Myeloma**.

356. Kumar SK, Kapoor P, Laplant B, Muchtar E, Buadi FK, Gonsalves WI, Dingli D, Go RS, Warsame R, Kourelis T, et al.: **Phase 2 Trial of Ixazomib, Lenalidomide, Dexamethasone and Daratumumab in Patients with Newly Diagnosed Multiple Myeloma**. *Blood* 2018, **132**:304.

357. Yimer H, Melear J, Faber E, Bensinger W, Burke JM, Narang M, Stevens D, Gunawardena SW, Lutska Y, Qi K, et al.: **Lyra: A Phase 2 Study of Daratumumab (Dara) Plus Cyclophosphamide, Bortezomib, and Dexamethasone (Cybord) in Newly Diagnosed and Relapsed Patients (Pts) with Multiple Myeloma (MM)**. *Blood* 2018, **132**:152.

358. Sanchez L, Wang Y, Siegel DS, Wang ML: **Daratumumab: a first-in-class CD38 monoclonal antibody for the treatment of multiple myeloma**. *Journal of hematology & oncology* 2016, **9**:51-51.

359. Dall'Ozzo S, Tartas S, Paintaud G, Cartron G, Colombat P, Bardos P, Watier H, Thibault G: **Rituximab-Dependent Cytotoxicity by Natural Killer Cells**. *Cancer Research* 2004, **64**:4664.

360. Wang Y, Zhang Y, Hughes T, Zhang J, Caligiuri MA, Benson DM, Yu J: **Fratricide of NK Cells in Daratumumab Therapy for Multiple Myeloma Overcome by Ex Vivo-Expanded Autologous NK Cells**.

361. Krejcik J, Frerichs KA, Nijhof IS, van Kessel B, van Velzen JF, Bloem AC, Broekmans MEC, Zweegman S, van Meerloo J, Musters RJP, et al.: **Monocytes and Granulocytes Reduce CD38 Expression Levels on Myeloma Cells in Patients Treated with Daratumumab**.

362. Drach J, McQueen T, Engel H, Andreeff M, Robertson KA, Collins SJ, Malavasi F, Mehta K: **Retinoic Acid-induced Expression of CD38 Antigen in Myeloid Cells Is Mediated through Retinoic Acid Receptor-α**. *Cancer Research* 1994, **54**:1746.

363. Huang H-W, Chen C-H, Lin C-H, Wong C-H, Lin K-I: **B-cell maturation antigen is modified by a single N-glycan chain that modulates ligand binding and surface retention**. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**:10928-10933.

364. Yu B, Jiang T, Liu D: **BCMA-targeted immunotherapy for multiple myeloma**. *Journal of Hematology & Oncology* 2020, **13**:125.

365. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M: **MUC1 and cancer**. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1999, **1455**:301-313.

366. Natoni A, Bohara R, Pandit A, O'Dwyer M: **Targeted Approaches to Inhibit Sialylation of Multiple Myeloma in the Bone Marrow Microenvironment**. *Frontiers in bioengineering and biotechnology* 2019, **7**:252-252.

367. Granzin M, Soltenborn S, Müller S, Kollet J, Berg M, Cerwenka A, Childs RW, Huppert V: **Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy**. *Cytotherapy* 2015, **17**:621-632.

368. Pomeroy EJ, Hunzeker JT, Kluesner MG, Lahr WS, Smeester BA, Crosby MR, Lonetree CL, Yamamoto K, Bendzick L, Miller JS, et al.: **A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy**.

369. van de Donk NWCJ, Usmani SZ: **CD38 Antibodies in Multiple Myeloma: Mechanisms of Action and Modes of Resistance**. *Frontiers in immunology* 2018, **9**:2134-2134.

370. Sanz MA, Fenaux P, Tallman MS, Estey EH, Löwenberg B, Naoe T, Lengfelder E, Döhner H, Burnett AK, Chen S-J, et al.: **Management of acute promyelocytic leukemia: updated recommendations from an expert panel of the European LeukemiaNet**. *Blood* 2019, **133**:1630-1643.

