TARGETING SIGLECS ON RECIPIENT ANTIGEN-PRESENTING CELLS WITH SIALIC ACID- MODIFIED ALLOANTIGEN TO PROMOTE TRANSPLANTATION TOLERANCE

MONICA SEN

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Abstract

Chronic transplant rejection remains a persistent barrier to transplant survival. One approach to tackle this is to target the indirect pathway of allorecognition, the major contributor to chronic mediated rejection. Modifying the recipient's antigen- presenting cells which have a major role of presenting graft-derived donor alloantigens to recipient T cells, which proliferate and differentiate into effector cells thereby leading to an immune attack against the transplanted organ, has been undertaken. Previous studies found that targeting alloantigens derived from BALB/c MHC Class I H-2K^{d} via specific cell surface receptors to immature dendritic cells (DCs), impaired indirect allorecognition leading to murine skin grafts survival. Siglec receptors have previously been targeted on murine DCs to induce tolerance in an autoimmune mouse model. These receptors specifically bind to sialylated ligands and have a role in down-regulating immune responses due to their immunoreceptor tyrosine-based inhibitory motif. To date, it has not been established whether targeting these receptors can induce transplantation tolerance; therefore our aim was to target Siglecs on a heterogeneous population of recipient DCs with sialylated H-2K^{d} alloantigen to modify the indirect pathway of allorecognition and to promote organ transplant survival.

In this study it was established Siglecs were expressed on DCs, B cells and macrophages. Targeting sialylated alloantigen to DCs impaired indirect alloreactive CD4^{+} T cell proliferation and cytokine production; and were able to induce/expand CD4^{+} Foxp3^{+} Tregs *in vitro* following targeting.

Additionally, we have demonstrated *in vivo* that sialylated alloantigen treatment prior to MHC-mismatch donor skin transplantation to wild-type B6 recipient mice resulted in graft prolongation and also a significant increase of CD4^{+} CD62L^{+} Foxp3^{+} Tregs in recipient blood, a possible contributor to the observed graft survival. In addition, there was a marked decrease of alloantibodies, signifying that alloreactive B cells activated via indirect pathway of allorecognition are being inhibited. This was attributed to Siglec expression on DCs requiring Batf3 transcription factor for their development (CD103^{+} and CD8α^{+} DCs). It was also observed that mice treated with sialylated alloantigen deleted indirect CD4^{+} T

cells *in vivo*, suggesting that this could be one of the mechanisms contributing to allograft survival.

In conclusion, targeting recipient DCs with sialylated alloantigen may represent a novel mechanism to regulate allorecognition and prolong allograft survival.

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Abbreviations

Ab	Antibody
ACK	Ammonium-Chloride-Potassium
Ag	Antigen
AICD	Activation-induced cell death
APC	Antigen presenting cell
APC	Allophycocyanin
ANOVA	Analysis of variance
Anti	Antibody
ATCC	American Type Culture Collection
B	B lymphocyte
B6	C57BL/6J
Batf3	Basic leucine zipper transcription factor
BCR	B cell receptor
BD	Becton Dickinson
BDCA2	Blood dendritic cell antigen 2
BM	Bone marrow
Bcl-xL	B-cell lymphoma-extra large
BM-DC	Bone marrow-derived dendritic cell
BM-Macs	Bone marrow-derived macrophages
Breg	Regulatory B cell
BV421	Brilliant violet 421
CD	Cluster differentiation
cDC	Conventional dendritic cell
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CO_2	Carbon dioxide
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
Dex-D3	Dexamethasone- Vitamin D3
DHRS9	Dehydrogenase/reductase 9
DNA	Deoxyribonucleic acid
DR5	Death receptor 5
DST	Donor-specific transfusion
EAE	Experimental autoimmune encephalomyelitis
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FAM5/6	5(6)-FAM (5(6)-Carboxyfluorescein
FasL	Fas ligand
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLT3-L	FMS-like tyrosine kinase 3 ligand
FO	Follicular
Foxp3	Forkhead box P3
FSC	Forward scatter
g	Times gravity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HEL	Hen egg white lysozyme
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$KHCO_3$	Potassium bicarbonate
HLA	Human leukocyte antigen
hNC16A	Human type XVII collagen
HPLC	High Performance Liquid Chromatography
ICOS-L	Inducible T cell costimulator ligand
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
I.P.	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ISD	Immunosuppressive drugs
I.V.	Intravenous
K^d	H-2K^d 54-68 peptide
KO	Knock-out
LN-DC	Lymph node-derived dendritic cell
LN	Lymph node
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
ME	2- Mercaptoethanol
MFI	Mean fluorescence cell sorting
MHC	Major histocompatibility complex
miRNA	MicroRNA
ml	Millilitre
MOG	Myelin oligodendrocyte glycoprotein
MST	Mean survival time

Mreg	Regulatory macrophage
MR1	CD40 ligand antibody
mTOR	Mammalian target of rapamycin
MZ	Marginal zone
Neu5Ac	N-acetyl neuraminic acid
NFAT-5	Nuclear factor of activated T-cells 5
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4Cl	Ammonium chloride
NK	Natural killer cell
NZW	New Zealand White
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
PE	R-phycoerythrin
PMA	Para-Methoxyamphetamine
PVDF	Polyvinylidene fluoride
RAG	Recombination-activating gene
RBC	Red blood cells
RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
SD	Standard deviation
SEB	Staphylococcal entertotoxin B
SEM	Standard error of the mean
Sia	Sialic acid
Siglec	Sialic acid-binding immunoglobulin-type lectin
SHP	Src homology region 2 domain-containing phosphatase
SPF	Specific pathogen free
SPLN	Spleen
SPLN-DC	Splenic-derived dendritic cell
SLO	Secondary lymphoid organs
SSC	Side scatter
T	T lymphocyte
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T Helper cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TolDC	Tolerogenic dendritic cell

TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
Tr1	Type 1 regulatory cells
TSDR	Treg-specific demethylated region
Tx	Transplant
T1	Transitional 1
T2	Transitional 2
U	Units
UK	United Kingdom
USA	United States of America
WT	Wild-type
3D	Three-dimensional
µg	Microgram
µl	Microlitre
µM	Micromolar
µCi	Microcurie

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Presentations

Oral Presentations

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- SIICA, EFIS, International retreat for PhD students in Immunology, Verona, Italy – *October 2017*
- School of Health, Sport and Biosciences Post-graduate research conference – *March 2018*
- Division of Transplantation Immunology and Mucosal Biology, King's College London, UK – *April 2018*
- AHC College Research Day (Three-minute Thesis competition) – *June 2018*
- Research Update Conference (RUC) for the School of Immunology & Microbial Sciences, King's College London, UK- *September 2018*.
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Publications

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CHAPTER 1:

INTRODUCTION

1.1 Historical outline of transplantation

Transplantation has been one of the most ground breaking medical interventions for organ failure for centuries, with procedures dated back to 600B.C (Barker & Markmann, 2013). The process of transferring a functional organ from one individual to another has been a sought after medical phenomenon for centuries and due to on-going research in the following years, successes have included heart, liver, intestinal and the most recent triumph, the full face transplant [Fig. 1.1] (Barret *et al*., 2011). However, successful organ transplantation has always been challenging.

A study by Voronoy (1937) highlighted this problem and demonstrated that human kidney transplants between individuals with major mismatched blood groups were quickly rejected (Barker & Markmann, 2013). Amongst the pioneers of transplantation, Peter Medawar paved the way in understanding the link between transplantation success and the graft recipient's immune response. He investigated skin allograft (donor organ from a genetically non-identical individual, derived from the same species) outcomes in rabbits and observed complete or partial degeneration of the architecture of rejected skin grafts (Medawer, 1944; Barker & Markmann, 2013). His study highlighted organ tissue destruction to be a possible outcome of acute inflammation induced by a large repopulation of recipient leucocytes within the genetically non-identical skin graft, an assessment made using histological analysis (Medawer, 1944; Barker & Markmann, 2013). Later, Medawar and Billingham performed skin transplants on larger animals, cows, in order to ascertain that genetically identical donor and recipients (such as twins) as opposed to genetically non-identical individuals, have a greater chance of transplant acceptance (Barker & Markmann, 2013).

This led to further research on the exchange of blood and stem cells between twins *in utero.* In 1954 Dr Murray and Dr Hume at Brigham Hospital in Boston, were able to successfully engraft kidneys between identical twins, marking this as the world's first successful kidney transplant (Delmonico, 2002). Medawer's pioneering research led to studies which determined whether modifying the transplant recipient prolonged skin graft survival. To assess this, recipient mice

foetuses were inoculated with donor spleen cells which led to skin graft acceptance (Billingham & Medawar, 1953; Barker & Markmann, 2013).

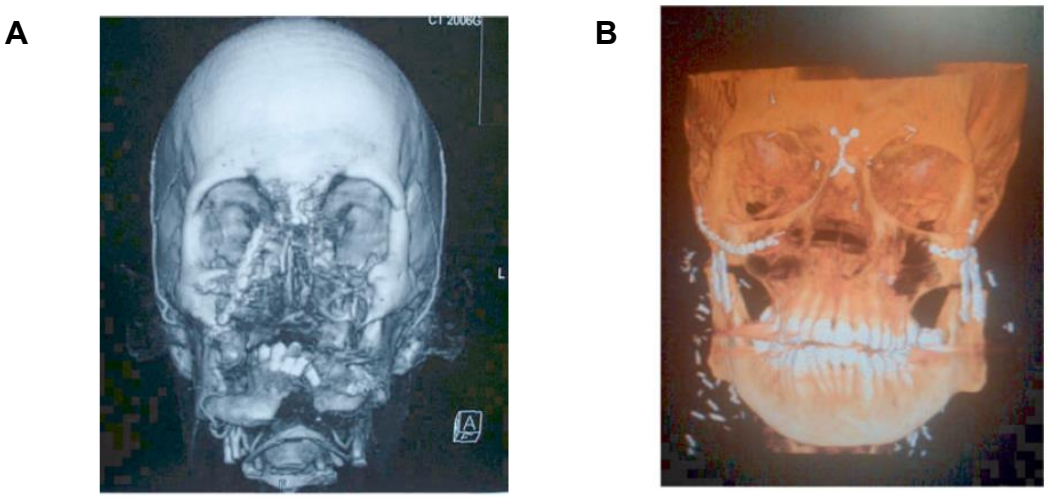


Figure 1.1. 3D CT scan of patient before and after receiving a full-face transplant. *A. Image taken before full-face transplant. B. Image taken 4 months post full-face transplant. Figure adapted from: Barret et al., (2011).*

1.2 Transplant rejection and allorecognition

Despite these successes, transplant rejection remains a persistent problem, particularly in non-identical individuals. This is due to the recipient's immune system. The immune system has developed the ability to distinguish ‘self’ (own proteins) and ‘non-self’ (foreign pathogens/ proteins) molecules. In the context of transplantation, a donor’s organ is recognised as non-self foreign 'invader', resulting in activation of the recipient's immune cells and the eventual destruction of the transplanted organ. The process whereby the recipient immune cells recognise antigens on donor tissue is known as allorecognition. Allorecognition is driven by recipient T cells, which recognise donor antigens presented via major histocompatibility complexes (MHC) on antigen presenting cells (APC).

1.2.1 Major histocompatibility complex and alloantigens

MHC molecules expressed on immune cells are the driving force for allorecognition and initiation of an immune response, following a transplant (Benichou *et al*., 2011). There are two types of MHC, MHC Class I (MHC I) which is present on all nucleated cells and its role is to present endogenous peptide antigen to $CD8^+$ T cells (cytotoxic T cells), whereas MHC Class II (MHC II) is mainly present on APCs and presents exogenous antigens to $CD4^+$ T cells (helper T cells) [Fig. 1.2] (Benichou *et al*., 2011).

MHC molecules are highly polymorphic and each individual possess multiple variants of genes that encode for their MHC. The genes that encode MHC is located on murine chromosome 17 (Stuart, 2015) and on human chromosome 6 (Francke & Pellegrino, 1977). The human MHC molecules are referred to as Human Leukocyte Antigen (HLA) (MHC I subclasses: HLA- A, B, and C and MHC II subclasses: HLA- DP,DM, DQ and DR) and in mouse as H-2 genes (MHC I subclasses: H-2D, K, L and MHC II subclasses: H-2A(I-A) or H-2E(I-E). In a transplant setting, the donor graft consists of cells expressing donor MHC molecules which the recipient immune cells will recognise as foreign leading to rejection- in this case the donor MHC (whether it be intact or processed into peptides) is then referred to as an alloantigen [Fig. 1.3]. To assess the likelihood of rejection, laboratory tests such as mixed lymphocyte reactions (MLRs) involving co-culture of recipient and donor lymphocytes and assessing their proliferative capacity will help decipher whether an immune response occurs, additionally, HLA-tissue typing is another method that determines the level of genetic similarity between the donor and the recipient thus providing an effective predictor for graft rejection. However, the presence of minor histocompatibility antigens (non-MHC derived such as placenta-derived Ags, H-Y Ags) (Linscheid & Petroff, 2013), which also contribute to rejection, make finding a complete match extremely challenging. The minor histocompatibility antigens are effective at mediating the indirect pathway of allorecognition, which is described in *section 1.2.2.2* (pg.8), and provides an explanation as to why MHC-matched skin grafts can be rejected (Warrens *et al*., 1994; Roopenian *et al*., 2002; Goulmy *et al*., 1997; Goulmy *et al*., 1995).

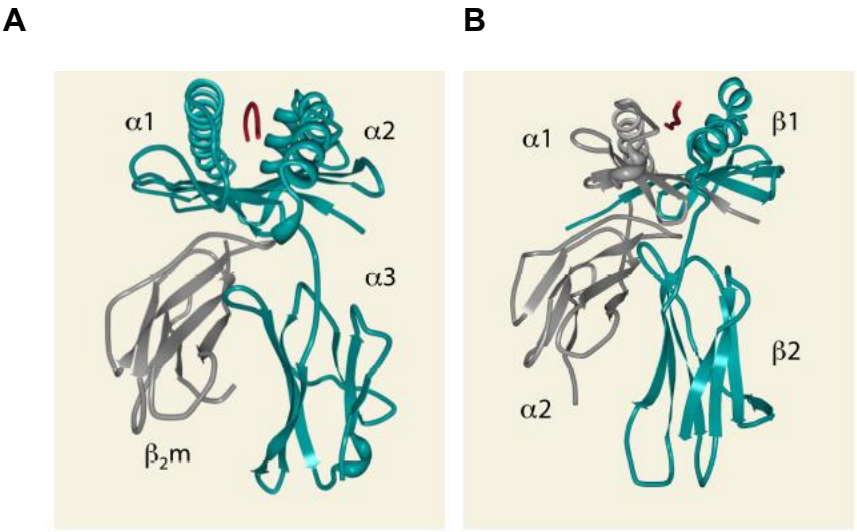


***Figure 1.2. 3D structures of MHC I and II**. A. 3D structure of MHC I molecule present on all nucleated cells containing α1, α2, α3 and β2-microglubulin subunits and a peptide binding groove with the capacity to bind to peptides approx. 8-11 amino acids in length. B. 3D structure of MHC II molecule present on APCs containing α1, β1, β2 and α2 subunits and a peptide binding groove with the capacity to bind to peptides approx. 13-25 amino acids in length. Image adapted from: Blum et al., (2013).*

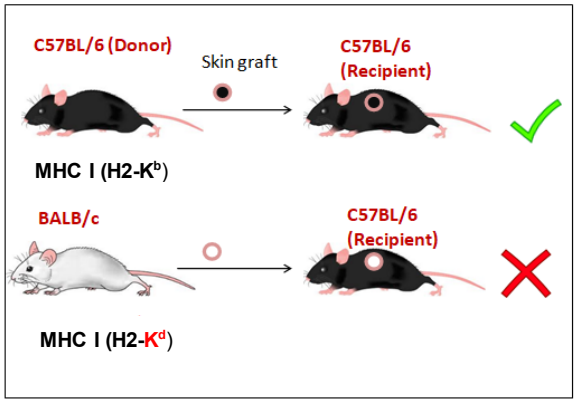


Figure 1.3. MHC genetic disparity can contribute to transplant rejection. *MHC mismatched skin graft between two genetically non-identical individuals can contribute to rejected allograft. Skin transplant between genetically identical individuals with matched MHC does not amount to transplant rejection. Transplant between unmatched MHC individuals (eg: H2-K^d donor and H2-K^b recipients) contributes to transplant rejection.*

1.2.2 Allorecognition and contribution to transplant rejection

Allorecognition consists of three pathways which include; direct, indirect and semi-direct pathways of allorecognition. All of which contribute towards rejection, however each pathway is characteristically different (Siu *et al.*, 2018).

1.2.2.1 Direct allorecognition

Direct allorecognition is defined by pre-existing alloreactive recipient T cells recognising donor MHC molecules on donor-derived DCs or 'passenger cells', (Boardman *et al.*, 2016). The aforementioned T cells make up approximately 10% of the T cells present within the recipient (Veerapathran *et al.*, 2011; Boardman *et al.*, 2016). In order to understand how these alloreactive T cells respond to donor MHC, one model proposed that the amino acid polymorphic regions of allogeneic MHC on the surface of donor DCs are recognised by recipient TCRs and elicit T cell activation- this is known as high determinant density model (Bevan, 1984). The second model, known as the multiple binary complex model, describes that the allopeptide bound to the donor MHC forms an allogeneic MHC-peptide complex which mimics self-MHC molecules so that

they can be recognised by a vast range of recipient TCRs (Matzinger & Bevan, 1977).

Earlier studies have demonstrated the importance of this pathway, for example Lechler and Batchelor (1982), demonstrated the role of donor-derived DCs in T cell activation which was confirmed in a rat kidney transplant model where graft survival was prolonged in recipients depleted of passenger leukocytes. Another study demonstrated that kidney allograft rejection was imminent in the presence of donor-derived DCs in irradiated recipient mice administered with direct-allospecific $CD4^+$ T cells (Braun *et al.*, 1993). In fact, it was recently shown that a particular subset of donor-derived skin $CD103^+$ DCs are able to present allogeneic MHC II to prime T cells with direct allorecognition and induce acute rejection (Borges *et al.*, 2018). The donor $CD103^+$ DCs were able migrate to host lymph nodes from the donor skin allograft and drive the direct pathway (Borges *et al.*, 2018). Additionally, when donor skin grafts were incubated with *M. tuberculosis* DnaK (DnaK) solution which is a bacterial ortholog of heat shock protein 70 which can decrease MHC II expression on skin donor $CD103^+$ DCs, followed by applying skin grafts onto mice, direct alloreactivity was reduced and thus improved murine skin graft survival (Borges *et al.*, 2018).

Effector $CD4^+$ and $CD8^+$ T cells are crucial for the establishment of direct allorecognition. One study using SCID and $Rag1^{-/-}$ mice demonstrated that cardiac allografts containing MHC II- expressing donor cells were acutely rejected in the presence of adoptively transferred $CD4^+$ T cells (Pierta *et al.*, 2000). On the contrary, the aforementioned recipient mice transplanted with donor heart grafts with MHC II-deficient cells and adoptively transferred $CD4^+$ T cells, also rejected the cardiac graft, but at a distinctly slower pace (Pierta *et al.*, 2000) demonstrating the requirement of both donor MHC II cells and $CD4^+$ T cells to initiate rejection (Pierta *et al.*, 2000). To further stress the importance of $CD4^+$ T cells in acute rejection, help from $CD4^+$ T cells that have been activated via the direct pathway, stimulate $CD8^+$ T cell cytotoxic immunity in response to MHC I expressing donor cells, resulting in allograft rejection (Taylor *et al.*, 2007).

Direct allorecognition gives rise to acute transplant rejection which is characterised by fast-paced onset of transplanted organ failure which develops days to a few weeks post transplant. This was attributed to MHC gene disparity between the donor and the recipient contributing to antibody-mediated rejection and cellular rejection (Benzimra *et al*., 2017). Acute cellular rejection is mediated by the recognition of donor MHC on APCs which present antigens to recruited effector T lymphocytes (Benzimra *et al*., 2017). This type of rejection can be identified quickly and alleviated through the use on immunosuppressive drugs (ISD) such as corticosteroids, however recurrent episodes of rejection would inevitably lead to chronic rejection.

1.2.2.2 Indirect allorecognition

In 1982, Lechler and Batchelor proposed the pathway of indirect allorecognition. The aforementioned study noticed that despite depletion of donor DCs that led to prolonged allograft survival, rat kidney grafts were still rejected (Lechler and Batchelor, 1982). It was then discovered that recipient APCs were able to present peptides derived from donor MHC from graft tissue to recipient T cells to stimulate allograft rejection- indirect allorecognition (Fangmann *et al*., 1992; Benichou *et al*., 1992; Auchincloss *et al*., 1993; Pierta *et al*., 2000; Benichou *et al.,* 1997). Benichou *et al*., (1992) demonstrated recipient APCs that presented peptide derived from polymorphic regions from the donor MHC where able to instigate proliferation of T cells derived from murine skin grafted recipients. It was published one year later in 1993 that skin grafts derived from donor MHC II knock-out mice and transplanted onto recipient mice with $CD4^+$ T cells (depleted of $CD8^+$ T cells), had rejecting skin grafts suggesting that donor Ags were presented via self-MHC II molecules, thus confirming the role of recipient APCs and $CD4^+$ T cells in indirect allorecognition (Auchincloss *et al*., 1993).

The $CD4^+$ T cell mediated direct allorecognition has been shown to be aggressive. Post transplantation, NK cells (Garrod *et al*. 2010) and $CD4^+$ T cells assist in the removal of donor cells, thereby limiting number of donor DCs to instigate direct allorecognition (Ali *et al*., 2016). The direct pathway only remained persistent for an extended period of time when immune cells such as NK cells and $CD4^+$ T cells were depleted (Ali *et al*., 2016). Within the recipient,

donor DCs are depleted by prolonged interactions with recipient NK cells, however it was shown that depleting donor DCs did not prevent allograft rejection (Garrod *et al*., 2010). One study demonstrated using CD11c-DTR mice, (B6 mice expressing the diphtheria receptor under the CD11c promoter where injection of diphtheria toxin (DT) into these mice leads to the transient loss of all $CD11c^+$ DCs and/or macrophages (van Blijswijk *et al*., 2013)), that heart allografts were prolonged when recipient DCs were depleted as opposed to just depleting donor DCs (Garrod *et al*., 2010).

Indirect allorecognition is known to be long-lived and can remain for many years post-transplant, thereby leading to chronic graft rejection that cannot be alleviated using ISD (Baker *et al*., 2001). Brennan *et al*., (2009), confirmed the contribution of indirect allorecognition to chronic transplant rejection, when comparisons were made with direct allorecognition in a murine skin graft model. Their study found that recipient T cells that were activated by donor APCs via direct allorecognition were not able to continue the allorecognition response when donor passenger cells were depleted (Brennan *et al*., 2009). However, in the absence of donor APCs, T cells that were activated via indirect allorecognition were still able to reject murine skin grafts, demonstrating the persistence of this response (Brennan *et al*., 2009).

Unlike direct allorecognition, indirect allospecific T cells are restricted to specific TCRs recognising specific alloantigens. The indirect alloresponse occurs in reaction to a few determinants which are the highly polymorphic regions on the donor MHC (Benichou *et al*., 1994; Benichou *et al*., 1997). In fact, Ali *et al*., 2016 published that murine MHC I alloantigen peptides presented via self-MHC II restricted DCs could increase proliferation of indirect $CD4^+$ T cells within the host, as opposed to MHC II- derived alloantigen peptide. They reasoned that this was due to the limited and short-lived presence of donor hematopoietic cells within the recipient which was the source of donor MHC II alloantigens (Ali *et al*., 2016). Whereas MHC I alloantigens were derived from the donor's organ parenchyma cells which are more persistent within the recipient (Ali *et al*., 2016). Therefore, the origin of alloantigens can contribute towards the aggressiveness of the indirect alloresponse.

Ali *et al*., (2016) study compared the duration of allorecognition pathways and determined that indirect allorecognition remained persistent in establishing rejection. Unlike acute rejection, chronic rejection is much more difficult to treat due to its persistence and can occur months-years post transplant. This disease results in complete tissue injury and loss of graft function, caused by the activation of alloreactive T and B cells and narrowing of graft blood vessels, thereby restricting blood-flow to the graft and scarring of graft tissue (Kloc & Ghobrial, 2014). This kind of rejection is particularly prominent in MHC-mismatched individuals.

1.2.2.2.1 Role of B cells in indirect allorecognition

B cells and APCs are known to express MHC molecules and are thus able to contribute to transplant rejection via their ability to activate T cells and production of donor-specific antibodies (alloantibodies) (Constant *et al*., 1995; Brandle *et al*., 1998). Antigen presenting B cells recognise Ags via their BCR, followed by processing and presenting of Ag via MHC II (Adler *et al*., 2017). Following this interaction, B cells present Ag via MHC II to the corresponding TCR resulting in T cell activation which is also supported by the engagement of B cells CD40 and CD40L on the T cell (Adler *et al*., 2017). They also have the ability to support the proliferation of memory T cells which in turn has shown to accelerate murine skin graft rejection as a consequence of B cell help (Ng *et al*., 2010).

The antigen presenting potential of B cells has been demonstrated by Rossetti *et al*., (2018), where B cells treated with anti-CD40 upregulated MHC II and stimulated T cell anti-tumour responses, whereas DCs were resistant to anti-CD40 stimulation. To support the role of B cells in rejection, a murine cardiac transplant model demonstrated that mice deficient of MHC II expressing B cells had prolonged allograft survival in comparison to wild type mice (Noorchashm *et al.,* 2006). Previous transplant regimens have adopted B cell depletion using anti-CD20 monoclonal antibodies for the purpose of suppressing the development of alloantibodies which contribute to organ rejection (Clark & Ledbetter, 2005). Production of donor-specific IgG antibodies or alloantibodies against donor MHC, has been shown to enhance heart and skin transplant

rejection in murine models. One study in particular demonstrated that anti-K^d alloantibodies act as opsonins that are acquired by APCs to enhance the indirect pathway of allorecognition by supporting proliferation of adoptively transferred CFSE- labelled K^d-specific T cells in B6 mice (Burns and Chong, 2011). They also observed that administering anti-K^d alloantibodies to BALB/c skin transplant recipients who also received anti-CD154 (or CD40L) and DST of splenocytes, acutely rejected transplants in comparison to mice that were not treated with anti-K^d (Burns and Chong, 2011). Overall, this demonstrates a role of B cells for stimulating transplant rejection and targeting these cells may help to promote tolerance.

1.2.2.3 Semi-direct allorecognition

It has been established that the indirect pathway is a $CD4^+$ T cell response; however, it was introduced by Matzinger (1977) that alloreactive $CD8^+$ cells could also be indirectly activated, therefore making indirect allorecognition less restricted to $CD4^+$ T cell activation (Benichou *et al.*, 2011). Theoretically, exogenous alloantigens are presented via MHC II to $CD4^+$ T cells to mediate indirect allorecognition. $CD8^+$ T cells require assistance from $CD4^+$ T cells in order for them to become activated. For this interaction to occur, two APCs are required; one recipient APC presenting alloantigen via MHC II to indirect allospecific $CD4^+$ T cells and one MHC I expressing donor APC to present Ag to direct alloreactive $CD8^+$ T cells, thus a total of four cells. This is also known as the 'four-cell' conundrum which led to a perplexing question regarding the logistics of this interaction. This conundrum was resolved during the discovery of the semi-direct pathway of allorecognition or 'cross-dressing', where one APC is required rather than two (Herrera *et al.*, 2004) [Fig 1.4C].

Transplant rejection is not only mediated via antigen presenting DCs, but also by antigen transporting DCs, where allogeneic intact MHC molecules are acquired by recipient DCs that then prime alloreactive T cells. This process is able to initiate both direct $CD8^+$ T cell and indirect $CD4^+$ T cell allorecognition pathways simultaneously on the same recipient DC: one by the transfer of intact MHC class I molecules by donor DCs/parenchyma to recipient DCs (direct pathway) and the other mechanism in which donor MHC class I molecules are

processed and presented as peptides via recipient DCs MHC class II (indirect pathway). Therefore, leading to the three-cell model and solving the 'four-cell conundrum'. The semi-direct pathway was first described by Herrera *et al*., (2004) as acquisition and presentation of the entire donor MHC molecules present on endothelial cells by recipient DCs and extended to acquisition of MHC on donor DCs (Smyth *et al*., 2017; Brown *et al*., 2008). They discovered *in vitro* that recipient DCs acquired donor MHC from allogeneic endothelial cells or DCs isolated from mice, this was also observed *in vivo* under proinflammatory conditions where mice where administered with IFN-γ to induce local inflammation followed by CFSE- labelled DCs (Herrera *et al*., 2004). Furthermore, Smyth *et al.,* (2017), demonstrated that recipient DCs were able to acquire MHC I from allogeneic skin graft which can be sustained for more than one month following murine skin transplantation, thereby demonstrating the persistence of $CD8^+$ T cell effector immune response and the semi-direct pathway (Smyth *et al*., 2017).

Recently, it has been shown that recipient DCs can acquire donor MHC through the uptake of extracellular vesicles (EVs). EVs are small vesicles that can be 50-100nm in size that may contribute to activation of graft specific T cells throughout the lifespan of the graft and to chronic rejection (Liu *et al*., 2001; Liu *et al.*, 2016; Morelli *et al.*, 2017). It is not only DCs that can acquire EVs, we have unpublished data demonstrating that murine B cells are able to acquire donor- DC-derived EVs, which can in turn stimulate proliferation of antigen-specific T cells *in vitro*. This would suggest that both DCs and B cells may play a role in semi-direct allorecognition, however, further studies would need to carried out to fully elucidate their contribution to rejection via this pathway.

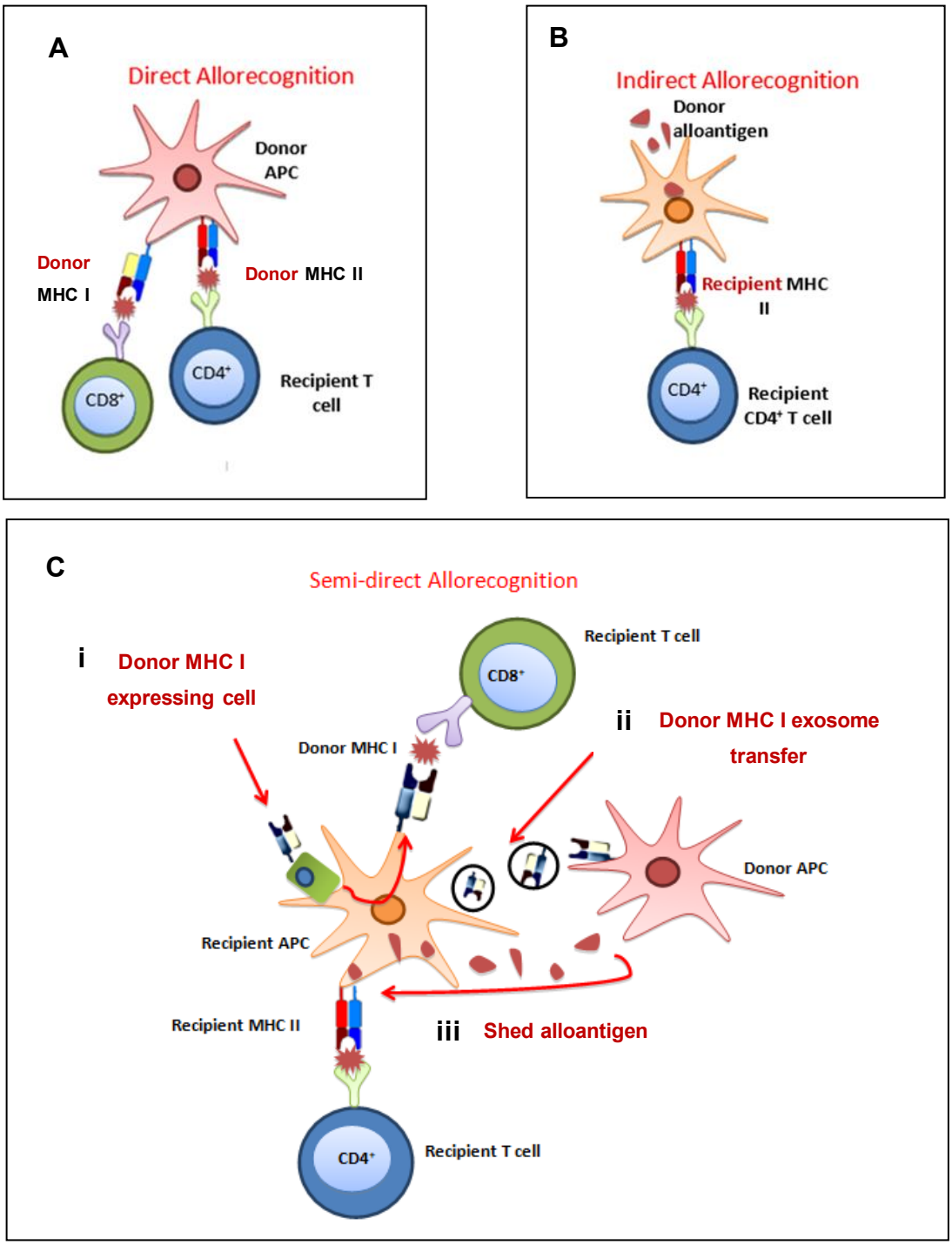


Figure 1.4. Three pathways of allorecognition. *A. Direct allorecognition- donor APCs present intact donor MHC to recipient CD4⁺ or CD8⁺ T cells. B. Indirect allorecognition- recipient APCs acquire donor alloantigens and are presented via self-MHC to recipient CD4⁺ T cells. C. Semi-direct allorecognition- Recipient APCs are able to acquire (i) donor MHC- expressing cells or (ii) uptake intact donor MHC molecules via vesicles, which are presented to CD4⁺ or CD8⁺ T cells (direct), (iii) Recipient APCs can also acquire alloantigens (donor MHC-derived) which have been shed by donor cells and are then presented via self-MHC to recipient CD4⁺ T cells (indirect).*

1.2.3. Immune responses that constitute allograft rejection

Effector immune responses occur almost immediately post transplantation. Donor DCs or passenger leukocytes migrate to the recipient's secondary lymphoid organs where they then activate naïve recipient T cells into alloreactive effector T cells. These effector T cells then go on to migrate towards the donor's transplanted organ where they initiate damage. Th1 cells contribute to acute rejection by the secretion of cytokines such as IFN-γ,TNF-α and IL-2 which helps for the recruitment of NK cells, macrophages and stimulate cytotoxic $CD8^+$ T cells (CTL). These CTLs release cytolytic component such as granzyme B and perforin which can induce cell apoptosis (Benichou *et al.*, 2011).

Memory T cells in particular are robust at maintaining rejection due to their ability to become activated by low antigen stimulation and resistance to immunosuppression which constitutes a major barrier to transplant tolerance (Benichou *et al.*, 2011). Memory $CD4^+$ T cells have various roles such as providing help to CTLs and/or assisting in the generation of pathogenic IgG alloantibodies despite costimulatory blockade using donor-specific cell transfusion and anti-CD40 ligand antibody (Chen *et al.*, 2004). In addition, B cells that generate IgG alloantibodies can be influenced by the release of IFN-γ by memory $CD4^+$ T cells (Gorbacheva *et al.*, 2015).

DCs can increase in numbers, recognise alloantigens and migrate to secondary lymphoid organs where they make contact and activate T cells, resulting in their differentiation to cytotoxic, T helper or memory effector T cells (Liu & Nussenzweig., 2010). Recipient DCs also have the capability to migrate and infiltrate the donor's organ to elicit rejection. One study tested whether recipient DCs simply circulate through the donor's organ and uptake alloantigens derived from the graft, which are then presented to recipient T cells in the secondary lymphoid organs, or the possibility that these DCs mediate effector function on T cells within the graft (Zhuang *et al.*, 2016). They discovered that within the donor organs, donor DCs were eventually replaced with recipient DCs, these recipient DCs were able to support proliferation and effector function of T cells

within the graft thereby supporting rejection of transplanted cardiac and kidney tissues in mice (Zhuang *et al.*, 2016).

1.2.4 The role of macrophages in rejection

The role of macrophages in transplant rejection is yet to be fully elucidated, however several studies have demonstrated the contribution by these cells to transplant rejection. It is established that macrophages are phagocytic cells with APC ability that can form as first-line defence to foreign pathogens as well as foreign allografts. The role of macrophages was confirmed when one study found that ablation of macrophages in CD11b-DTR mice (diphtheria toxin receptor gene under CD11b reporter so that administration of DT can ablate $CD11b^+$ macrophages) led to reduced infiltration of macrophages towards the donor kidney and less incidences of acute allograft rejection (Qi *et al.*, 2008). Extensive macrophage proliferation at transplant sites can release a variety of proinflammatory mediators resulting in local inflammation that gives rise to donor organ damage (Grau *et al.*, 1997). Once macrophages have infiltrated the organ, they can produce large amounts of proinflammatory cytokines such as TNF-α, IFN-γ, IL-1, IL-6 and IL-12 which can lead to tissue damage to the organ (Li *et al.*, 2019). With regard to IL-12, Yang *et al.*, (2003) demonstrated the importance of this cytokine in chronic rejection and observed that blocking the action of IL-12 which is a cytokine required for Th1 development, reduced allograft injury. In fact IL-12 KO mice were more resistant to renal ischemia and reperfusion injury, thus demonstrating the importance of this cytokine in rejection (de Paiva *et al.*, 2009). The specific subset of macrophages which are known to produce these cytokines upon stimulation such as LPS are known as M1 macrophages, that are thought to support Th1 responses as opposed to M2 which are regarded as anti-inflammatory characterised by the release of IL-10 and TGF-β (Martinez and Gordon, 2014).

Macrophages extend their role in transplant rejection by acting as APCs. IFN-γ released by activated T cells upregulate expression of MHC II on their surface and present antigens which have been phagocytosed by the macrophage to $CD4^+$ T cells. In fact macrophages have the ability to phacogytose materials of the graft and present these to $CD4^+$ T cells, leading to anti-donor T cell

responses (Wyburn *et al*., 2004). In 2018, one study demonstrated the importance of Nuclear factor of activated T-cells 5 (NFAT5) for the expression of MHC II on the surface of macrophages (Buxadé *et al*., 2018). OVA peptide loaded- NFAT5 deficient macrophages reduced their capability to stimulate OT-II $CD4^+$ T cell proliferation, in addition skin derived from $NFAT5^{-/-}$ donor mice delayed allograft rejection when transplanted onto recipient WT mice (Buxadé *et al*., 2018). Therefore, this would suggest that macrophages with antigen presenting ability can instigate rejection. Nonetheless, macrophages can also be considered as tolerance inducers given their ability to present commensal bacteria and host antigens to $CD4^+$ T cells and their tolerogenic capacity will be discussed in later sections (Shouval *et al*., 2014).

1.3 Mechanisms of peripheral tolerance and contribution to transplant tolerance

1.3.1 Immune tolerance mechanisms to contain allorecognition

Peripheral tolerance occurs outside of the thymus and can be described as the unresponsiveness to self-antigens presented via self-MHC restricted APCs. The outcome is the prevention of the development of self-reactive T cells, which were not negatively selected in the thymus. Deletion, anergy and the induction/ expansion of regulatory T cells (Tregs) are all mechanisms identified to maintain peripheral tolerance (Sakaguchi *et al*., 1995; Shaban *et al*., 2018) and all of which have been utilised for promotion of transplant tolerance. The definition of transplant tolerance was introduced by Medawer as unresponsiveness to antigens (Billingham *et al*., 1953) or has been described as tolerance when recipients are able to maintain stable allografts once weaned from ISD for more than a year (Feng *et al*., 2012). Several mechanisms that induce peripheral tolerance have been described [Fig. 1.5].

1.3.1.1 Deletion

T cell clonal deletion occurs in the thymus when TCRs expressed on thymocytes bind strongly to self-Ag MHC complex, leading to apoptosis. This plays a key role in central tolerance. It is not only in the periphery where

alloreactive T cell deletion supports allograft survival, intrathymic deletion can also prolong transplant survival (Manilay *et al*., 1998) such as one study that injected antigen-pulsed APCs that promote tolerance, or also known as, 'tolerogenic APCs', via intrathymic injection and noticed donor type allografts were indefinitely accepted (Garrovillo *et al*., 1999).

This mechanism is also employed in inducing peripheral transplantation tolerance by T cell apoptosis, such as activation induced cell death (AICD), which can occur after repetitive antigen exposure. Deletion is crucial for the control of clonal expansion alloreactive T cells so that the population size of these T cells are small and can in turn be easily controlled via other peripheral tolerance mechanisms.

Wells *et al*., (1999), demonstrated the importance of passive cell death for transplant tolerance by utilising B-cell lymphoma-extra large (Bcl-xL) transgenic mice which over-express Bcl-x_L gene on T cells, which prevents apoptosis of alloreactive T cells by cytokine deprivation. Chronic rejection and arteriosclerosis of MHC-mismatched cardiac transplant was observed in mice over-expressing Bcl-x_L and could not be abrogated in the presence of tolerance induction protocols such as (Cytotoxic T-lymphocyte-associated protein 4) CTLA-4 – immunoglobulin (Ig) or anti-CD154 blockade (Wells *et al*., 1999). Therefore, this demonstrates the importance of alloreactive T cell deletion for the promotion of transplant tolerance.

1.3.1.2 Anergy

The term anergy can be described as long-term hyporesponsiveness caused by partial activation, leading to the failure of T cells to proliferate or produce cues to support T cell proliferation- this can be utilised to support allograft survival (Lechler *et al*., 2001; Chen *et al*., 2017). One study discovered that pancreatic islet transplant recipient mice were rendered tolerant to the allograft following treatment with an anti-CD3 antibody which contained alloantigen-specific $CD4^+$ T cells (Besançon *et al*., 2017). These $CD4^+$ T cells were anergic and displayed high expression of PD-1 inhibitory receptor and $CD73^{hi}FR4^{hi}$, two markers known to be expressed on anergic T cells (Besançon *et al*., 2017). The former

study also found that along with anergy, Tregs assisted in murine pancreatic islet allograft survival, thereby demonstrating that multiple peripheral tolerance mechanisms could contribute to allograft survival (Besançon *et al.*, 2017).
CTLA-4 is known to interact with CD80/86 which has been shown to promote T cell anergy (Greenwald *et al.*, 2008). CTLA-4 Ig in particular has the ability to prevent CD28 interaction with CD80/86, thus impairing T cell activation and has demonstrated its use in transplant tolerance induction of murine cardiac allograft survival (Pearson *et al.*, 1994; Schwarz *et al.*, 2016).

1.3.1.3 Regulatory T cells

Tregs maintain self-tolerance, protecting the body from disorders such as autoimmune disease by suppressing self-reactivity through the recognition of self-antigen (Ag) (Corthay, 2009). Tregs are a heterogeneous population of $CD4^+$ and $CD8^+$ T cells, the former subdivided into thymic derived Tregs (tTregs) which constitute 5-10% of $CD4^+$ T cells and peripheral Tregs (pTregs) which develop in the periphery upon Ag-recognition and cytokine stimulation such as transforming growth factor β (TGFβ) and are the main drivers of peripheral tolerance (Issa and Wood, 2012). Tregs are known to constitutively express high levels IL-2 receptor α-chain (CD25) and require absorption of proinflammatory IL-2 for their survival and development and limiting the availability of IL-2 for effector T cells to utilise (Sakaguchi *et al.*, 1995; Fan *et al.*, 2018). They also express transcription factor Forkhead box P3 (Foxp3) and the loss in function of this transcription factor has been known to break tolerance and cause autoimmune diseases (Brunkow *et al.*, 2001; Wildin *et al.*, 2001). This transcription factor has also shown to be crucial for the suppression of proinflammatory cytokines such as IL-2 and IFN-γ (Khan, 2016). As mentioned previously, Tregs constitute of naturally occurring Tregs derived from the thymus (tTregs) or peripherally derived Tregs (pTregs) and markers such as Neuropillin-1 (Yadav *et al.*, 2012; Weiss *et al.*, 2012) and Helios (Thornton *et al.*, 2010) help to establish differences between the two. In addition, Tregs generated *ex vivo* are known as induced Tregs (iTreg). Subsets of murine Tregs include Treg type 1 cells (Tr1) which lack Foxp3 expression, inducible costimulator ($ICOS^+$) Tregs, and double negative Tregs- all types secrete

immunosuppressive cytokines, IL-10 and TGFβ (Zhang *et al.*, 2014) (Juvet & Zhang, 2012; Zhang *et al.*, 2015). A subset of $CD8^+$ T cells also has suppressive properties; the $CD8^+$ Tregs (Guillonneau *et al.*, 2010).

Human Tregs are also considered a highly heterogeneous population. They express markers such as CD4, CD25 and express low levels of CD127 (an IL-7 receptor α chain) and Foxp3. Level of Foxp3 expression in human Tregs can vary; such as Tregs which are naïve or resting are $CD45RA^+$ $Foxp3^{low}$, Tregs that are effector or activated Tregs are $CD45RA^-Foxp3^{high}$. However, it becomes further complicated in humans as conventional and activated $CD4^+CD25^-$ T cells can also develop Foxp3 expression, therefore in order to characterise $Foxp3^+$ Treg cells, the methylation state on Treg specific demethylation regions (TSDR) on the Foxp3 gene can be assessed, where suppressive $Foxp3^+$ Tregs have demethylated TSDR (Toker *et al.*, 2013). Other subsets of Tregs exist including Th3 which can be induced from $CD4^+$ $CD25^-$ cells by TGF-β and IL-4 cytokines (Weiner *et al.*, 2001) and similarly to mouse, humans comprise of Tr1 Tregs in the presence of IL-10 and IFN-α (Levings *et al.*, 2001).