371. Huang ME, Ye Yc Fau - Chen SR, Chen Sr Fau - Chai JR, Chai Jr Fau - Lu JX, Lu Jx Fau - Zhoa L, Zhoa L Fau - Gu LJ, Gu Lj Fau - Wang ZY, Wang ZY: **Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia**.

372. Casneuf T, Xu XS, Adams HC, 3rd, Axel AE, Chiu C, Khan I, Ahmadi T, Yan X, Lonial S, Plesner T, et al.: **Effects of daratumumab on natural killer cells and impact on clinical outcomes in relapsed or refractory multiple myeloma**. *Blood advances* 2017, **1**:2105-2114.

373. Naeimi Kararoudi M, Nagai Y, Elmas E, de Souza Fernandes Pereira M, Ali SA, Imus PH, Wethington D, Borrello IM, Lee DA, Ghiaur G: **CD38 deletion of human primary NK cells eliminates daratumumab-induced fratricide and boosts their effector activity**. *Blood* 2020, **136**:2416-2427.

374. Vey N, Karlin L, Sadot-Lebouvier S, Broussais F, Berton-Rigaud D, Rey J, Charbonnier A, Marie D, André P, Paturel C, et al.: **A phase 1 study of lirilumab (antibody against killer immunoglobulin-like receptor antibody KIR2D; IPH2102) in patients with solid tumors and hematologic malignancies**. *Oncotarget* 2018, **9**:17675-17688.

375. Carlsten M, Korde N, Kotecha R, Reger R, Bor S, Kazandjian D, Landgren O, Childs RW: **Checkpoint Inhibition of KIR2D with the Monoclonal Antibody IPH2101 Induces Contraction and Hyporesponsiveness of NK Cells in Patients with Myeloma**. *Clinical Cancer Research* 2016, **22**:5211.

376. Benson DM, Cohen AD, Jagannath S, Munshi NC, Spitzer G, Hofmeister CC, Efebera YA, Andre P, Zerbib R, Caligiuri MA: **A Phase I Trial of the Anti-KIR Antibody IPH2101 and Lenalidomide in Patients with Relapsed/Refractory Multiple Myeloma**. *Clinical Cancer Research* 2015, **21**:4055.

377. Robbins M, Jure-Kunkel M, Dito G, Andre P, Zhang H-f, Bezman N, Graziano RF: **Effects of IL-21, KIR Blockade, and CD137 Agonism on the Non-Clinical Activity of Elotuzumab**. *Blood* 2014, **124**:4717-4717.

378. Sola C, Blery M, Bonnafous C, Bonnet E, Fuseri N, Graziano RF, Morel Y, André P: **Lirilumab Enhances Anti-Tumor Efficacy of Elotuzumab**. *Blood* 2014, **124**:4711-4711.

379. Romagné F, André P, Spee P, Zahn S, Anfossi N, Gauthier L, Capanni M, Ruggeri L, Benson DM, Jr., Blaser BW, et al.: **Preclinical characterization of 1-7F9, a novel human anti–KIR receptor therapeutic antibody that augments natural killer–mediated killing of tumor cells**. *Blood* 2009, **114**:2667-2677.

380. Pazmany L, Mandelboim O, Valés-Gómez M, Davis DM, Reyburn HT, Strominger JL: **Protection from Natural Killer Cell-Mediated Lysis by HLA-G Expression on Target Cells**. *Science* 1996, **274**:792.

381. Rouas-Freiss N, Marchal RE, Kirszenbaum M, Dausset J, Carosella ED: **The α₁ domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: Is HLA-G the public ligand for natural killer cell inhibitory receptors?** *Proceedings of the National Academy of Sciences* 1997, **94**:5249.