1.3.1.3.1 How do Regulatory T cells mediate immunosuppressive functions?

Tregs have the ability to suppress immune cells in various ways. They have the ability to suppress T effector cells via cytokines IL-10, IL-35 and TGF-β release (Burrell *et al.* 2012). Tregs also have role in suppressing costimulatory molecules, CD80 and CD86 on APCs, which in turn makes them poor stimulators of effector T cells. One study reported bone marrow (BM) and spleen (SPLN)- derived DCs that were co-cultured with Tregs resulted in down-regulation of CD80/86, mediated via CTLA-4 expressed on Tregs, resulting in inhibited T cell proliferation (Oderup *et al.*, 2006). A mechanism of how CTLA-4 down-regulates CD80/86 expression was described by Qureshi *et al.*, (2011) where CTLA-4 interacted with CD80/86 on opposing APCs by mediating trans-endocytosis, resulting degradation of these costimulatory molecules.

As well as CTLA-4, Tregs also express lymphocyte activation gene-3 (LAG-3) which has ability to bind to MHC II on murine BM-DCs, and as a result can inhibit DC activation (Liang *et al.*, 2008). The aforementioned study assessed *in*

vitro that LAG-3 deficient OT-II Tregs were not as suppressive as WT OT-II-specific Tregs when co-cultured with OT-II peptide- pulsed BM-DCs. In addition maturation of MHC $II^{-/-}$ BM-DCs, as measured by CD86 expression, was increased when co-cultured with Tregs (WT or LAG-$3^{-/-}$ Treg), as opposed to Treg co-culture with WT BM-DCs (Liang *et al*., 2008). Therefore, LAG-3 on Tregs and MHC II on BM-DCs interactions are required to prevent maturation of BM-DCs (Liang *et al*., 2008).

Another function of Tregs is cytolysis, described as the killing of target cells. Granzyme B is a serine protease present is cytolytic NK cells, $CD8^+$ T cells and $CD4^+$ Tregs where the latter can promote apoptosis of effector T cells (Cao *et al*., 2007). One study found that Tregs that had a loss of Granzyme B function were not able to induce indefinite murine skin allograft survival as opposed to normal Tregs (Gondek *et al*., 2008). Another apoptosis inducing mechanism is Fas/FasL interaction, where one study demonstrated that interaction between FasL expressing Tregs with Fas expressing DCs, mediated DC apoptosis and limited their antigen-presenting capacity to initiate effector $CD8^+$ T cell immune responses (Gorbachev and Fairchild, 2010). In addition to the aforementioned mechanisms, Galectin-9 interaction with Tim-3 has been found to enhance the induction of $Foxp3^+$ Tregs from conventional $CD4^+$ $CD25^-$ T cells in humans, and reduced expression of both Galectin-9 and Tim-3, as seen in intracranial aneurysm patients, significantly impaired Treg induction (Zhao *et al*., 2018; Madireddi *et al*., 2017).

Tregs have a role of depriving effector T cells of cytokines. As mentioned previously, Tregs express high levels of CD25, IL-2 receptor, which has shown to utilise IL-2 for their survival and in turn deprive this cytokine from proliferating effector T cells and/or lead to apoptosis of effector T cells (Sakaguchi *et al*., 1995; Padiyan *et al*., 2007; Bull *et al*., 1990). Another mechanism for how Tregs mediate apoptosis of target cells is via tumour necrosis factor-related apoptosis inducing ligand (TRAIL)/ death receptor 5 (DR5) pathway (Ren *et al*., 2007). In fact, blocking of this receptor using DR5 blocking antibodies led to decrease of $CD4^+$ effector T cell apoptosis *in vitro* and reduced murine skin allograft survival when this antibody was mixed with adoptively transferred Tregs (Ren *et al*., 2007).

Human and murine Tregs have the ability to make extracellular vesicles (EVs) that are able to suppress T cells activation and cytokine production. For example, EVs known as exosomes derived from Tregs have been shown to contain MicroRNA (miRNA), particularly miR-150-5p and miR-142-3p, which were shown to be taken up by DCs, rendering them tolerogenic indicated by an increase of immunosuppressive IL-10 cytokine secretion (Tung *et al.*, 2018). Furthermore, Tregs have the ability to disrupt metabolic pathways by expressing ectoenzymes CD73 and CD39, their expression generates anti-inflammatory adenosine known to suppress effector T cells proliferation (Deaglio *et al.*, 2007). In fact exosomes derived from murine Tregs were shown to secrete secreting CD73 to mediate immune suppression (Smyth *et al.*, 2013).

Given that Tregs have the ability to modify immune responses, there has been a growing interest in their role in transplantation with respect to immune tolerance. As Tregs were present in accepted grafts [Table 1], many researchers have focused on increasing the number of Tregs in the transplant by either administering Tregs (exogenous) or by manipulating DCs to expand or induce (endogenous) Tregs. The next section focuses on the role of Tregs in murine transplant models.

1.3.1.3.2 Regulatory T cells in murine transplant models [Table. 1]

During allorecognition in murine models, Tregs elicit their suppressive function by initially migrating to the donor organ and then to the draining lymph nodes (dLN) to continually dampen effector alloreactivity and promote tolerance, (Zhang *et al.*, 2009). In the study by Zhang *et al.*, (2009), adoptively transferred PKH26 (red fluorescent dye that labels the cell membrane) labelled Tregs were administered locally to the islet allograft and were monitored in recipient mice, the authors noticed that in mice with surviving allografts, Tregs were able to migrate towards the afferent lymphatics and to the dLN to mediate suppression of effector T cells (Zhang *et al.*, 2009).

Alloantigen-specific Tregs can also be generated *in vitro* and adoptively transferred *in vivo* to prolong allograft survival (Yu *et al.*, 2011; Nikolouli *et al.*, 2017). Alloantigen-specific Tregs generated by co-culture of Foxp3^{+} Tregs with

donor SPLN-DCs treated with vitamin C were able to demonstrate stable Foxp3 expression, as well as increased murine skin graft survival when administered to recipient mice (Nikolouli *et al*., 2017). In another murine study, Tregs with indirect specificity which have been *ex vivo* expanded by donor MHC- specific DCs, when administered to murine recipients in combination with anti-CD8 depletion (to deplete $CD8^+$ direct alloreactive cells) and Rapamycin, induced indefinite cardiac allograft survival (Tsang *et al*., 2009).

In addition to administering *ex vivo* generated Tregs, endogenous Tregs may be increased to improve transplant survival (Ochando *et al*., 2006; Battaglia *et al*., 2006). As an example, Magee *et al*., (2019), recently showed that blocking Notch-1 signalling using an anti-Notch-1 antibody, significantly prolonged MHC-mismatched allograft survival. These authors showed that this was due to reduced graft-infiltrating conventional T cells and an increase of $Foxp3^+$ Tregs.

It is established that Tregs are important in prolonging transplant survival and there are protocols to increase the amount of Tregs using APCs such as DCs as targets which are explained in the next sections (Marin *et al.,* 2018).

Table. 1: Murine Regulatory T cell subsets and examples for their contribution to transplant tolerance

Subset	Phenotypic markers	Examples of function in transplantation
Natural Treg	CD4, CD25, Foxp3	Sorted naïve $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ from CBA mice were administered to recipient CP1-CBA mice, followed by B10.BR skin (Graca *et al*., 2002). Mice co-transferred with naïve CD4+ CD25+ T cells had skin graft prolongation, suggesting that naturally-derived Tregs also have the potential to promote tolerance (Graca *et al*., 2002).
Induced Treg	CD4, CD25, Foxp3	pDCs presenting donor alloantigens induced alloantigen $CD4^+$, $CD25^+$, $Foxp3^+$ Tregs via TGF-β which mediated cardiac allograft survival (Ochando *et al*., 2006). Tregs with indirect allospecificity were generated and expanded *ex vivo* using autologous DCs pulsed with donor alloantigen. When these Tregs were introduced into recipient mice along with IST, indefinite fully MHC- mismatched heart allograft survival as induced (Tsang *et al*., 2009). Another study demonstrated the potential of expanding adoptively transferred Tregs *in vivo* by administering low dose IL-2; this was shown to prolong murine skin allograft survival (Kulachelvy *et al*., 2019).
Tr1	CD4, CD25	These Tregs are also induced in the periphery. In an murine islet allograft model, mice treated with IL-10 and rapamycin were able to promote tolerance as a result of induced Tr1 Tregs (Battaglia *et al*., 2006).
$CD8^+$ Treg	CD8, CD25, Foxp3	Studies have found that $CD8^+$ Tregs, along with low dose of immunosuppressant, were able to prevent allograft rejection (Lin *et al*., 2009). $CD8^+$Tregs have shown to be cross-immunosuppressive towards $CD4^+$ effector T cells and can be induced from $CD4^+$ T cell activation (Yunliang *et al*., 2016).

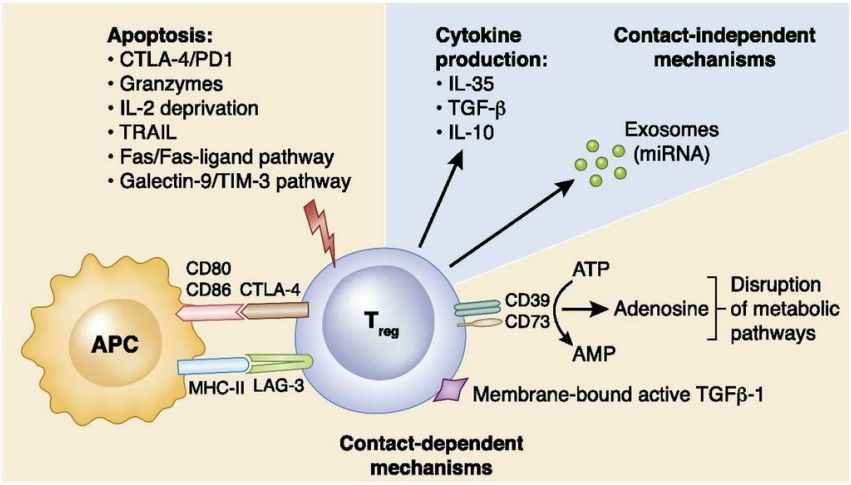


Figure 1.5. Inhibitory function of regulatory T cells*: production of anti-inflammatory cytokines, cell-death as a consequence of cytolysis, competition for interaction with DCs with effector T cells, metabolic disruption, modifying DCs and miRNA released from Treg exosomes. Image adapted from: Martin-Moreno et al., (2018).*

1.4 Using DCs, B cells and Macrophages for transplant tolerance

Given the immunoregulatory role of Tregs in transplantation, strategies have been developed to increase the number of these cells by targeting specific cells such as APCs. APCs (such as DCs, B cells and macrophages) are some of the key players in allorecognition and rejection. DCs in particular have been modified and administered to recipients, or have been targeted *in situ* in order to modify the recipient's immune system to favour transplant survival.

1.4.1 Dendritic cells

DCs have a crucial role in stimulating immune responses as well as promoting tolerance. *In vivo,* steady-state DCs promote tolerance to self-antigens - these are known as tolerogenic DCs (TolDCs) (Morelli & Thomson 2011). These TolDCs have an inability to initiate effector T cell responses, due to their low expression of MHC I and II as well as costimulatory molecules (CD80/86), however, they have the ability to induce/ expand Tregs (Lutz, *et al.*, 2004; Marin *et al.*, 2018) [Fig. 1.6].

1.4.1.1 Mechanisms used by TolDCs to mediate tolerance

As previously mentioned, peripheral tolerance can be induced via deletion, anergy or Treg induction/ expansion. DCs express many surface markers, including some that directly interact with cognate receptors on T cells to promote tolerance. For example, DCs promote T cell deletion following interaction of Fas-L on DCs and Fas on T cells. Murine BM-DCs transfected to express Fas-L induced apoptosis of Fas^+ T cells *in vitro* and when administered into fully MHC-mismatched cardiac transplanted recipients, grafts prolongation was observed (Min *et al.*, 2000). Another surface marker expressed on DCs, Programmed death-ligand 1 (PD-L1), also impairs effector T cell responses. Administering kidney MHC-mismatched allograft rat recipients with BM-DCs transfected with adenovirus expressing PD-L1, resulted in transplant survival as a result of impaired $CD8^+$ T cell proliferation, as well as IL-2 and IFN-γ production (Peng *et al.*, 2011). PD-L1 expressing murine BM-DCs are also able to promote expansion of Tregs, which induced islet allograft tolerance (Wu *et al.*, 2013).

Cell surface markers expressed on DCs such as ICOS-L and Ig-like transcript (ILT) 2, 3 and 4 can promote T cell anergy. Following interaction of ICOS-L on human monocyte-derived DCs and ICOS expressing T cells, $CD4^+$ T cell anergy was observed which was blocked when ICOS expression on T cells was removed (Tuttenberg *et al.*, 2009). In addition to ICOS-L, Ig-like transcript (ILT)2, 3, and ILT4, two of which are expressed on tolerogenic DCs, have been shown to promote T cell anergy (Chang *et al.*, 2002). DCs that express these markers were shown to promote anergic T cells, preventing their ability to proliferate (Chang *et al.*, 2002). To support the aforementioned author's findings, one study reported that DCs isolated from human PBMCs were rendered tolerogenic under the influence of IL-10 and 1α, 25-dihydroxyvitamin D3 (D3) and identified increased expression of ILT3 and ILT4 as a consequence of IL-10 treatment (Manavalan *et al.*, 2003).

DCs have been reported to release immunomodulatory cytokines such as IL-10 and TGF-β to promote tolerance (Comi *et al.*, 2018; Laouar *et al.*, 2008). IL-10 is a cytokine known to down-regulate proinflammatory responses and

impair antigen presentation. A subset of DCs known to produce IL-10 present in humans, known as DC10, are able to induce hyporesponsive T cells as well as induce Tr1 Tregs, therefore establishing themselves as a regulatory DC (Comi *et al.*, 2018). TGF-β is a cytokine produced by DCs with a role in tolerance induction by impairing T cell proliferation and inducing Tregs from $CD4^+$ T cells. Transgenic mice expressing an inactivated TGF-β receptor on DCs have exacerbated autoimmunity in an EAE model with strong inflammation and T cell proliferation in the central nervous system (Laouar *et al.*, 2008).

DCs also impair effector immune responses by modulating metabolic activity via vitamin D3 (Gregori *et al.*, 2001), Indoleamine 2,3- dioxygenase (IDO) (Xie *et al.*, 2015), inducible nitric oxide synthase (iNOS) and arginase (Arg) (Simioni *et al.*, 2017). Vitamin D3 is known to be involved in calcium and bone metabolism as well as impairing effector immune responses (Casteels *et al.*, 1995), along with mycophenolate mofetil (MMF) which inhibits B and T cell activation, were able to modify DCs in favour of a tolerogenic phenotype (Gregori *et al.*, 2001). Administration of vitamin D3 and MMF to islet allograft murine recipients displayed allograft survival and increase in Tregs in tolerant mice (Gregori *et al.*, 2001). DCs from mice treated with vitamin D3 and MMF were tolerogenic, demonstrated by down-regulation of costimulatory CD80/86 molecules and dampened production of IL-12 (Gregori *et al.*, 2001). Another nutrient deprivation mechanism is though IDO, which is an enzyme that assist with tryptophan catabolism and deprives T cells of tryptophan resulting in their suppression. IDO expressing DCs were shown to suppress T cell proliferation *in vitro* and mice receiving IDO treated DCs in a murine small bowel transplantation model, had surviving allografts (Xie *et al.*, 2015).

Overall, given that steady state/immature DCs have the ability to perform peripheral tolerance by some of the mechanisms named above, these APCs have become a prime target to prevent unwanted aberrant immune responses, and have therefore established themselves as cellular immunotherapy to battle transplant rejection.

1.4.1.2 Use of tolerogenic DCs in human clinical trials

Extensive preclinical research using animal models have paved the way to the use of TolDCs in human clinical trials. The ONE study was a collaborative effort by laboratories based in the UK, US, Germany, France and Italy, to develop immunotherapeutic approaches to tackle kidney allograft rejection without the reliance of ISD (Geissler, 2012). Amongst the regulatory cell types studied, DCs were one of cells tested, particularly monocyte-derived DCs (Geissler, 2012). Research has suggested that certain IL-10 producing monocyte-derived human DCs have the ability to induce Tregs and suppress effector T cell proliferation (Boks *et al*., 2012; Naranjo-Gomez *et al*., 2011).

TolDC immunotherapeutic approach can not only be used to overcome transplant rejection, but also other autoimmune disorders such as rheumatoid arthritis (RA), Chron's disease, etc. (Joo *et al*., 2014; Jauregui-Amezaga *et al*., 2015). A study by Harry *et al*., (2010), assessed the stability of TolDCs in RA patients and established TLR 2 expression as a marker for TolDCs due to its high expression in these DCs and low expression in mature DCs. In addition, a Phase 1 clinical trial in 2014 demonstrated a good outcome of RA patients that were administered with autologous DCs pulsed with recombinant peptidylarginine deiminase 4 (PAD4), heterogeneous nuclear ribonucleoprotein A2/B1(RA33), citrullinated-filaggrin (cit-FLG) and vimentin antigens, demonstrating an increase in Tregs in peripheral blood (Joo *et al*., 2014).

1.4.1.3 Modifying and targeting alloantigens to DCs to promote transplant tolerance

1.4.1.3.1 Modifying donor-derived DCs and recipient-derived DCs to promote graft prolongation

Given the promising data obtained by the aforementioned clinical trials (Benham *et al*., 2015; Joo *et al*., 2014; Jaregui-Amezaga *et al*., 2015), TolDCs, either of donor or recipient origin, may prove to be an effective immunotherapeutic approach to battle unwanted immune responses such as transplant rejection as discussed in a review by Moreau *et al*., (2012).

Various studies have determined the potential of treating transplant recipients with donor-derived DCs as a means of promoting allograft survival (Lutz *et al*., 2000; Lu *et al*., 1997; DePaz *et al*., 2003; O'Flynn *et al*., 2013). Particularly, Lutz *et al*., (2000) demonstrated that donor-derived murine BM-DCs could be rendered tolerogenic *ex vivo* by growing DCs in the presence of low dose granulocyte-macrophage colony-stimulating factor (GM-CSF). These DCs were phenotypically immature and were insufficient at activating an effector T cell response *in vitro* as opposed to BM-DCs treated with higher concentration of GM-CSF. When these low-dose GM-CSF-treated donor BM-DCs (derived from C57BL/10J mice) were introduced into CBA recipient mice, 7 days prior to C57BL/10J cardiac transplantation, allograft prolongation or indefinite skin graft was induced (Lutz *et al*., 2010). This response was identified to be antigen-specific as third-party NZW/LacJ cardiac allografts were rejected (Lutz *et al*., 2010).

Smyth *et al*., (2013) investigated whether donor-derived DCs can modulate indirect allorecognition in favour of allograft survival in a murine MHC-mismatched transplant model. Dexamethasone (Dex)-D3 treated donor-derived BM-DCs, which *in vitro* were resistant to activation via proinflammatory stimuli (LPS, anti-CD40, TNF-α), promoted antigen specific T cell hyporesponsiveness to alloantigen rechallenge and expanded Tregs *in vitro*. However, these DCs failed to prolong MHC I-mismatched skin transplant survival *in vivo* even in the absence of $CD8^+$ T cells (Smyth *et al*., 2013). In fact these authors observed that administration of donor TolDCs prior to transplant led to accelerated graft rejection. To explain the rejection observed, it was established that MHC alloantigens, derived from the drug-treated donor DCs, were presented to alloreactive T cells by recipient DCs and primed $CD4^+$ T cell responses via the indirect pathway (Smyth *et al*., 2013). These authors observed transplant survival using Dex and D3 treated donor DCs, but only when recipient mice were devoid of cross-presenting recipient $CD8\alpha^+$ DCs, suggesting a role of $CD8\alpha^+$ DCs in priming alloantigen specific T cells (Smyth *et al*., 2013).

In addition to the aforementioned, there are several other disadvantages of administering donor-derived DCs, one being the risk of donor DCs depleted by host NK cells after administration, which has been shown by Yu *et al*., (2006)

and may enhance the risk of sensitization. In addition, *in vitro* generated tolerogenic donor DCs therapy is only applicable to live donors, whereas tolerogenic DCs derived from the live recipient can be generated at any time point (Morrelli and Thomson, 2007). Therefore, an alternative to administering 'tolerogenic' donor DCs, would be the administration of 'tolerogenic' recipient-derived DCs (Peche *et al*., 2005; Bériou *et al*., 2005). One study aimed to address the efficacy of recipient DCs, in comparison to donor DCs, in a rat cardiac transplant model (Peche *et al*., 2005). They identified that an adherent population of recipient derived rat BM-DCs, generated *in vitro* with low dose of GM-CSF and IL-4, were tolerogenic. These BM-DCs which were administered one day before a mismatched heart transplant, led to improved allograft survival (Median survival: 22.5 days) far better than recipient rats receiving donor-derived BM-DCs (Median survival: 16.5 days). Thus, this adherent population of recipient derived BM-DCs have proven to be successful in allograft survival (Peche *et al*., 2005; Hill *et al*., 2011), and multiple studies have opted for the use of injecting recipient DCs for transplant immunotherapy (Bass *et al*., 2014; Segovia *et al*., 2014; Garrovillo *et al*., 1999).

1.4.1.3.2 Pulsing recipient DCs with alloantigens

Treating transplant recipients with donor Ag-pulsed recipient DCs in order to induce tolerance via inhibiting the indirect allorecognition pathway has also been studied. Garrovillo *et al*., intrathymically injected recipient Lewis rats with allogeneic MHC class I (RT1.A^{u}) peptide only or RT1.A^{u} peptide- pulsed recipient DCs, followed by a Wistar Furth (WF, RT1^{u}) rat heart graft 7 days post infusion (Garrovillo *et al*., 1999). They showed that targeting recipient rats with donor Ag-pulsed recipient DCs intrathymically or administering native donor-Ag, with complementary transient antilymphocyte serum immunosuppressant, led to long-term allograft survival (Garrovillo *et al*., 1999). Interestingly, in comparison to Smyth *et al.,* (2013) study, the aforementioned found that Ag-pulsed recipient-derived DCs significantly prolonged heart allograft survival, therefore suggesting that thymus targeting may be useful for targeting indirect allorecognition (Garrovillo *et al*., 1999). However, it should be taken into account that both studies are different in terms of transplant models and routes of DC administration (Garrovillo *et al*., 1999; Smyth *et al*., 2013).

In a murine heart transplant model, rapamycin (RAPA) treated recipient-derived BM-DCs help to prolong allograft survival (Taner *et al*., 2004) due to their poor capacity to stimulate T cells, and their resistance to maturation stimuli such CD40 stimulation (Turnquist *et al*., 2007). These RAPA-DCs when pulsed with donor alloantigen and infused into murine recipients, with a short course of RAPA post-transplant, led to indefinite allograft survival accompanied by an increase in Treg numbers (Turnquist *et al*., 2007). The loading of recipient DCs with donor alloantigen was extended to non-human primate models. Ezzelarab *et al*., (2017) investigated renal transplant tolerance in Indian rhesus macaques following administration of donor alloAg-pulsed recipient monocyte-derived DCs. In this study recipient macaques received untreated or donor Ag-pulsed monocyte-derived DCs, which were pulsed via cell-membrane vesicles derived from donor peripheral blood mononuclear cells (PBMCs), one day before an MHC-mismatched kidney graft (Ezzelarab *et al*., 2017). Recipient-derived, donor alloAg-pulsed DCs, in addition to low-dose immunosuppressant rapamycin treatment, improved transplant survival by an increase in mean survival time of 27 days in comparison to transplant recipients treated with unpulsed DCs. In addition this treatment decreased donor-reactive IL-17^{+} T cells, which were associated with human renal allograft rejection (Ezzelarab *et al*., 2017; Krummey *et al*., 2014).

Another issue to administering 'tolerogenic' DCs is that they appear to be short-lived within the host and rather than performing their role of presenting antigens, administered DCs are in fact re-processed by recipient APCs (Divito *et al*., 2010; Smyth *et al*., 2013). Therefore, targeting endogenous DCs at quiescent state *in situ* may be more advantageous. The obvious disadvantage to in *vitro*-generated DCs for clinical use is the time it takes to generate 'tolerogenic' DCs which can take several days, this is not an issue when DCs are targeted *in situ*.

One way to promote alloantigen specific tolerance is by targeting alloantigen to specific cell surface receptors expressed by different DC subsets [Table 2]. An example; includes targeting recipient CD11c^{high} CD8α^{+} and CD8α^{-} DC *in situ* using donor-derived apoptotic cells given intravenously, promoted aortic

allograft survival (Wang *et al*., 2009). Along with allograft survival, T cell deletion and $Foxp3^{+}$ cells were induced as well as a decrease of alloantibodies, suggesting reduced indirect allorecognition response (Wang *et al*., 2009).

Table 2: Examples of murine DC subsets (modified from Eisenbarth, 2019.

DC subset	Example of cell surface markers	Examples of their roles in the immune system	Ref.
cDC1	CD11c, XCR1, CD8α, DC-SIGN, CLEC9A, CD103, Langerin	Require Batf3, IRF8 for their development. CD8α and C103 DCs have the ability to cross- present Ags to $CD8^+$ T cells and are also known to express Langerin.	Hildner *et al*., 2008; Sichien *et al*., 2016 ; den Haan *et al*., 2000; Haniffa *et al*., 2012; Sanchez-Paulete *et al*., 2017; Bigley *et al*., 2014.
cDC2	CD4, DCIR2, signal regulatory protein α (SIRPα), CD11b, CD301b	IRF4- dependent. This is subset may be involved in $CD4^+$ T cell priming	Suzuki *et al*., 2004; Gao *et al*., 2014; Williams *et al*., 2013.
Plasmacytoid DC	Siglec H, CD317, B220	Requires IRF8 for their development. Release Type 1 Interferons and is associated with fighting viral infections.	Sichien *et al*., 2016; Asselin- Paturel *et al*., 2001; Swiecki *et al*., 2010.
Langerhan cells	Langerin, EPCAM, CD301b	Constitute a population of tissue-resident macrophages that serve as APCs within skin by priming naïve T cells against foreign Ags.	Indoyaga *et al*. 2011; West and Bennet, 2017.

1.4.1.3.3 Targeting alloantigens to cell surface receptors expressed on DCs to induce tolerance

Antigens can be targeted to specific receptors *in vivo* when coupled to a monoclonal antibody directed towards a specific cell surface marker [Table. 3]. Targeting cell surface receptors present on DCs with antigens, to induce peripheral tolerance was extensively reviewed by Steinman *et al*., (2003). For

example, CD205 (DEC-205), abundantly found on CD8α$^+$ CD4$^-$DCs within lymphoid tissues, is a well-studied receptor to assess tolerance induction. It has been shown that targeted CD8α$^+$ DCs *in vitro* and *in vivo* with ovalbumin protein antigen (OVA) coupled to a monoclonal antibody recognising DEC-205, rendered OVA-specific CD8$^+$ T cells unresponsive to OVA (Bonifaz *et al*., 2000). A study by Hawiger *et al*., (2001) also found that targeting DEC-205 expressing DCs *in vivo*, with a DEC-205 specific monoclonal antibody fused with hen egg white lysozyme (HEL) protein antigen, resulted in reduced T cell activation 7 days post infusion (Hawiger *et al*., 2001). They also found that T cells from these mice could not be reactivated when re-challenged with HEL peptide in Complete Freund's adjuvant (CFA) 7 or 20 days later (Hawiger *et al*., 2001). Whereas mice that were treated with saline and rechallenged with HEL peptide/ CFA, showed strong HEL-specific T cell proliferation *ex vivo* (Hawiger *et al*., 2001). Therefore highlighting that this targeting regimen induced antigen-specific tolerance.

In addition, alloantigen coupled to DC receptor specific antibodies, have proven to be an effective immunotherapeutic approach to combat transplant rejection. A mouse donor MHC Class I alloantigen (K^d) conjugated to a monoclonal antibody directed towards DCIR2 (33D1), a receptor present on murine CD4$^+$ CD8$^-$ DCs, was shown to induce indirect pathway-mediated antigen-specific tolerance in a mouse skin transplant model (Tanriver*, et al*., 2010). These authors demonstrated indefinite transplant survival in mice treated with this construct, however only when mice were depleted on CD8$^+$ T cells (Tanriver, *et al*., 2010). They also observed that unlike alloreactive CD4$^+$ T cells, which were deleted following targeting, Tregs numbers remained intact. In addition to targeting DCIR2 subset of DCs, targeting the immunodominant domain of human type XVII collagen (hNC16A) to DEC-205 receptors expressed on CD8α DCs using a DEC-205 Ab (DEC-hNC16A) significantly prolonged hNC16A expressing skin allograft survival to murine recipients (Ettinger *et al*., 2012) [Table. 3].

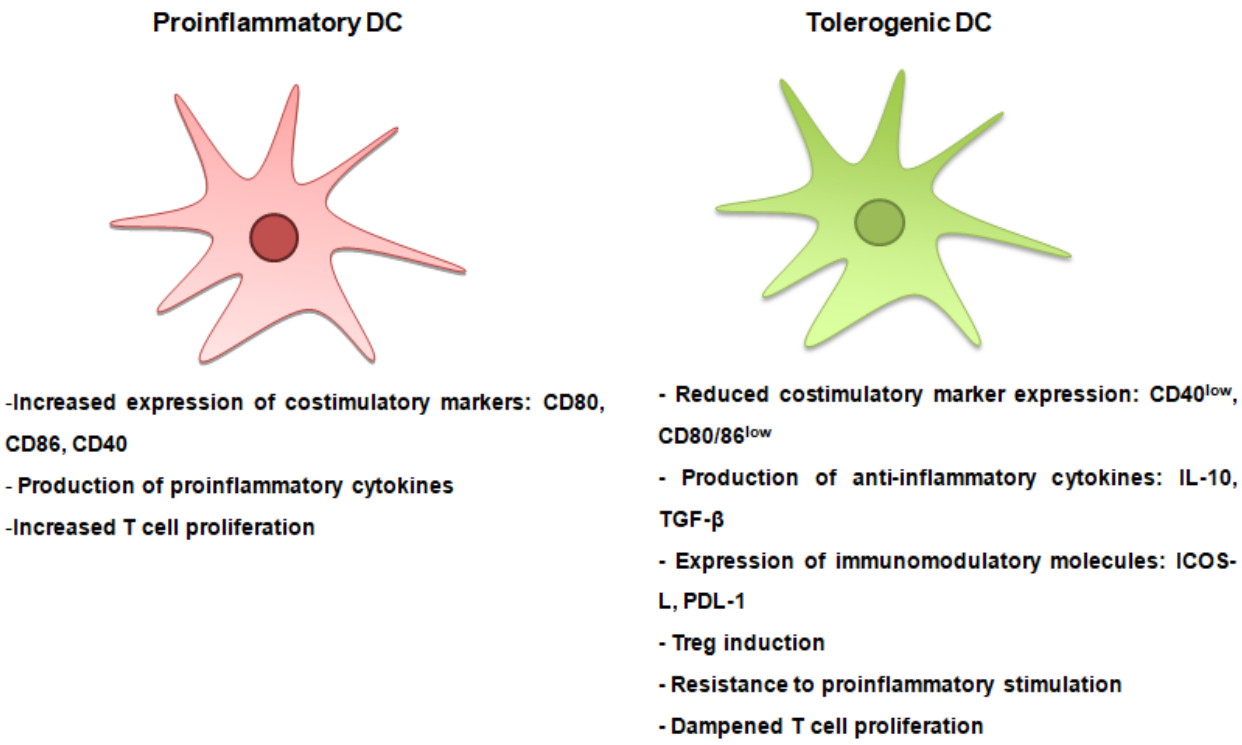


Figure 1.6. Phenotypic profile of mature DCs versus immature tolerogenic DCs. *Image modified from: Marin et al., (2018).*

Table 3: Targeting murine recipient DC in situ to promote transplant tolerance.

DC subset	Specific marker targeted	Method	Outcome	Ref.
cDC1	DEC 205	DEC-205 murine DCs were targeted by administering immunodominant domain of human type XVII collagen, hNC16A, which was conjugated to DEC-205 Ab (DEC-hNC16A) to naive B6 mice 14 days prior to skin transplant.	Skin transplants from human type XVII collagen transgenic mice, were either indefinitely prolonged or transplant survival was increased following DEC-hNC16A treatment	Ettinger *et al*., 2012.
cDC2	DCIR2	33D1- MHC I (H2-K^d) monomer was intravenously administered to target DCIR2 DCs in B6 mice, 14 days prior to MHC I mismatched skin transplant.	B6.K^d skin transplant was only indefinitely prolonged with administration of anti-CD8 to abrogate $CD8^+$ T cell responses and was accompanied by a reduction in alloantibodies.	Tanriver *et al*., 2010.

1.4.2. B cells and their role in transplant tolerance

Transitional B cells are a specific subset of B cells present at the intermediate stages of B cell development between the bone marrow and the spleen. After development in the bone marrow, immature B cells are developed in the periphery through various stages and can be divided into transitional phases and cell populations: transitional 1 (T1) ($IgM^{high}IgD^{-}CD21^{-}CD23^{-}$), transitional 2 (T2) ($IgM^{high}IgD^{+}CD21^{+}CD23^{+}$) (Chung *et al*., 2003; Allman and Pillai, 2008) and mature B cells constituting of marginal zone (MZ) B cells ($IgM^{high}CD21^{hi}CD23^{-}$) (Zouali and Richard, 2011) which make up 5% of the B cell population in the spleen and are known to be efficient antigen presenters to $CD4^{+}$ T cells (Attanavanich and Kearney, 2004) and follicular (FO) B cells ($IgM^{low}CD21^{+}CD23^{+}$) (Petro *et al*., 2002; Allman and Pillai, 2008), which can interact with $CD4^{+}$ helper T cells via CD40/CD40L and differentiate into plasma cells or memory B cells (Steele *et al*., 1996). The stages of development begin from the bone marrow to then migration to the spleen which are characterised by T1 B cells, followed T2 and finally mature B cells (Petro *et al*., 2002). T1 are thought to undergo apoptosis following BCR engagement (Petro *et al*., 2002) whereas T2 B cells are more responsive to antigen stimulation (Chung *et al*., 2002).

A particular subset of B cells described T2, are believed to be regulatory and promote allograft survival (Moreau *et al*., 2014). When T2 B cells isolated from mice that were rendered tolerant to MHC I mismatched skin graft were adoptively transferred to murine skin transplant recipients, prolonged allograft survival was observed (Moreau *et al*., 2014). These as well as T1 B cells were also found to contribute to allograft survival in mice that were housed under non-SPF conditions (Alhabbaba *et al*., 2015). In humans, IL-10 producing Transitional B cells were found in recipients that were tolerant to kidney allografts as opposed to recipients experiencing chronic rejection (Lamperti *et al*., 2016). Another subtype of murine regulatory B cells (Bregs) known as $CD5^{+}CD1d^{hi}$ B10 cells, has been shown to produce IL-10 even in the presence of LPS, PMA/ Ionomycin stimulation, thus demonstrating their anti-inflammatory potential (Yanaba *et al*., 2008; Mauri and Bosma, 2012).

Volchenkov *et al.,* (2013) showed that Dex-D3 treated IL-10 producing human TolDCs, were capable of inducing Tr1 Tregs and surprisingly, Bregs (Volchenkov *et al.,* 2013). Interestingly, it was recently discovered that IL-10 producing Bregs were able to form strong interactions with antigen-specific effector $CD8^+$ and $CD4^+$ T cells impairing their function. As a consequence, this Breg: T interaction inhibited subsequent DC: T interactions, thereby limiting T cell responses (Mohib *et al*., 2020). Using microscopic imaging studies, they demonstrated that in the presence of conventional B cells, OVA pulsed BM-DCs were able to make contact with OVA-specific $CD8^+$ T cells (OT-I), whereas in the presence of Bregs, contact between OT-I T cells and DCs were impaired (Mohib *et al*., 2020). Whether this interaction between Breg: T cells interferes with TolDCs interaction with T cells has yet to be determined.

1.4.3. Macrophages and their role in transplant tolerance

Macrophages also have a regulatory role and have been shown to promote transplant tolerance in human and murine recipients (Hutchinson *et al*., 2011; Riquelme *et al*., 2013). Like DCs, macrophages display various functions depending on their activation status (M1, M2), from killing invading microbes/antigens to regulating the immune responses (Mregs). This plasticity in function is dependent on the immune microenvironment. Mregs are known to produce IL-10 and low levels of IL-12 and can also be induced by co-culturing human macrophages with Tregs (Tiemessen *et al*., 2007). There have been several studies that have targeted/modified macrophages to promote transplantation tolerance (Zhao *et al*., 2018; Conde *et al*., 2015). Recently Zhao *et al*., (2018) showed that inhibiting the mammalian target of rapamycin (mTOR) in M2 anti-inflammatory macrophages induced expression of PD-L1 and prevented chronic cardiac allograft rejection. In addition macrophages express specific receptors that promote tolerance, one being DC-SIGN, and it has been demonstrated that these particular macrophages were able to support the accumulation of Tregs and promoted heart allograft survival (Conde *et al*., 2015). Overall, macrophages make an interesting target to improve graft survival with experiments and trials being undergone (Hutchinson *et al*., 2011; Riquelme *et al*., 2017) and markers such as dehydrogenase/reductase 9 (DHRS9) expressed on human regulatory macrophages make it feasible to

identify and target these cells to promote tolerance (Riquelme *et al.*, 2017).

1.5 Sialic acid-binding immunoglobulin-like lectins

Siglecs are type 1 transmembrane proteins found on human and mouse cells which consist of an extracellular N-terminal V-set immunoglobulin domain, designated for sialic acid binding, and an immunoreceptor tyrosine-based inhibitiory motif (ITIM) receptor in its cytoplasmic portion [Fig.1.7-1.8] (Jandus *et al.*, 2011). Siglecs E, F, G, and CD169 (Siglec H does not bind to sialic acids) are able to bind to sialic acid containing glycans or sialylated ligands and are present on the surface of leucocytes such as DCs, macrophages, B cells, eosinophils and T cells (Jandus *et al.*, 2011).

There are two types of immunoreceptor tyrosine motifs, one is an immunoreceptor tyrosine-based activation motif (ITAM) and the inhibitory counterpart is known as an ITIM [Fig. 1.7] (Barrow & Trowsdale, 2006). Siglec receptors inhibit certain immune response, due to their ITIM in the cytosolic region which down-regulates cell signalling [Fig.1.7-1.8] (Avril *et al.*, 2004; Paulson *et al.,* 2012). ITIMs upon phosphorylation by Src tyrosine kinases act as docking sites for SHP or Src homology 2 (SH2)-domain cytoplasmic phosphatases such as SHP-1 or SHP-2 [Fig. 1.7] (Barrow & Trowsdale, 2006). This interaction leads to dampening of intracellular signalling pathways and immune responses as outlined in Table 4.

Sialic acids are a group of sugars composed of 9 carbon residues derived from neuraminic acids and within this group, N-acetyl neuraminic acid (Neu5Ac) is primarily found in humans. Sialic acids are known to be distributed at the end of glycoproteins present on the cells surface, making them available for interactions with cognate cells (Cohen and Varki, 2010). Sialic acids have an importance role in immunoregulation, with deficiencies in sialic acids causing autoimmunity, immunological abnormalities such as deficiencies in T and B cell proliferation in animals (Jenner *et al.,* 2006). In addition, sialic acids binding to Siglecs lead to the concept of sialic acids acting as self-associated molecular patterns (SAMPs), making Siglecs receptors for ‘self’ recognition (Varki, 2011).

Siglecs have specificity for different configurations of sialic acids such as α2,3, α2,6 and α2,8 linked sialic acids, where the α2,8- linked sialic acids are a combination of α2,3 and α2,6 linked, configurations (Pillai *et al*., 2012). Siglecs present on DCs and macrophages have been shown to have specificity for α2,3 and/or α2,6 sialic acids, whereas α2,6 linked sialic acids are mostly recognised by Siglecs CD22 and Siglec G expressing B cells, however some Siglec G expressing- B cells can also bind to α2,3 sialic acids (Pillai *et al*., 2012).

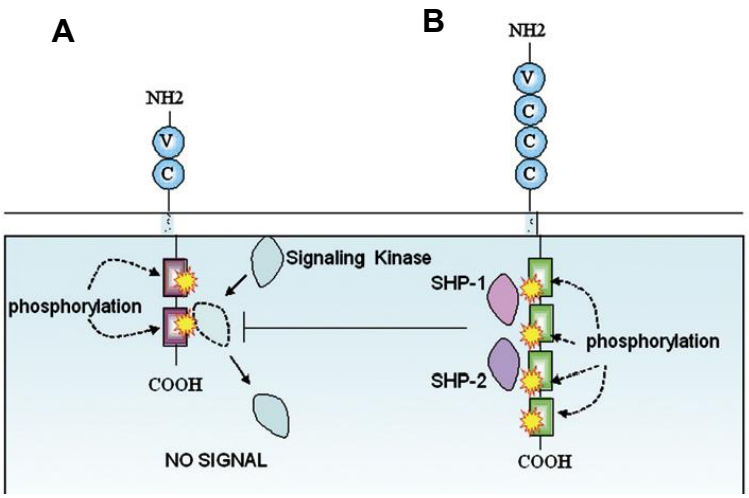


Figure 1.7. Structure and function of ITAM and ITIM*. A. Activatory ITAM. B. Inhibitory ITIM can counteract activatory ITAM upon tyrosine phosphorylation and inhibit cellular processes. Image adapted from Munitz & Levi-Schaffer (2007).*

1.5.1. Targeting ITIM-bearing Siglecs to impair aberrant immune responses

Several pathogens as well as certain tumours, have exploited Siglec receptors; sialic acid interactions to evade host immune defences (Laübli *et al*., 2014). In a mouse metastasis model, blocking Siglecs on neutrophils enhanced anti-tumour responses (Laübli *et al*., 2014). In fact Siglec E knockout mice, which is a homologue of human Siglec 9, showed enhanced killing of tumour cells, demonstrating that hypersialylated tumours and interactions with Siglecs can dampen the immune system, thus allowing the tumour to survive (Laübli *et al*., 2014). In addition to hypersialylated tumours, pathogens expressing sialic acid structures have also been shown to evade the host immune system. Sialylated

structures on the flagellum of *Campylobacter jejuni*, are able to engage with Siglecs on host human monocyte-derived DCs leading to a profound increase of IL-10 supporting an anti-inflammatory environment favouring the survival of *C.jejuni* within the host (Stephenson *et al*., 2014).

Due to the modulatory characteristic of these Siglecs, Siglec-sialic acid interaction has been utilised to inhibit the immune system. Recently, Perdicchio *et al*., (2016) targeted Siglecs on BM-DCs, SPLN-DCs and endogenous DCs in mice to induce tolerance (Perdicchio *et al.,* 2016). These authors adopted two different mouse models to address their investigation: OVA protein and an encephalitogenic peptide derived from myelin oligodendrocyte glycoprotein (MOG). In the OVA model, these authors observed that murine SPLN-DCs were able to induce tolerance *in vitro* and *in vivo* following α2,3 or α2,6-linked sialylated OVA (Sia-OVA) treatment (Perdicchio *et al*., 2016). This was demonstrated in mixed lymphocyte reactions whereby OVA specific $CD4^+$ and $CD8^+$ effector T cells (isolated from OT-II and OT-I mice, which express a transgenic receptor specific for OVA, respectively) stimulated with *ex vivo* isolated mouse SPLN-DCs pulsed with either α2,3 or α2,6 Sia-OVA, had reduced production of pro-inflammatory cytokines (IFN-γ and TNF-α) and increased proportions of $Foxp3^+$ Tregs as compared to untreated and non-sialylated OVA pulsed DCs (Perdicchio *et al*., 2016). In order to assess whether targeting endogenous DCs using sialylated antigen can promote tolerance *in vivo*, the authors of the aforementioned study administered 50μg of α2,6 Sia-OVA, followed by priming of mice with a subcutaneous injection of OVA/polyinosinic–polycytidylic acid [poly(I:C)]/anti-CD40 antibody (Perdicchio *et al*., 2016). In mice treated with sialylated OVA as opposed to OVA alone, the authors observed a significant reduction of IFN-γ producing $CD8^+$ and $CD4^+$ T cells, which were also accompanied by an increased frequency of $Foxp3^+$ Tregs. This observation suggests that sialylated antigen treatment promoted an immunosuppressive environment, indicated by a reduction of effector T cells and an increase in Tregs, *in vivo* (Perdicchio *et al*., 2016). These authors suggested that their findings were mediated via Siglec E. When they targeted Sia-OVA to Siglec E knock out BM-DCs, the efficiency of Tregs induction was reduced *in vitro* as opposed to Sia-OVA targeted WT BM-DCs (Perdicchio *et al*., 2016). Lastly and importantly, these authors also described that targeting

sialylated-peptide antigen rather than native antigen also induced antigen-specific tolerance *in vitro* (Perdicchio *et al*., 2016). DCs pulsed with Sia-MOG peptide and co-cultured with MOG-specific $CD4^+$ T cells (2D2 T cells), induced these cells to express Foxp3 and reduced differentiation of 2D2 T cells into IFN-γ producing effector cells (Perdicchio *et al*., 2016). However the author did not present data on the effects of sialylated peptide antigens *in vivo*, which is what the current thesis will address. Overall their study highlighted that targeting Siglecs on recipient DCs with a sialylated protein or peptide, can induce antigen-specific tolerance *in vivo* (Perdicchio *et al*., 2016).