382. Villa-Álvarez M, Sordo-Bahamonde C, Lorenzo-Herrero S, Gonzalez-Rodriguez AP, Payer AR, Gonzalez-Garcia E, Villa-Álvarez MC, López-Soto A, Gonzalez S: **Ig-Like Transcript 2 (ILT2) Blockade and Lenalidomide Restore NK Cell Function in Chronic Lymphocytic Leukemia**. *Frontiers in Immunology* 2018, **9**.

383. Soulas C, Remark R, Brezar V, Lopez J, Bonnet E, Caraguel F, Lalanne A, Hoffmann C, Denis C, Arnoux T, et al.: **Abstract 2714: Combination of monalizumab and durvalumab as a potent immunotherapy treatment for solid human cancers**. *Cancer Research* 2018, **78**:2714.

384. Ohaegbulam KC, Assal A, Lazar-Molnar E, Yao Y, Zang X: **Human cancer immunotherapy with antibodies to the PD-1 and PD-L1 pathway**.

385. Garcia-Diaz A, Shin DS, Moreno BH, Saco J, Escuin-Ordinas H, Rodriguez GA, Zaretsky JM, Sun L, Hugo W, Wang X, et al.: **Interferon Receptor Signaling Pathways Regulating PD-L1 and PD-L2 Expression**.

386. Chen L, Byers LA, Ullrich S, Wistuba II, Qin X-F, Gibbons DL: **CD38 as a novel immune checkpoint and a mechanism of resistance to the blockade of the PD-1/PD-L1 axis**. *Journal of Clinical Oncology* 2017, **35**:79-79.

387. Bezman NA, Kinder M, Jhatakia AD, Mattson BK, Pizutti D, Thompson EW, Capaldi DA, Mendonca MW, Anandam A, Dhar G, et al.: **Abstract 1727: Antitumor activity associated with dual targeting of CD38 and programmed death-1 (PD-1) pathways in preclinical models**. *Cancer Research* 2018, **78**:1727.

388. Jelinek T, Paiva B, Hajek R: **Update on PD-1/PD-L1 Inhibitors in Multiple Myeloma**. *Frontiers in immunology* 2018, **9**:2431-2431.

389. Daly J, Carlsten M, O'Dwyer M: **Sugar Free: Novel Immunotherapeutic Approaches Targeting Siglecs and Sialic Acids to Enhance Natural Killer Cell Cytotoxicity Against Cancer**. *Frontiers in immunology* 2019, **10**:1047-1047.

390. Deuss FA, Watson GM, Fu Z, Rossjohn J, Berry R: **Structural Basis for CD96 Immune Receptor Recognition of Nectin-like Protein-5, CD155**. *Structure* 2019, **27**:219-228.e213.

391. Jin H-S, Park Y: **Hitting the complexity of the TIGIT-CD96-CD112R-CD226 axis for next-generation cancer immunotherapy**. *BMB reports* 2021, **54**:2-11.

392. Minnie SA, Kuns RD, Gartlan KH, Zhang P, Wilkinson AN, Samson L, Guillerey C, Engwerda C, MacDonald KPA, Smyth MJ, et al.: **Myeloma escape after stem cell transplantation is a consequence of T-cell exhaustion and is prevented by TIGIT blockade**. *Blood* 2018, **132**:1675-1688.

393. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, Ritchie DS, Colonna M, Andrews DM, Smyth MJ: **The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions**.

394. Miller JS, Tessmer-Tuck J, Blake N, Lund J, Scott A, Blazar BR, Orchard PJ: **Endogenous IL-2 production by natural killer cells maintains cytotoxic and proliferative capacity following retroviral-mediated gene transfer**. *Experimental hematology* 1997, **25**:1140-1148.

395. Matosevic S: **Viral and Nonviral Engineering of Natural Killer Cells as Emerging Adoptive Cancer Immunotherapies**. *Journal of immunology research* 2018, **2018**:4054815-4054815.

396. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P: **CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes**. *Science* 2007, **315**:1709.

397. Shabbir MAB, Shabbir MZ, Wu Q, Mahmood S, Sajid A, Maan MK, Ahmed S, Naveed U, Hao H, Yuan Z: **CRISPR-cas system: biological function in microbes and its use to treat antimicrobial resistant pathogens**. *Annals of Clinical Microbiology and Antimicrobials* 2019, **18**:21.

398. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD: **Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin**.

399. Huai C, Li G, Yao R, Zhang Y, Cao M, Kong L, Jia C, Yuan H, Chen H, Lu D, et al.: **Structural insights into DNA cleavage activation of CRISPR-Cas9 system**. *Nature communications* 2017, **8**:1375-1375.

400. Miyaoka Y, Berman JR, Cooper SB, Mayerl SJ, Chan AH, Zhang B, Karlin-Neumann GA, Conklin BR: **Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing**. *Scientific Reports* 2016, **6**:23549.

401. Rautela J, Surgenor E, Huntington ND: **Efficient genome editing of human natural killer cells by CRISPR RNP**. *bioRxiv* 2018:406934.

402. Lambert M, Leijonhufvud C, Segerberg F, Melenhorst JJ, Carlsten M: **CRISPR/Cas9-Based Gene Engineering of Human Natural Killer Cells: Protocols for Knockout and Readouts to Evaluate Their Efficacy**.

403. Huang R-S, Shih H-A, Lai M-C, Chang Y-J, Lin S: **Enhanced NK-92 Cytotoxicity by CRISPR Genome Engineering Using Cas9 Ribonucleoproteins**. *Frontiers in Immunology* 2020, **11**.

404. Duggan AJ: **Silencing the CD96 immune checkpoint receptor on natural killer cells enhances their cytotoxic potential against multiple myeloma**. Edited by National University of Ireland GCoMT. Galway: Thesis (M.Sc.)

National University of Ireland, Galway, 2018.; 2018.

405. Prescher H, Frank M, Gütgemann S, Kuhfeldt E, Schweizer A, Nitschke L, Watzl C, Brossmer R: **Design, Synthesis, and Biological Evaluation of Small, High-Affinity Siglec-7 Ligands: Toward Novel Inhibitors of Cancer Immune Evasion**. *Journal of Medicinal Chemistry* 2017, **60**:941-956.

406. Angelo LS, Banerjee PP, Monaco-Shawver L, Rosen JB, Makedonas G, Forbes LR, Mace EM, Orange JS: **Practical NK cell phenotyping and variability in healthy adults**. *Immunologic research* 2015, **62**:341-356.

407. Carlsten M, Childs RW: **Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications**. *Frontiers in immunology* 2015, **6**:266-266.

408. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, Ritchie DS, Colonna M, Andrews DM, Smyth MJ: **The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions**. *Nature Immunology* 2014, **15**:431-438.

409. Choi YH, Lim EJ, Kim SW, Moon YW, Park KS, An H-J: **IL-27 enhances IL-15/IL-18-mediated activation of human natural killer cells**. *Journal for ImmunoTherapy of Cancer* 2019, **7**:168.

410. Harjunpää H, Guillerey CA-O: **TIGIT as an emerging immune checkpoint**.

411. Li L, Liu Ln Fau - Feller S, Feller S Fau - Allen C, Allen C Fau - Shivakumar R, Shivakumar R Fau - Fratantoni J, Fratantoni J Fau - Wolfraim LA, Wolfraim La Fau - Fujisaki H, Fujisaki H Fau - Campana D, Campana D Fau - Chopas N, Chopas N Fau - Dzekunov S, et al.: **Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method**.

412. Nakai R, Maniwa Y Fau - Tanaka Y, Tanaka Y Fau - Nishio W, Nishio W Fau - Yoshimura M, Yoshimura M Fau - Okita Y, Okita Y Fau - Ohbayashi C, Ohbayashi C Fau - Satoh N, Satoh N Fau - Ogita H, Ogita H Fau - Takai Y, Takai Y Fau - Hayashi Y, et al.: **Overexpression of Necl-5 correlates with unfavorable prognosis in patients with lung adenocarcinoma**.