Below we highlight murine Siglecs and give examples of their expression and known inhibitory immune responses.

1.5.2 Examples of murine Siglecs and their inhibitory roles

- **Siglec F**

Examples of Siglec F- expressing cells: Eosinophils, DCs, Macrophages
Siglec F $^{-/-}$ mice challenged with an allergen had enhanced lung eosinophillic inflammation in a lung allergy model, suggesting that ITIM containing Siglec F has a role in modulating allergic responses *in vivo* (Tateno *et al*., 2007). Siglec F is also present on macrophages in murine alveoli and expression can be induced following GM-CSF treatment (Guilliams *et al*., 2013) (Tateyama *et al.,* 2019). The exact role of Siglec F on murine macrophages remains to be determined, however by Liu *et al* (2019), identified that Siglec F engagement in murine alveolar macrophages does not affect their phagocytic ability. It has been reported that Siglec F is expressed on both BM-DCs and BM-DMs, however the authors did not present this data (Tetano *et al*., 2007).

- **Siglec E**

Examples of Siglec E- expressing cells: DCs and macrophages
Sialylated *Trypansoma cruzi* are able to evade the host immune system through interaction with Siglec E expressing DCs, resulting in decreased production of the proinflammatory cytokine IL-12 and reduced Th1 responses (Erdmann *et al*., 2009). Siglec E- expressing murine macrophages targeted with α2,8 sialic

acid decorated nanoparticles reduced production of proinflammatory cytokines in response to LPS stimulation and were found to be beneficial in treating a pulmonary injury model in mice (Spence *et al*., 2015).

- **Siglec G**

Examples of Siglec G- expressing cells: DCs and B cells

Siglec $G^{-/-}$ B cells display enhanced BCR signalling and increased accumulation of B1a cells, which resulted in increased humoral immunity (Pillai *et al*., 2013). Siglec G has also been studied as an inhibitor of DAMPs; CD24 is a sialoglycoprotein which associates with Siglec ligands as side chains, particularly Siglec G (Pillai *et al*., 2013). CD24 forms a complex with HMGB1 (high mobility group protein box 1), which is a DAMP released in response to tissue damage, and in turn associates with Siglec G/ 10 to produce reduced NF-kB activation and suppressed immune response towards HMGB1, thus preventing uncontrolled inflammation (Chen *et al*., 2009). In fact, Siglec $G^{-/-}$ mice displayed an aberrant increase of proinflammatory cytokines and acute organ failure in response to HMGB1 in an acetaminophen (AAP)-induced liver necrosis model (Chen *et al*., 2009). In relation to transplantation, this CD24-Siglec G interaction has been shown to be a negative regulator of DAMP release and adaptive T cell allorecognition in a graft-versus-host disease mouse model (Toubai *et al*., 2014). $CD24^{+}$ donor T cells are able to interact with Siglec G recipient DCs to mitigate the severity of GVHD in comparison to donor $CD24^{-/-}$ T cells and recipient Siglec $G^{-/-}$ DCs *in vivo* (Toubai *et al*., 2014). Furthermore, Siglec G expression is not only limited to the surface of immune cells but can additionally be found within murine $CD8\alpha^{+}$ DC phagosome (Ding *et al*., 2016). Siglec G can inhibit $CD8^{+}$ T cell adaptive immune response by impairing DC cross presentation in response to pathogenic antigens (Ding *et al.,* 2016). Ding *et al*., (2016) demonstrated how Siglec G interaction with *Listeria monoyctes* inhibited MHC I- peptide complex formation by impairing NADPH oxidase 2 (NOX 2) activation thus acidifying the DC phagosome, which in turn causes excessive antigen degradation within the DC phagosome and inhibited antigen presentation (Ding *et al*., 2016).

❖ Siglec H

Examples of Siglec H- expressing cells: Plasmacytoid dendritic cells (pDCs),
Siglec H in comparison to other Siglecs, has two major differences. Firstly, it does not need to recognise sialic acid to function and secondly it contains an ITAM instead of an ITIM (Zhang *et al.,* 2006). Although, these differences suggest that a sialylated ligands might not interact with Siglec H, this Siglec has been shown to inhibit immune responses. For example, Loshko *et al*., (2007) inhibited Ag-specific Th responses in an autoimmune mouse model by targeting Siglec H- antigen cross-linking Abs to Siglec H^+ pDCs *in vivo* (Loschko *et al*., 2007).

❖ CD22

Examples of Siglec CD22- expressing cells: B cells
CD22 has a high affinity for α2,6-linked sialic acids. It is highly expressed on mature B cells and is one of the most studied amongst all the Siglecs (Mahajan *et al*., 2016). CD22 and Siglec G are known to inhibit immune responses by downregulating B cell receptor (BCR) signalling, either by BCR crosslinking interaction of α2,6 sialic acids present on the same surface (*cis* interaction) or via α2,6 sialylated ligands present on target cells (*trans* interaction) (Jellusova *et al*., 2011).

❖ CD169 (Sialoadhesin)

Examples of Siglec CD169- expressing cells: Macrophages
CD169 is an endocytic receptor expressed on macrophages. Its main role is internalising and transporting cargo as it lacks an ITIM or ITAM (Jandus *et al.*, 2011). In fact several studies have suggested a proinflammatory role for this receptor. These include impairing the proliferation of Tregs and interacting with cross-presenting DCs to support $CD8^+$ effector T cell immunity (Wu *et al*., 2009; van Dinther *et al*., 2018). The role of Siglecs and macrophages varies however; some studies have found that $CD169^+$ macrophages are involved in preventing viral dissemination by capturing the virus, transferring them to conventional DCs which then present these to CTLs for anti-viral immunity (Uchil *et al*., 2019).

Additionally, CD169^{+} macrophages have a role in self-tolerance by removing apoptotic cell material and presenting them to cDC1s. Depletion of these macrophages resulted in a break in self-tolerance (Miyake *et al.*, 2007). TIM-4^{hi} (T-cell immunoglobulin mucin protein-4) CD169^{+} cells which are a subset of M2 macrophages have been found to promote induction of Tregs and cardiac allograft survival (Thornley *et al.*, 2014).

Unlike mouse, human Siglecs are denoted numerically. Some of these receptors are briefly described, along with their immunoregulatory effects, in Table 4.

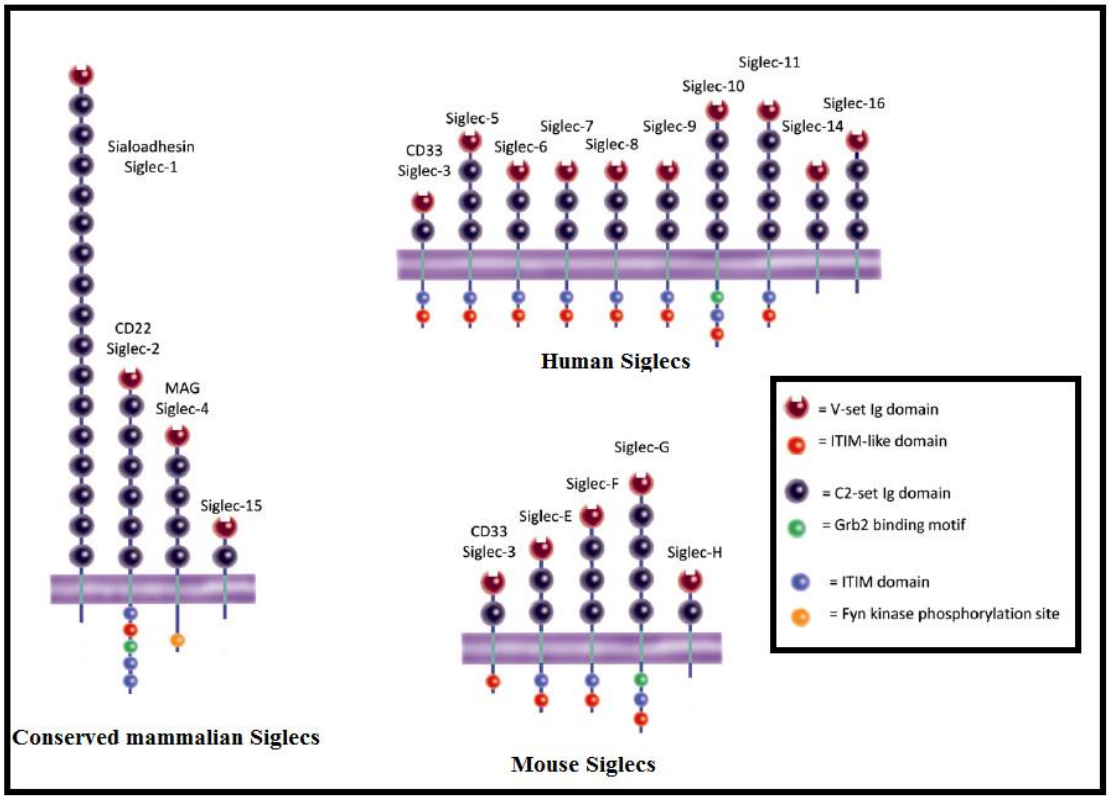


***Figure 1.8. Structures of Siglecs conserved in mammals and present in human or mouse**. Adapted from Jandus et al., (2011).*

Table 4. Examples of Human Siglecs and some of their roles in immunoregulation

Human Siglec	Examples of their inhibitory roles	Ref.
Siglec 8 (mouse homologue, Siglec F)	The human homologue of Siglec F is Siglec 8, both of which are known to be expressed on eosinophils and impair effector functions of eosinophils allergic inflammation.	Gao *et al*., 2010; Kiwamoto *et al*., 2014.
Siglec 10 (mouse homologue, Siglec G)	Siglec 10 expressed on human DCs has been shown to interact with DAMP receptor, CD24, to negatively regulate DC inflammatory immune responses towards molecules released by damaged cells in response to tissue injury (DAMPs).	Chen *et al*., 2009.
Siglec 9 (mouse homologue, Siglec E)	Siglec 9 activation on human neutrophils can trigger apoptosis of neutrophils. This receptor is also expressed on human macrophages, and has demonstrated potential to down-regulate CCR7 and regulate genes associated with inflammation in the presence of proinflammatory stimuli. Siglec 9 is expressed on human immature DCs. Ligation of tumour-produced mucins with Siglec-9 expressed on DCs, in the presence of LPS, resulted in down-regulation of IL-12, but no decrease of IL-10. Therefore, suggesting that under proinflammatory stimulus, immune responses are down-regulated which suggest a role of this Siglec in evading anti-tumour attacks.	Zhang *et al*. 2000; vonGunten *et al*., 2005; Higuchi *et al*., 2016; Ohta *et al*., 2010.
Siglec 2 (mouse homologue, CD22)	Similarly to murine CD22 Siglecs on B cells, Siglec 2 has been shown negatively regulate BCR activation and reducing $Ca2^{+}$ signalling which is associated with BCR activation.	Kimura *et al*., 2007.

1.6 Aims of the investigation

Targeting alloantigens to DCs using monoclonal antibodies, specific for molecules present on DC subsets has led to improved transplant outcomes. However, these studies are restricted to looking at one subset of DCs and indefinite survival was not observed. What may be required to improve this strategy is to target many subsets of DCs, given that targeting multiple DC subsets have proven to promote tolerance. Targeting Siglec receptors, which are expressed on many DC subsets, with sialylated antigens, including peptide, has been shown to render DCs 'tolerogenic' leading to the expansion/ induction of Tregs. The aim of the work presented in this thesis was to address whether targeting sialylated donor alloantigen peptides to Siglecs expressed on DCs leads to prolongation of graft survival.

The specific objectives of this investigation are;

1. To test the possibility of improving skin transplant survival by targeting Siglec-expressing cells using Sia-alloantigen (K^d) peptide as model alloantigens.
2. To identify whether specific DC subsets, B cells or macrophages are targeted by sialylated alloantigen and whether this contributes to transplantation outcome.
3. If improved transplant outcome occurs, to assess what are the mechanisms of 'tolerance' following targeting recipient APCs with sialylated alloantigen.

Importance of the research: The information that we obtained from this thesis will directly inform the design of the protocols for the induction of tolerance in the context of transplantation. In the future, if successful, this strategy may be applied to patients receiving a transplant organ from deceased or live donors.

Overall, this study could contribute to the Phase 1 trials of tolerogenic APC therapy aimed at reducing the development and incidences of chronic rejection by targeting Ag-specific immune responses.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Mice [Table. 5]

Wild type (WT) mice used in this study included C57BL/6J (B6, H-2^b) and BALB/c (H-2^d). Both of which were purchased from Charles River Laboratories (Margate, UK).

B6.Rag-$2^{-/-}$ (H-2^b) mice which contains a disruption in the Rag 2 gene, resulting in impaired V(D)J recombination and therefore no generation of B and T lymphocytes, and B6.Batf3$^{-/-}$ mice, which lack exons 1 and 2 of the basic leucine zipper transcription factor, ATF-like 3 gene (Batf3), which is a gene required for development of $CD11c^+CD8\alpha^+$ and $CD11c^+CD103^+$ DCs (Hildner *et al.*, 2008) were used.

Transgenic lines used in this study included; (i) TCR75 Rag$^{-/-}$ mice: B6 mice express a TCR that recognises processed BALB/c MHC I peptide ($K^{d\ 54-68}$) presented by B6 MHC II I-A^b (Honjo *et al.*, 2000) and (ii) B6.K^d mice: B6 expressing a transgene encoding BALB/c MHC I (H-2K^d) (Honjo *et al.*, 2000). Both lines were kind gifts from Pat Bucy (Honjo *et al.*, 2000).

All transgenic, knockout and WT mice were sex and age matched. Wild-type mice, TCR75 Rag$^{-/-}$, B6.Rag-$2^{-/-}$, B6.K^d, and B6.Batf3$^{-/-}$ mice were bred and housed at the Biological Services Unit, King's College London under specific pathogen free (SPF) conditions and were kind gifts from Professor Giovanna Lombardi, King's College London, UK. All procedures involving mice were carried out in accordance with the institutional and Home Office Animals Scientific Procedures Act (1986) under the Home Office Project Licence: 709066. Mice were sacrificed under Schedule 1 protocol by exposure to CO_2 at a rising concentration.

Table 5: Wild-type and genetic strains used in this study

Strain	Details	MHC haplotype antigens
C57BL/6J	Wild- type	MHC I: K^b D^b L^{null} MHC II: I-A^b I-E^{null}
BALB/c	Wild-type	MHC I: K^d D^d L^d MHC II: I-A^d I-E^d
B6. Rag $2^{-/-}$ (Brennan *et al.,* 2009)	B and T cell deficient	MHC I: K^b D^b L^{null} MHC II: I-A^b I-E^{null}
B6.TCR75 Rag $1^{-/-}$ (Honjo *et al*., 2000)	TCR transgenic strain (Vα1.1, Vβ8.3). Consist of $CD4^+$ T cells with TCR that specifically recognises K^d peptide 54-68 (QEGPEYWEEQTQRAK) derived from MHC I H-$2K^d$ in the context of MHC II I-A^b	MHC I: K^b D^b L^{null} MHC II: I-A^b I-E^{null}
B6. K^d (Honjo *et al*., 2000)	B6 mice expressing K^d transgene	MHC I: K^{bd} D^b L^{null} MHC II: I-A^b I-E^{null}
B6. Batf3$^{-/-}$ (Hildner *et al*., 2008)	B6 mice lacking batf3 transcription factor. Devoid of $CD103^+$ and $CD8\alpha^+$ DCs.	MHC I: K^b D^b L^{null} MHC II: I-A^b I-E^{null}

2.2 Cell culture media

Cells were maintained in complete media consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Paisley, UK) without L-glutamine supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (ThermoFisher, Paisley, UK), 50 mM 2- Mercaptoethanol (ME) (ThermoFisher) and 10% heat-inactivated FCS (PAA, Biopath stores, Cambridge, UK) or 10% FCS (ThermoFisher). Cell cultures were maintained at 37°C in 5% CO_2 incubator (Galaxy R+, RS Biotech).

2.3 Cell isolation

2.3.1 Preparation of DCs from mouse bone marrow (BM) and spleen (SPLN)

2.3.1.1 Bone marrow derived DCs (BM-DCs)

BM-DCs were prepared according to a protocol published by Smyth ***et al***., (2013) [Fig 2.1]. Briefly, B6 mice were sacrificed and the hip, femur and tibia bones were harvested by dissection. Sides of the bones were cut on each side so that the BM can be harvested by flushing bones with fresh RPMI media using a 27 ½ G needle (Appleton Woods, Birmingham, UK) and a 20ml syringe (Appleton Woods). BM cells were then passed through a 70µm cell strainer (Appleton Woods) to obtain a single-cell suspension, before being washed with 20ml of RPMI and spun (Eppendorf, Stevenage, UK) for 5 mins at 4°C at 562*g*. The cell pellet was then treated for 30 seconds at room temperature with 500µl of Ammonium-Chloride-Potassium (ACK) buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2- EDTA, pH 7.2-7.4) to lyse red blood cells (RBCs) and washed with RPMI. Next, RBC-depleted cells were incubated, with constant shaking for 30 mins at 4°C, with 300µl of each the following hydridoma culture supernatants: YTS 191 (rat anti-CD4; American Type Culture Collection (ATCC), Manassas, Virginia, USA), YTS 169 (rat anti-CD8, ATCC), M5/114 (rat anti-Class II. ATCC), RA3-3A1 (rat anti-B220, ATCC) all of which were kind gifts from Prof. Giovanna Lombardi, for the depletion of $CD4^+$, $CD8^+$, MHC II expressing cells and B cells, respectively. After incubation, cells were washed with RPMI to remove unbound antibodies and incubated with pre-washed polyclonal sheep anti-rat IgG coated magnetic Dynabeads® (Invitrogen, ThermoFisher) for 30 min at 4°C, followed by magnetic separation using DynaMag™-15 Magnet (ThermoFisher). BM-DC progenitor cells were collected via negative selection, and cultured in complete media supplemented with 4ng/ml GM-CSF (kind gifts from Prof. Giovanna Lombardi) in 24 well plates (Appleton Woods) for 7 days. Cell media was replaced with 1ml of fresh media on day 2 and 4 of culture. Purity of BM-DCs was assessed by $CD11c^+$ antibody staining and flow cytometry [Fig. 2.2].

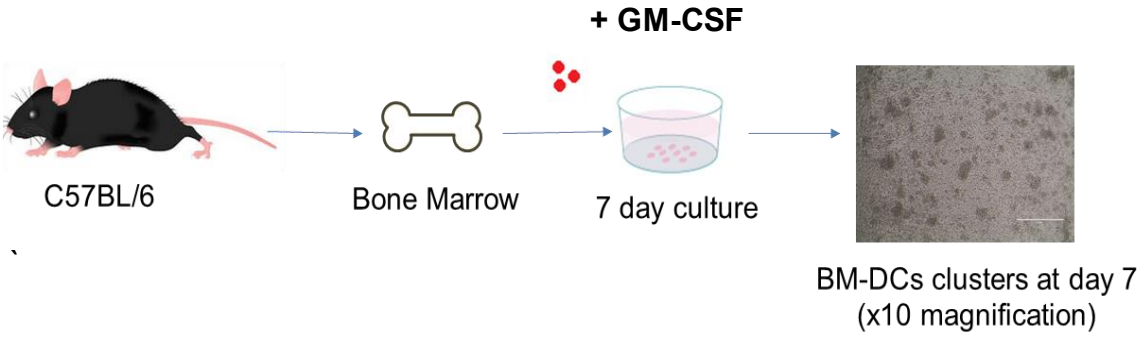


Figure 2.1 Schematic for the generation of BM-DCs. *Bone marrow was isolated from mouse tibia, femur and hip bones and DC progenitors cultured with GM-CSF for the expansion of BM-DCs. After 7 days BM-DCs appear in clusters (x10 magnification).*

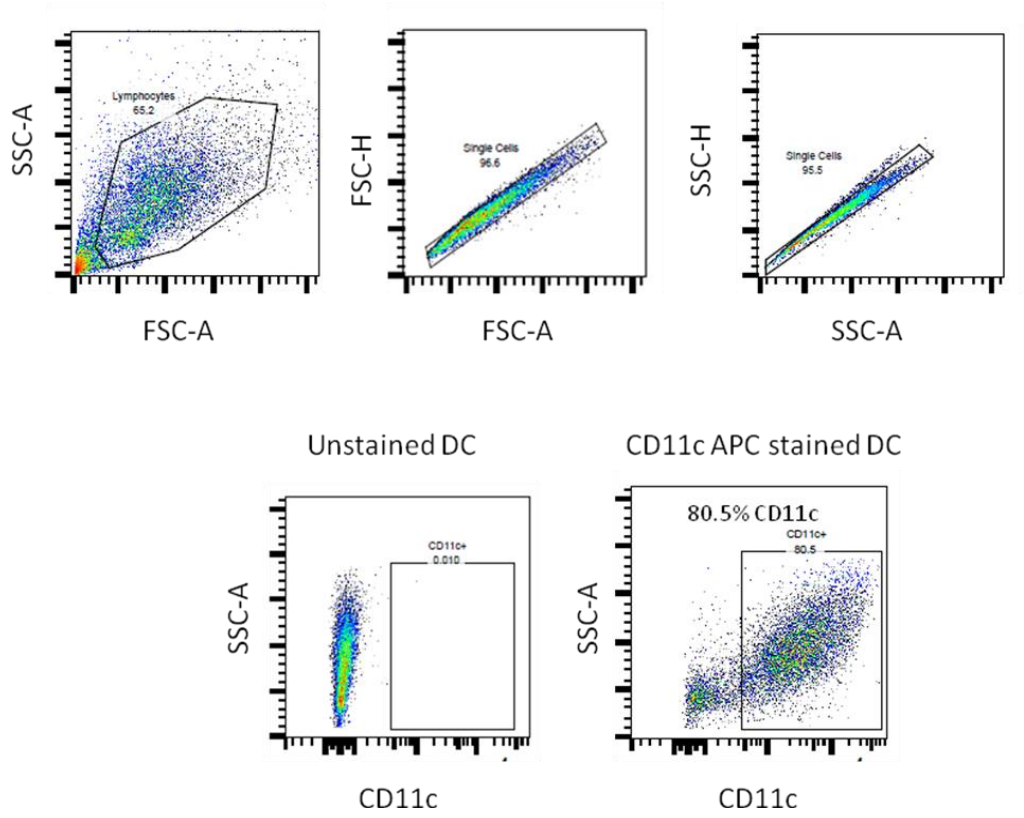


Figure 2.2. Gating strategy for assessing CD11c⁺ BM-DCs via flow cytometry. *BM-DCs were stained with anti-CD11c specific antibodies, followed by flow cytometry analysis. Cells were gated on live cells (FSC vs. SSC) and doublets were excluded. FACS plot is a representative for one out of 5 experiments.*

2.3.1.2 Spleen

For splenic DC isolation, B6 and B6.Batf3$^{-/-}$ mice were sacrificed, and spleens harvested and diced into small sections using Swann-Morton® sterile blades (Appleton Woods) in non-treated Petri-dishes (Appleton Woods). A single cell suspension was made by digesting the spleen pieces with 0.5 mg/ml collagenase (Sigma, Dorset, UK) in the presence of 10µM/ml DNase (Sigma, Dorset, UK) for 30 mins at 37°C. Disaggregated splenic cell suspension was collected from the Petri-dishes and passed through a 70 µm cell strainer to obtain a single-cell suspension before RBCs were lysed with 1ml of ACK buffer followed by washing with RPMI. DCs were isolated from splenocytes using CD11c microbeads, MS/LS columns and MACS (Miltenyi Biotech, Cologne, Germany) according to manufacturer's protocol. Briefly, splenocytes were counted, using Trypan Blue (Sigma, Dorset, UK) and 10^8 splenocytes were resuspended in 400µl in isolation buffer (2% FCS, 2mM EDTA/PBS) incubated with 100µl of CD11c Microbeads (volumes of beads were adjusted according to number of cells). After 10-minute incubation at 4°C, cells were placed into prewashed LS column and placed on an OctoMACS magnet (Miltenyi Biotech). Splenic DCs were isolated by positive selection, with the column being washed twice to remove contaminant cells. After all the unlabelled cells were removed (non-DCs), bead-bound DCs were flushed and isolated from the column using isolation media and a plunger.

Purity of splenic DCs was assessed by CD11c$^+$ antibody staining and flow cytometry [Fig. 2.3].

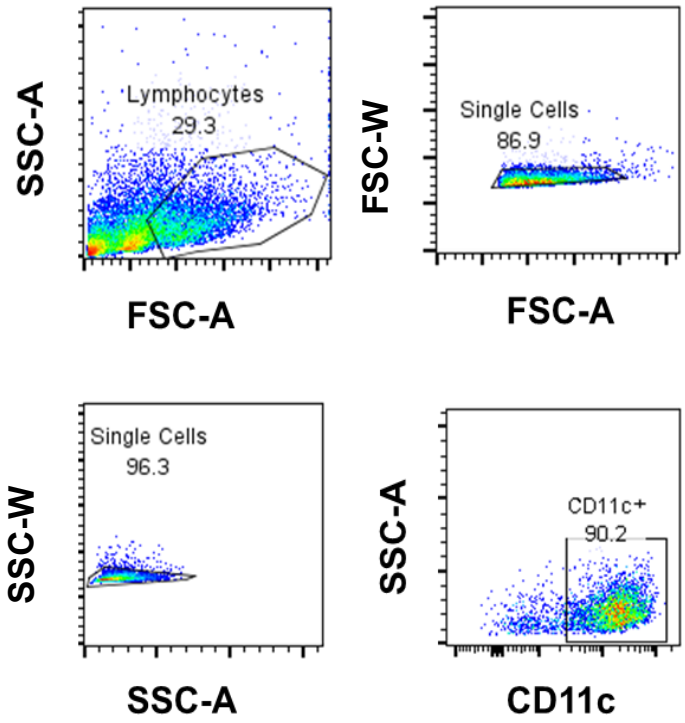


Figure 2.3. Gating strategy for the analysis of CD11c⁺ SPLN-DCs by flow cytometry. *SPLN-DCs were stained with CD11c, followed by flow cytometry analysis. Cells were gated on live cells (FSC vs. SSC) and doublets were excluded. FACS plot is a representative for one experiment out of three experiments.*

2.3.2 Isolation and expansion of macrophages from mouse BM (BM- DMs)

Mice were sacrificed, bones dissected, and RBCs depleted BM cells were prepared as previously described in section *2.3.1.1*. (pg. 50). BM-DMs were isolated using a protocol described by Trouplin *et al.*, (2013). Briefly, $1x10^7$ RBC-depleted BM cells were re-suspended in 10ml of complete media supplemented with 10% FCS (Gibco, ThermoFisher), RPMI without L-glutamine supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (ThermoFisher), 2 mM L-glutamine, 10mM HEPES, (ThermoFisher) 50 mM 2-ME (ThermoFisher) and 50ng/ml M-CSF (Biolegend, California, US) and placed in 10cm non-TC treated Petri-dishes. BM-DMs were grown for 7 days at 37°C in 5% CO_2. Fresh complete media (10ml) supplemented with M-CSF media was

added on day 3 of culture. Purity of cells was determined by assessing F4/80 expression by flow cytometry [Fig. 2.4].

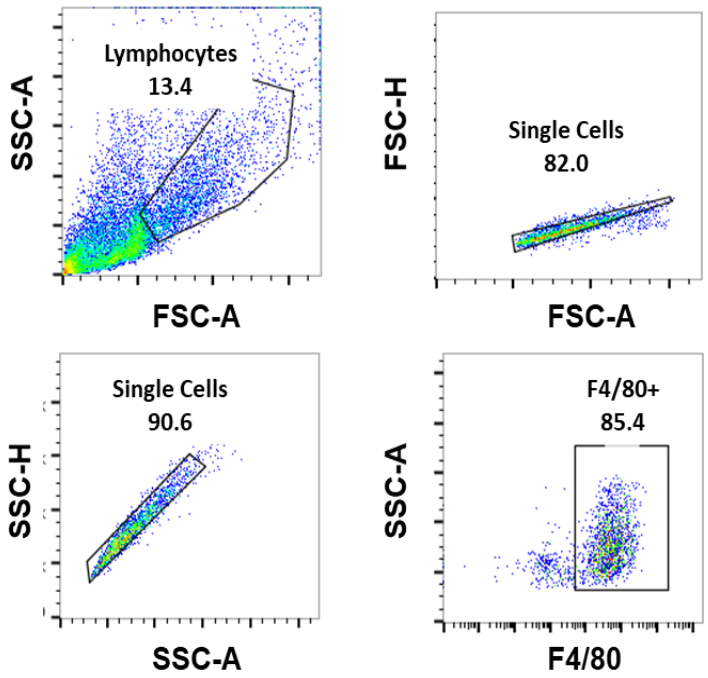


Figure 2.4. Gating strategy for the analysis of F4/80 BM-DMs by flow cytometry. *BM-DMs were stained with F4/80, followed by flow cytometry analysis. Cells were gated on live cells (FSC vs. SSC) and doublets were excluded. FACS plot is a representative for one experiment out of three experiments.*

2.3.3 Preparation of B cells from mouse spleen

Spleens were harvested from B6 mice, and a splenocyte single cell suspension was obtained by disaggregating organ through a 70µm cell strainer. Next, a RBCs free splenocyte suspension was obtained, as previously described, and B cells isolated through negative selection using a Dynabeads™ Mouse CD43 Untouched™ B Cells kit (Invitrogen, Thermofisher) according to manufacturer's instructions. Briefly, 5 x 10^7 splenocytes were resuspended in 1ml isolation buffer (2% FCS, 2mM EDTA/PBS) were incubated with 125µl of prewashed anti-CD43 coated magnetic beads for 20 minutes at 18-25°C, constant rotation. After incubation, the cells and bead mixture was placed onto a DynaMag™-15

Magnet (ThermoFisher) and 'untouched' B cells were isolated by negative selection. Dynabeads containing the non-B cells were removed using the magnet. B cell purity was measured by assessing $B220^+$ expression by flow cytometry [Fig. 2.5].

B cell subsets were assessed by IgM, CD21 and CD23 expression by flow cytometry [Table. 6].

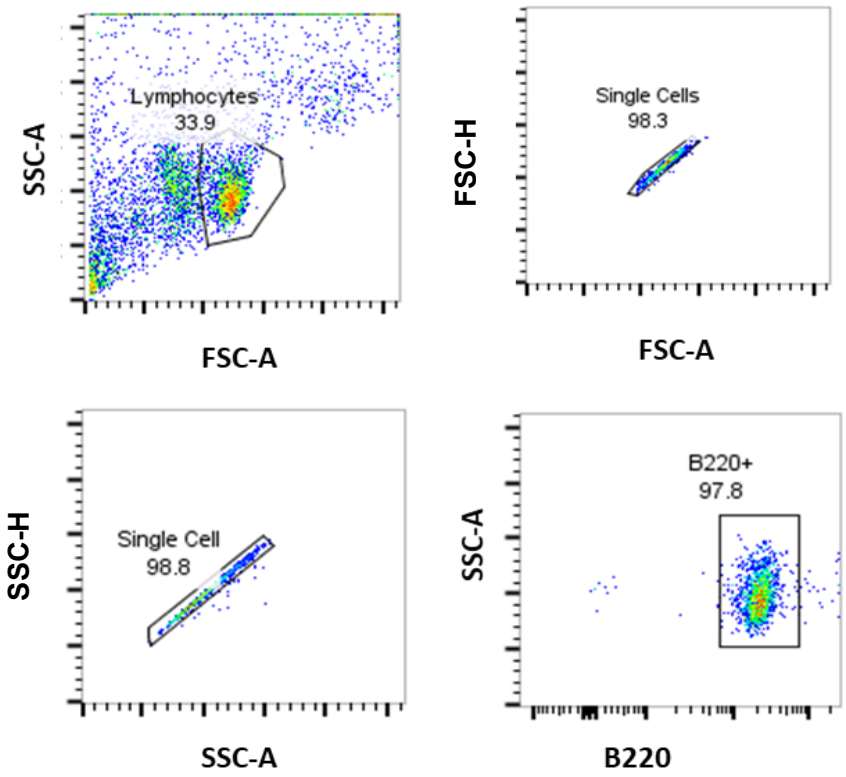


Figure 2.5. Gating strategy for the analysis of $B220^+$ B cells by flow cytometry. *B cells were stained with B220-APC, followed by flow cytometry analysis. Cells were gated on live cells (FSC vs. SSC) and doublets were excluded. FACS plot is a representative for one experiment out of three experiments.*

2.3.4 Preparation of T cells from mouse spleen

$CD4^+$ T cells were isolated from the spleen of TCR75 $Rag^{-/-}$ mice. A single-cell RBC- free cell suspension was prepared as described in section *2.3.1.2* (pg. 52). Following counting, cells were resuspended in complete medium at a 10^6/ml or 10^7/ml cell density suspension and purity of >90% $CD4^+$ T cells.

2.3.4.1 CD4$^+$ T cell isolation for in vivo adoptive transfer

In some experiments T cells were administered. For adoptive transfer to B6.Rag2$^{-/-}$ transplant recipients, CD4$^+$ T cells were isolated from B6 spleens using CD4 Dynabeads™ Untouched™ isolation kit (Thermo Fisher Scientific) following manufactures instructions. Recipient mice received 0.5x 10^6 B6 CD4$^+$ T cells in 200µl saline via intravenous injection one day before skin transplantation.

2.4 Antibodies

Fluorescent and non-fluorescent monoclonal antibodies were used for flow cytometry [Table. 6] and were purchased from a number of suppliers.

2.5 Flow cytometry

Cells were analysed using either a LSR Fortessa™, BD FACSCelesta™ or BD Accuri C6™ flow cytometer. Acquired data was analysed using FlowJo (TreeStar, Ashland, Oregon, USA) or BD Accuri C6 software.

2.5.1 Extracellular staining: phenotypic analysis of mouse cells

All immunostaining was performed using the following buffer; phosphate buffered saline (PBS) supplemented with 2% FCS and 2mM EDTA (all Thermo Fisher, known as FACS buffer). Cells were washed with FACS buffer and pelleted at 562*g* for 5 minutes before staining. Staining was performed using between 0.5x10^6-1x10^6 cells in a volume of 100µl of FACS buffer. Firstly, to block Fc receptors and reduce non-specific antibody binding, cells were incubated with anti-CD16/CD32 antibody (clone 93), 1/100 dilution) for 20 minutes at 4°C. The cells were then stained with fluorescently-conjugated antibodies specific to the antigens of interest for 30 minutes at 4°C. Appropriate dilutions of fluorochrome-conjugated antibodies used was according to manufactures protocols, see *Table 6*. After immunostaining cells were washed twice with FACS buffer and resuspended in 200µl of FACS buffer before being acquired. In some experiments, cells were fixed using BD CellFIX™

(ThermoFisher). Briefly, following the final wash, cells were resuspended in 100ul of 1x BD CellFix and incubated for 10 minutes in the dark at room temperature before being washed, twice, with FACS buffer and resuspend as above.

2.5.2 Intracellular staining for Foxp3 expression

Intracellular staining for Foxp3 expression was performed following manufactures instructions (eBioscience, Thermofisher). After cell surface staining, cells were fixed and permeabilised using 200µl of eBioscience Fixation and Permeabilisation buffer (ThermoFisher) for 30 minutes at room temperature. Cells were then washed twice with permeabilisation buffer and resuspended in 100µl of anti-Foxp3-APC conjugated antibody for 60 minutes at room temperature. After staining, cells were washed twice with permeabilisation buffer, before being resuspended in 200µl FACS buffer for flow cytometry analysis.

2.5.3 CFSE labelling

To assess T cell proliferation, we used Carboxyfluorescein succinimidyl ester (CFDA SE or CFSE) labelling of responder T cells. $CD4^+$ T cells were isolated from mice, washed and resuspended in PBS before being labelled, in the dark, with 0.5µM Vybrant™ CFDA SE (ThermoFisher) for 15 minutes at 37°C water bath. Labelling was stopped by the addition of 10X excess of FCS containing complete media and cell were pelleted and counted.

Table 6: Summary of Monoclonal antibodies used for flow cytometry

Antibody specificity	Flourochrome	Clone	Working dilution	Source
CD3ε	PE	145-2C11	1/50	eBioscience
CD4	FITC	RM4-5	1/100	eBioscience
CD4	PE	RM4-5	1/100	eBioscience
33D1	PE	33D1	1/100	Biolegend
XCR1	PE	REA707	1/50	MACS Miltenyi
CD62L	PE	MEL-14	1/100	eBioscience
CD11c	APC	N418	1/200	eBioscience
CD11c	APC	N418	1/200	Biolegend
CD11c	PE	N418	1/100	eBioscience
F4/80	PE	BM8	1/100	Biolegend
B220	APC	RA3-6B2	1/100	Bioscience
B220	FITC	RA3-6B2	1/100	eBioscience
CD23	PerCP Cy5.5	B3B4	1/100	Biolegend
CD21/35	PE	CR2/CR1	1/100	Biolegend
IgM	BV421	RMM-1	1/100	Biolegend
IgG	FITC	-	1/200	Sigma- Aldrich
Siglec E	FITC	8D2	1/100	MBL
Siglec F	PE	ES22-10D8	1/50	MACS Miltenyi
Siglec G	APC	SH2.1	1/100	eBioscience
Siglec H	PE	551.3D3	1/50	MACS Miltenyi
CD169 (Sialoadhesin-1)	PE	REA197	1/100	MACS Miltenyi
CD22	PE	Cy34.1	1/50	MACS Miltenyi
Foxp3	APC	FJK-16s	1/100	eBioscience
CD90.1 (Thy 1.1)	PE	HIS51	1/100	eBioscience
CD275 (ICOS-L)	PE	HK5.3	1/100	eBioscience
CD274 (PD-L1)	PE	MIH5	1/100	eBioscience
H-2K^{b}	FITC	28-14-8	1/200	eBioscience
I-A/E^{b}	FITC	AF6-120.1	1/200	eBioscience
CD80	FITC	16-10A1	1/100	eBioscience
CD86	FITC	GL1	1/100	eBioscience
CD16/32 Fc Block™	-	93	1/200	eBioscience

2.6 H-2K^{d} $^{54-68}$ peptide: sialylated and non-sialylated conjugates

2.6.1. K^{d} peptide derived from BALB/c MHC I H-2K^{d} molecule is an established alloantigen peptide

As mentioned in Chapter 1, the intact MHC molecule or the peptides derived from the MHC can act as highly immunogenic alloantigens that can stimulate allorecognition and transplant rejection. The importance of the MHC to graft rejection was investigated when transplants were carried out on genetically identical or congenic mice, and it was found that the MHC region on chromosome 17 was responsible for rejection (Gorer *et al*., 1938; Gorer *et al*., 1948). It has been said that the highly polymorphic transplantation genes of MHC I, which are known to mediate transplant rejection, are denoted by letters, K, D and L which are located in the H-2 region of MHC I (Hood *et al*., 1983). In a review by Hood *et al*., (1983), they explain that each mouse has a combination of alleles (haplotype) associated with transplant genes K or D. For example, in BALB/c MHC I, one haplotype is denoted as d (lower-case and superscript) and its gene that it is associated with would be denoted in a capital letter, e.g. K, so the overall gene combination to describe MHC I would be called H-2K^{d} (K^{d}) [Fig. 2.7] (Hood *et al*., 1983). K^{d} 54-68 peptide, derived from H-2K^{d} has been established as a highly stimulatory allopeptide in comparison to other peptide sequence derived from the α1 hypervariable region of BALB/c MHC I and has been used as an alloantigen in transplant models since the early 2000s [Fig. 2.6] (Honjo *et al.*, 2000). Given that the aforementioned K^{d} peptide derived from BALB/c MHC I H-2K^{d} molecule is an established alloantigen peptide this was used in our studies [Fig.2.7].

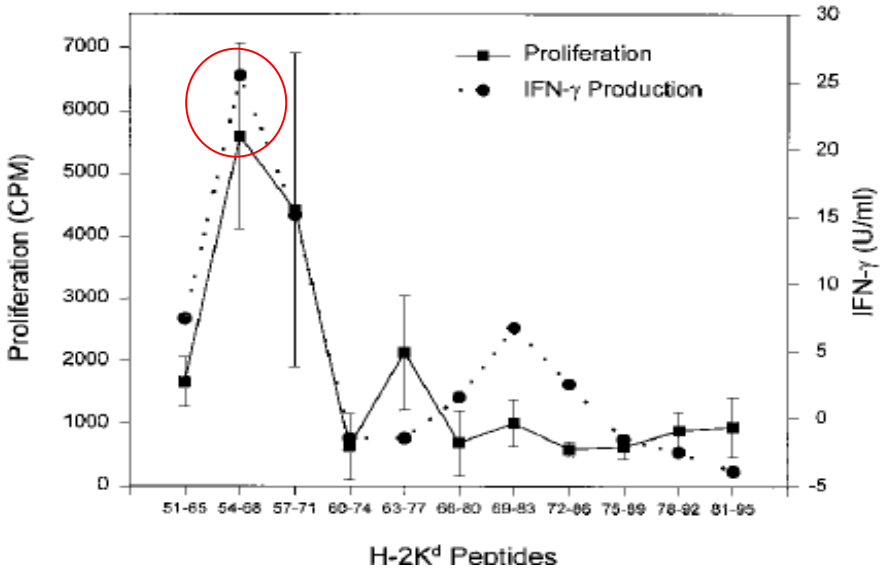


Figure. 2.6. *K^d 54-68 is most stimulatory peptide out of the entire MHC I H-2K^d molecule by increasing proliferation of alloreactive cells and increased production of IFN-γ - image acquired from Honjo et al., (2000).*

All K^d peptides were manufactured by Professor Yvette van Kooyk lab (VU University Medical Centre, Netherlands), sialylated peptides were produced using a patented procedure mentioned by Perdicchio *et al.*, (2016) [Fig. 2.8]. Briefly, malemide-activated Neu5Acα2,3Galβ1,4Glc or Neu5Acα2,6Galβ1,4Glc was conjugated to thio-activated K^d peptide to form α2,3 Sia-K^d or α2,6 Sia-K^d, respectively (Perdicchio *et al.*, 2016). Some peptides were also supplied with FAM5/6 (FITC) conjugation for the assessment of peptide binding [Fig. 2.8]. The structures show that the initial Cysteine residue (C) was used for coupling of the sialic acid glycan on the N terminus. The FAM 5/6 fluorochromes was attached to the C terminus.

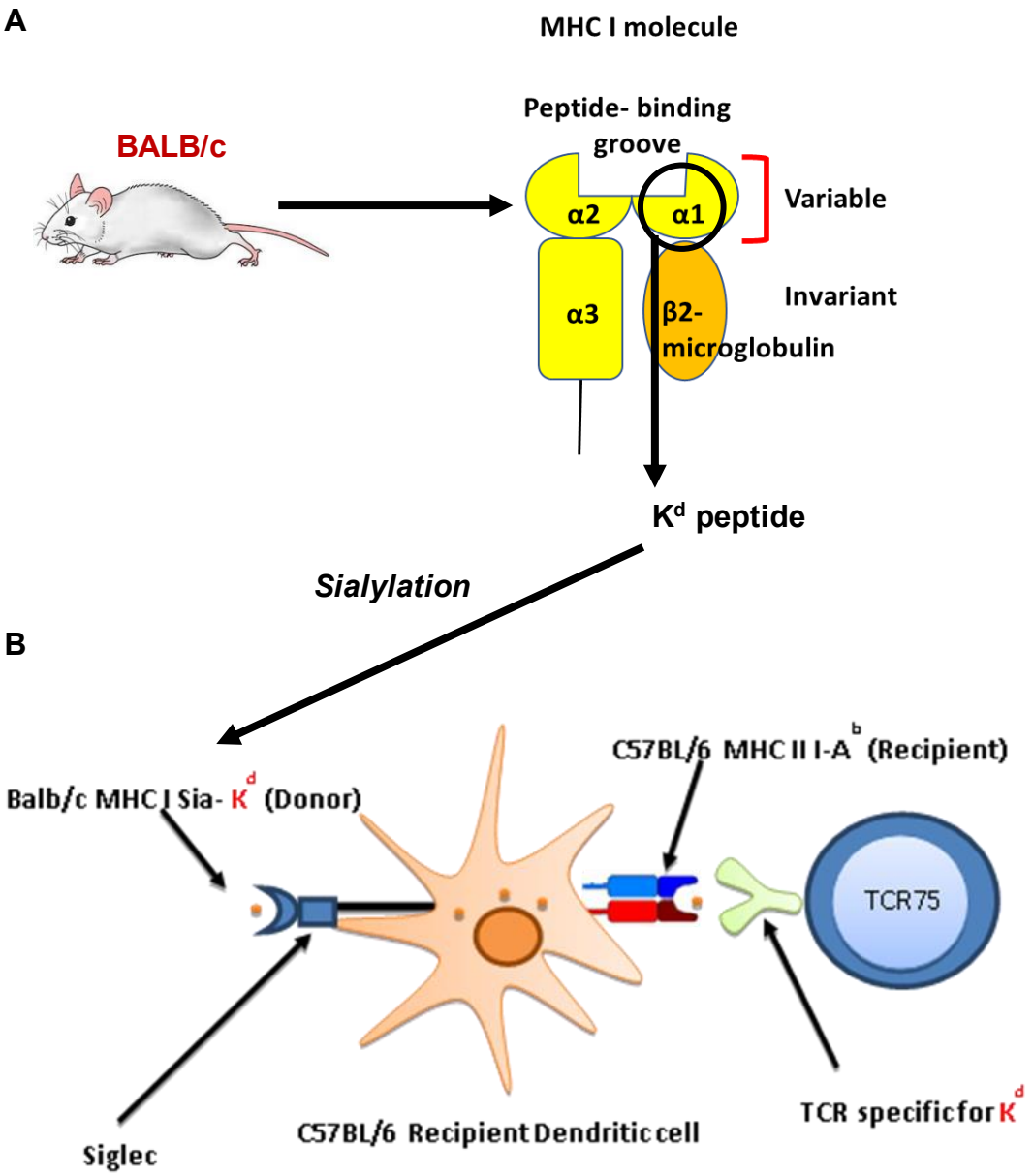


Figure 2.7. H-2K^d alloantigen peptide. *A. K^d peptide is derived from H-2K^d molecule derived from donor BALB/c strains. B. Indirect allorecognition of Sia-K^d by TCR75 T cells. This model proposes that sialylated K^d will bind to Siglec-expressing recipient B6 DC (or B cell/ macrophage), followed by antigen presentation via MHC II to TCR75 T cells. The T cells are isolated from TCR transgenic mice (TCR75 Rag$^{-/-}$) that have a high specificity for a particular K^d alloantigen and are an excellent tool for measuring rates of transplant rejection and alloantigen-specific alloresponses.*

The following K^d peptides 54-68 [Fig. 2.8] plus their 16 amino acid sequences were used:

1. *Unlabeled*

K^d: CQEGPEYWEEQTQRAK.
α2,3 Sia-K^d: Sia-α2,3- CQEGPEYWEEQTQRAK.
α2,6 Sia-K^d : Sia-2,6- CQEGPEYWEEQTQRAK.
α2,3L Sia-K^d : LSTd-2,3- CQEGPEYWEEQTQRAK.

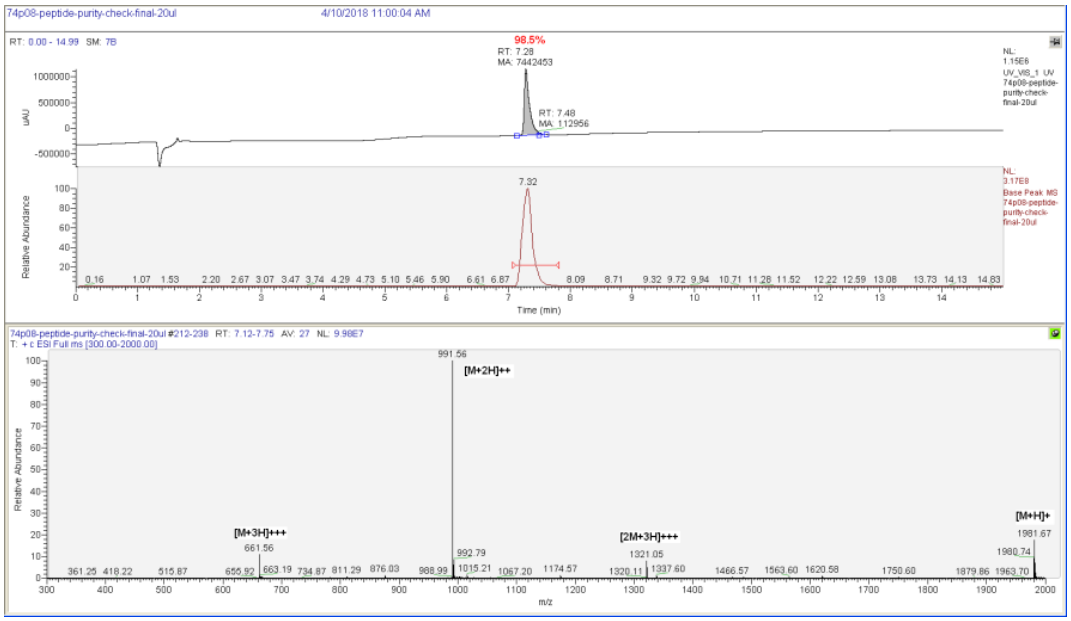
2. *FAM5/6 labeled*

K^d- FITC: CQEGPEYWEEQTQRAK-FAM5/6 (FITC).
α2,3 Sia-K^d- FITC: Sia-α2,3- CQEGPEYWEEQTQRAK-FAM5/6 (FITC).
α2,6 Sia-K^d- FITC: Sia-α2,6- CQEGPEYWEEQTQRAK-FAM5/6 (FITC).

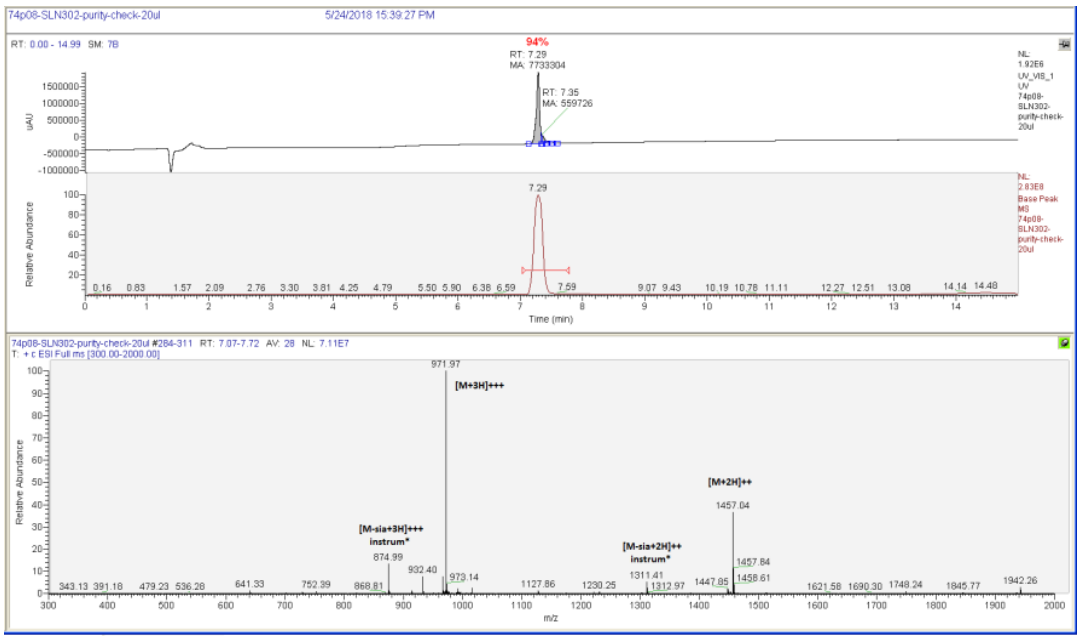
2.6.2 Purity of peptides

The purity of the peptides was assessed and analysed by Prof Yvette van Kooyk's lab at VUmc using HPLC, where each peak represents a component within the peptide sample and purity was deemed as >90%. Mass spectrometry was used to assess the mass of the product [Fig. 2.8].

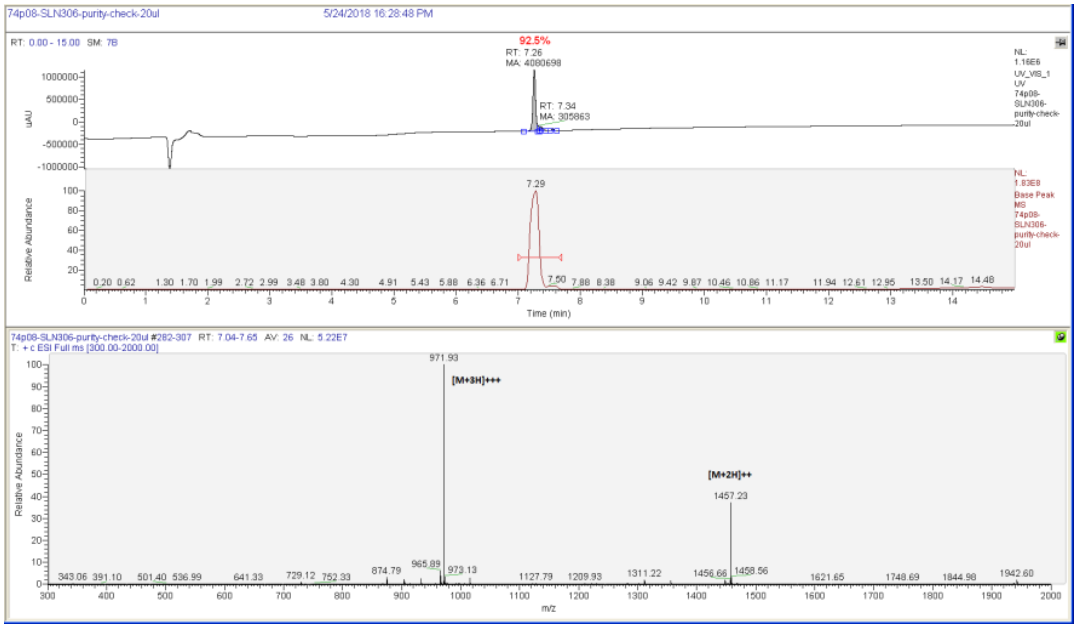
A **K^d: CQEGPEYWEEQTQRAK**



B **α2,3 Sia-K^d: Sia-α2,3- CQEGPEYWEEQTQRAK**

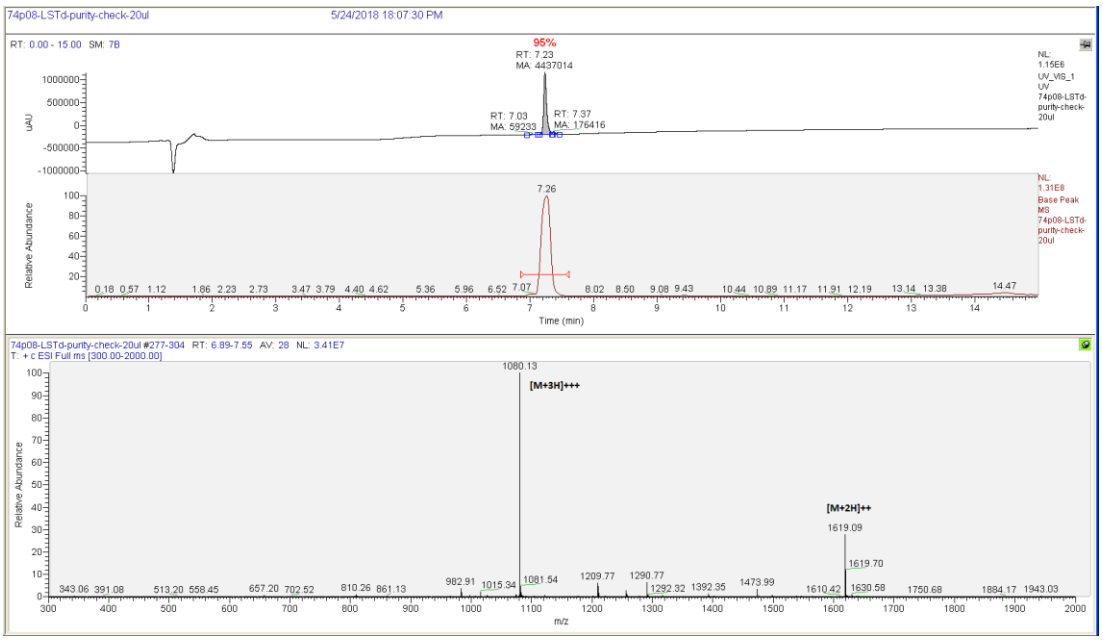


C **α2,6 Sia-K^{d} : Sia-2,6- CQEGPEYWEEQTQRAK**



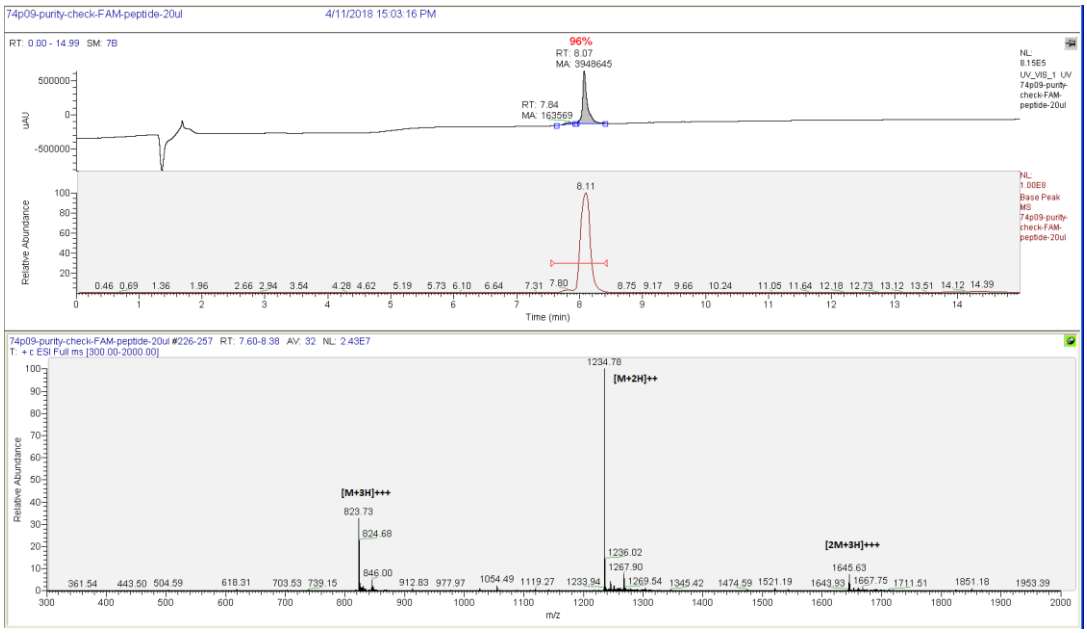
D

α2,3L Sia-K^{d} : LSTd-2,3- CQEGPEYWEEQTQRAK



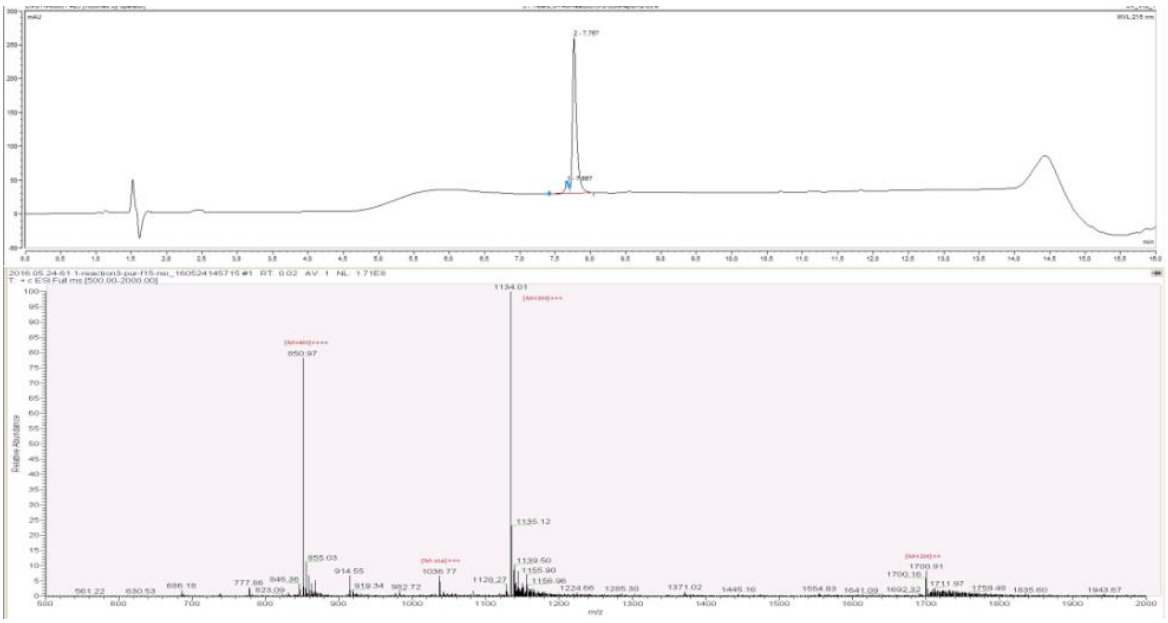
E

K^d- FITC: CQEGPEYWEEQTQRAK-FAM5/6 (FITC)



F

α2,3 Sia-K^d- FITC: Sia-α2,3- CQEGPEYWEEQTQRAK-FAM5/6 (FITC)



G α2,6 Sia-K^d- FITC: Sia-α2,6- CQEGPEYWEEQTQRAK-FAM5/6 (FITC)

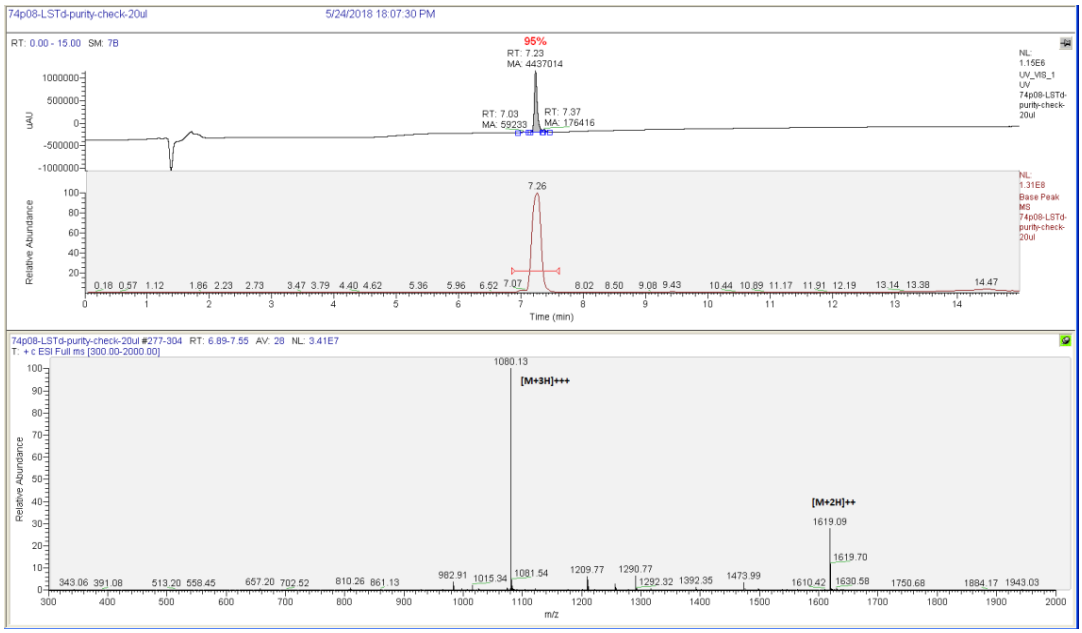


Figure: 2.8. Purity of sialylated and non-sialylated alloantigen K^d peptides*: HPLC (upper plot) and mass spectrometry (lower plot) analysis of sialylated and non-sialylated K^d alloantigen peptides. A. K^d peptide, B. α2,3 Sia-K^d, C. α2,6 Sia-K^d , D. α2,3L Sia-K^d, E. K^d-FITC, F. α2,3 Sia-K^d-FITC, G. α2,6 Sia-K^d-FITC. This data was generated by Dr Martino Ambrosini from VUmc, Amsterdam, NL.*

2.7 Binding of FAM5/6 labelled K^d alloantigen constructs to APCs

2.7.1 In vitro

Bone marrow-derived cells, BM-DCs and BM-DMs, were gently harvested from the tissue culture plastic, using a rubber plunger from a 1ml syringe, after 7 days of culture. For splenic-derived B cells and DCs, cells were isolated as previously mentioned in *section 2.3.* (pg. 50) and used immediately. Binding of labelled K^d peptide conjugates to APCs (BM-DCs, SPLN-DCs, BM-DMs and B cells) isolated from B6 and/or B6.Batf3$^{-/-}$ mice were performed by incubating 0.5

x 10^6- 1 x 10^6 cells in 100µl of complete media with either 10µg/ml K^d-FAM5/6, or sialylated K^d-FAM5/6 fluorescent peptides for 4 hours at 37°C 5% CO_2 in a 24-well flat bottom TC-treated plate. APCs left untreated were the negative controls. At the end of the incubation step excess peptide was removed by harvesting and washing cells twice with FACS buffer. Cells were counted and 0.2 x 10^6 cells were incubated firstly with anti-CD16/32 antibody, as described above, followed by either anti-CD11c-APC conjugated antibody (for DCs), anti-F4/80-APC (for macrophages) and anti-B220-APC conjugated antibodies (for B cells). For B cell subset stain, cells were stained with anti-B220-APC, IgM-BV421, CD21-PE or CD23- PerCP Cy5.5. Cells were assessed by flow cytometry. Expression was compared to an unstained control and single stained controls (no peptide).

2.7.2 In vivo

B6 and B6.Batf3$^{-/-}$ mice were placed into a 38°C heated incubator for 10 minutes to allow their tail veins to dilate and become visible. Once warm, mice were transferred into a mouse restrainer and their tail cleaned with a 70% ethanol sterile cotton swab. Intravenous administration (i.v.) of 10µg of peptides in 200µl of saline/mice was achieved using a 0.3 ml BD MicroFine™ syringe and 30G needle (Medisave UK Ltd, Dorset, UK). After 2 hours, mice were sacrificed and both spleens and lymph nodes (axillary, brachial, inguinal, mesenteric) were harvested and digested with 0.5 mg/ml collagenase in the presence of 10µM/ml DNase for 30 mins at 37°C, as previously mentioned, in section *2.3.1.2* (pg. 52). Cell suspensions were then passed through a 70 µm cell strainer to obtain a single-cell suspension before the RBCs were lysed. RBC-free cells were washed in FACS buffer, counted and 2 x 10^6 cells were stained with in 100µl of the aforementioned, following a blocking step, with conjugated antibodies specific to CD11c, F4/80 or B220. Cells were assessed by flow cytometry. Expression was compared against an unstained control and single stained controls (no peptide).

2.8 DC microscopic imaging

BM-DCs (2x 10^6/ml) were grown (similarly to the protocol mentioned in *section 2.3.1.1,* pg. 50) onto circular cover slips (Appleton Woods) placed into wells of a 24 well plate for 7 days and pulsed with 10µg/ml with K^d- FITC or sialylated-K^d-FITC for 2 hours at 37°C 5% CO_2. The wells containing DCs were washed for 5 minutes with 1ml of PBS under gentle agitation; this was repeated 3 times to remove complete media. Cells were then treated with 4% paraformaldehyde (Sigma, Dorset, UK) for 5 minutes at room temperature. Cover slips containing cells were carefully transferred to a 24-well plate, followed by adding 300µl of 300 nM DAPI (ThermoFisher) /PBS solution onto the coverslip ensuring that slide is fully covered and after 5 minutes the slides were washed 3 times with 6 ml PBS. Coverslips were mounted onto slides using 1 drop of prolong gold antifade (ThermoFisher). Slides were visualised at 200x magnification using Nikon microscopic imaging (Olympus BX51 Fluorescence Microscope).

2.9 LPS stimulation of BM-DCs and BM-DMs

On day 6 of culture, BM-DCs and BM-DMs were harvested and counted. 0.5 x 10^6 BM-DCs and BM-DMs were pulsed with 10µg/ml unsialylated or sialylated K^d peptide in 1ml of complete media for 4 hours at 37°C/ 5% CO_2, or left untreated. Some cells were treated for 24 hours/37°C with either 100ng/ml or 200ng/ml LPS derived from *E.coli* (serotype: O111:B4) (Sigma, Dorset, UK). Negative controls were cells that were not treated with peptide or LPS. Before the cells were harvested for flow cytometry analysis, 50µl of culture supernatants from each conditioned well was collected and stored at -20°C for subsequent cytokine analysis. 0.2 - 0.5 x 10^6 cells were stained with fluorescently conjugated antibodies to MHC class I-FITC and II-FITC, CD80-FITC, CD86-FITC and CD11c- PE/ APC or F4/80- PE/ APC. To assess surface marker expression, cells were gated on CD11c or F4/80 to identify the DC and macrophage population respectively, followed by measuring the MFI of FITC to determine, MHC I/II, CD80/86 expression.

2.10 T cell activation assays

Splenic-derived B6 B cells and BM-DCs were pulsed with various concentrations of K^d or sialylated-K^d peptide (1µg/ml, 5µg/ml or 10µg/ml) for 4 hours/ 37°C, before being washed twice, counted and resuspended at 1 x 10^6 cells/ml in complete medium. DCs and T cells were co-cultured at different ratios in triplicate in a 96-well round bottom plate (Appleton Woods) (a total volume of 250µl complete media) for 3 days at either 1:1, 1:5 and 1:10 DC:T cell ratios. In these experiments TCR75 $Rag^{-/-}$ $CD4^+$ T cells were used. Unpulsed APCs and TCR75 $Rag^{-/-}$ $CD4^+$ T cells, as well as T cells alone were used as negative controls. Fifty microlitres of supernatants were collected on day 2 of co-culture for cytokine analysis. Proliferation of TCR75 $Rag^{-/-}$ $CD4^+$ T cells was assessed using two methods. The first was via a thymidine incorporation assay. On day 2 of cultures, cells were pulsed with 1µCi per well of ^{3}H-thymidine (Perkin Elmer UK, Beaconsfield) and on day 3, thymidine incorporation was measured by liquid scintillation (Perkin Elmer UK) counting using a β-plate counter (Perkin Elmer UK). Samples were carried out in triplicates and proliferation was assessed as counts per minute (CPM).

The second method used to assess $CD4^+$ T cell proliferation was by CFSE labelling the responder T cells. $CD4^+$ T cells were isolated from TCR75 $Rag^{-/-}$ mice as previously mentioned in *section 2.3.4* (pg. 55) and were CFSE labelled. CFSE labelled $CD4^+$ T cells were co-cultured with either alloantigen-pulsed DCs (10µg/ml) or alloantigen-pulsed B cells (10µg/ml) for 3 days or 5 days at 37°C 5% CO_2. Unpulsed APCs and CFSE labelled TCR75 $Rag^{-/-}$ $CD4^+$ T cells, as well as unlabelled T cells alone were used as negative controls. Following incubation, cells were harvested and counted and 0.2- 0.5 x 10^6 cells were stained with anti-CD4 antibody conjugated to PE and analysed via flow cytometry. To assess proliferation of TCR75 $Rag^{-/-}$ $CD4^+$ T cells, cells were gated on CD4 PE and proliferation was observed on a histogram plot against FL-1 channel [Fig. 2.9].

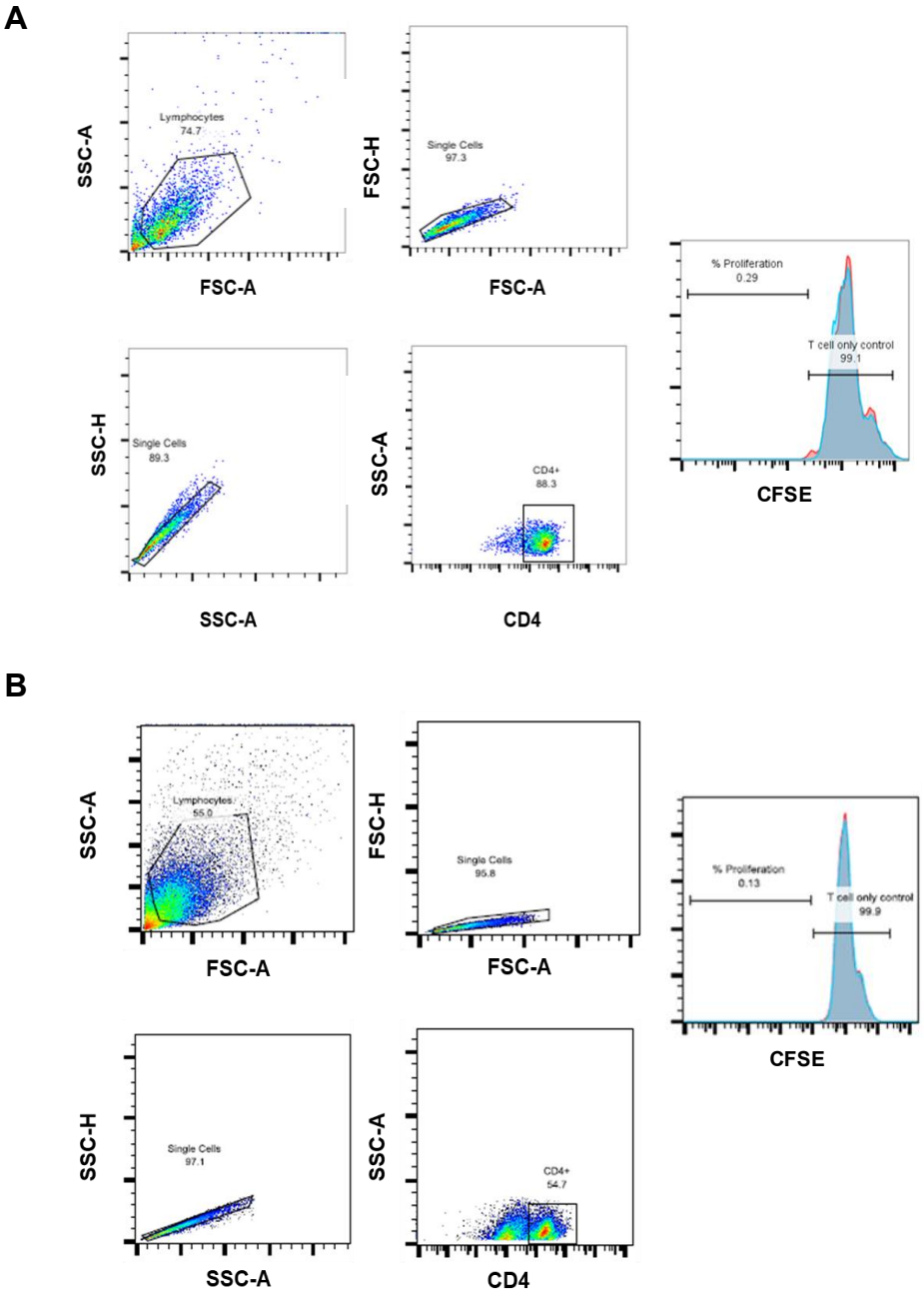


Fig. 2.9. Gating strategy for T cell proliferation assays. *B6 BM-DCs or B cells were cocultured with CFSE labelled TCR75 CD4$^+$ T cells at 1:10 ratio. After 3 days, cells were surface stained with CD4 antibody, followed by analysis of CFSE proliferation. Cells were gated on live cells (FSC vs. SSC); doublets were excluded followed by gating on CD4$^+$ T cells that were CFSE$^+$. A. Gating strategy for DC/T co-cultures and. B. Gating strategy for B/T co-cultures in the absence of peptide.*

2.11 *In vitro* Treg induction assay

B6 BM-DCs and B cells were treated with 10µg/ml of peptides as previously mentioned and co-cultured with CFSE labelled TCR75 $Rag^{-/-}$ $CD4^{+}$ T cells at a ratio of 1:10 (APC: T cell) for either 3 or 5 days in 96 U-bottom plates at 37°C (a total volume of 250µl complete media). IL-2 (5 U/ml) was added to cells at the beginning of the culture on day 0. Supernatants were harvested for IL-10 cytokine analysis and cells were stained with anti-CD4-FITC or PE conjugated antibody. Intracellular Foxp3 expression was assessed using intracellular staining with anti-Foxp3-APC conjugated antibody (ThermoFisher), as mentioned in section *2.5.2* (pg. 57). Expression was assessed using flow cytometry and gating on live cells, followed by CD4 vs. Foxp3 gating.

2.12 Enzyme-Linked Immunosorbent Assay

After the incubation period on the co-cultures, 50 -100 µl of supernatants were harvested for ELISA. IL-10 and IL-12 production by BM-DCs and BM-DMs were assessed using a Mouse IL-10 sandwich ELISA (ThermoFisher) and IL-12 sandwich ELISA (Biolegend, California, US) respectively following manufactures instructions. To measure IL-2 and IFN-γ cytokine production from T cells, IL-2 and IFN-γ sandwich ELISAs were used following manufactures instructions (ThermoFisher). All supernatants were diluted 1:10 and each sample was measured in duplicates or triplicates. Absorbance was read using Multi-Mode Reader (Synergy HTX) (BioTek, US).

2.13 Skin transplants

Skin transplant were performed as previously described by Golshayan *et al*., (2007). All surgery was done using sterile suture packs and autoclaved surgical scissors and forceps to ensure minimal infection. In brief, B6.K^{d} or BALB/c donor mice were sacrificed on the day of transplant and tail skin was immediately harvested using sterile forceps and stored in a sterile Petri-dish containing saline. Skin was cut using sterile forceps (Sigma) into 1-1.5cm^{2} sections and stored at 4^{o}C until used. Before skin transplantation recipient B6, B6.$Batf3^{-/-}$ and B6.$Rag2^{-/-}$ mice were administered with 2µg/mouse Vetergesic®

analgesia (Ceva, Amersham, UK) diluted in saline (i.p.) and the back of the animal shaved prior to surgery under anaesthesia using IsoFlo® isoflurane (Zoetis, Kalamazoo, Michigan) and oxygen (3.5 L/min). Once shaved, recipient mice were placed on a heat mat and kept under anaesthesia using a face cone. Mice were prepared for surgery by washing the shaved skin with Vetasept povidone-iodine antiseptic solution (Animalcare Ltd, York, UK) and draped with sterile robes leaving a small section on the dorsal thorax of recipient mice exposed for surgery. A small section, similar to the size of donor skin, was excised and donor skin was sutured onto the recipient using 45mm polyamide Ethilon sutures (Ethicon Inc, Edinburgh, Scotland). All 4 corners of the donor skin were sutured onto the recipient skin, to ensure vascularisation, before the transplant area was secured with a waterproof Elastoplast plaster (Hamburg, Germany) wrapped around the mid section of the mouse. This was then secured in place with autoclave masking tape. After surgery, mice were kept in a 29°C heated incubator for recovery until mice were fully awake and mobile. Mice were checked daily to ensure that the plaster remained in place and were recast if the plaster fell off.

On day 7 the plaster and tape were removed and grafts were observed daily thereafter to measure rejection. Rejection was considered when there was >90% necrosis of donor tissue. Skin grafts that had not engrafted after 7 days were treated as a failed experiment and were excluded from analysis.

2.13.1 Alloantigen peptide administration intravenously

In some experiments, mice received 10µg of either K^d, α2,3 Sia-K^d, α2,3L Sia-K^d or α2,6 Sia-K^d peptides administered intravenously in 200µl of saline either 1 or 10 days prior to skin transplant. This peptide concentration was chosen based on experiments published by Tanriver *et al*., (2010). Untreated mice were injected with 200µl of saline only. All injections were into the tail vein of recipient mice.

2.13.2 $CD8^+$ cell depletion

To deplete $CD8^+$ cells in B6 recipient mice, 250µg of anti-CD8 antibody (YTS169) in 100µl of saline was given via i.p on various days mentioned in Chapter 5-6 (Smyth *et al*., 2013).

2.14 Treg analysis in transplant recipients

To analysis Treg populations in transplant recipients, mice were bled 14 days post transplant. To collect blood, mice were placed in a 38°C heated incubator for 10 minutes and blood extracted from the tail vein using a 26½ G needle into a Microvette® CB 300 tube (Sarstedt, Numbrecht, Germany), lined with EDTA to prevent clotting. To isolate cells, blood was diluted into 15ml of PBS and spun at 562*g* for 5 minutes to obtain a cell pellet. Following RBCs lysis, leucocytes were stained with anti-CD4 FITC and anti-CD62L PE antibodies followed by intracellular staining for Foxp3, as previously described. Percentages of $CD4^+CD62L^-$ $Foxp3^+$ cells and $CD4^+CD62L^+$ $Foxp3^+$ cells were measured using BD Fortessa Flow Cytometer and analysed by FlowJo.

2.15 Alloantibody detection in skin transplant recipients

To measure anti-K^d alloantibody production by transplant recipients, blood samples were collected from the tail vein 4 weeks post-transplant (i.v). Blood was stored overnight at 4°C before being spun for 3 minutes at 2419*g* to pellet lymphocytes and RBCs. Serum was collected and stored at -20°C until further analysis. To detect K^d-specific IgG alloantibodies. RBC lysed splenocytes were isolated from B6.K^d (positive control) and B6 (negative control) mice as previously described. Fc receptors were blocked with anti-CD16/32 antibodies/ 5% goat serum/ PBS for 20 mins at 4°C, before staining with anti-CD3ε antibodies PE for a further 20 mins at 4°C. After incubation, cells were washed with FACS buffer; splenocytes were then resuspended in FACS buffer supplemented with transplant recipient's serum at a 1:20 dilution for 20 minutes at 4°C. Serum Ab-bound splenocytes were washed in FACS buffer, followed by addition of goat anti-mouse IgG FITC (Sigma-Aldrich) at a 1:100 dilution in FACS buffer for a further 20 mins at 4°C. Cells were washed twice in FACS buffer and assessed by flow cytometry. To quantify IgG alloantigens within the sample, B6 or B6.K^d CD3-PE^+ cells were gated, followed by histogram plots to assess mean fluorescence intensity (MFI) of IgG FITC using BD Accuri C6 flow cytometer.

2.16 Antigen specific T cell deletion and Treg expansion *in vivo*

For *in vivo* deletion experiments, $CD4^+$ T cells were isolated from spleens of TCR75 $Rag^{-/-}$ mice (Thy1.1) as previously described in *section 2.3.4* (pg. 55). Purity of $CD4^+$ T cell was assessed as >90% $CD4^+$ total cell population. Approximately, 2 x 10^6 cells/ 200µl saline were intravenously injected into B6 or B6.$Batf3^{-/-}$ mice and 10 days later LNs and spleens were harvested. A RBC-free single-cell suspension was made and counted as previously mentioned. After washing cells twice with FACS buffer, approximately 0.5 x10^6- 1 x10^6 cells were stained with anti-mouse Thy1.1- PE antibody (ThermoFisher) to assess the recovery of TCR75 $CD4^+$ T cells. Cells were also stained with anti-mouse CD4 FITC (ThermoFisher) and anti-mouse Foxp3 APC (ThermoFisher) to observe Treg induction/ expansion. After staining, cells were gated on live cells (FSC vs. SSC), doublets were excluded, cells were gated on CD4 followed by Thy1.1 to assess recovery of TCR75 T cells, or gated on CD4 and Foxp3 to assess induction/ expansion of Tregs.

2.17 Statistical analysis

In vitro experiment: Statistical analysis was performed using unpaired Student's t tests and One-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism (GraphPad Software, California, US).

In vivo experiments: The mean survival time (MST) of skin grafts was calculated using Mantel Cox and log rank test using GraphPad Prism. Data shown is mean ± standard error of the mean (SEM). Statistical significance was expressed as follows;$p<0.0001$**** $p<0.001$***, $p<0.01$**, $p<0.05$*, $p>0.05$= NS.

CHAPTER 3:

TARGETING RECIPIENT APCs WITH α2,3 SIA-K^d AND α2,3L SIA-K^d ALLOANTIGEN PEPTIDES DAMPENS INDIRECT ALLOREACTIVE IMMUNE RESPONSES *IN VITRO*

3.1 Introduction

3.1.1 Dendritic cells

Immature/ semi-mature DCs and those maintained at a steady-state are capable of promoting tolerance as opposed to matured/ activated DCs. Immature/ semi-mature DCs display a 'tolerogenic' phenotype, demonstrated by their inability to initiate an effector immune response due to their low expression of MHC molecules and co-stimulatory molecules such as CD80/86, which gives rise to insufficient co-stimulation, and their ability to induce/ expand Tregs (Lutz, *et al.*, 2004; Marin *et al.*, 2018).

As outlined in *section 1.4.1* (pg. 24); e*x vivo* generated tolerogenic DCs can exhibit an immunoregulatory role. In one report by Lee *et al.*, (2017), antibiotic Minocycline and steroid Dexamethasone treated BM-DCs displayed reduced levels of MHC II and CD80/86 molecules in comparison to DCs that were matured with proinflammatory stimuli IFN-γ and TNF-α (Lee *et al.*, 2017). These tolerogenic DCs also had the ability to induce $CD4^+$ $Foxp3^+$ Tregs *in vitro* and additionally, when these Minocycline/Dexamethasone treated DCs were pulsed with MOG antigens and introduced into mice, there was reduced clinical signs of induced EAE in a mouse model, therefore supporting the use of tolerogenic DCs to promote tolerance *in vivo* (Lee *et al.*, 2017).

Multiple publications have described various strategies to deliver antigens to endogenous DCs *in vivo* in order to promote antigen-specific tolerance. These include the use of vehicles such as nanoparticles or specific receptor targeting (Steinman *et al.,* 2003; Tacken *et al.*, 2006; Tacken *et al.,* 2007; Ding *et al.*, 2008; Kovacsovics- Bankowski *et al.*, 1993). Targeting DC cell surface receptors such as DCIR2, expressed on $CD8^-$ DCs using DCIR2-specific fusion antibodies has been shown to prolong murine skin allograft survival and ameliorate EAE, (Tanriver *et al.*, 2010; Tabanksy *et al.*, 2018), whereas, targeting DEC-205, expressed on resident $CD8^+$ DCs and migratory $CD103^+$ DCs using DEC-205 specific antibodies was shown to promote peripheral $CD8^+$ T cell tolerance (Bonifaz *et al.*, 2002) and the expansion of Tregs.

Tanriver *et al*., (2010) identified targeting DCIR2^{+} endogenous DCs using a murine MHC class I (K^{d})-monomer conjugated to an anti-33D1-antibody, prolonged MHC-mismatched skin transplant survival in the absence of CD8^{+} T cells, demonstrating that targeting an endogenous DC subset can promote tolerance. Their regimen is limited to targeting one specific DC subset and given that multiple DC subsets are known to promote tolerance (e.g. DEC-205-Bonifaz *et al*., 2002), there is a need to find specific receptors that are expressed on multiple DC subsets to optimise antigen-specific tolerance.

Siglec receptors are expressed on different DC subsets as well as, macrophages and B cells. Given their ITIM in their cytosolic portion and convenient location on the cell's surface, these inhibitory receptors are known to inhibit/ modulate immune responses. Siglec interactions have been targeted on DCs, macrophages and B cells for the purpose of inhibiting immune responses. Perdicchio *et al*., (2016) demonstrated *in vitro*, that antigen specific tolerance was achieved following engaging Siglecs expressed on BM-DCs and SPLN-DCs with α2,3 sialic acid-conjugated antigen. Another study found that interaction of Siglec E on BM-DCs with the sialic acids present on *E.coli* led to endocytosis of TLR4, thereby decreasing their immune response to *E.coli* infection (Wu *et al*., 2016). These results were not achieved when sialic acids were removed from the surface of *E.coli* using sialidase or when Siglec E$^{-/-}$ BM-DCs were used (Wu *et al*., 2016). In the latter, persistent expression of TLR4 on the surface was observed resulting in the release of high levels of pro-inflammatory cytokines such as TNF-α and IL-6 following exposure to *E.coli* (Wu *et al*., 2016). This would suggest that Siglecs on BM-DCs are able to modify TLR 4 responses upon sialic acid interaction (Wu *et al*., 2016). Contrary to this study Nagala *et al*., (2018) published that Siglec E on both BM-DMs and BM-DCs were not involved in TLR4 signalling in response to LPS, which leaves the mechanism of whether Siglecs modulate TLR4 signalling a on-going question. Altogether, this supports the idea of targeting Siglecs on DCs can impair aberrant effector immune responses. This chapter will focus on targeting DCs to determine whether they display a tolerogenic phenotype by assessing MHC and costimulatory marker expression and cytokine production, whether these DCs can impair CD4^{+} effector T cell alloreactivity and possibly promote Treg induction/ expansion.

3.1.2 B cells

Similarly to DCs, targeting Siglec G expressed on B cells with α2,3 sialic acid has been shown to down-regulate BCR signalling by decreasing intracellular Ca^{2+} mobilization (Hutzler *et al.*, 2014). This makes Siglecs on B cells an attractive target as it is known that B cells contribute to transplant rejection via their APC ability to stimulate $CD4^+$ T cells via the indirect pathway of allorecognition and production of alloantibodies (Noorchashm *et al.,* 2006; Conlon *et al.*, 2012). There are certain B cell subsets which have shown to be regulatory these include transitional B cells (T1 ($IgM^{high}CD21^-CD23^-$), and T2 ($IgM^{high}CD21^+CD23^+$)) and some studies have found that these subsets are required for transplant survival (Moreau *et al.*, 2014). Upon BCR engagement, transitional B cell subsets are known to undergo apoptosis or cell cycle arrest making them an ideal cell subset for tolerance induction by preventing their ability to present alloantigens and stimulate Th1 effector alloreponses. As mentioned in the introduction (*section 1.4.2*, pg. 36), T1 B cells are thought to undergo apoptosis following BCR engagement (Petro *et al.*, 2002) whereas T2 B cells are more responsive to antigen stimulation (Chung *et al.*, 2002). They also found that these transitional B cells, whether they are $CD23^+$ or $CD23^-$, do not upregulate co-stimulatory molecules upon BCR engagement (Cook *et al.*, 1998; Chung *et al.*, 2002). One study in particular identified that T2 B cells are believed to be regulatory and promote allograft survival (Moreau *et al.*, 2014). Therefore, this chapter will focus on targeting B cells to determine whether they can impair $CD4^+$ effector T cell alloreactivity and possibly promote Treg induction and also determine which B cells subsets the sialylated and non-sialylated constructs bind to, should these targeted B cells have a regulatory role.