413. Sloan KE, Eustace BK, Stewart JK, Zehetmeier C, Torella C, Simeone M, Roy JE, Unger C, Louis DN, Ilag LL, et al.: **CD155/PVR plays a key role in cell motility during tumor cell invasion and migration**. *BMC cancer* 2004, **4**:73-73.

414. Masson D, Jarry A Fau - Baury B, Baury B Fau - Blanchardie P, Blanchardie P Fau - Laboisse C, Laboisse C Fau - Lustenberger P, Lustenberger P Fau - Denis MG, Denis MG: **Overexpression of the CD155 gene in human colorectal carcinoma**.

415. Pearce OMT, Läubli H: **Sialic acids in cancer biology and immunity**. 2016, **26**:111-128.

416. Liu S, Galat V, Galat Y, Lee YKA, Wainwright D, Wu J: **NK cell-based cancer immunotherapy: from basic biology to clinical development**. *Journal of Hematology & Oncology* 2021, **14**:7.

417. Miyazaki K, Sakuma K, Kawamura YI, Izawa M, Ohmori K, Mitsuki M, Yamaji T, Hashimoto Y, Suzuki A, Saito Y, et al.: **Colonic Epithelial Cells Express Specific Ligands for Mucosal Macrophage Immunosuppressive Receptors Siglec-7 and -9**. *The Journal of Immunology* 2012, **188**:4690.

418. Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker PR: **Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms**. *European Journal of Immunology* 2003, **33**:1642-1648.

419. DeRogatis JM, Viramontes KM, Neubert EN, Tinoco R: **PSGL-1 Immune Checkpoint Inhibition for CD4+ T Cell Cancer Immunotherapy**. *Frontiers in Immunology* 2021, **12**.

420. Tinoco R, Bradley LM: **Targeting the PSGL-1 pathway for immune modulation**. *Immunotherapy* 2017, **9**:785-788.

421. Frerichs KA, Minnema MC, Levin M-D, Broyl A, Bos G, Kersten MJ, Mutis T, Verkleij CPM, Bosman PWC, Klein SK, et al.: **Efficacy and Safety of Daratumumab Combined with All-Trans Retinoic Acid in Relapsed/Refractory Multiple Myeloma; Results of the Phase 1/2 Dara/ATRA Study**. *Blood* 2019, **134**:1826-1826.

422. Rajkumar SV: **Daratumumab in multiple myeloma**. *Lancet* 2016, **387**.

423. Nijhof IS, Casneuf T, van Velzen J, van Kessel B, Axel AE, Syed K, Groen RWJ, van Duin M, Sonneveld P, Minnema MC, et al.: **CD38 expression and complement inhibitors affect response and resistance to daratumumab therapy in myeloma**. *Blood* 2016, **128**:959-970.

424. Cao Y, Wang X, Jin T, Tian Y, Dai C, Widarma C, Song R, Xu F: **Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy**. *Signal Transduction and Targeted Therapy* 2020, **5**:250.

425. Zanwar S, Nandakumar B, Kumar S: **Immune-based therapies in the management of multiple myeloma**. *Blood Cancer Journal* 2020, **10**:84.

426. Mateos M-V, Orlowski R, Siegel D, Reece D, Moreau P, Ocio E, Shah J, Rodríguez-Otero P, Munshi N, Avigan D: **Pembrolizumab in combination with lenalidomide and low-dose dexamethasone for relapsed/refractory multiple myeloma: final efficacy and safety analysis**. *J Clin Oncol* 2016, **34**:8010a.

427. O. Bénac MGWO-HHRBMOEBAMRL-DCSSCRRG, M. Phelipot SBFRALMBCBAMBRLGCPEVYMI: **Preclinical development of first-in-class antibodies targeting Siglec-9 immune checkpoint for cancer immunotherapy** edn Supplement. Edited by. American Association for Cancer Research; 2018. vol 78.]