3.1.3 Macrophages

Macrophages also play a role in transplant rejection, making these cells a valid target for transplant tolerance. Macrophages can become activated upon recognition of cellular damage via DAMPS caused by the transplantation procedure; this activation allows macrophages to phagocytose antigens and act as APCs in the context of MHC and stimulate T cells (Li *et al.*, 2019). Similar

activation also occurs in response to TLR activation (eg; via LPS stimulation) which can result in the production of proinflammatory cytokines such as IL-12. Little is known about the role of macrophages and their contribution to chronic rejection, however, given their ability to promote inflammation it is not a surprise that one study found that accumulation of macrophages were associated with cardiac rejection in a rat model (Kanno *et al*., 2001). Macrophages can contribute to chronic transplant rejection through the release of proinflammatory cytokines, eg: TNF- α and IFN- γ, which were shown to cause transplanted tissue fibrosis and vascular injury (Mitchell, 2009). Similarly to DCs and B cells, macrophages also have a regulatory role (Mregs). One study demonstrated the importance of macrophages in a cardiac murine transplant model where $CD8^+$ T cells immune responses were inhibited whereas $CD4^+$ $Foxp3^+$ Tregs were promoted in the presence of DC-$SIGN^+$ macrophages (Conde *et al*., 2015). Deletion of these macrophages resulted in reduced allograft survival (Conde *et al*., 2015). Therefore, this chapter also determines whether targeting recipient macrophages has the ability to dampen proinflammatory cytokines following sialylated alloantigen targeting.

The focus of this chapter is to determine whether targeting sialylated alloantigen to Siglec-expressing DCs and B cells can impair indirect allorecognition effector immune responses and promote Treg induction *in vitro*. In addition, the targeting of macrophages and DCs will also be assessed to determine if these cells are resistant to LPS maturation. Overall, should these Siglec expressing-APCs be regulatory upon sialylated alloantigen targeting, this targeting regimen can be used as a prospective cellular immunotherapy for transplantation. Either by administering antigen treated APCs to transplant recipients or by targeting endogenous APCs by directly administering these sialylated alloantigen (Sia-K^d) peptides into transplant recipients.

3.2 Aims

In this chapter targeting sialylated alloantigen, α2,3 Sia-K^d and a longer construct, α2,3L Sia-K^d, to APCs such as DCs, B cells and macrophages, will be undertaken to determine whether targeting Siglecs leads to tolerogenic APCs, impaired indirect $CD4^+$ T cell allorecognition and effector immune responses using various *in vitro* assays.

Objectives

1. Determine what Siglecs are expressed on different APCs including DCs, B cells and macrophages.
2. Identify whether sialylated alloantigens bind to Siglecs expressed on DCs, B cells (and subsets) and macrophages *in vitro*.
3. Determine whether targeting sialylated alloantigen to Siglecs expressed on DCs, B cells and macrophages *in vitro* promotes an immunosuppressive phenotype
4. Assess whether targeting DCs and B cells influences dampened allogeneic T cell responses and/or induced/ expanded $CD4^+$ $Foxp3^+$ Tregs *in vitro.*

To achieve the objectives, K^d alloantigen peptide, derived from mouse MHC class I H-2K^d (CQEGPEYWEEQTQRAK) and was chosen for sialylation (α2,3 Sia-K^d and α2,3L Sia-K^d). Prof Y van Kooyk (VUMC, collaborator) generated the Sia-K^d conjugated with or without a FAM5/6 (FITC) label using an existing patented method for this project (Perdicchio *et al.,* 2016). The justification for using this MHC class I K^d peptide (derived from donor BALB/c mice) as a major alloantigen was that this peptide when presented by MHC class II I-A^b (B6 self-MHC II), is recognised by a transgenic T cell receptor (TCR) expressed by $CD4^+$ T cells isolated from TCR75$Rag^{-/-}$ transgenic mice. Thus recreating the indirect allorecognition response where recipient APCs present donor Ags to recipient $CD4^+$ T cells (Ali *et al.,* 2016; Honjo, *et al*., 2000). This established 'indirect allorecognition model' has been used to assess indirect allorecognition *in vitro* and *in vivo* in murine transplant models (Chai *et al.,* 2015; Ali *et al.,* 2015; Tanriver *et al*., 2010).

3.3 Results

3.3.1 BM-DCs express well characterised DCs surface markers following GM-CSF expansion

The current study used *ex vivo* expanded BM-DCs for the analysis of tolerance induction *in vitro*, which is standard practice in the DC field (Helft *et al*., 2015; Smyth *et al*., 2013). Bone marrow progenitor cells were isolated and expanded with GM-CSF using a protocol published by Smyth *et al*., (2013) and described in detail in the methods section (*section 2.3.1.1,* pg. 50). These cells were stained with fluorochrome-conjugated antibodies to the following cell surface markers commonly found on BM-DCs; CD11c, MHC II, MHC I, and co-stimulatory molecules, CD86 and CD80. As expected, viable cells as assessed by FSC vs. SSC [Fig.2.2] expressed CD11c, [Fig. 3.1A-B], as previously described (Smyth *et al*., 2013). In addition they expressed, well-characterised DCs markers including MHC II, MHC I, and the co-stimulatory molecules CD80 and CD86 [Fig. 3.1B-C]. Given the aforementioned, it can be concluded that the cells isolated from these cultures are immature bone marrow- derived DCs, which expressed high levels of MHC II (Hoffmann *et al*., 2005; Smyth *et al*., 2013) [Fig. 3.1].

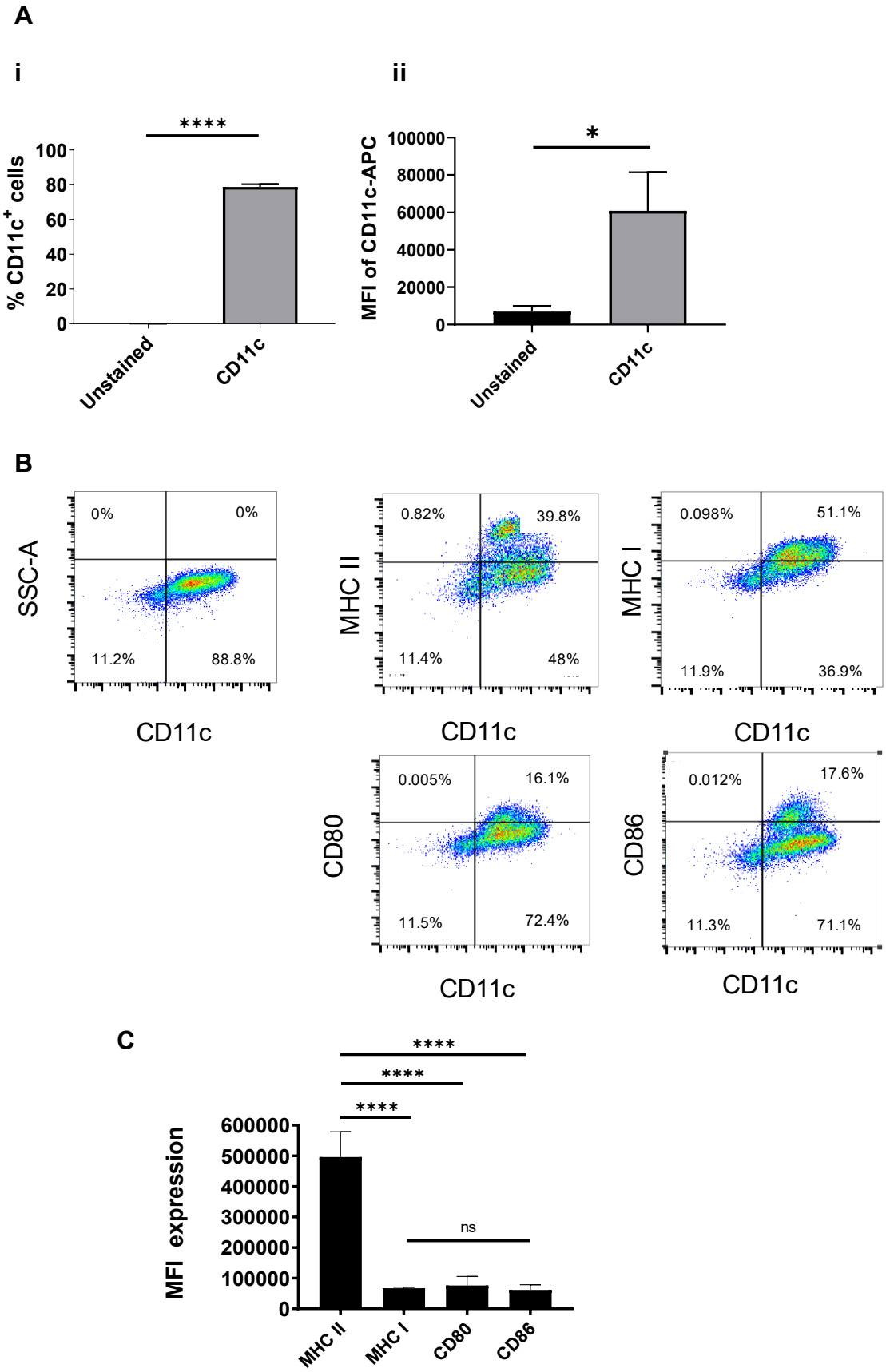


Figure 3.1. BM-DC express CD11c and are MHC II^{high}, MHC I$^{low/int}$, CD80$^{low/int}$ and CD86$^{low/int}$. *GM-CSF expanded B6 derived BM-DCs were harvested on day 7 of culture and the expression of CD11c, MHC II, MHC I,*

*CD80 and CD86 was assessed using specific fluorochrome-conjugated antibodies and flow cytometry. Gating strategy for CD11c$^+$ BM-DCs is demonstrated in Fig. 2.2. A. (i) Percentage of CD11c$^+$ BM-DCs. Unstained cells served as a control and data was pooled from 3 independent experiments. (ii) MFI for CD11c expression on BM-DCs. Unstained cells served as a control. Data was pooled from 5 individual experiments. Bar charts representing the means +/- SEM. Statistical comparisons performed using unpaired two-tailed Student's t-test B. Data shown are representative pseudocolour dot plots from one out of 3 experiments demonstrating phenotype of ex-vivo generated BM-DCs gated on live cells. C. Bar charts representing the mean MFI expression +/- SEM of MHC II, MHC I, CD80 and CD86 on CD11c$^+$ BM-DCs. Data was pooled from three independent experiments. Statistical analysis was performed by One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p< 0.0001****, p<0.05*, NS = p>0.05.*

3.3.2. Siglec- expressing BM-DCs and SPLN-DCs bind α2,3 Sia-K^d alloantigen peptide in vitro

Siglecs are found on DCs although, according to different studies, the expression of Siglec receptors differs between murine endogenous DCs and *ex vivo* generated BM-DCs. For example, Ding *et al*., (2016), identified Siglec G on splenic CD8α$^+$ DCs, Loscko *et al*., (2011) discovered Siglec H on pDCs and Perdicchio *et al*., (2016) identified Siglec E on BM-DCs. In order to confirm the presence of Siglecs on GM-CSF expanded CD11c$^+$ BM-DCs, cells were stained with Siglec specific antibodies, and analysed using flow cytometry. Siglec F and Sialoadhesin- 1 (CD169), which is an endocytic receptor that is not considered inhibitory due to a lack of an ITIM, were highly expressed on BM-DCs, whereas there was moderate expression of Siglec E, G and H [Fig. 3.2A]. To assess Siglec expression on CD11c$^+$ endogenous spleen derived DCs (SPLN-DCs), DCs from B6 spleen were isolated as mentioned in the methods in *section 2.3.1.2* (pg. 52) and stained with Siglec E, F, G, H and CD169 antibodies as mentioned previously. Similarly to BM-DCs, Siglecs E, F, G and CD169 were expressed on SPLN-DCs, with Siglec H being expressed the least, however this marker is known to be primarily expressed on plasmacytoid DCs [Fig. 3.2A-B].

Interestingly, Siglec F was highly expressed in both BM- and SPLN-DCs [Fig. 3.2].

In order to determine whether the sialylated alloantigen peptide bind to these Siglec expressing-DCs *in vitro*, B6 derived BM-DCs and SPLN-DCs were pulsed with FITC-conjugated α2,3 Sia-K^d (10µg/ml) for 4 hours at 37°C before cells were assessed for peptide binding via flow cytometry. BM-DCs pulsed with non-sialylated K^d-FITC and untreated DCs served as controls. MFI of FITC expression signified peptide-FITC binding on these $CD11c^+$ DCs. Both the K^d-FITC and α2,3 Sia-K^d-FITC peptides were capable of binding to $CD11c^+$ BM-DCs and SPLN-DCs, as compared to unpulsed controls [Fig. 3.3 A-B]. However, α2,3 Sia-K^d binding to BM-DCs was significantly greater ($p= 0.01$) to K^d peptide binding, perhaps reflecting that each peptide binds to different receptors [Fig. 3.3A]. For example, K^d peptide has the ability to bind to MHC molecules expressed by these DCs, whereas α2,3 Sia-K^d may be binding to both the MHC and Siglec receptors, however this has yet to be confirmed. Similar binding results were found using SPLN-DCs, although binding levels between sialylated and unsialylated peptides was not statistically different ($p=0.81$), however there was a trend for greater binding of α2,3 Sia-K^d peptide as compared to K^d (Fig. 3.3B).

To confirm binding, BM-DCs were visualised using microscopic imaging following FITC-peptide incubation. To achieve this cells were grown on cover slips as mentioned in *section 2.8* (pg. 68) and treated with either K^d-FITC or α2,3 Sia-K^d-FITC conjugates as per above. DCs were further stained with DAPI, to identify the nucleus. In comparison to the non treated DCs [Fig. 3.3C (i)], as expected and as previously demonstrated in Fig. 3.3A both K^d-FITC and α2,3 Sia-K^d-FITC peptides were bound to the BM-DCs [Fig. 3.3C (ii-iii)]. Overall, we can conclude that sialylated alloantigens bind to Siglec-expressing B6 BM-DCs and SPLN-DCs *in vitro* which were confirmed using flow cytometry and using microscopic imaging.

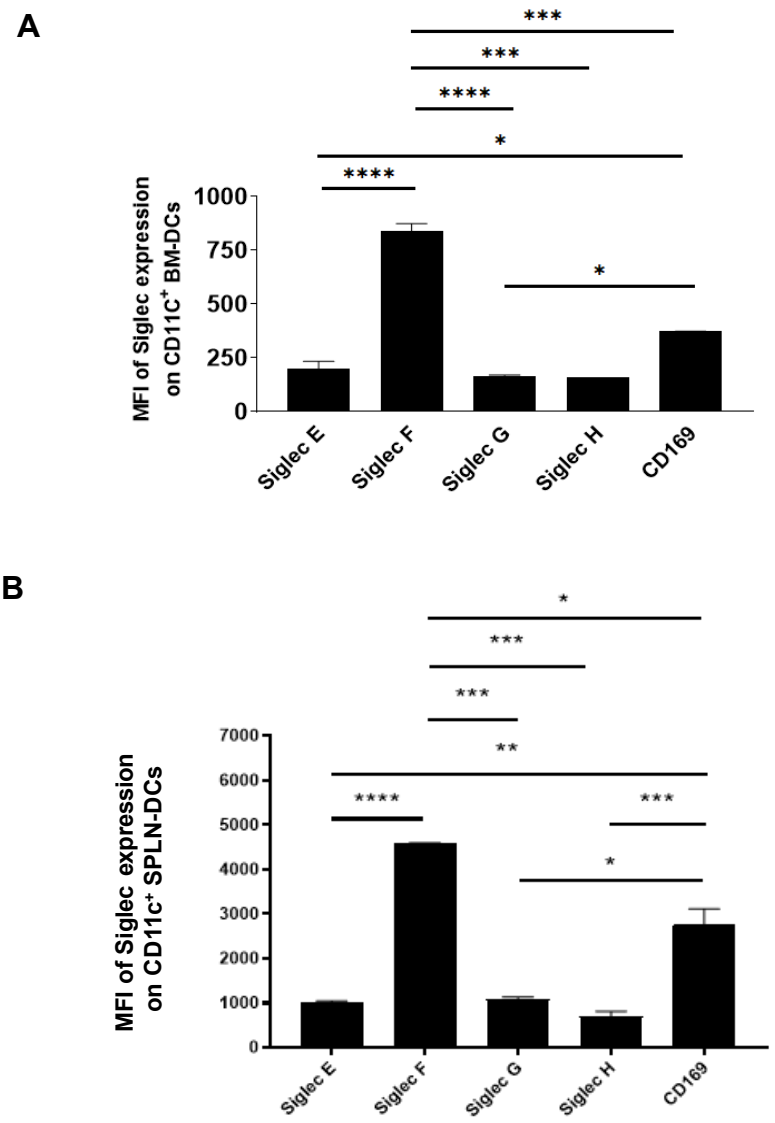


Figure. 3.2. Siglecs are expressed on B6 BM-DCs and SPLN-DCs. *For Siglec expression, day 6 or 7 B6 BM-DCs were stained using anti-mouse CD11c APC/PE and either anti-mouse Siglec E- FITC, Siglec F- PE, Siglec G-APC, Siglec H-PE and CD169-PE. Live cells were gated on FSC, SSC, followed by doublet exclusion and CD11c$^+$ cell gating [Fig. 2.2]. Siglecs were then gated against CD11c$^+$ DCs according to the gating strategy in Appendix Fig.1. (A). MFI of Siglec-expression on CD11c$^+$ BM-DCs. Data was pooled from 3 independent experiments. (B). MFI of Siglec expression on CD11c$^+$ SPLN-DCs. Data was pooled from 3 independent experiments. Statistical analysis was performed by One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p< 0.0001****, p<0.001***, p<0.01**, p<0.05*. Comparisons that were not significantly significant (NS) are not presented on the graph*.

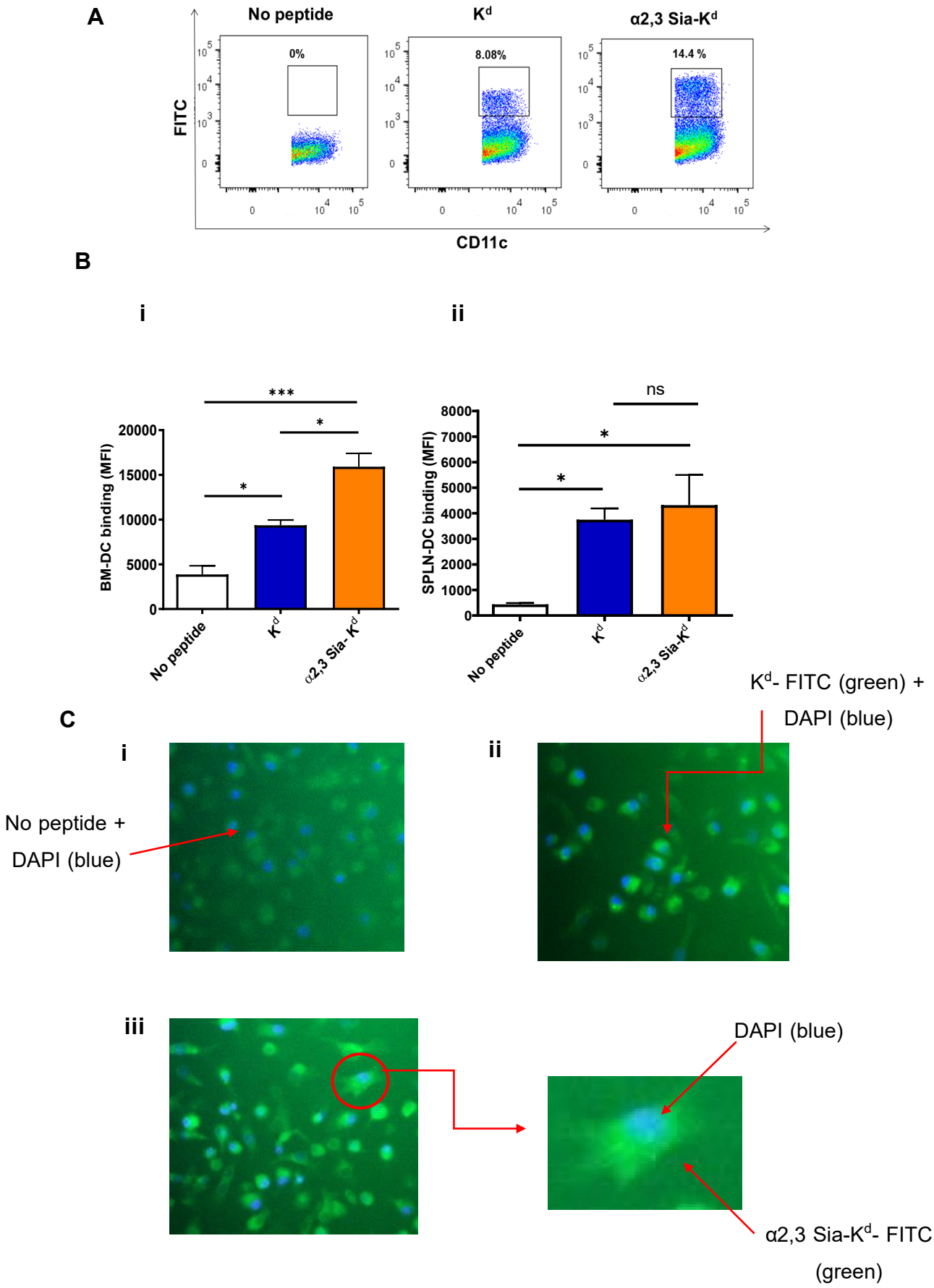


Figure 3.3. α2,3 Sia-K^{d-} FITC binds to BM- and SPLN-DC in vitro. *For peptide binding studies, B6 BM-DCs and or B6 SPLN- DCs were pulsed with*

*either 10 µg/ml K^d-FITC or α2,3 Sia-K^d- FITC peptide. A. (i) Cells were gated on $CD11c^+$ cells and presence of FITC staining assessed using flow cytometry. Plot is a representative for one experiment out of four. Unpulsed cells were the negative controls. B. (i) Peptide binding to BM-DCs, data presented was pooled from four independent experiments. (ii) Peptide binding to SPLN-DCs, data presented was pooled from 3 independent experiments. Each bar presents MFI of FITC +/- SEM. Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.001***, p<0.05*, NS = p>0.05. C. Microscopic image of peptide-pulsed B6 BM-DCs at x200 magnification. BM-DCs grown on coverslips were pulsed with peptides followed by DAPI staining (blue). (i) pulsed with either no peptide, (ii) 10 µg/ml K^d FITC (green) or (iii) 10 µg/ml α2,3 Sia-K^d FITC (green) peptides (image on left) and a larger image of one DC (image on right). Images represents 1 experiment. Comparisons were made against the no peptide control.*

3.3.3 Engaging Siglecs with α2,3 Sia-K^d peptide down-regulated MHC expression on BM-DCs

As mentioned in *sections 1.4.1- 1.4.1.1 (pg. 24-25),* tolerogenic DCs are able to promote an immunosuppressive response through the low expression of co-stimulatory molecules and production of immunosuppressive cytokines such as IL-10 (Marin *et al.*, 2018). Therefore, in order to determine whether Siglec engagement with α2,3 Sia-K^d peptide modified BM-DCs creating an immature/semi-mature 'tolerogenic' DC the levels of MHC I/II and CD80 and CD86 co-stimulatory molecules, as well as IL-10 and IL-12 production were measured. BM-DCs were pulsed with 10µg/ml α2,3 Sia-K^d peptides for 4 hours, K^d-pulsed and unpulsed DCs acted as controls, and cells were stained with antibodies to MHC II, MHC I, CD80 and CD86 before being analysed via flow cytometry. BM-DCs treated with α2,3 Sia-K^d peptides expressed significantly less MHC I in comparison to K^d treated DCs (13.2% decrease, p=0.01) and no peptide controls (13.5% decrease, p=0.01), and significantly less MHC II molecules in comparison to K^d treated DCs (24.2% decrease, p=0.02) and no peptide controls (23.9% decrease, p=0.02) [Fig. 3.4A-B]. In addition, there was a trend for less CD80 expression following Siglec engagement, although this

was not significant. No difference in CD86 expression following α2,3 Sia-K^d treatment DCs was observed [Fig. 3.4C-D]. Lastly, no significant increase in IL-10 or IL-12 production was observed following treatment, however there appears to be a trend indicating lower IL-12 production following sialylated alloantigen targeting in comparison to K^d targeted BM-DCs [Fig. 3.5A].

Overall, targeting sialylated alloantigen to Siglecs on BM-DCs leads to down-regulation of MHC molecules, and does not induce maturation of the DCs.

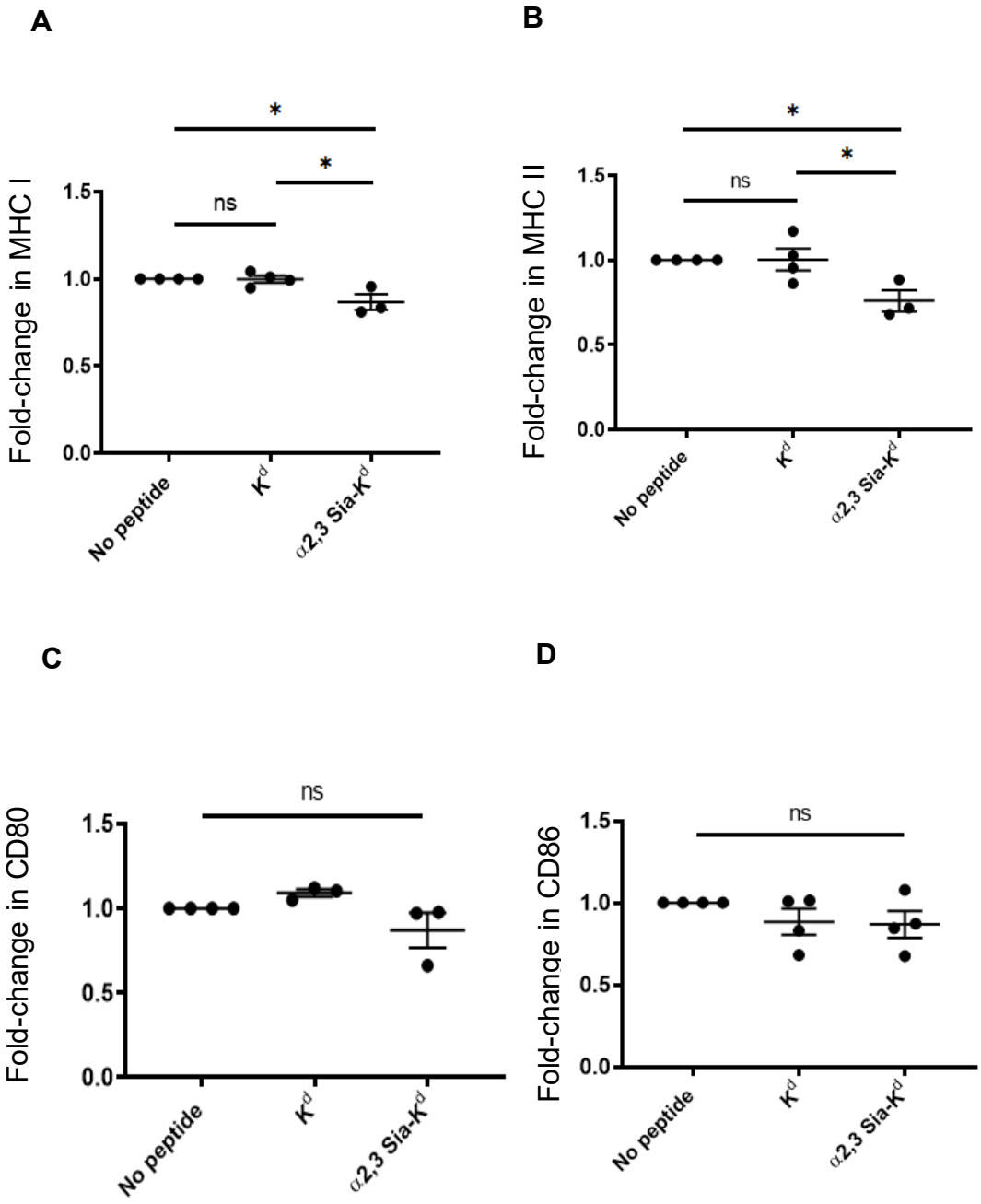


Figure 3.4. α2,3 Sia-K^d treatment down-regulates MHC I and MHC II on BM-DCs. *B6 BM-DCs were harvested and pulsed with 10μg/ml of either peptide on day 6 of culture for 4 hours. Cells were stained for CD11c, MHCI/II or CD80 and CD86 fluorochrome-conjugated antibodies and analysed via flow cytometry. Cells were gated on live (FSC vs. SSC) CD11c$^+$ cells. A. Fold-change for MFI for MHC I expression. B. Fold-change for MFI for MHC II expression. C. Fold-change for MFI for CD80 expression. D. Fold-change MFI for CD86 expression. Each point represents one experiment and data was pooled from 3-4 independent experiments. Fold-change was calculated by comparing the MFI of peptide treated DCs to unpulsed DCs (set to a value of 1). All statistical analysis was determined using One-way ANOVA and Tukey's multiple comparison test. Data shown is mean +/- SEM. Statistical significance was expressed as follows; p<0.05*. NS= p>0.05.*

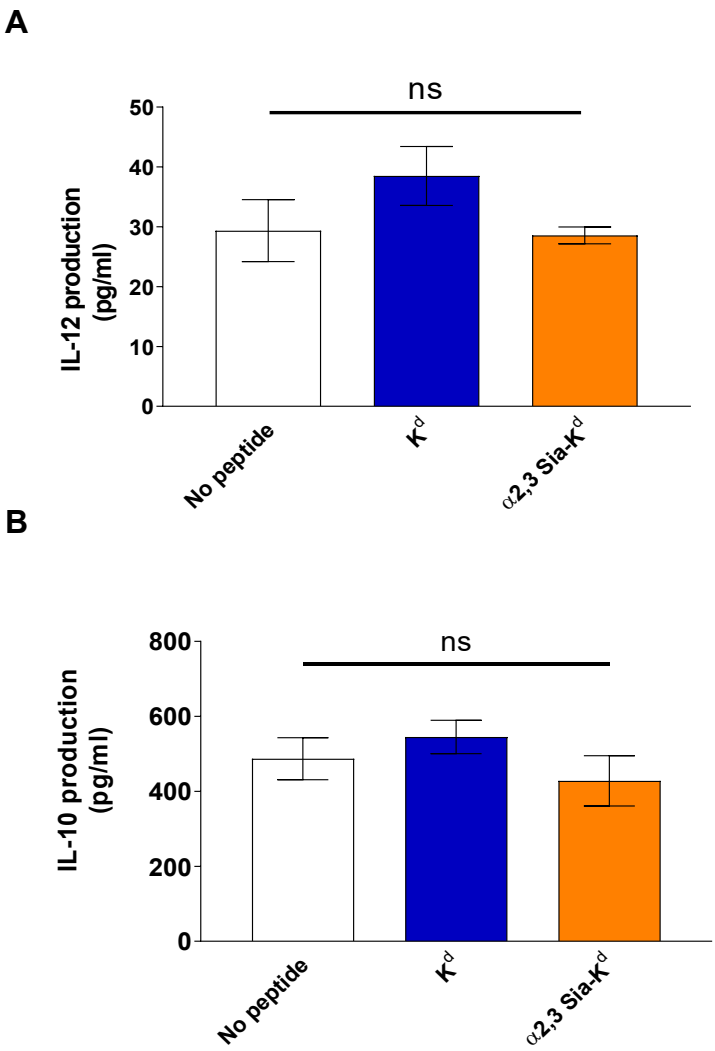


Figure 3.5. K^d or Sia-K^d treatment does not increase significant IL-10 or IL-12 production from BM-DCs. *B6 BM-DCs were pulsed with 10μg/ml of described peptide on day 6 of culture for 4 hours. A. IL-12 and B. IL-10 production by BM-DCs was assessed using a sandwich ELISA. Data is a representative of cytokine production (pg/ml) from 3 pooled independent experiments and is shown as mean +/- SEM. All statistical analysis was determined using One-way ANOVA and Tukey's multiple comparison test. Statistical significance was expressed as follows; NS= p>0.05. NS was observed between all controls.*

3.3.4 Siglec engagement with a2,3 Sia-K^d does not inhibit BM-DCs responses to LPS

It has been shown previously that murine DCs treated with Sia-OVA and Sia-MOG peptides were resistant to LPS maturation as measured via reduction of intracellular expression of TNF-α and IL-6 following treatment (Perdicchio *et al*., 2016). Therefore, suggesting that sialylated antigen targeted DCs had an 'immature phenotype' under proinflammatory stimulus (Perdicchio *et al*., 2016). This finding was in line with others who have found that 'tolerogenic' DCs are resistant to maturation (see *section 1.4.1,* pg. 24). In one of these studies, BM-DCs rendered tolerogenic by drug treatment had low expression of MHC class II, co-stimulatory molecules and was in favour of producing IL-10 rather than IL-12 in comparison to control BM-DCs following LPS/ anti-CD40 or TNFα stimulation (Smyth *et al*., 2013). As the Perdicchio *et al*., (2016) study did not present data on the aforementioned, the expression of MHC and co-stimulatory molecules as well as IL-12/IL-10 production following LPS stimulation was assessed in sialylated alloantigen treated BM-DCs via flow cytometry and ELISA, respectively.

Surprisingly, both MHC I and II levels were not increased in un-pulsed BM-DCs after LPS stimulation [Fig. 3.6A-B]. This was an unexpected finding and is conflicting to Smyth *et al*., (2013) and Kelleher & Beverley (2001) studies, where they found that untreated BM-DCs stimulated with LPS had increased expression of MHC molecules. In contrast, CD80 and CD86 levels were increased following LPS stimulation in untreated cells (no peptide/LPS p=0.01). Pre-treatment with both unsialylated or sialylated peptides did not modify the increase in CD80 however, CD86 was slightly reduced by peptide treatment (K^d/LPS vs. untreated/LPS, p=0.13) (α2,3 Sia-K^d /LPS vs. untreated/LPS, p =0.10) [Fig.3.6 C-D].

The supernatants from these treated BM-DCs were collected for the assessment of IL-12 and IL-10 production. Both IL-12 and IL-10 levels were increased in response to LPS for all conditions [Fig. 3.6 E-F]. However, compared to K^d pulsed and LPS-treated control BM-DCs, α2,3 Sia-K^d treatment did not modify cytokine production [Fig. 3.6E].

In conclusion, whether sialylated alloantigen targeted BM-DCs are refractory to LPS stimulation remains inconclusive given no significant differences in MHC expression and cytokine production in response to LPS.

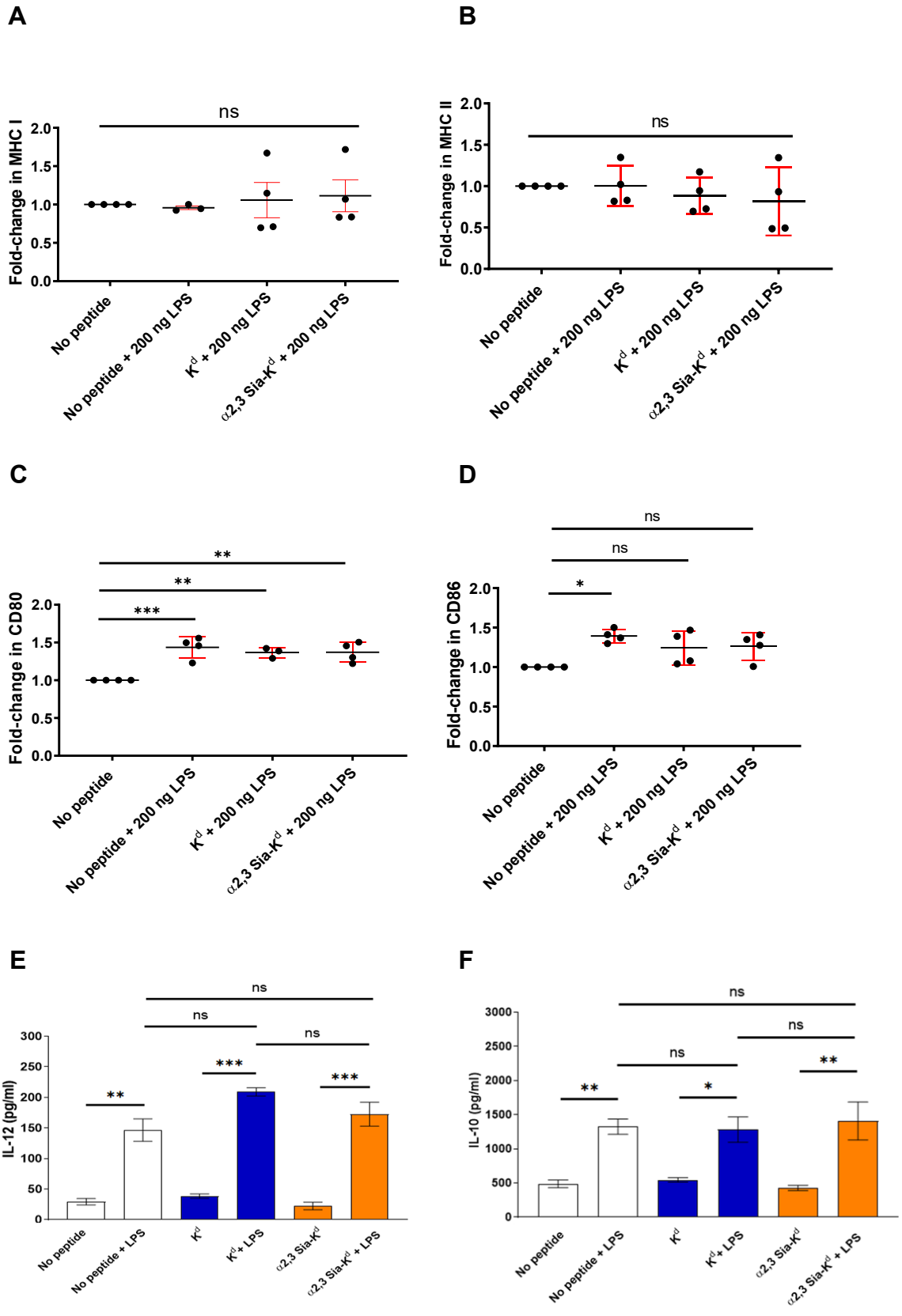


Figure 3.6. MHC, co-stimulatory molecules, IL-10 and IL-12 remain unchanged between peptide and unpulsed BM-DCs. *B6 BM-DCs were harvested and pulsed with 10μg/ml peptide on day 6 of culture for 4 hours and subsequently treated with 200ng/ml LPS for 24 hours. IL-12 and IL-10 was*

*measured in the culture supernatants using a sandwich ELISA. Cells were also stained with anti CD11c, MHCI/II or CD80 and CD86 flurochrome-conjugated antibodies and assessed by flow cytometry, to determine MHC and costimulatory marker expression. For expression of MHC and costimulatory molecules, each dot represents one experiment. Overall, data was pooled from 4 independent experiments and the error bars represent +/- SEM. Data is presented as fold-change of MFI for MHCI/II or CD80/86 expression, in comparison to BM-DCs treated with no peptide or LPS (set to a value of 1): A. MFI for MHC I expression. B. MFI for MHC II expression. C. MFI for CD80 expression. D. MFI for CD86 expression. E. Bar chart showing the mean +/- SEM concentration of IL-12 (pg/ml) and F. IL-10 (pg/ml) produced in the absence or presence of LPS. Data was pooled from 3 independent experiments. All statistical analysis was determined using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*, NS= p>0.05.*

3.3.5 In vitro targeting of α2,3 Sia-K^d to B6 BM-DC reduces proliferation as well as IL-2 and IFN-γ production of K^d alloantigen- specific CD4$^+$ T cells

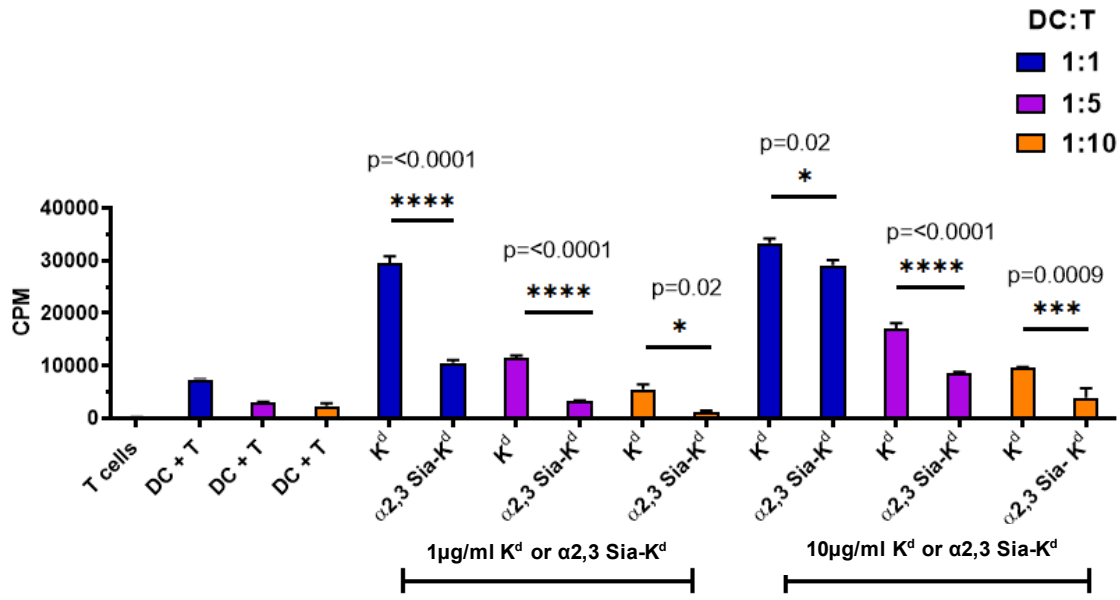
Perdicchio *et al.*, (2016), observed that Sia-OVA antigen pulsed DCs significantly impaired proliferation of antigen specific responder T cells in contrast to DCs pulsed with unsialylated OVA (Perdicchio *et al.*, 2016). To determine whether pulsing DCs with a sialylated-alloantigen resulted in a similar observation, K^d specific T cells were isolated from TCR75 transgenic Rag $^{-/-}$ mice and stimulated with B6 derived BM-DCs pulsed with either 1 or 10 µg/ml of K^d or α2,3 Sia-K^d peptides. As mentioned earlier TCR75 $CD4^+$ recognises K^d alloantigen presented via B6 I-A^b, modelling demonstrating an indirect T cell responses. Controls were DC and T cell co-cultured in the absence of antigen and T cells cultured alone. Different DC: T cell ratios were used to optimise the assay. T cell proliferation was measured on day 3 via thymidine incorporation and IL-2 and IFN-γ cytokine production was measured using IL-2 and IFN-γ specific sandwich ELISAs [Fig.3.7].

In comparison to T cells co-cultured with unpulsed DCs, proliferation of TCR75 T cells was observed in the presence of BM-DCs treated with either 1 and 10 µg/ml K^d at all DC: T cell ratios [Fig. 3.7A]. By contrast, T cell proliferation to BM-DCs pulsed with 1 µg/ml of α2,3 Sia-K^d pulsed BM-DC was significantly reduced, however in the presence of BM-DCs pulsed with 10 µg/ml α2,3 Sia-K^d an increase in proliferation was seen at the 1:1 and 1:5 DC:T cell ratios suggesting that the level of proliferation of T cells is dependent on the concentration of the peptide [Fig. 3.7A]. However, comparing T cell proliferation induced by both the peptide pulsed BM-DCs indicated that pre-treatment with α2,3 Sia-K^d in comparison to K^d peptide resulted in significantly less T cell activation [Fig. 3.7A] (See appendix Fig. 2 for all data).

In addition, and complementary to the T cell proliferation observed, IL-2, a cytokine involved in T cell expansion, and IFN-γ, an effector T cell cytokine, were produced by TCR75 T cells activated with K^d pulsed DCs. Interestingly, co-cultures containing BM-DCs pulsed with 1 µg/ml of peptides at a ratio of 1:10 suggested no significant differences in IL-2, although there was a trend of less IL-2 in the presence of sialylated alloantigen [Fig. 3.7B]. Overall, in the presence of 1 or 10 µg/ml α2,3 Sia-K^d, there was a significant reduction of IL-2 in comparison to K^d [Fig. 3.7B]. The low levels of IL-2 may be a result of induced/ expanded Tregs utilising this cytokine for their survival. This could explain the proliferation data, which may be utilising the IL-2 for their expansion [Fig. 3.7B]. This was assessed and the data will be discussed in *section 3.3.7*. Compared to K^d pulsed DCs, reduced IFN-γ levels were also observed when T cells were cocultured with BM-DCs pulsed with 10 µg/ml α2,3 Sia-K^d [Fig. 3.7C].

Therefore, despite high levels of Sia-K^d binding to BM-DCs [Fig. 3.3] there is significant suppression of antigen specific T cell proliferation and cytokine production. Taken together this data suggests that engaging Siglecs expressed on DCs with sialylated K^d peptide resulted in suppression of the expansion and effector function of indirect alloreactive T cells.

A


DC:T
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1:5
1:10
CPM
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30000
20000
10000
0
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p=<0.0001
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*
p=0.02
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T cells
DC + T
DC + T
DC + T
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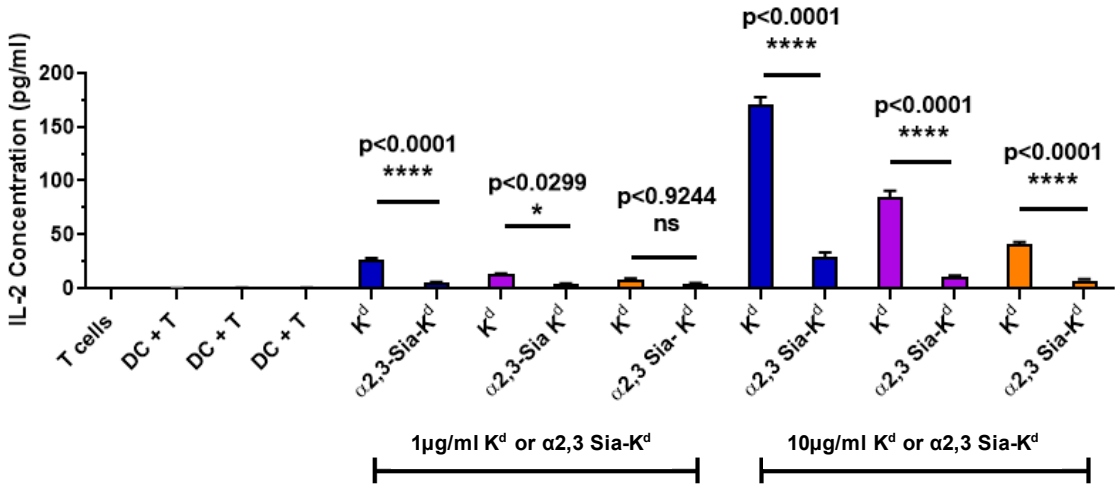
B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150
100
50
0
p<0.0001
****
p<0.0299
*
p<0.9244
ns
p<0.0001
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p<0.0001
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p<0.0001
****
T cells
DC + T
DC + T
DC + T
Kd
α2,3-Sia-Kd
α2,3-Sia Kd
α2,3 Sia- Kd
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10μg/ml Kd or α2,3 Sia-Kd

C

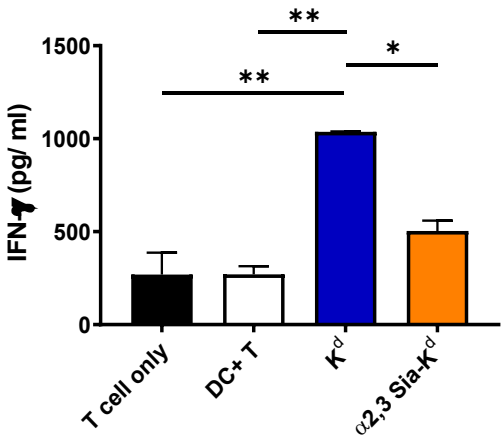
IFNγ (pg/ ml)
1500
1000
500
0
**
**
*
T cell only
DC+ T
Kd
α2,3 Sia-Kd

Figure 3.7. Targeting BM-DC Siglecs with Sia-K^d lead to impaired TCR75 CD4$^+$ T cell proliferation as well as IL-2 and IFN-γ production in vitro. *B6 BM-DCs were pulsed with 1 or 10μg/ml concentrations of either K^d or α2,3 Sia-K^d peptide and co-cultured with CD4$^+$ cells from TCR 75 Rag $^{-/-}$ mice at 1:1, 1:5 or 1:10 DC: T ratios. A. T cell proliferation was assessed on day 3 following the addition of ^{3}H thymidine for the last 18hrs of the culture. Proliferation is expressed as counts per minute (CPM) +/- SD. Data represents one experiment out of 3 performed. Each bar represents a technical triplicate. B. IL-2 cytokine present in culture supernatants 3 days after co-culture was determined using an IL-2 sandwich ELISA. Data is representative for one experiment out of three experiments, +/- SD. C. IFN-γ production of TCR75 CD4$^+$ T cells stimulated with BM-DCs pulsed with either 10μg/ml K^d or α2,3 Sia-K^d peptides at a 1:10 DC:T ratio were assessed using a specific ELISA. Controls included T cells and unpulsed BM-DCs. Each bar is a representative of pooled data from 2 independent experiments, +/- SEM. One-way ANOVA and Tukey's multiple comparisons test was used to compare the means and statistical significance was expressed as follows; p<0.0001****, p<0.001***, p<0.01**, p<0.05*, NS= p>0.05.*

3.3.6 Long construct of α2,3 sialylated alloantigens (α2,3L Sia-K^d) impairs proliferation and IL-2 production of indirect alloreactive CD4$^+$ T cells

We also tested the effect of a longer sialylated alloantigen construct known as α2,3L Sia-K^d which was provided to us later on in the project. We were informed via personal communication with Prof. van Kooyk lab (Vumc), that the longer constructs produced IL-10, these constructs were in the initial stages and required further investigation. BM-DCs were treated with either 10 μg/ml of K^d or α2,3L Sia-K^d and subsequently co-cultured with CFSE labelled TCR75 T cells. Controls were DC and T cell co-culture in the absence of antigen and T cells cultured alone. The BM-DC: T ratio of 1:10 was chosen based on the previous data in *Figure 3.7* where decreased CD4$^+$ T cell proliferation, IL-2 and IFN-γ was found with this ratio. Co-cultured cells were incubated for 3 days, after this time supernatants were harvested for the assessment of IL-2 cytokine

production using an IL-2 specific sandwich ELISA, and proliferation assessed by measuring CFSE dilution using flow cytometry.

Like α2,3 Sia-K^d, the α2,3L Sia-K^d peptide impaired indirect allorecognition *in vitro*. Reduced TCR75 $CD4^+$ T cell proliferation was observed in the presence of α2,3L Sia-K^d pulsed BM-DCs, in comparison to K^d, controls with responses being similarly to the shorter sialylated alloantigen peptide [Fig. 3.7-8]. As expected, T cells produced significantly less IL-2 in the presence of α2,3 Sia-K^d ($p=0.0009$) and α2,3L Sia-K^d ($p=0.0005$) as compared to K^d-treated BM-DCs, with no significant difference in IL-2 production between the sialylated alloantigens being observed ($p=0.9$) [Fig. 3.8B]. Therefore, both the short and long α2,3 Sia-K^d peptides impair indirect alloreactive T cell proliferation and IL-2 production.

A

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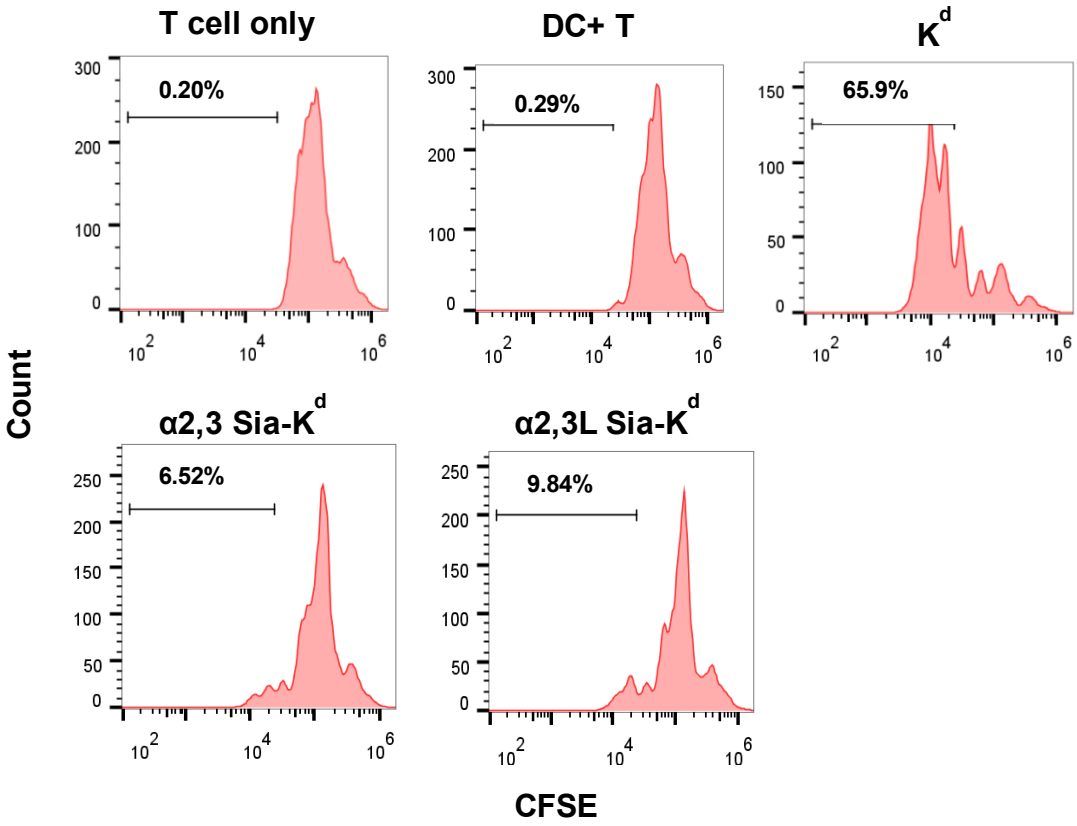
T cell only
DC+ T
K^d
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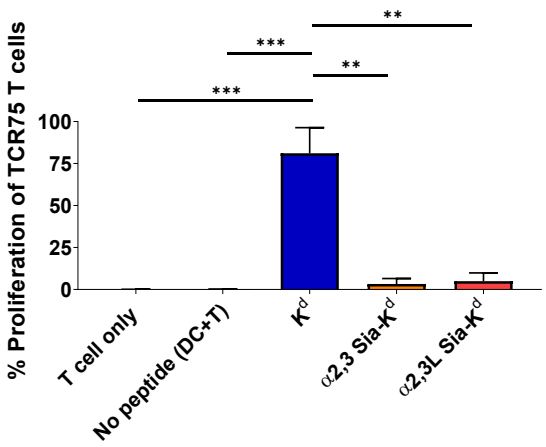
0.29%
65.9%
α2,3 Sia-K^d
α2,3L Sia-K^d
6.52%
9.84%
Count
CFSE

ii


% Proliferation of TCR75 T cells
T cell only
No peptide (DC+T)
K^d
α2,3 Sia-K^d
α2,3L Sia-K^d

B

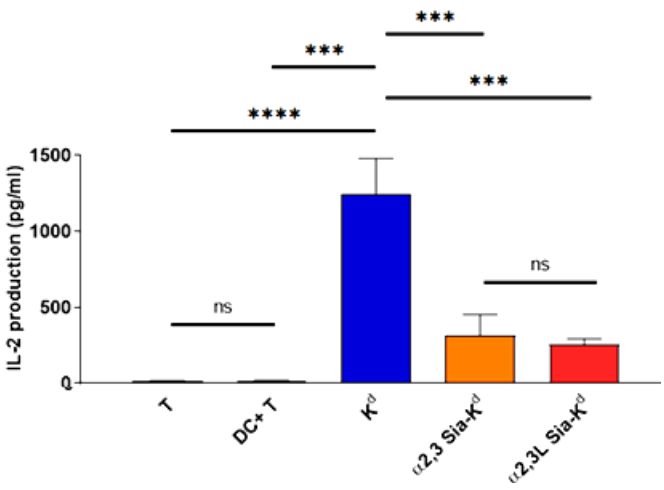

IL-2 production (pg/ml)
ns
ns
T
DC+ T
K^d
α2,3 Sia-K^d
α2,3L Sia-K^d

Figure 3.8. Targeting B6 BM-DCs with α2,3 Sia-K^d and α2,3L Sia-K^d suppressed proliferation of TCR75 CD4$^+$ T cells *B6 BM-DCs were pulsed with 10μg/ml peptide and co-cultured with CFSE labelled TCR75 CD4$^+$ T cells at 1:10 ratio. After 3 days, supernatants were collected and cells were surface stained with CD4 antibody, followed by analysis of CFSE proliferation. A. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on CD4$^+$ T cells that were CFSE$^+$ [Fig. 2.9A]. (i) Histogram plots demonstrate CFSE dilution of CD4$^+$ TCR75 T cells. Data is a representation of one out of three independent experiments. (ii). Histograms represent the mean +/- SEM percentage of T cell proliferation. B. IL-2 production: supernatants were collected in order to determined IL-2 cytokine release using IL-2 sandwich ELISA. Error bars demonstrate +/- SEM. Data was pooled from 3 experiments. Statistical comparisons were performed using One-way ANOVA and Tukey's multiple comparisons. Statistical significance was expressed as follows; p<0.0001****, p<0.001***, p<0.01**, p<0.05*. NS= p>0.05*

3.3.7 Targeting α2,3 or α2,3L Sia-K^d to Siglecs leads to DCs with a greater ability to expand/induce Tregs

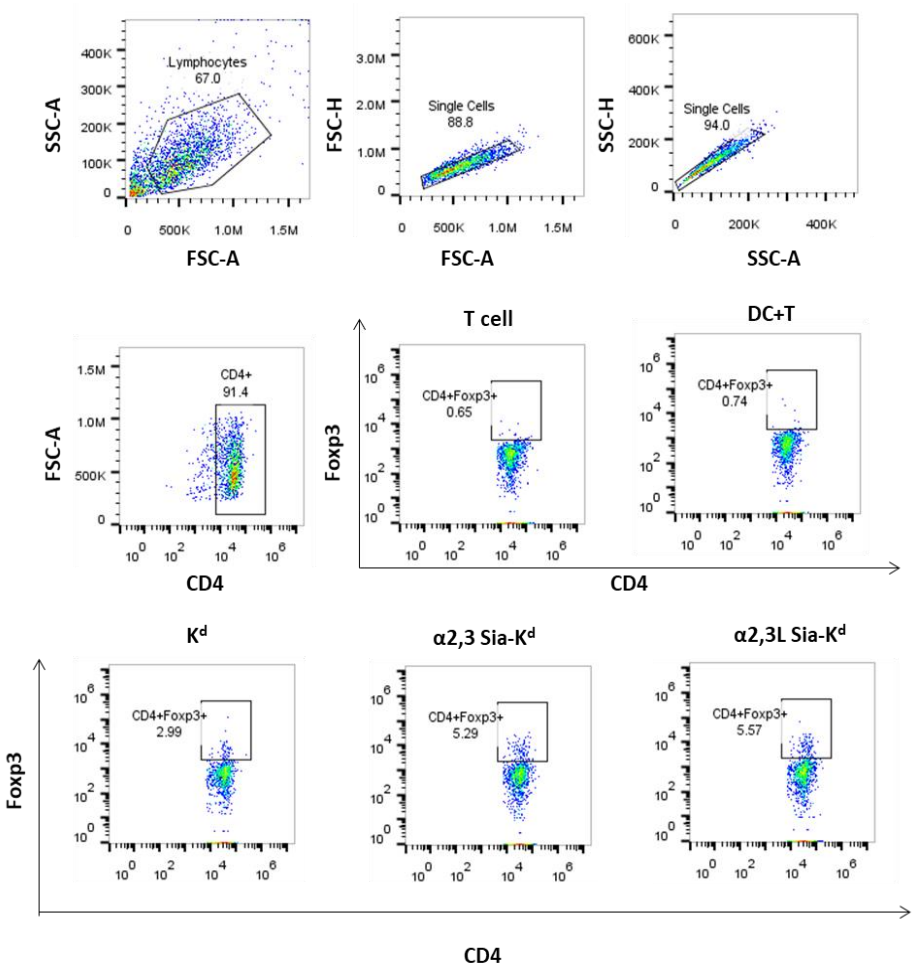
In the previous section we highlighted that targeting Siglecs on DCs can impair CD4$^+$ T cell proliferation and effector cytokine production, confirming previous studies showing that activation through Siglec leads to DCs with a 'tolerogenic' function. One way 'tolerogenic' DCs promote tolerance is through the induction/ expansion of Tregs, as mentioned earlier in *section 1.4.1* (pg. 24), and by Perdicchio *et al.,* (2016), who observed that α2,3 Sia-OVA/ MOG peptide pulsed DCs were capable of inducing/expanding Tregs (Perdicchio *et al*., 2016). This section focuses on whether the hyporesponsive state of TCR75 T cells observed was accompanied by the induction/ expansion of Tregs. TCR75 CD4$^+$ T cells were co-cultured with B6 derived BM-DCs pulsed with either 10 μg of K^d, α2,3 Sia-K^d or α2,3L Sia-K^d in the presence of IL-2. We initially assessed Treg induction using 10μg/ml peptide at 1:5 and 1:10 DC: T ratio, and found that 10ug/ml peptide at 1:10 ratio gave optimal results [Appendix fig. 4]. The percentage of Tregs in the culture was measured 3 days later via flow cytometry using a Foxp3 specific antibody. Like the aforementioned study, it was

observed that more Foxp3$^+$ Tregs were induced/expanded when TCR75 CD4$^+$ T cells were co-cultured with DCs pulsed with either of the Sia-K^d peptides in comparison to no peptide (unpulsed controls) and K^d-treated DCs, however there were no significant differences in Treg levels between the two sialylated alloantigens (p=0.09) [Fig. 3.9B].

T cells co-cultured with sialylated MOG treated- DCs were unable to produce significant levels of IL-10, an immunosuppressive cytokine produced by Tregs, in contrast to native MOG peptide (Perdicchio *et al*., 2016). In order to investigate whether sialylated K^d treated DCs influenced T cells to produce more IL-10, we measured levels of this cytokine from Treg induction co-cultures. In line with Perdicchio *et al*., (2016) findings, there was no significant production of IL-10 between K^d and both α2,3 Sia-K^d treated DC:T cell co-cultures (α2,3 Sia-K^d p=0.9) (α2,3L Sia-K^d p=0.4) [Fig. 3.9C] as compared to T cell only controls. Interestingly, there was a significant increase of IL-10 with K^d (p=0.01) and α2,3 Sia-K^d (p= 0.04) in comparison to DC+ T only control, however, as there were no differences in comparison to T cells, it cannot be concluded that IL-10 is produced in response to alloantigens [Fig. 3.9C].

Taken together with our previous *in vitro* observations, the data demonstrates that treatment of DCs with sialic acid modified alloantigen may lead to a 'tolerogenic' DC phenotype which results in suppression of alloantigen reactive T cells with indirect specificity and induction/expansion of Tregs. We mentioned Treg induction as well as expansion as Tanriver *et al*., (2010), discovered that a small endogenous proportion of CD4$^+$ Foxp3$^+$ Tregs (1.74% Tregs) are present within TCR75 mice, therefore, Treg induction as well as expansion may be of consequence. Overall, this may help to induce tolerance *in vivo* and will be explored in later chapters.

A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CD4+
91.4
FSC-A
CD4
T cell
CD4+Foxp3+
0.65
Foxp3
CD4
DC+T
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0.74
Kd
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2.99
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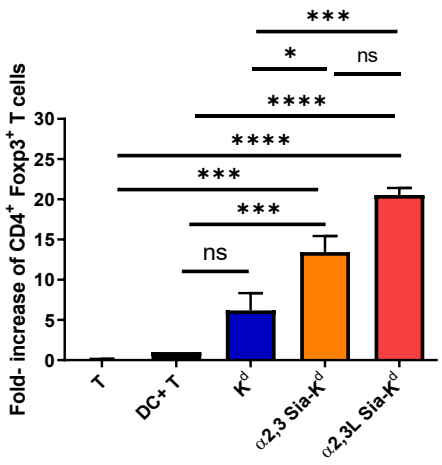
Fold- increase of CD4+ Foxp3+ T cells
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*
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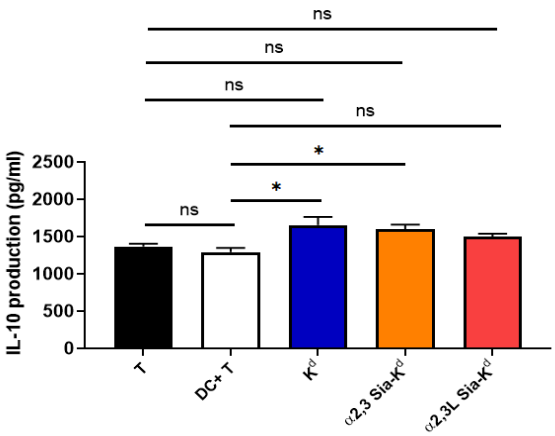
T
DC+ T
Kd
α2,3 Sia-Kd
α2,3L Sia-Kd

C


IL-10 production (pg/ml)
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ns
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*
ns
T
DC+ T
Kd
α2,3 Sia-Kd
α2,3L Sia-Kd

Figure 3.9. α2,3 and α2,3L Sia-K^{d} targeted BM-DCs induced CD4^{+} Foxp3^{+} Tregs in vitro. *B6 BM-DCs were pulsed with 10μg/ml peptide and co-cultured with TCR75 CD4^{+} T cells at 1:10 ratio with subsequent addition of 5U IL-2 at day 0. After 3 days, Foxp3 expression in CD4^{+} T cells was measured following intracellular staining and subsequent flow cytometry. A. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on CD4+ versus Foxp3^{+}. B. The numbers indicate fold- increase of CD4^{+} Foxp3^{+} T cells over DC+ T controls, which was set to a value of 1. C. IL-10 production (pg/ml) as measured using a sandwich ELISA. Data was pooled from three independent experiments. Error bars demonstrate +/- SEM. Statistical comparisons between samples were made using One-way ANOVA and Tukey's multiple comparisons test to assess significance between means of samples. Statistical significance was expressed as follows; p< 0.0001**** p<0.001***, p<0.01**, p<0.05*, NS=p>0.05.*

3.3.8 Immunomodulatory molecules ICOS-L and PDL-1 are not induced following Siglec ligation

Tolerogenic DCs can decrease T cell proliferation through various cell surface marker interactions such as programmed-death-ligand 1 (PD-L1) and inducible T-cell costimulator-ligand (ICOS-L), both of which are highly expressed on DCs (Tuettenberg *et al.*, 2009; Tanaka *et al.*, 2008). Having demonstrated that Sia-K^d treatment impaired BM-DCs ability to activate $CD4^+$ T cell proliferation we measured the expression of the aforementioned molecules to assess whether expression of these inhibitory receptors were involved. BM-DCs were treated for 4 hours with 10µg/ml K^d, α2,3 Sia-K^d, or α2,3L Sia-K^d and negative control DCs remained unpulsed. Cells were then stained with anti-ICOS-L or anti-PD-L1 conjugated antibodies and surface expression was analysed using flow cytometry. The data suggests that there were no significant changes in PD-L1 [Fig. 3.10A] and ICOS-L [Fig. 3.10B] expression following exposure to either Sia-K^d or K^d peptide. Expression of these molecules was the same as untreated controls [Fig. 3.10 A and B].

Taken together, Sia-K^d treated BM-DCs do not mediate impaired T cell proliferation of Tregs induction/expansion via PDL-1 or ICOS-L expression.

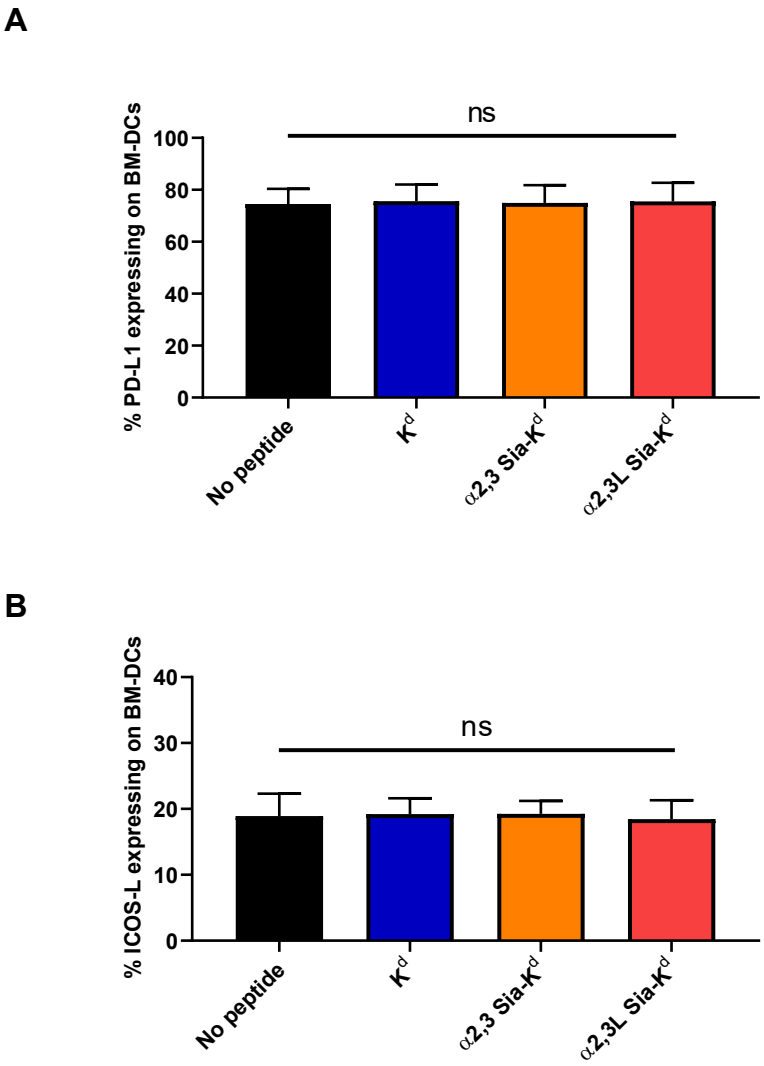


Figure 3.10. Sialylated K^d peptide and Siglec interaction on BM-DCs does not affect ICOS-L or PD-L1 expression. *BM-DCs were pulsed with either K^d, α2,3 Sia-K^d or α2,3L Sia-K^d DC for 4 hours with 10 µg/ml peptide and analysed using flow cytometry. No peptide treated DCs were used as a negative control. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on $CD11c^+$ DCs [Appendix Fig. 5]. Unstained and single stained controls were used as negative controls. A. Percentage of $CD11c^+$ BM-DCs expressing ICOS-L. B. Percentage of $CD11c^+$ BM-DCs expressing and PD-L1. Data was pooled from 3 independent experiments. Statistical comparisons between samples were made using One-way ANOVA and Tukey's multiple comparisons test to assess significance between means of samples. Error bars represent +/- SEM, NS= p>0.05.*

3.4 Targeting B6 B cells with α2,3 and α2,3L sialylated alloantigen

3.4.1 Siglecs expressed on B cells bind to α2,3 Sia-K^d alloantigen

As previously mentioned, B cells are actively involved in allorecognition (Firl *et al*., 2017). Given that these cells also express Siglecs, CD22 and Siglec G, targeting alloantigens to these receptors may facilitate transplant tolerance (Meyer *et al*., 2018). To confirm the presence of the aforementioned Siglecs on resting splenic derived B cells, cells were stained with anti-CD22 or anti-Siglec G antibodies and assessed using flow cytometry. *Figure. 3.11* highlights that resting B cells express Siglec G and CD22, as expected. It should be noted that statistical analysis comparison between B cells Siglec expression could not be performed as CD22 expression staining was conducted once, however, we and several publications have confirmed expression of this Siglec on B cells (Pillai *et al*., 2012; Torres *et al*., 1992; Pfrengle *et al*., 2013; Hutzler *et al*., 2014).

Next to assess the interaction between these Siglecs and the sialylated allopeptide, B cells were incubated with either α2,3 Sia-K^d or control K^d -FITC fluorescent conjugates (10μg/ml for 4 hours at 37°C) before uptake was assessed via flow cytometry. In comparison to control unpulsed B cells both the K^d-FITC and α2,3 Sia-K^d- FITC peptides were capable of binding to B cells [Fig. 3.12]. Although we observed moderate expression of Siglec G and CD22 on B cells, α2,3 Sia-K^d peptide binding to these cells appears to be slightly less efficient than that seen with the BM-DCs in *Figure 3.3A* (1.5 fold increase of α2,3 Sia-K^d than K^d- targeted B cells and 1.7 fold-increase binding of α2,3 Sia-K^d than K^d- targeted BM-DCs). Given the role of transitional B cell subsets in promoting murine allograft survival (Moreau *et al*., 2014), we next assessed which B cell subsets were binding to α2,3 Sia-K^d peptide. B cell subsets were identified using IgM, CD21 and CD23 surface markers (Chung *et al*., 2003) following the gating strategy by Zhang *et al*., (2004); Teague *et al*., (2007) and Ledesma-Soto *et al*., (2012). From the data shown in *Fig 3.13*, α2,3 Sia-K^d and K^d peptide bind to T1 (IgM^{high}CD21$^-$CD23$^-$), T2 (IgM^{high}CD21$^+$CD23$^+$), MZ (IgM^{high}CD21^{hi}CD23$^-$) and FO (IgM^{low}CD21$^+$ CD23$^+$) B cells (Petro *et al*., 2002; Allman and Pillai, 2008; Zouali and Richard, 2011), with increased FITC staining being seen on these cells compared to the no peptide control. No significant

difference in peptide binding preferences was observed between the B cell subsets.

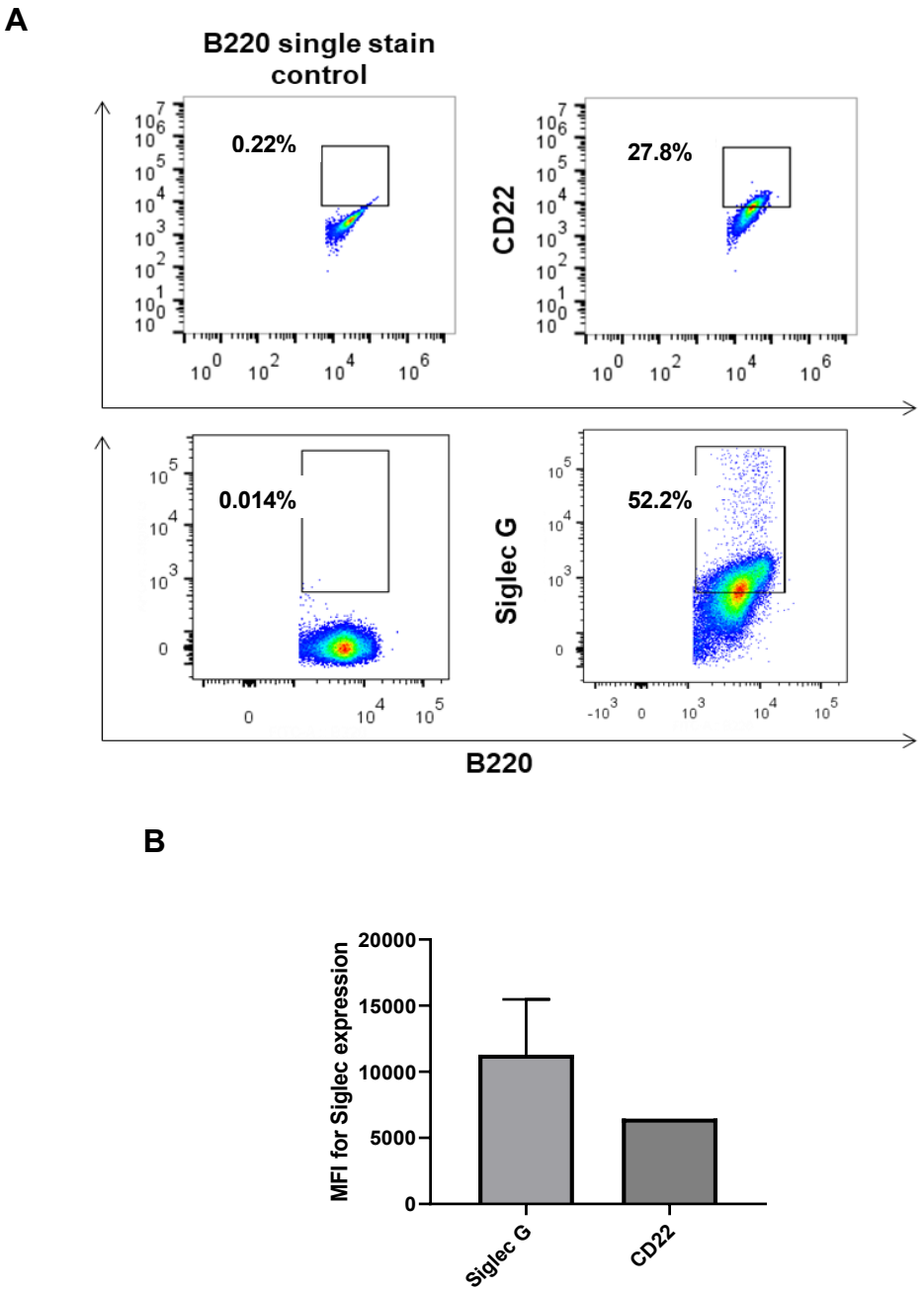


Figure 3.11. Siglec G and CD22 are expressed on B6 B cells. *B cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on B220⁺ B cells [Fig. 2.5]. A. FACS plot demonstrating Siglec G and CD22 expression on B220⁺ B cells and B220-FITC single stained control. B. MFI of Siglec G and CD22 on B220⁺ cells. Siglec G expression was assessed in 3 individual experiments whilst CD22 expression was assessed once.*

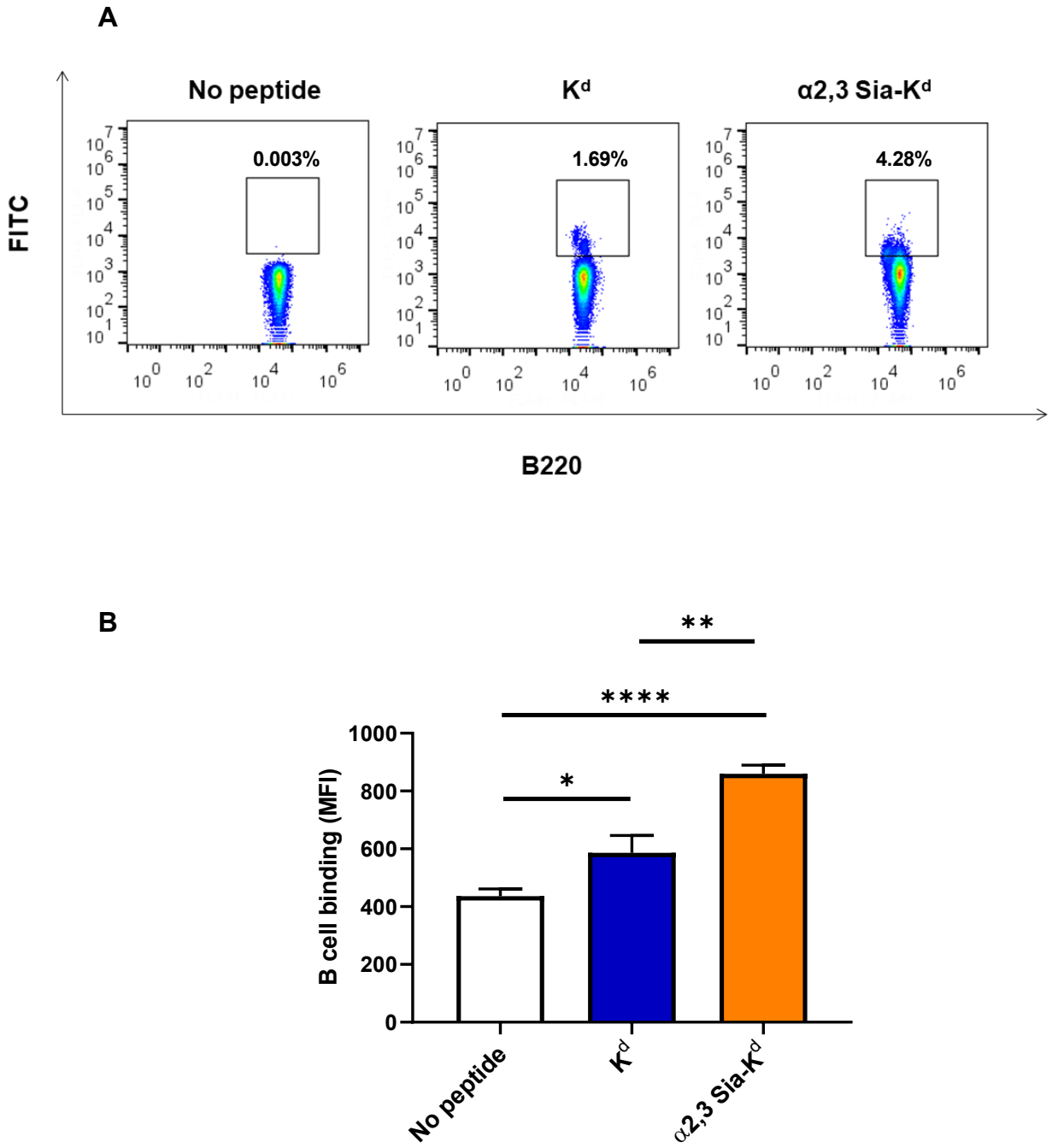
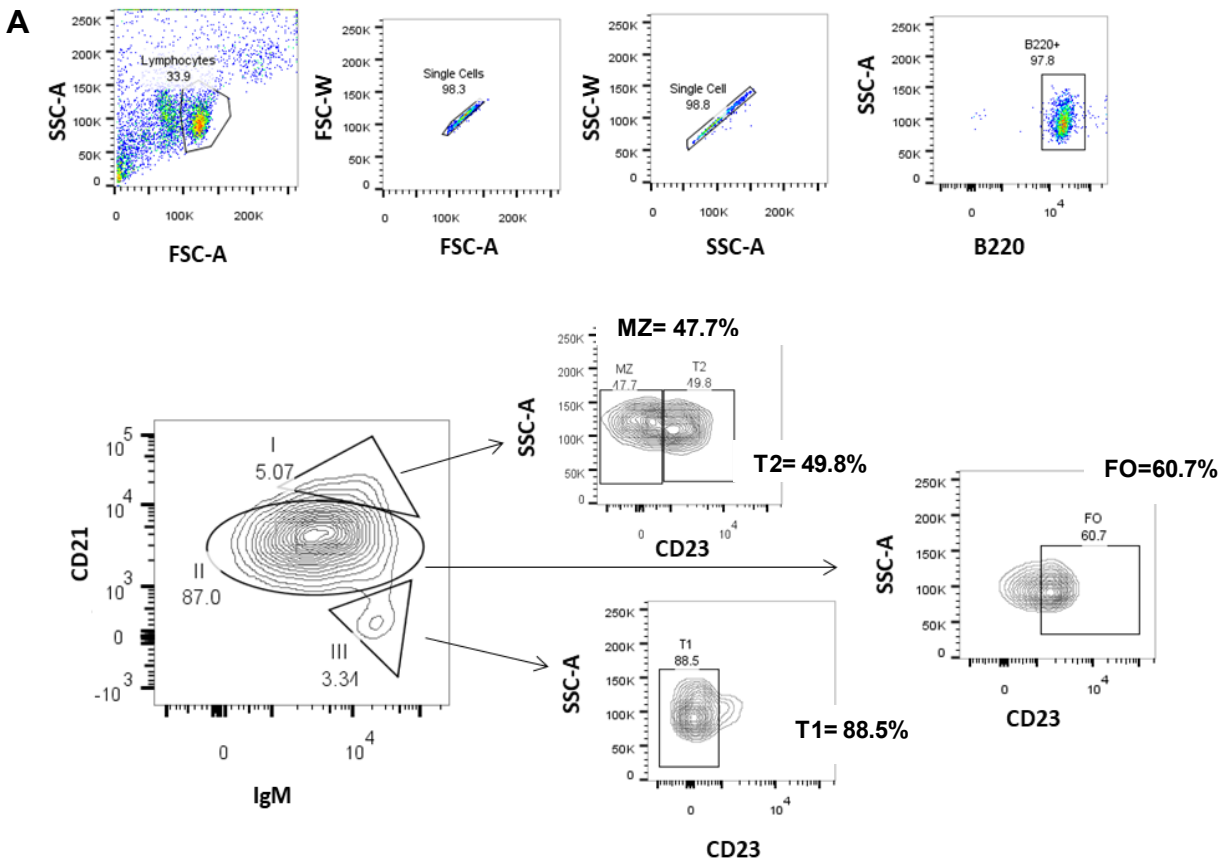


Figure 3.12. α2,3 Sia-K^d binds to Siglec expressing B cells in vitro. *For peptide binding studies, B6 spleen derived B cells were pulsed for 4 hours with either 10 µg/ml K^d -FITC or α2,3 Sia-K^d -FITC. Control (no peptide) B cells were left untreated. A. Live cells were gated on FSC-A vs. SSC-A [Fig. 2.5] and analysed for peptide binding by gating on B220$^+$ cells SSC and measuring the B. MFI of FITC using flow cytometry. Data from 3 independent experiments were pooled and statistically compared using One-way ANOVA and Tukey's multiple comparisons test. Data shown is mean ± SEM. Statistical significance was expressed as follows; p< 0.0001****, p<0.01**, p<0.05.*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FSC-A
Single Cells
98.3
FSC-W
FSC-A
Single Cell
98.8
SSC-W
SSC-A
B220+
97.8
SSC-A
B220
CD21
IgM
I
5.07
II
87.0
III
3.31
MZ= 47.7%
MZ
47.7
T2
49.8
SSC-A
CD23
T2= 49.8%
FO=60.7%
FO
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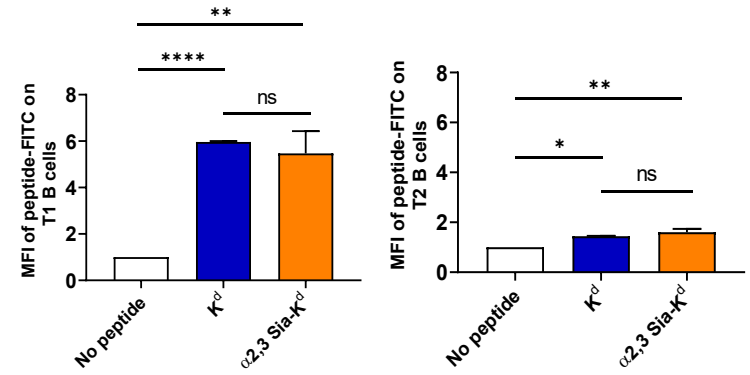
SSC-A
CD23
T1= 88.5%


B
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No peptide
Kd
α2,3 Sia-Kd
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ns
C
MFI of peptide-FITC on T2 B cells
No peptide
Kd
α2,3 Sia-Kd
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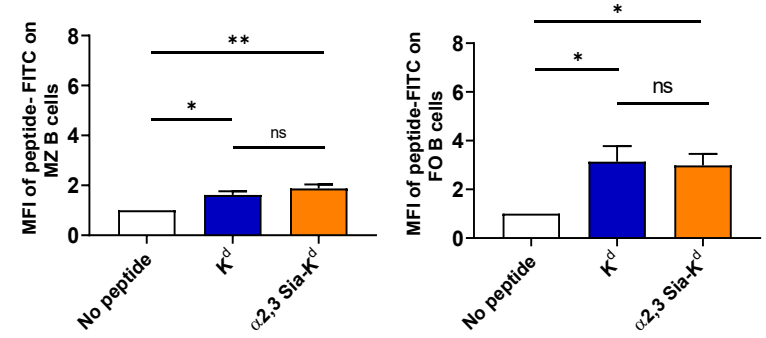
D
MFI of peptide- FITC on MZ B cells
No peptide
Kd
α2,3 Sia-Kd
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*
ns
E
MFI of peptide-FITC on FO B cells
No peptide
Kd
α2,3 Sia-Kd
*
*
ns

Figure. 3.13 Sialylated and non-sialylated alloantigens bind to transitional, MZ and FO B cell subsets *A. Gating strategy for B6 spleen derived B cells and B cell subset analysis determined by staining cells with antibodies to B220, IgM, CD23 and CD21. Live cells were gated on FSC vs. SSC, and B220$^+$ B cells. Data is represented as contour plots and representative of one out of three independent experiments. For peptide binding analysis, B cells were pulsed for 4 hours with either 10 µg/ml K^d -FITC or α2,3 Sia-K^d -FITC. MFI of FITC expression is shown as a fold-change over the no peptide (set to a value of 1). Pulsed B cell subset controls: B. MFI of peptides-FITC bound to T1 B cells. C. MFI of peptides-FITC bound to T2 B cells. D. MFI of peptides-FITC bound to MZ B cells. E. MFI of peptides-FITC bound to FO B cells. Data shown is mean ± SEM and was pooled from 3 independent experiments with statistical comparisons being performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.0001 ****, p<0.01**, p<0.05*. NS = p>0.05.*

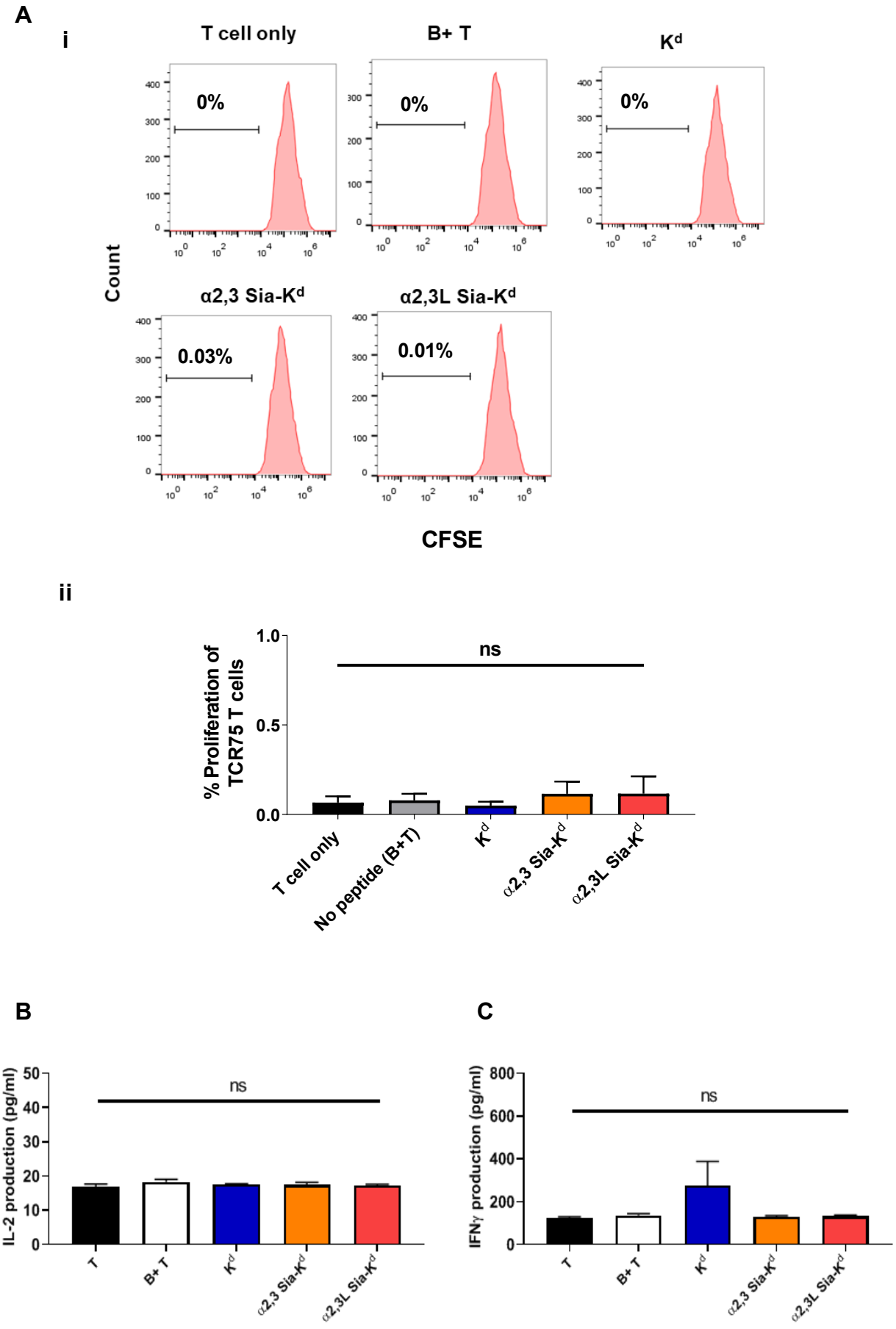
3.4.2 In vitro targeting of K^d peptide, α2,3 Sia-K^d or α2,3L Sia-K^d to B6 B cells does not stimulate proliferation of antigen specific CD4$^+$ T cells

As previously shown (*Section 3.3.5,* pg. 94), reduced proliferation and cytokine release of K^d- specific CD4$^+$ T cells was observed in the presence of BM-DCs pulsed with α2,3 Sia-K^d or α2,3L Sia-K^d peptide. To assess whether targeting Siglecs on B cells using the aforementioned peptides, also reduced T cell responses, B6 B cells were incubated with 10 µg/ml of either the sialylated-alloantigens or control K^d peptide before being co-cultured with TCR75 CD4$^+$ T cells at a 1:10 ratio. Controls included B and T cell co-culture in the absence of peptide and T cells alone. Unlike the strong T cell proliferation observed with K^d pulsed DCs (*section 3.3.5,* pg. 94), B cells treated with K^d or sialylated K^d peptide did not induce CD4$^+$ T cell proliferation suggesting that naïve B cells were unable to activate these T cells [Fig. 3.14 A]. This was supported by a lack of IL-2 or IFN-γ production [Fig. 3.14 B-C].

Despite these findings, the induction/expansion of Tregs by Sia-K^d pulsed B cells was assessed. TCR75 CD4$^+$ T cells were co-cultured with B6 derived B cells pulsed with 10 µg of K^d or α2,3 Sia-K^d or α2,3L Sia-K^d at a 1:10 B:T ratio.

The percentage of Tregs in the culture was measured 3 days later as previously described. No Treg induction was observed in any conditions tested [Fig 3.15A]. Next, IL-10 production was assessed. Surprisingly, TCR75 T cells co-cultured with K^d- pulsed B cells, produced significantly more IL-10 in comparison to T cell only ($p=0.003$) and B and T control, ($p=0.002$) [Fig. 3.15 B]. In comparison to the T cells only control, this was not observed with sialylated alloantigen treated B cells (α2,3 Sia-K^d $p= 0.35$, α2,3L Sia-K^d $p=0.53$), however T cells cultured with B cells pulsed with α2,3 Sia-K^d produced more IL-10 in comparison to B and T control ($p=0.04$) [Fig. 3.15 B]. Overall, T cells released more IL-10 when co-cultured with K^d treated B, in comparison to B cells treated with sialylated alloantigen (α2,3 Sia-K^d $p= 0.01$, α2,3L Sia-K^d $p=0.02$) [Fig. 3.15 B].

Taken together, sialylated or unsialylated alloantigen treated naïve B cells do not stimulate $CD4^+$ T cell proliferation or induce Tregs, however K^d targeted B cell appear to produce more IL-10 in comparison to the negative and sialylated alloantigen-targeted controls.


A
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B+ T
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α2,3L Sia-Kd
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0.01%
CFSE
ii
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ns
T cell only
No peptide (B+T)
Kd
α2,3 Sia-Kd
α2,3L Sia-Kd
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IL-2 production (pg/ml)
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Kd
α2,3 Sia-Kd
α2,3L Sia-Kd
C
IFNγ production (pg/ml)
ns
T
B+ T
Kd
α2,3 Sia-Kd
α2,3L Sia-Kd

Figure 3.14 Targeting alloantigens to B6 B cells do did not stimulate alloantigen-specific CD4$^+$ T cells proliferation or IL-2 and IFN-gamma production*. B6 B cells were pulsed with 10µg/ml peptide and co-cultured with CFSE labelled TCR75 CD4$^+$ T cells at 1:10 ratio. After 3 days, supernatants were collected and cells surface stained with anti-CD4-APC antibody. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on CD4$^+$ T cells and CFSE$^+$ dilution assessed [Fig. 2.9B]. A. (i) Histogram FACS plots demonstrating the percentage of CD4$^+$ TCR75 T cells proliferation. Data is a representation for one out of three independent experiments. (ii) Percentage proliferation of TCR75 T cells in response to targeted B cells. Error bars demonstrate +/- SEM pooled from 3 independent experiments. B. IL-2 (pg/ml) and C. IFN- γ (pg/ml) present in culture supernatants were analysed for using either an IL-2 or IFN- γ specific sandwich ELISA. Data represent mean ± SEM cytokines from 3 experiments and statistical analysis was performed using One-way ANOVA and Tukey's multiple comparisons test. NS = p>0.05.*

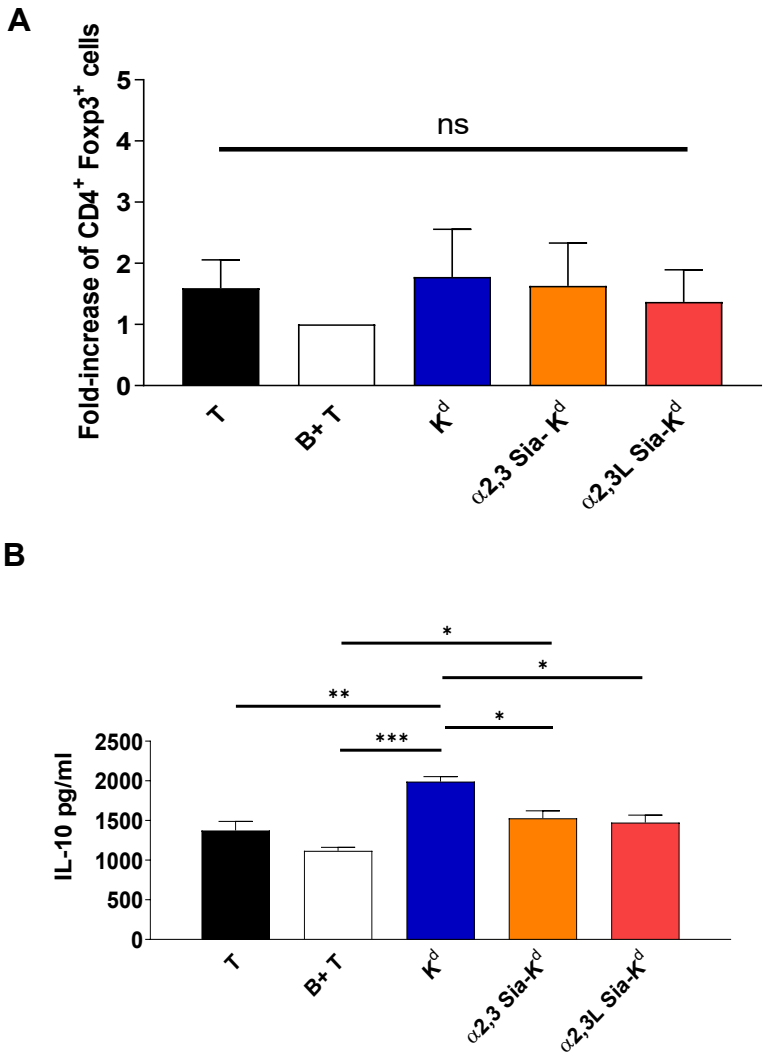

Figure 3.15. α2,3 Sia-K^d targeted B cells do not induce CD4$^+$ Foxp3$^+$ Tregs in vitro. *B6 B cells were pulsed with 10μg/ml peptide and co-cultured with TCR75 CD4$^+$ T cells at 1:10 ratio with subsequent addition of 5U IL-2 at day 0. After 3 days, expression of Foxp3 was measured by intracellular antibody staining and subsequent flow cytometry analysis. A. Fold-change of CD4$^+$ Foxp3$^+$ T cells in comparison to B+T controls (set to a value of 1). B. IL-10 release from T cells co-cultured with pulsed/unpulsed B cells was assessed using a IL-10 specific ELISA. Data is a representative of three pooled experiments. Statistical comparisons were made using a One-way ANOVA and Tukey's multiple comparisons test to determine statistical significance between the means, error bars present +/- SEM p<0.001***, p<0.01**, p<0.05*, NS= p>0.05.*

3.5 Targeting B6 macrophages with α2,3 and α2,3L sialylated alloantigen

3.5.1 Targeting alloantigen to Siglec-expressing macrophages reduced IL-12 production in presence of LPS

Aside from DCs and B cells, macrophages also play a role in transplant rejection. These cells express Siglecs (Pillai *et al.*, 2012) such as Siglec F, which was the predominant Siglec expressed on M-CSF expanded BM-derived macrophages (BM-DM) [Fig 3.16A]. However they also express CD169 and low levels of Siglec E and H, as assessed using flow cytometry [Fig 3.16A]. Like the aforementioned cells in this chapter, both K^d and α2,3 Sia-K^d FITC peptides bind to macrophages [Fig. 3.16B]. Surprisingly, we noticed that K^d peptide, and not α2,3 Sia-K^d, preferentially binds to macrophages, although not significant, which is opposite to what we have observed with DCs and B cells [Fig. 3.16B] [Fig.3.12] [Fig.3.3 A-B]. This was quite an interesting finding given that Siglec expression between DCs and macrophages were quite similar. It would be interesting to determine whether the same Siglecs expressed on different APCs, have a particular binding affinity/strength to different structures of sialic acids.

To determine whether targeting Siglecs on macrophages with α2,3 Sia-K^d or α2,3L Sia-K^d could render them refractory to LPS stimulation, macrophages were pulsed with α2,3 Sia-K^d, α2,3L Sia-K^d or K^d peptides whilst control cells were left untreated [Fig. 3.16C]. After 4 hours, cells were treated for 24 hours with 200 ng/ml of LPS. Supernatants from the cultures were collected and IL-12, which is produced by proinflammatory macrophages, was measured using a sandwich ELISA. Without LPS stimulation, no differences in the production of IL-12 were observed between the control and peptide targeted BM-DMs [Fig. 3.16C]. Following LPS stimulation, untreated (no peptide) BM-DMs ($p=0.0003$), BM-DMs treated with K^d ($p=0.04$) and α2,3L Sia-K^d ($p=0.04$) produced more IL-12 in response to LPS stimulation, however, this was not observed in α2,3 Sia-K^d pulsed macrophages ($p=0.25$) [Fig 3. 16C]. This would suggest that α2,3 Sia-K^d targeted macrophages may be resistant to LPS maturation [Fig. 3.16C]. Amongst the LPS-treated BM-DMs, there was also a general decrease in IL-12 production for peptide targeted BM-DMs in comparison to no peptide-treated BM-DMs. [Fig.3. 16C].

Overall, Siglec-expressing macrophages can bind to sialylated alloantigen leading to modification of their cytokine production following LPS stimulation in the case of α2,3 Sia-K^d. Whether these targeted macrophages can impair indirect alloreactive T cell proliferation and promote Treg induction *in vitro* remains to be determined.

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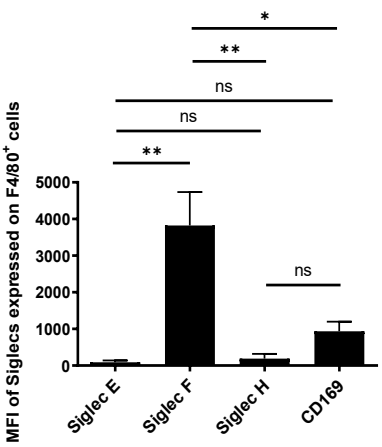
MFI of Siglecs expressed on F4/80+ cells
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Siglec E
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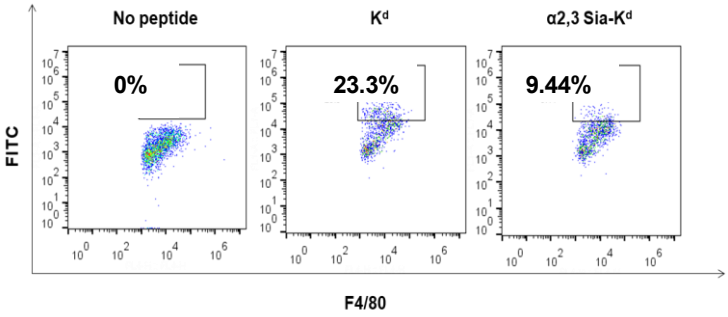
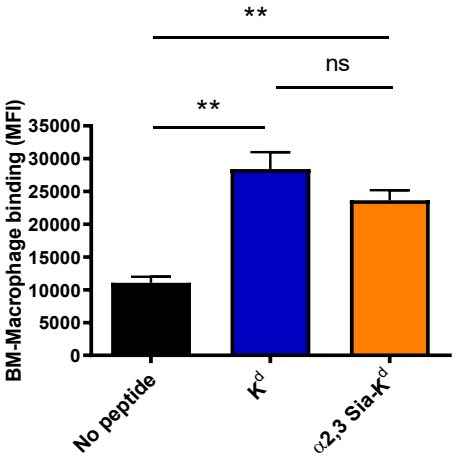
Siglec H
CD169

B

i


No peptide
Kd
α2,3 Sia-Kd
0%
23.3%
9.44%
FITC
F4/80

ii


BM-Macrophage binding (MFI)
35000
30000
25000
20000
15000
10000
5000
0
**
ns
**
No peptide
Kd
α2,3 Sia-Kd

C

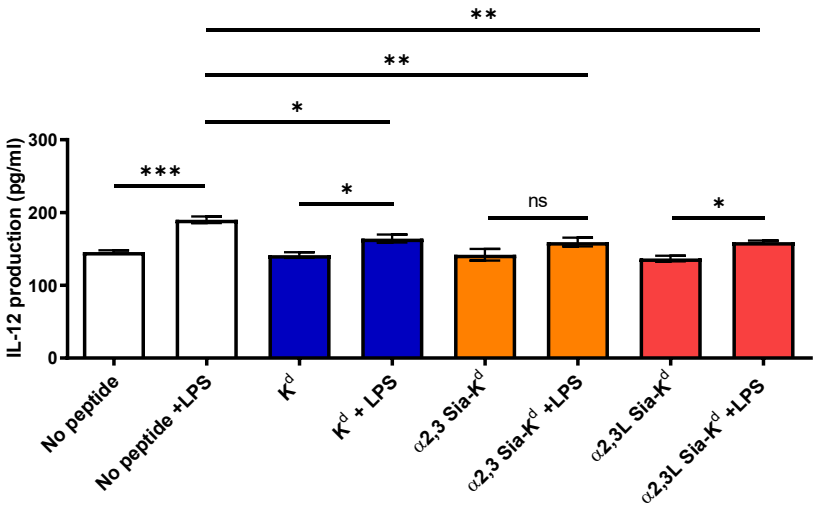

IL-12 production (pg/ml)
300
200
100
0
**
**
*
***
*
ns
*
No peptide
No peptide +LPS
Kd
Kd + LPS
α2,3 Sia-Kd
α2,3 Sia-Kd +LPS
α2,3L Sia-Kd
α2,3L Sia-Kd +LPS

Figure 3.16. Sia-K^d binds to Siglec expressing macrophages in vitro. *A. Siglec expression on F4/80$^+$ BM-DMs was assessed via flow cytometry using antibodies specific to mouse Siglec E, F, H and CD169. Live cells were gated on forward scatter (FSC), side scatter (SSC) [Fig. 2.4]. B. For peptide binding studies, B6 BM-DMs were pulsed with either 10 µg/ml K^d -FITC or α2,3 Sia-K^d -FITC and (i) peptide binding assessed by gating on F4/80$^+$ cells and measuring the MFI of FITC expression using flow cytometry. Controls cell were untreated BM-DMs.(ii) Each bar represent pooled data for MFI of FITC and error bars demonstrate +/- SEM, n=3. C. IL-12 production (pg/ml) produced by day 6 BM-DMs pulsed with 10 µg/ml of the various peptides shown in the presence or absence of 200 ng/ml LPS for 24 hours. Unpulsed (no peptide) served as negative control. Each bar represents pooled cytokine data from 3 independent experiments. Statistical comparisons were made using a One-way ANOVA and Tukey's multiple comparisons test. Data shown is mean ± SEM. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*. NS= p>0.05.*

3.6 Discussion

The main focus of this chapter was to determine whether α2,3 Sia-K^d or the longer α2,3 Sia-K^d constructs (α2,3L Sia-K^d) target Siglecs expressed on DCs, B cells and macrophages and whether this interaction can modulate indirect allorecognition *in vitro*. The data in this chapter suggests that targeting these Siglec receptors using sialylated alloantigens modifies both innate and adaptive immunity leading to a hyporesponsive environment.

We initially addressed whether Siglecs were expressed on DCs, B cells and macrophages and confirmed that Siglec F, CD169 and Siglec E were predominantly expressed on BM-DCs, SPLN- DCs and BM-DMs and Siglec G and CD22 were expressed on B cells (Pillai *et al*., 2012). In fact, we found that Siglec F was highly expressed on the aforementioned DCs and macrophages. Currently, there are very few publications demonstrating Siglec F expression and the functional role of this receptor on BM-DCs. Siglec F expression is mostly associated with macrophages present in the lung and eosinophils (Feng & Mao, 2012; Zhang *et al*., 2004). It has been reported that Siglec F is expressed on BM-DCs, SPLN-DCs as well as BM-DMs, however the authors did not present this data (Tetano *et al*., 2007). One study confirmed expression of Siglecs E on both murine splenic DC and macrophages, but did not observe Siglec F expression on either B6 or BALB/c cells (Zhang *et al*., 2004). Similarly to their study, Siglec expression on cells derived from B6 mice which were housed under SPF-free conditions was assessed using flow cytometry and we also confirmed Siglec E expression on macrophages and splenic DCs. It remains uncertain as to why Siglec F expression is varied amongst studies, possibly due to the varied methodologies for generating *ex vivo* expanded BM-DCs.

We did not assess Siglec G expression on BM-DMs, however according to previous literature, we expect our macrophages to also express Siglec G (Chen *et al*., 2014). In line with previous literature we were able to confirm that Siglecs CD22 and Siglec G were expressed on splenic B cells (Meyer *et al*., 2018; Müller & Nitschke, 2014). We did not examine expression of these Siglecs on B cell subsets, however Zhang *et al*., (2004) confirmed their expression, using

flow cytometry, on transitional and MZ/ FO B cells subsets. These authors demonstrated low but detectable expression of Siglec E on T2 and MZ B cells, while Siglec E expression on T1 B cells were negligible. Siglec F was not detected in any of the B cells subsets. It has yet to be determined whether Siglec E receptor has any functional role on B cells, but the former study did observe that Siglec E levels remained unchanged when B cells were stimulated *in vitro* (findings were not published) (Zhang *et al*., 2004). As future work, Siglec E levels could be assessed on B cells subsets to confirm Zhang *et al*., (2004) study.

CD169 is a Siglec expressed on murine macrophages (van Dinther *et al*., 2018), and has also been reported to be expressed on human DCs, (Puryear *et al*., 2013). We confirmed CD169 expression on BM-DMs and surprisingly on BM-DCs. This raises the question of the phenotype similarity of *ex vivo* generated BM-DMs and BM-DCs. Both cells are known to express CD11c; however F4/80 is predominantly expressed on macrophages. We also observed CD169 expression on SPLN-DCs, therefore, by gating on $CD11c^+$ cells, it may be a possibility that macrophages are being pooled within the DC population during analysis; this could explain CD169 expression detected. Others have also questioned the purity of GM-CSF expanded BM-DCs. Helft *et al*., (2015) published phenotype data of GM-CSF expanded murine BM-DCs and concluded that these cells were comprised of a heterogeneous population of $CD11c^+$ MHC II^+ DC and macrophages. There are several distinctions in the preparation of BM-DCs culture between the current and aforementioned study, one being that in our study, BM cells were initially depleted of $B220^+$, MHC II^+, $CD4^+$ and $CD8^+$ cells prior to culture with GM-CSF, this was not mentioned in Helft *et al*., (2015) study. Additionally, our BM cells were cultured in DC media which was fully replenished on day 2 and 4 as opposed to the protocol in Helft *et al*., (2015). Therefore, given the differences in the protocols used, it cannot be fully confirmed that our *ex-vivo* expanded BM-DCs comprise of a heterogeneous population of DCs and macrophages, and therefore expression of CD169 on murine BM-DCs may be a novel finding to this study. As future work, $F4/80^+$ cells can be measured using flow cytometry to determine purity of this study's *in vitro* generated BM-DCs.

In addition, α2,3 Sia-K^d or α2,3L Sia-K^d alloantigens targeted to BM-DCs were unable to stimulate TCR75 T cell proliferation or effector cytokines IL-2 and IFN-γ production in comparison to K^d- pulsed BM-DCs. Sialylated alloantigen treated BM-DCs were dominant at inducing/ expanding $Foxp3^+$ Tregs, this finding was considerably opposite to what was observed with Sia-K^d treated B cells. Unlike BM-DC targeting, B cells were not able to stimulate T cell proliferation or Treg induction/ expansion; however, there was an increase of IL-10 released from TCR75 T cells cultured with K^d-pulsed B cells.

A study by Perdicchio *et al.*, (2016) confirmed that sialylated OVA protein antigen had the capability of binding to Siglec E expressed on BM-DCs. In their study, they were able to confirm a decrease in sialylated antigen binding to Siglec $E^{-/-}$ DCs in comparison to Siglec E expressing WT DCs, whereas no significant difference in binding was observed between the Siglec $E^{-/-}$ and WT DCs when non-sialylated antigen was tested (Perdicchio *et al.*, 2016). In fact, these authors observed that there was preferential binding of sialylated antigen to B6 BM-DCs in comparison to non-sialylated antigen. In keeping with their data, we also observed that α2,3 Sia-K^d preferentially binds to Siglec-expressing BM-DCs and SPLN-DCs in comparison to K^d. However we did not assess whether this was via Siglec E or the other Siglecs found- this can be addressed as future work using Siglec KO mice. Given the differential binding between K^d and α2,3 Sia-K^d, the data suggests that the K^d peptide may have the ability to bind to MHC molecules whereas α2,3 Sia-K^d, may be binding to both the MHC and Siglec receptors.

We extended the work of Perdicchio *et al.,* (2016) and assessed the binding of α2,3 sialylated peptide antigen to Siglecs expressed on B cells and macrophages. α2,3 sialylated alloantigens were preferentially expressed by BM-DCs and B cells in comparison to the unsialylated equivalent, however, this was not the case with BM-DMs. This was an intriguing observation given that Siglec expression found on DCs and macrophages were similar. This may possibly suggest that Siglecs expressed on different APCs, have a particular binding affinity/strength to different structures of sialic acids. It appears that the

level of α2,3 Sia-K^d binding may also correlate with the Siglec expression. It was suggested in a review that Siglec E binds moderately to α2,3 and α2,6 sialic acids, whereas Siglec F binds moderately to α2,3 sialic acids but weakly to α2,6 sialic acids (Crocker *et al.*, 2007). In addition, Siglec G also has the ability to bind to both linkages of sialic acids (Chen *et al.*, 2014), but in order to fully confirm which Siglecs the peptides are binding to it would be important to utilise Siglec E, F and G knock-out mice or use blocking antibodies.

As mentioned in Chapter 1 (*section 1.4.1.1*, pg. 25) DCs can be rendered 'tolerogenic' in a number of ways, Tol-DCs generally express low levels of MHC and co-stimulatory molecules, inhibitory receptors and produce anti-inflammatory cytokines. Interestingly, treatment with sialylated alloantigen but not alloantigen resulted in the down-regulation of MHC molecules and no increase in PD-L1 and ICOS-L, suggesting that PD-L1 and ICOS-L receptors are not modulated following α2,3 sialylated alloantigen targeting. It is known that DCs express sialic acids on their surface where they are conveniently placed for interactions with receptors such as Siglecs (Silva *et al.*, 2016). One study demonstrated the importance of sialic acids and tolerogenic DCs; by removing sialic acids on the surface of DCs using sialidase, murine splenic DCs became mature (Silva *et al.*, 2016). Their study also compared expression of MHC I/II and CD80/86 on splenic DCs and discovered that DCs treated with sialidase had increased expression of these molecules (Silva *et al.*, 2016). This would suggest that sialic acids are required for the maintenance of tolerogenic quiescent DCs and this would support our findings where MHC I and II molecules were down-regulated upon sialylated alloantigen targeting. Unlike Silva *et al.*, (2016) study, we did not notice any changes in CD80/86 expression, however, differences in CD80/86 were only noticed following LPS treatment. Our findings are somewhat in line to Perdicchio *et al.*, (2016) study, where they observed no changes of co-stimulatory marker expression as a consequence of Sia-antigen uptake by DCs- although their study did not publish this data but did mention this in their discussion. They still found that Sia-antigen –treated DCs were able to promote tolerance *in vitro* and *in vivo* despite DCs not having the typical 'tolerogenic profile' (Perdicchio *et al.*, 2016).

Heavily sialylated pathogens down-regulate pro-inflammatory immune responses following interaction with Siglecs- this is their mechanisms to evade the host's immune systems for their survival. For example, sialylated *Trypansoma cruzi* are able to evade the host immune system through interaction with Siglec E expressing DCs leading to significant reduction in IL-12 (Erdmann *et al*., 2009). This inhibitory effect was reversed when *T.cruzi* was desialylated (Erdmann *et al*., 2009). Unlike the former study, we observed no differences in this pro-inflammatory cytokine production from BM-DCs in response to sialylated alloantigen. It is a possibility that IL-12 is not the correct readout for assessing sialylated antigen targeted DC's capacity to become refractory to LPS stimulation. One study demonstrated that sialylated OVA antigens targeted to DCs under LPS stimulation were able to impair proinflammatory cytokines such intracellular TNF-α and IL-6 (Perdicchio *et al*., 2016). In fact previous literature states that Siglec $E^{-/-}$ BM-DCs stimulated with *E.Coli* LPS, sustained high levels of TNF-α and IL-6, suggesting that these cytokine levels are modulated via Siglec E (Wu *et al*., 2016). Therefore and as a future investigation TNF-α and IL-6 could be assessed.

It was recently demonstrated that the sialylated structures on the flagellum derived from *Campylobacter jejuni*, were able to engage with Siglec 10 on host human monocyte-derived DCs and Siglec G on murine BM-DCs (Stephenson *et al*., 2014). This interaction led to a profound increase of IL-10 mediated by MyD88 and p38 MAPK signalling, which supported an anti-inflammatory environment favouring the survival of *C.jejuni* within the host (Stephenson *et al*., 2014). Therefore these studies demonstrate that targeting Siglecs on DCs that are stimulated via TLRs, are able to dampen proinflammatory immune responses. It appears that Siglec G and Siglec E have a role in controlling IL-10 and IL-12 from DCs in response to TLR signalling, however, our results demonstrate quite low levels of Siglec E and Siglec G expression on BM-DCs. Therefore, this may explain the little difference in IL-12/ IL-10 release when Siglecs are engaged with Sia-K^d and stimulated with LPS. On the other hand, Stephenson *et al*., (2014) and Erdmann *et al*., (2009) studies are based on sialylated pathogens and their interactions with DCs, which is significantly different to the current study (targeting DCs with sialylated alloantigens and LPS derived from *E.coli*). First of all, in the current study BM-DCs were treated with

peptide for 4 hours prior to LPS stimulation which lasted for 24 hours, as opposed to Stephenson *et al.*, (2014), where cytokines were measured 8 hours post infection. The next issue is that the "stimulatory capacity' of the LPS used is questionable, given that MHC molecules were not up-regulated in response to LPS, as a result we cannot conclude that DCs were fully activated in response to the LPS used.

Boyd *et al.*, (2009), demonstrated that inhibitory receptors Siglec E present on macrophages dampens TLR signalling as a result of NF-*κ*B attenuation in the presence of LPS stimulation and Siglec E cross-linking Ab targeting. NF-kB is a transcription factor that is upregulated following TLR4 activation, this results upregulation of genes encoding for proinflammatory cytokines such as TNF-α or IL-6 (Zhang and Ghosh, 2001). We have seen that BM-DMs treated with α2,3 Sia-K^d and LPS, released less IL-12 in comparison to BM-DMs treated with LPS alone, a finding similar to all other peptide plus LPS treated BM-DMs. However, the difference is that BM-DMs targeted with α2,3 Sia-K^d alloantigen and LPS, displayed similar low levels of IL-12 in comparison to BM-DMs treated with α2,3 Sia-K^d alone, which was not observed in the other peptide treated BM-DMs. Therefore, α2,3 Sia-K^d targeted macrophages can suppress IL-12 production under LPS stimulation. What surprising was that unlike α2,3 Sia-K^d, BM-DMs treated α2,3L Sia-K^d and LPS displayed increased levels of IL-12 in comparison to BM-DMs treated with α2,3L Sia-K^d alone . This finding reflects what was found by Nagala *et al.*, (2018), where no significant role of Siglec E in TLR4 signalling was identified in response to LPS. To fully confirm this, other read-outs may be necessary such as NF-*κ*B signalling given Boyd *et al.*, (2009) findings.

Targeting antigens to specific cell surface receptors on DCs has been shown to dampen antigen specific effector T cell responses through T cell deletion or anergy as well as inducing/ expanding Tregs. For example, $DCIR2^+$ DCs targeted using an anti-DCIR2 antibody fused to MOG peptide promoted tolerance through expansion of antigen specific Tregs (Tabansky, *et al.*, 2018). Chappell *et al.*, (2014) showed when targeting antigen to blood dendritic cell antigen 2 (BDCA2) expressed on plasmacytoid DCs led to impaired antigen-

specific $CD4^+$ T cell proliferation even following antigen re-challenge (Chappell, *et al*., 2014). Therefore, it was not surprising that targeting Siglecs on BM-DCs with α2,3 Sia-K^d and α2,3L Sia-K^d would lead to impaired K^d- alloantigen specific T cell (TCR75 $CD4^+$ T cells) responses and the induction/expansion of Tregs. As mentioned before, the $CD4^+$ T cell indirect alloresponse plays a vital role in chronic-mediated rejection (Ali *et al*., 2015). $CD4^+$ T cells from TCR75 $Rag^{-/-}$ mice were especially useful at determining whether targeting Siglecs impaired effector T cells responses that contribute to chronic rejection mediated by indirect allorecognition. Using these TCR75 $CD4^+$ T cells as a model for indirect allorecognition, our data provides strong support to a study by Perdicchio *et al.,* (2016), where they demonstrated that targeting SPLN-DCs with sialylated OVA protein and MOG peptide impaired antigen specific T cell proliferation and effector cytokine production and induce antigen-specific Tregs. It should be taken to account that the aforementioned study identified their findings by targeting Siglecs on SPLN-DCs, whereas we identified similar T cell responses by targeting BM-DCs. Therefore, the data is this chapter can be extrapolated to the findings of Perdicchio *et al*., (2016) study. It was found in this study following α2,3 sialylated alloantigen targeting, IL-10 production was increased but at low levels, this may be due to the lack of increased ICOS-L levels of Sia-K^d treated BM-DCs (Tuettenberg *et al*., 2009). In addition, IL10-producing Tr1 cell may not contribute significantly to the population of Tregs that have been induced.

B cells are also known for their ability to present alloantigen indirectly to T cells as demonstrated by Reichardt *et al*., (2007), who found that pulsing naïve B cells with OVA antigen *in vitro* promoted the induction of antigen specific Tregs (Reichardt *et al*., 2007). In the peptide binding experiments for this study, it was observed that all the alloantigen peptides (sialylated and un-sialylated) were capable of binding to T1, T2, MZ and FO B cells subsets with no significant differences in binding between the peptides. This may be a possible result of the α2,3 Sia-K^d peptide binding to the MHC instead of the Siglec. B cell targeted with alloantigens did not stimulate T cell proliferation or effector cytokine production. $CD4^+$ $Foxp3^+$ Tregs were not induced/ expanded, however T cell co-cultured with K^d-targeted B cells produced the most IL-10. An explanation to this outcome may be due to sialic acids linkage that is being targeted to the B cells,

as Siglec CD22 preferably binds to α2,6 Sia and Siglec G has the ability to bind to α2,6 sialic acids, we will address whether the former targeting will provide a more substantial immune response in the next chapter.

Overall, targeting Siglec-expressing DCs with α2,3 Sia-K^d or α2,3L Sia-K^d impairs $CD4^+$ T cell proliferation, effector cytokine production, and induction $CD4^+$ $Foxp3^+$ Tregs, this was not observed when Siglecs were targeted on B cells. An explanation as to why α2,3 Sia-K^d and α2,3L Sia-K^d targeted DCs have an increased capability to induce Tregs in comparison to targeted B cells, could be that B cell Siglec CD22 can sometimes be 'masked' by other α2,6 sialylated ligands present on the surface of the same cell in *cis* interaction. This therefore limits their availability to bind to other ligands such as our α2,3 Sia-peptide (Razi and Varki, 1998), however this requires further investigation. It is not yet established as to whether Siglec G has a similar 'masking' mechanism.

The next chapter will focus on targeting DCs, B cells and macrophages with α2,6 sialylated alloantigens. As mentioned earlier in Chapter 1 (*section 1.5*, pg. 38), certain Siglecs expressed on different cells have preference for sialic acids linkages, for example Siglec E binds to α2,3 Sia and α2,6 Sia, Siglec F prefers to bind to α2,3 Sia and CD22 only binds to α2,6 Sia (Crocker *et al.*, 2007). Targeting using different sialic acids linkages will be appeal to a larger variety of Siglecs expressed on different cells, thus increasing the potential to hit various inhibitory receptors on DCs, B cells and macrophages.

CHAPTER 4:

TARGETING DCs WITH α2,6 SIA-K^d ALLOANTIGEN PEPTIDES LEADS TO REDUCED ALLOGENEIC RESPONSES *IN VITRO*

4.1 Introduction

As described in Chapter 3, Siglecs F, E, CD169 and G were expressed on DCs and macrophages, whilst CD22 and Siglec G were found on B cells. Also mentioned previously, these Siglec receptors have a specificity for certain linkages of sialic acids such as α2,3 and/or α2,6 or α2,8-linked sialic acids. For example, Crocker *et al*., (2007), suggested that Siglec E has moderate binding to both α2,3 and α2,6 sialic acids, whilst Siglec F has a preference for α2,3 sialic acids, CD22 binds to α2,6 sialic acids and Siglec G has the capacity to recognise α2,3 and/or α2,6 sialic acids (Crocker *et al*., 2007; Pillai *et al.,* 2012). It is also established that CD22 on B cells interact with α2,6- linked sialic acids which can impair BCR signalling by decreasing Ca^{2+} mobilization (Hutzler *et al*., 2014). Therefore, targeting APCs with different sialylated constructs may optimise the inhibitory function of these peptides by targeting multiple Siglec receptors.

Given the findings from Chapter 3 demonstrating that targeting Siglec expressing DCs with α2,3 sialylated alloantigen impaired proliferation of indirect alloreactive T cells and induced Tregs, we investigated whether α2,6-linked sialic acid- modified K^d alloantigen have the same efficacy. Similarly to Chapter 3, the findings from this chapter will determine whether targeting APCs using α2,6 Sia-K^d could be a potential targeting regimen for preventing chronic rejection.

4.2 Aims:

Similarly to Chapter 3, this chapter focuses on targeting Siglecs expressed on DCs, B cells and macrophages using an α2,6-linked sialic acid modified K^d alloantigen, to observe whether this targeting regimen can modulate indirect alloresponses *in vitro* and induce Tregs.

Objectives

1. Identify whether α2,6 Sia-K^d alloantigens bind to recipient DC, B cells and macrophages *in vitro*.
2. Determine whether targeting Siglecs with α2,6 Sia- alloantigen peptides on these APCs dampened allogeneic T cell responses *in vitro* and promotes Treg induction as seen with α2,3 Sia peptides.

4.3 Results

4.3.1. α2,6 Sia-K^d binds to Siglec-expressing BM-DCs

It has been reported that α2,6-linked sialic acids preferentially bind to Siglecs expressed on B cells, however according to Perdicchio *et al*., (2016), these sialic acids are also able to bind to Siglec-expressing BM-DCs and promote a tolerogenic immune response. In fact, these authors reported that α2,6-linked sialic acid conjugated OVA antigen was able to bind to Siglec E on DCs (Perdicchio *et al*., 2016). Given their data, the binding of α2,6 Sia-K^d-FITC alloantigen peptides to Siglecs expressed on BM-DCs was assessed *in vitro*. B6 derived BM-DCs were incubated with FAM5/6 (FITC) fluorochrome-conjugated alloantigen peptides; either K^d or α2,6 Sia-K^d (10µg/ml for 4 hours at 37°C) before cells were assessed via flow cytometry. Untreated BM-DCs served as a control. Peptide binding was determined by assessing the MFI of FITC expressed on CD11c$^+$ DCs. As expected both the K^d-FITC and α2,6 Sia-K^d-FITC peptides were capable of binding to CD11c$^+$ DCs. Compared to controls (no peptide treatment), there was a 2.8 fold increase in FITC expression for K^d treated BM-DCs and a 3.2 fold increase for α2,6 Sia-K^d pulsed BM-DCs [Fig. 4.1]. However, unlike the α2,3 Sia-K^d-FITC (please refer to Fig. 3.3B (i)), no significant difference in the amount of K^d-FITC and α2,6 Sia-K^d binding was found [Fig.4.1].

In conclusion, α2,6 sialylated alloantigens bind to Siglec expressing BM-DCs, although there is less binding of α2,6 sialylated alloantigen as compared to α2,3 sialylated alloantigen. This may reflect the type and the abundance of Siglec receptors expressed as well as reflecting their binding capacity [Fig 3.3A, Fig. 4.1], eg: Siglec F which preferentially binds to α2,3 sialylated molecules.

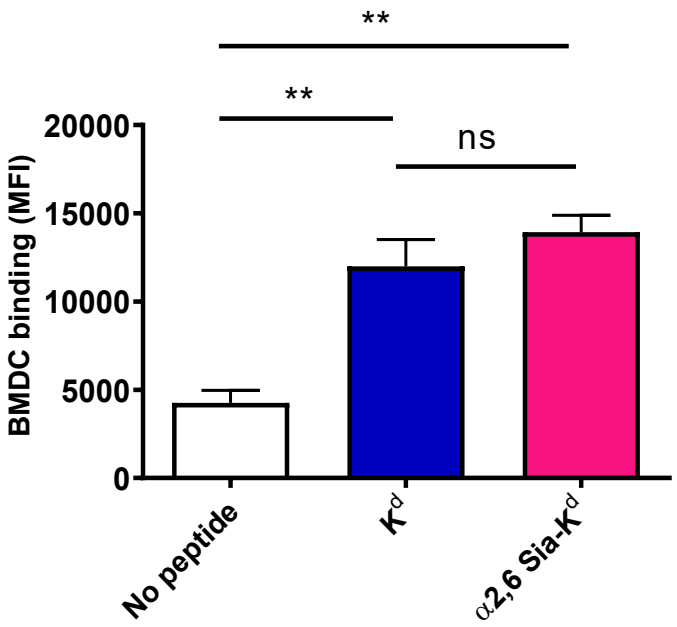


Figure. 4.1. α2,6 Sia-K^d binds to Siglec-expressing BM-DCs in vitro. *For peptide binding studies, B6 BM-DCs were pulsed for 4 hours with either 10 µg/ml K^d - FITC or α2,6 Sia-K^d FITC at 37° C incubation and analysed for peptide binding by gating on CD11c$^+$ cells [Fig. 2.2] and measuring the MFI of FITC using flow cytometry. Data was pooled from 3 independent experiments. No peptide treated DCs were included as controls. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.01**, NS= p>0.05.*

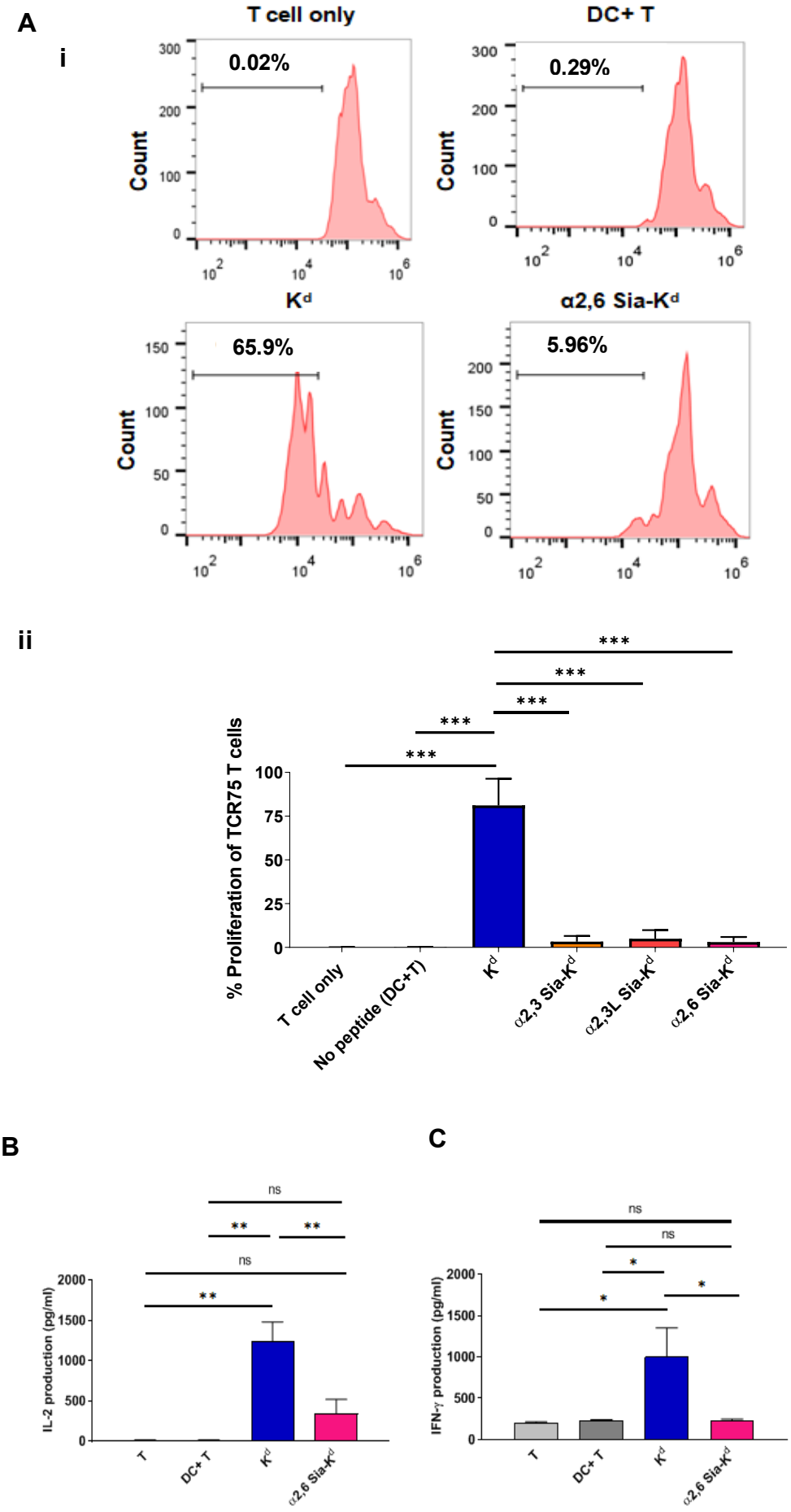
4.3.2 α2,6 Sia-K^d targeted DCs impair indirect allorecognition and induce Tregs

Perdicchio *et al.*, (2016), reported that B6 SPLN-DC targeted with α2,6 Sia-MOG peptide were capable of inducing/expanding more Foxp3$^+$ Tregs from MOG antigen-specific CD4$^+$ T cells (2D2 T cells) than DCs treated with α2,3 Sia-MOG. They also found no difference in IL-10 production when 2D2 T cells were co-cultured with α2,6 Sia-MOG, α2,3 Sia-MOG or MOG peptide treated DCs. However, a decrease in IFN-γ production was observed when these T cells were stimulated with α2,3/ α2,6 sialylated MOG pulsed DCs in comparison to MOG peptide alone (Perdicchio *et al.*, 2016). Taken together, this data highlighted that both α2,6 and α2,3 Sia-MOG have the ability to reduce antigen-specific effector responses and induce Foxp3$^+$ Tregs (Perdicchio *et al.*, 2016). To address whether targeting Siglecs on BM-DCs with α2,6 Sia-K^d impair,

antigen specific $CD4^+$ T cell proliferation, cytokine production (IL-2 and IFN-γ) and were also capable of inducing/ expanding Tregs, the following experiments were conducted: CFSE labelled TCR75 T cells were co-cultured with B6 derived BM-DCs pulsed with either 10µg of α2,6 Sia-K^d or control K^d peptide at a 1:10 DC:T ratio for 3 days and T cell proliferation, CFSE dilution, was investigated. Culture supernatants were harvested to allow measurement of IL-2 and IFN-γ production. In addition, and in separate cultures, Tregs induction/expansion was measured as previously described. Supernatants from these co-cultures were harvested to measure IL-10 using sandwich ELISA.

As expected, and as previously shown, proliferation of TCR75 T cells was observed when these $CD4^+$ T cells were co-cultured with K^d pulsed BM-DCs (Fig 4.2A). T cell proliferation was impaired when these cells were co-cultured with α2,6 Sia-K^d pulsed BM-DCs, which was similar to what was seen following α2,3 Sia-K^d DC treatment [Fig. 4.2A (ii)]. In addition, both IL-2 and IFN-γ production by TCR75 T cells was limited in response to α2,6 Sia-K^d pulsed DCs [Fig. 4.2B-C]. No T cell proliferation or cytokine production was observed in control groups; T cell plus unpulsed DCs and T cell alone.

As expected $Foxp3^+$ Tregs were induced/expanded when K^d specific $CD4^+$ T cells were co-cultured with DCs pulsed with α2,6 Sia-K^d peptides, however there were no significant differences in IL-10 production between groups [Fig.4.3]. This finding was similar to what was observed by Perdicchio *et al*., (2016), where IL-10 production from 2D2 $CD4^+$ T cells in response to sialylated and non-sialylated MOG was not significant [Fig. 4.3]. Overall, it appears that all the sialylated constructs are able to impair alloreactive T cell responses *in vitro* and induce/ expand $CD4^+$ $Foxp3^+$ Tregs.


A
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DC+ T
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0.29%
Count
Kᵈ
α2,6 Sia-Kᵈ
65.9%
5.96%
ii
% Proliferation of TCR75 T cells
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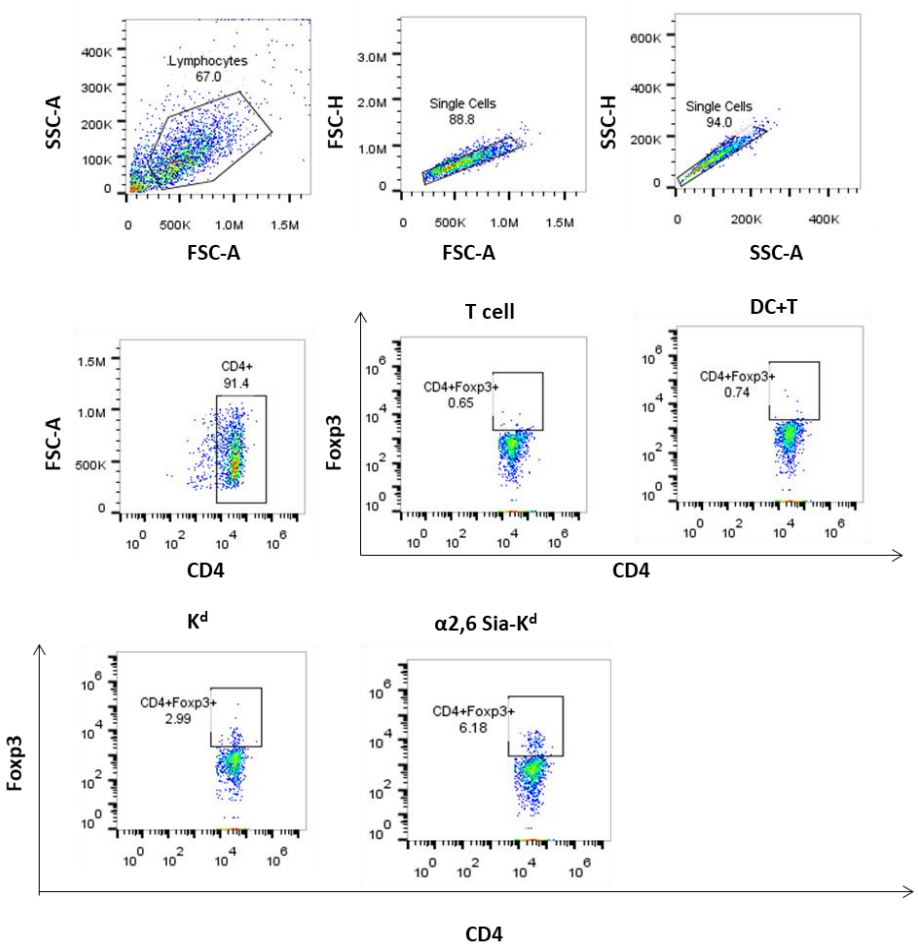
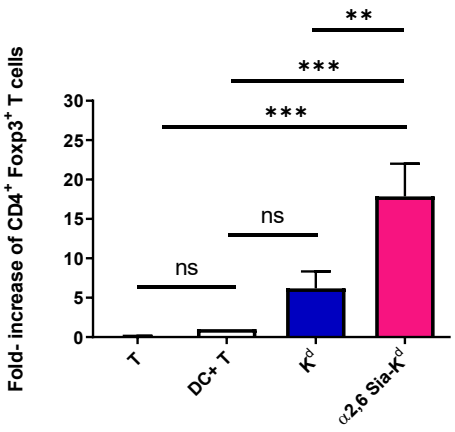
T cell only
No peptide (DC+T)
Kᵈ
α2,3 Sia-Kᵈ
α2,3L Sia-Kᵈ
α2,6 Sia-Kᵈ
B
IL-2 production (pg/ml)
ns
**
C
IFN-γ production (pg/ml)
*

Figure 4.2. Targeting B6 BM-DCs with α2,6 Sia-K^d suppressed proliferation and cytokine production of TCR75 $CD4^+$ T cells *B6 BM-DCs were pulsed with 10μg/ml peptide and co-cultured with CFSE labelled TCR75 $CD4^+$ T cells at 1:10 ratio. After 3 days, supernatants were collected and cells surface stained with CD4 antibody, followed by analysis of CFSE proliferation. A. Cells were gated on live cells (FSC vs. SSC); doublets were excluded followed by gating on $CD4^+$ T cells that were $CFSE^+$ [Appendix Fig. 3A]. (i) Histogram plots demonstrate CFSE dilution of $CD4^+$ TCR75 T cells. Data is a representation of one out of three independent experiments. (ii). Plot demonstrates a graphical comparison of TCR75 $CD4^+$ T cell proliferation with all sialylated constructs (B6 BM-DCs pulsed with α2,3 Sia-K^d, α2,3L Sia-K^d or α2,6 Sia-K^d). Data represents the mean +/- SEM percentage of T cell proliferation and was pooled from 3 independent experiments. B. IL-2 production: supernatants were collected in order to determined IL-2 cytokine release using IL-2 sandwich ELISA. Error bars demonstrate +/- SEM. Data was pooled from 3 experiments. C. Supernatants were collected in order to determined IFN-γ cytokine release using IFN-γ sandwich ELISA. Error bars demonstrate +/- SEM. Data was pooled from 3 experiments. Statistical comparisons were performed using One-way ANOVA and Tukey's multiple comparisons. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*. NS= p>0.05.*

A


Lymphocytes
67.0
SSC-A
FSC-A
Single Cells
88.8
FSC-H
Single Cells
94.0
SSC-H
SSC-A
CD4+
91.4
CD4
T cell
CD4+Foxp3+
0.65
Foxp3
DC+T
CD4+Foxp3+
0.74
Kd
CD4+Foxp3+
2.99
α2,6 Sia-Kd
CD4+Foxp3+
6.18

B


Fold- increase of CD4+ Foxp3+ T cells
**
***
***
ns
ns
T
DC+ T
Kd
α2,6 Sia-Kd

C

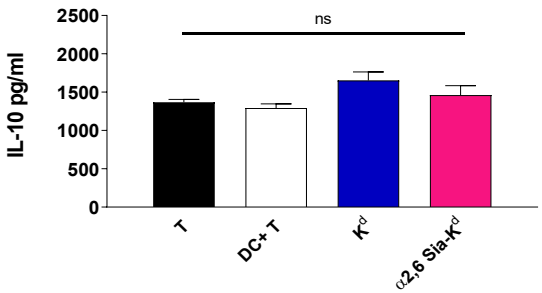

IL-10 pg/ml
ns
T
DC+ T
Kd
α2,6 Sia-Kd

Figure 4.3. Siglec targeting with α2,6 Sia-K^d induces CD4$^+$ Foxp3$^+$ Tregs in vitro. *B6 BM-DCs were pulsed with 10μg/ml peptide and co-cultured with TCR75 CD4$^+$ T cells at 1:10 ratio with subsequent addition of 5U IL-2 at day 0. A. After 3 days, the expression of Foxp3 was measured via intracellular staining and subsequent flow cytometry. FACS plot is a representative for one of 4 experiments. B. The numbers indicate fold-increase of CD4$^+$ Foxp3$^+$ T cells compared to DC+T cultures (set to a value of 1). Data was pooled from 4 independent experiments. C. IL-10 release from T cells co-cultured with pulsed/unpulsed B cells. IL-10 production was assessed using sandwich ELISA. Data is a representative of three pooled experiments and error bars present +/- SEM. Statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test and was expressed as follows; p<0.001***, p<0.01**, ns=p>0.05.*

4.3.3. ICOS-L and PD-L1 expression remain unchanged following α2,6 Sia-K^d targeting

As mentioned in *section 3.3.8* (pg. 104), levels of ICOS-L and PD-L1 on DCs were not affected by treatment with α2,3 Sia-K^d or α2,3L Sia-K^d sialylated alloantigens. Next we assessed whether Siglec:α2,6 Sia-K^d interaction could increase the expression of these molecules. Untreated BM-DCs and DCs treated with 10μg/ml K^d or α2,6 Sia-K^d were stained with ICOS-L or PD-L1 conjugated antibodies and analysed using flow cytometry. The results were in line with our previous findings. No significant difference in PD-L1 and ICOS-L expression was observed following the addition of the α2,6 sialylated peptide, and there was no significant difference with unsialylated peptide [Fig. 4.4]. In conclusion, ICOS-L and PD-L1 expression does not play a role in limiting T cell responses upon Siglec targeting with sialylated alloantigen.

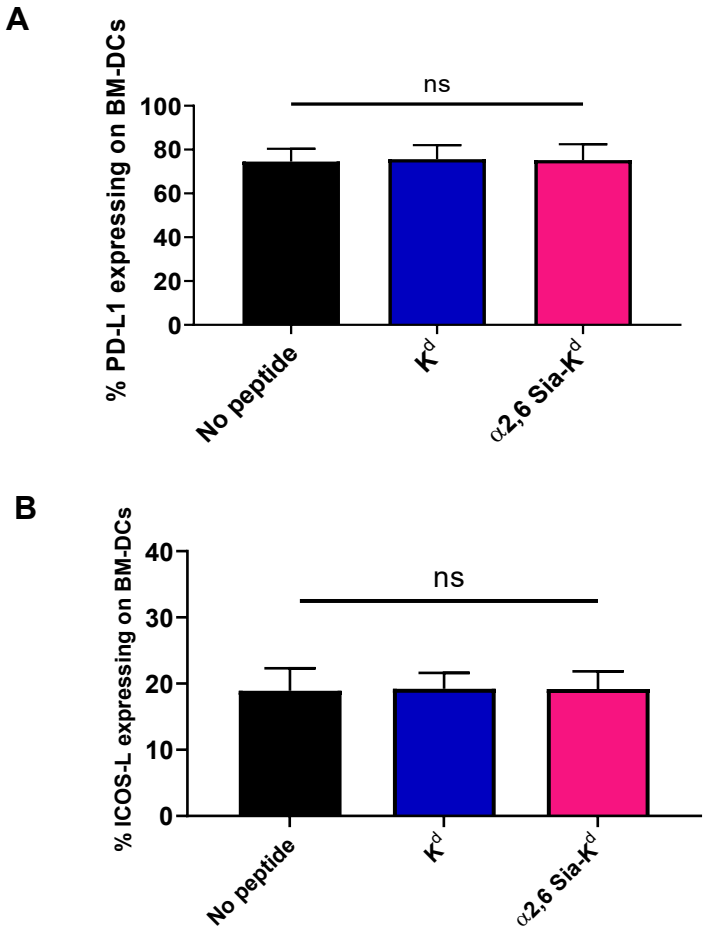


Figure 4.4. α2,6 Sia-K^d peptide treated BM-DCs do not affect ICOS-L or PD-L1 expression. *B6 derived BM-DCs targeted with no peptide, K^d or α2,6 Sia-K^d were pulsed for 4 hours with 10 μg/ml peptide at 37°C incubation and analysed using flow cytometry. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on CD11c$^+$ DCs [Fig. 2.2]. A. Numbers indicate the percentages of the total cell population of cells expressing CD11c and PD-L1 or B. ICOS-L. Data was pooled from 3 independent experiments. Error bars represent +/- SEM. Statistical comparisons were made use One-way ANOVA and Tukey's multiple comparisons test and was expressed as follows; NS =p>0.05.*

4.4 Targeting B6 B cells with α2,6 sialylated alloantigen

4.4.1 α2,6 Sia-K^d binds to B cells

CD22 and Siglec G present on B cells are capable of binding to α2,6 sialic acid (Pillai *et al*., 2012; von Guten *et al*., 2008; Meyer *et al*., 2018). Therefore, we wanted to determine whether α2,6 Sia-K^d was capable of binding these receptors. B6 splenic derived B cells were incubated with either α2,6 Sia-K^d-FITC or control K^d-FITC fluorescent conjugates and binding was assessed via flow cytometry. As expected, in comparison to unpulsed B cells, alloantigens K^d and α2,6 Sia-K^d significantly bound to B cells (p=0.0084 and p=<0.0001, respectively). However, α2,6 Sia-K^d preferentially binds to B cells in comparison to native K^d (p=<0.0001), suggesting that the α2,6 sialic acid linkage to the alloantigen may allow this constructs to bind to Siglecs (Siglec G and CD22) with a specific preference for this sialic acid [Fig. 4.5]. Overall, we have shown that in line with the published literature (Mahajan *et al*., 2016; Razi *et al*., 1998), α2,6 sialic acid-conjugated alloantigens bind to Siglec-expressing B cells [Fig 4.5].

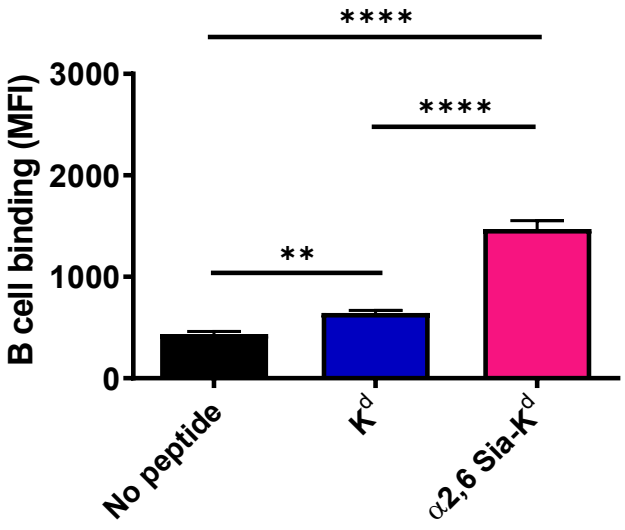
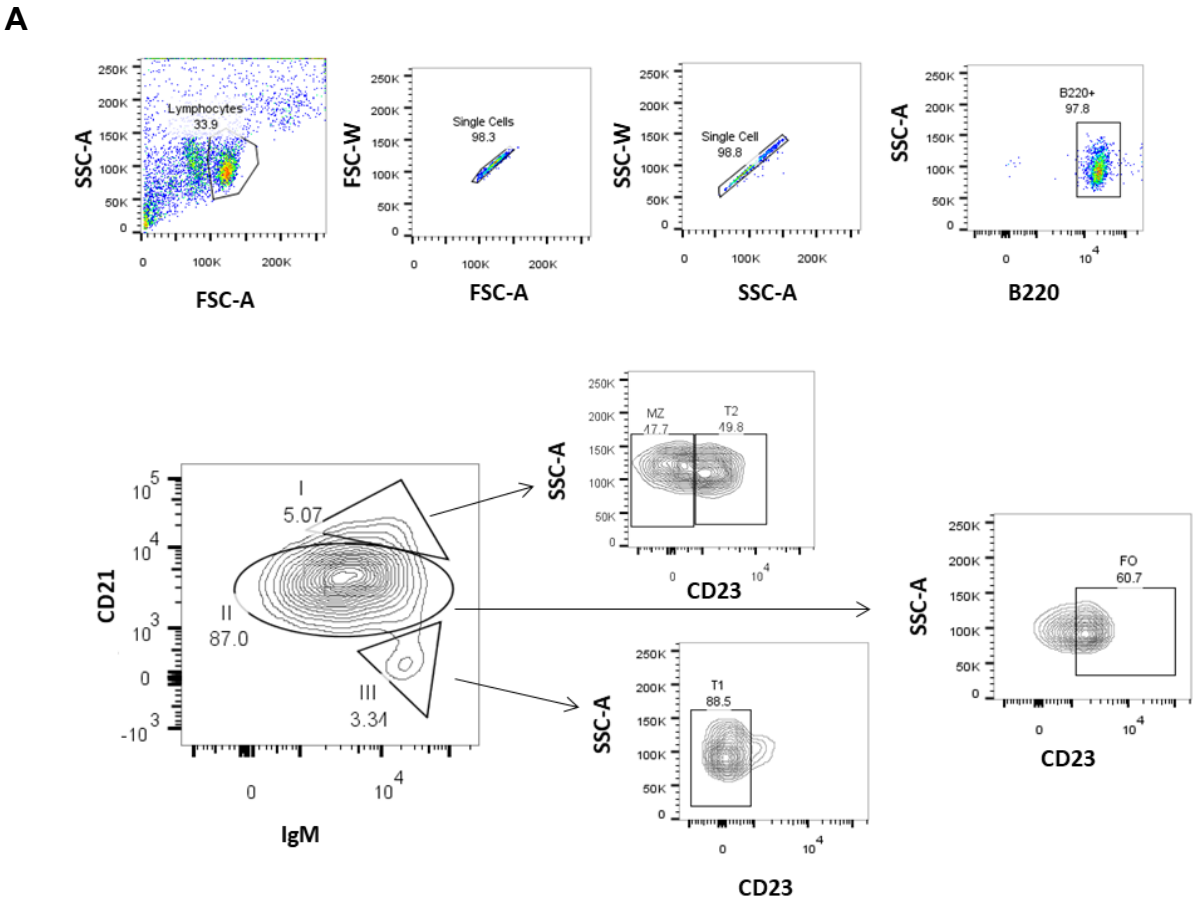


Figure 4.5. α2,6 Sia-K^d binds to Siglec expressing B cells in vitro. *B6 B cells were isolated from spleens, followed by treatment with or without peptide. For peptide binding studies, B6 B cells were pulsed for 4 hours with either 10 μg/ml K^d FITC or α2,6 Sia-K^d FITC at 37°C incubation and analysed for peptide*

*binding by gating on B220^{+} cells and measuring the MFI of FITC using flow cytometry. For the negative controls, cells were not treated with peptide. Live cells were gated on forward scatter (FSC), side scatter (SSC) [Fig. 2.5]. Statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test and data was pooled from 3 independent experiments (n=3). Data shown is mean ± standard deviation (SEM). Statistical significance was expressed as follows; p<0.0001****, p<0.01**.*

Next we determined which specific subset of B cells α2,6 Sia-K^{d} peptide binds to. Previous experiments in Chapter 3 demonstrated that α2,3 Sia-K^{d} binds to T1 (IgM^{high}CD21^{-}CD23^{-}), T2 (IgM^{high}CD21^{+}CD23^{+}), MZ (IgM^{high}CD21^{hi}CD23^{-}) and FO (IgM^{low}CD21^{+}CD23^{+}) B cell subsets. To assess whether Siglecs expressed on these B cell subsets also recognised the α2,6 Sia-K^{d} peptide, B6 splenic derived B cells were incubated with either α2,6 Sia-K^{d}-FITC or control K^{d}-FITC fluorescent conjugates and uptake assessed via flow cytometry. Like the α2,3 sialylated peptide, α2,6 Sia-K^{d} peptide significantly binds to T1, FO and MZ B cell subsets [Fig. 4.6], however it appears that only α2,6 Sia-K^{d} binds significantly to T2 as opposed to K^{d} (p=0.03) and unpulsed B cells (p=0.006) [Fig. 4.6C]. These observations suggest that the high level of α2,6 Sia-K^{d} binding, seen in *Fig 4.5* may be specific to a few B cell subsets (particularly T2 B cell subsets) perhaps reflecting the level of Siglec expression on these subsets.


A
Lymphocytes
33.9
SSC-A
FSC-A
Single Cells
98.3
FSC-W
FSC-A
Single Cell
98.8
SSC-W
SSC-A
B220+
97.8
SSC-A
B220
CD21
IgM
I
5.07
II
87.0
III
3.34
MZ
17.7
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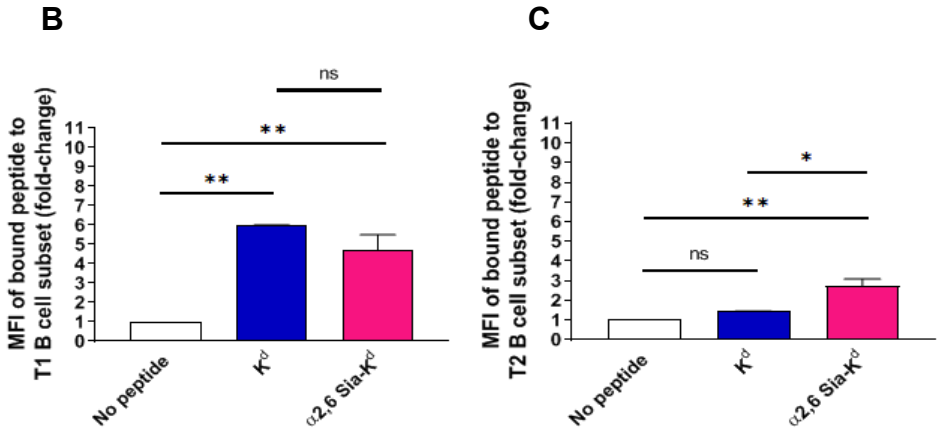
FO
60.7
SSC-A
CD23


B
MFI of bound peptide to T1 B cell subset (fold-change)
ns
**
**
No peptide
Kd
α2,6 Sia-Kd
C
MFI of bound peptide to T2 B cell subset (fold-change)
*
**
ns
No peptide
Kd
α2,6 Sia-Kd

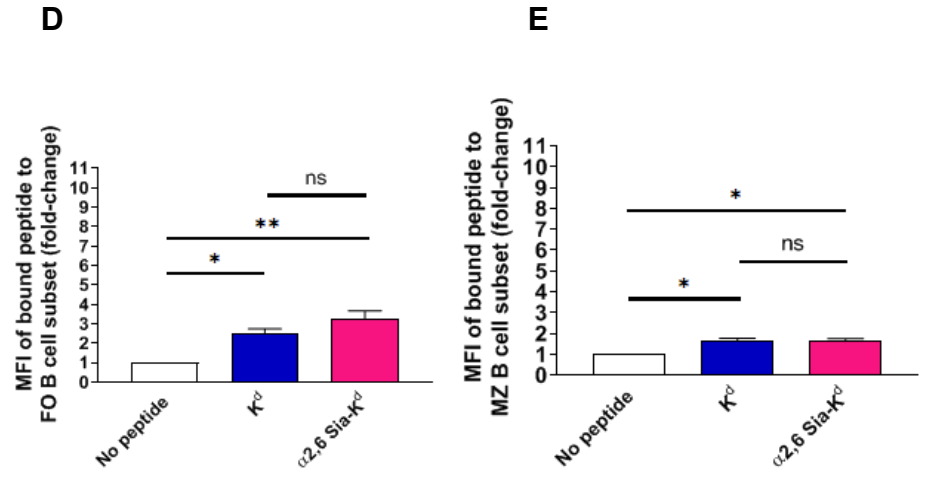

D
MFI of bound peptide to FO B cell subset (fold-change)
ns
**
*
No peptide
Kd
α2,6 Sia-Kd
E
MFI of bound peptide to MZ B cell subset (fold-change)
*
ns
*
No peptide
Kd
α2,6 Sia-Kd

Figure. 4.6 Sialylated and non-sialylated alloantigens bind to T1, T2, MZ and FO B cell subsets *A. Gating strategy for B cells subset analysis. Live cells were gated on forward scatter (FSC), side scatter (SSC) and B220^{+} B cells. Subsets were determined by staining cells with antibodies to IgM, CD23 and CD21. Data is represented as contour plots and representative one out of three independent experiments. (B-D) B cells were pulsed for 4 hours with either 10 µg/ml K^{d} FITC or α2,6 Sia-K^{d} FITC and B cell subsets B. T1 B cells. C. T2 B cells. D. MZ B cells. E. FO B cells were analysed for peptide binding by gating on B220^{+} , IgM, CD23 and CD21 B cells and measuring the MFI of FITC using flow cytometry. Controls cell were not treated with peptide. No peptide control was set to a value of 1 and peptide targeted B cells were compared against no peptide control as fold-change. Data was pooled from 3 independent experiments to look for B cell binding and statistical comparisons were made use One-way ANOVA and Tukey's multiple comparisons test (n=3). Data shown is mean ± SEM). Statistical significance was expressed as follows; p<0.01**, p<0.05*, NS= p>0.05.*

4.4.2 Targeting Siglecs on B6 B cells with α2,6 Sia-K^{d} peptides in vitro does not stimulate T cell proliferation or induce/expand Tregs

We have previously demonstrated in Chapter 3 that engaging Siglecs on naïve B cells with α2,3 Sia-K^{d} led to B cells with no capacity to activate antigen specific T cells or induce/expand Tregs. In order to assess whether α2,6 Sia-K^{d} treatment resulted in a similar or different observation, CFSE- labelled TCR75 T cells were stimulated with B6 derived B cells at a 1:10 B: T ratio and B cells were pulsed with K^{d} or α2,6 Sia-K^{d} peptides. T cell activation was assessed by CFSE dilution and the production of cytokines IL-2 and IFN-γ. Tregs induction/expansion was measured as previously described.

As expected naïve B cells did not induce T cell proliferation or cytokine release regardless of the presence of sialylated or non-sialylated alloantigens [Fig.4.7A]. In addition, α2,6 Sia-K^{d} and K^{d}-treated B cells did not induce Tregs [Fig. 4.8A], however and similarly to the data in Chapter 3, TCR75 T cells cocultured with K^{d}-pulsed B cells produced significantly more IL-10 in comparison to T cells activated with unpulsed B cells (p=<0.0001) [Fig. 4.8B]. In

conclusion, B cells targeted with K^d peptide favour IL-10 production more than α2,6 Sia-K^d-treated B cells, however naïve B cells do not activate TCR75 cells in response to any of the alloantigens.

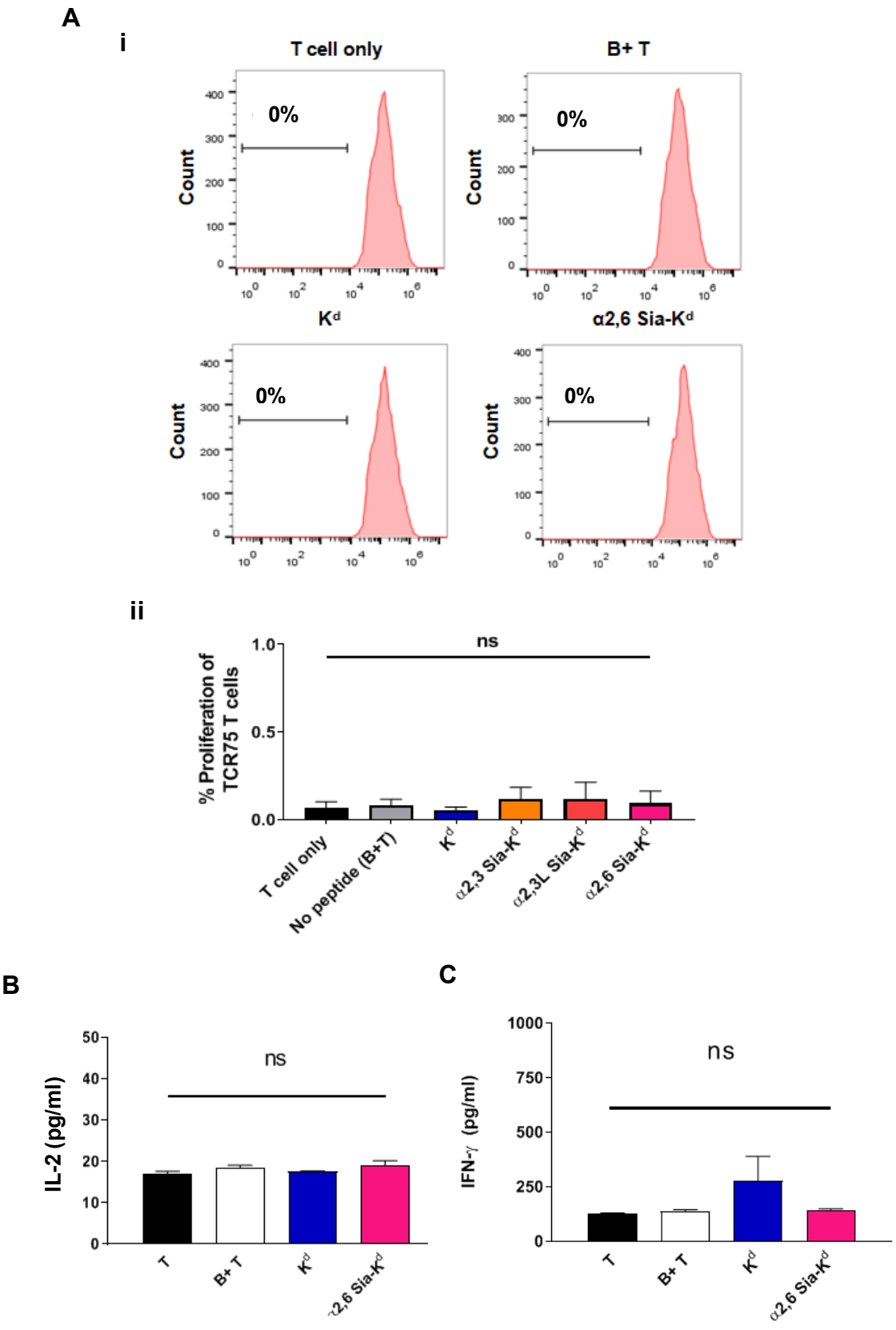


Figure. 4.7. Targeting Siglecs on B6 B cells does not stimulate alloantigen-specific CD4⁺ T cells proliferation. *B6 B cells were pulsed with 10μg/ml peptide and cocultured with CFSE labelled TCR75 CD4⁺ T cells at 1:10 ratio. After 3 days, supernatants were collected and cells surface stained with*

CD4 antibody, followed by analysis of CFSE proliferation. A. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on $CD4^+$ T cells that were $CFSE^+$ [Fig. 2.9 B]. (i) Histogram plots demonstrate $CFSE^+$ $CD4^+$ T cell proliferation. Data is a representation for one out of three independent experiments. (ii). Percentage of T cell proliferation. Error bars demonstrate +/- SEM and data represents 3 independent experiments. B. Supernatants were analysed for IL-2 using a sandwich ELISA. C. Supernatants were analysed for IFN- γ production using sandwich ELISA. ELISA data was pooled from 3 independent experiments, n=3. Data shown is mean ± SEM. Statistical comparisons were performed using One-way ANOVA and Tukey's multiple comparisons test and was expressed as follows; NS=p>0.05.

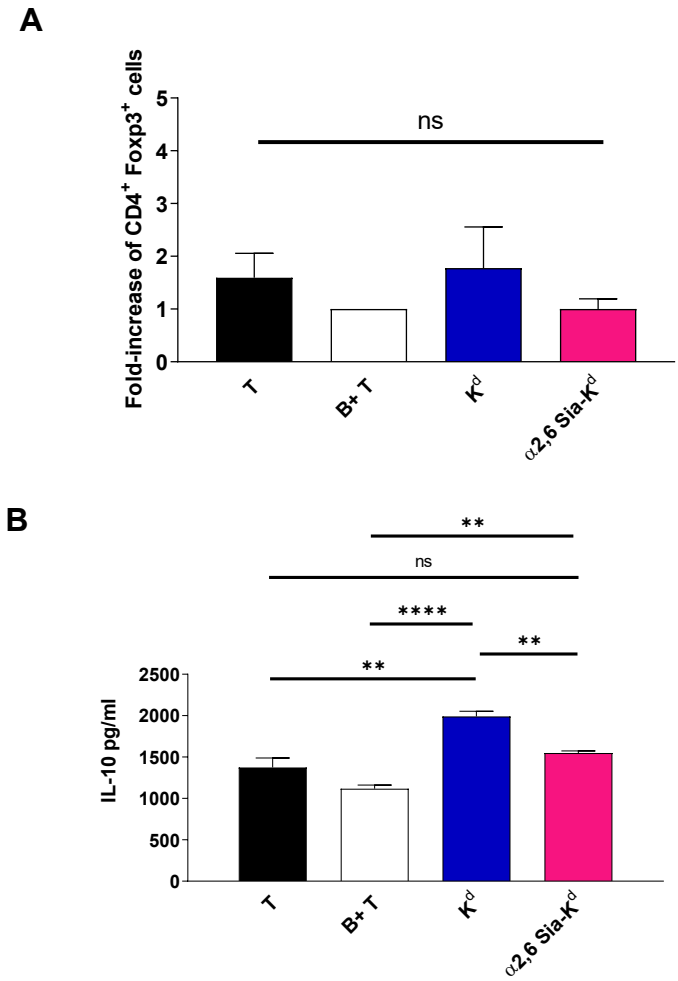


Figure. 4.8. K^d targeted B cells produce more IL-10 in vitro. *B6 B cells were pulsed with 10µg/ml peptide and cocultured with TCR75 $CD4^+$ T cells at 1:10 ratio with subsequent addition of 5U IL-2 at day 0. After 3 days, expression of Foxp3 was measured by intracellular staining and subsequent flow cytometry. A. The numbers indicate fold- increase of $CD4^+$ $Foxp3^+$ T cells from B+ T control. Data was pooled from 3 independent experiments (n=3). B. IL-10 release from T cells co-cultured with pulsed/unpulsed B cells. IL-10 production was assessed using sandwich ELISA. Data is a representative of three pooled experiments and error bars present +/- SEM. Statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test and was expressed as follows; p<0.0001****, p<0.01**, NS= p>0.05.*

4.5 Targeting B6 macrophages cells with α2,6 sialylated alloantigen

4.5.1 α2,6 Sia-K^d peptides bind Siglec-expressing macrophages

We have previously observed that α2,3 Sia-K^d binds to Siglec-expressing BM-DMs which did not result in IL-12 cytokine production following LPS treatment (Chapter 3). To assess whether α2,6 Sia-K^d peptide binds to B6 macrophages and whether this binding impacted IL-12 production, BM-DMs were targeted as previously mentioned in *section 4.3.1* (pg. 130). Binding of α2,6 Sia-K^d and K^d to B6 derived BM-DMs *in vitro* was observed by flow cytometry [Fig.4.9A]. Unlike α2,3 Sia-K^d targeting, there were no significant differences in binding between the peptides [Fig 4.9A].

Next, to determine whether targeting macrophages with α2,6 Sia-K^d altered IL-12 cytokine profile in the presence of LPS stimulation, macrophages were pulsed with K^d or α2,6 Sia-K^d peptides (unpulsed BM-DMs were controls), followed by a 24 hour LPS stimulation at a concentration of 200 ng/ml. Supernatants from the cultures were collected in order to measured IL-12 via ELISA. Interestingly, BM-DMs stimulated with LPS and K^d or α2,6 Sia-K^d alloantigens released less IL-12 in comparison to BM-DMs pulsed with LPS alone, suggesting that peptide treatment affected BM-DMs ability to respond to LPS stimulation (K^d, p= 0.02, α2,6 Sia-K^d p= 0.02) [Fig. 4.9B].

In summary, peptide targeted BM-DMs that were also stimulated with LPS produced less IL-12 in comparison to BM-DMs treated with LPS alone, a finding similar to Chapter 3, *section 3.5.1,* pg. 115. However, targeting Siglecs on BM-DMs using α2,6 sialylated alloantigens does not limit IL-12 production, demonstrated by an increase of this proinflammatory cytokines in response to LPS, thus demonstrating an opposite finding to α2,3 Sia-K^d targeted BM-DMs [Fig. 3.16C].

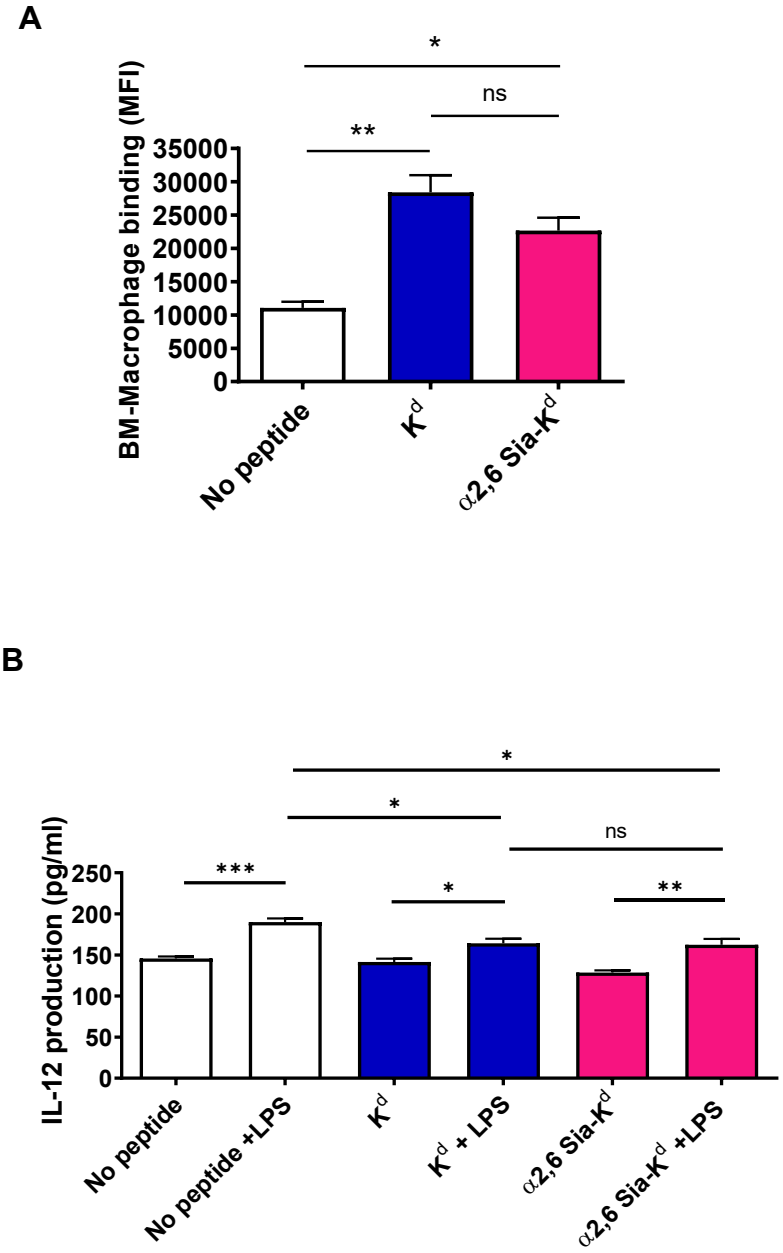


Figure. 4.9. α2,6 Sia-K^d binds to Siglec expressing macrophages in vitro and produces less IL-12 when treated with alloantigens. *A. For peptide binding studies, B6 BM-DMs were pulsed for 4 hours with either 10 μg/ml K^d FITC or α2,6 Sia-K^d FITC and peptide binding analysed by gating on F4/80$^+$ cells and measuring the MFI of FITC using flow cytometry. For the negative controls cell were not treated with peptide. Live cells were gated on forward scatter (FSC), side scatter (SSC) [Fig. 2.4]. Bar graphs showing the MFI of FITC expression on F4/80$^+$ BM-DMs following peptide binding. Error bars demonstrate +/- SEM and data represents 3 pooled independent experiments. B. BM-DMs were pulsed with 10μg/ ml peptide for 4 hours and some cells received 200 ng/ml LPS for 24 hours. After 24 hours, supernatants cells from*

*macrophages in culture were harvested for the measurement of IL-12 using sandwich ELISA. Each bar represents mean ± SEM IL-12 (pg/ml) pooled data from 3 independent experiments (n=3). Statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test to determine statistical significance. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*, ns=p>0.05.*

4.6 Discussion

The data in this section demonstrated that targeting B6 BM-DCs with α2,6 Sia-K^d impaired T effector cell proliferation, induced Tregs and decreased proinflammatory cytokines production. However, these findings were not due to ICOS-L and PD-L1 expression on BM-DCs. These findings were not found when B cells were targeted with α2,6 Sia-K^d. In addition, Siglec expressing macrophages targeted with α2,6 Sia-K^d are not resistant to LPS maturation as demonstrated by the IL-12 readout.

Different Siglecs are expressed between DCs, B cells and macrophages, for example Siglecs CD22 and Siglec G are expressed on B cells, whereas Siglecs CD169, E, F, G are expressed on DCs and macrophages (Pillai *et al.*, 2012). The abundance and expression of various Siglecs on one cell remains to be determined, but given that some of these Siglecs share the same specificity for α2,6 sialic acids, we can assume that differential levels of α2,6 Sia-K^d binding will be expected amongst the APCs. As expected and due to the expression of Siglecs G and CD22 on B cells which have specificities to α2,6 sialic acids, there was significant and preferential binding of α2,6 Sia-K^d to B cells in comparison to the unsialylated peptide. This was not seen when BM-DCs and BM-DMs were tested. Siglecs F and CD169 are highly expressed on both cells (Chapter 3) and these Siglecs are known to preferentially bind to α2,3-linked sialic acids, and Siglec F in particular binds weakly to α2,6-linked sialic acids (Crocker *et al.*, 2007), therefore the binding profiles may reflect the type of Siglecs that are present on BM-DCs and BM-DMs. Siglec E is said to have the ability to bind moderately to both α2,3/ α2,6 linked sialic acids. Although we have confirmed expression of Siglec E on DCs, their expression levels were not as high as Siglec F and CD169. Therefore, the expression of Siglecs and their specificity towards sialic acid linkages may provide some explanation as to why α2,6 Sia-K^d binding to BM-DCs and BM-DMs was not as prominent as α2,3 Sia-K^d binding, when comparisons are made to K^d-peptide binding (Chapter 3). Regardless of this result, we still assessed the capacity of α2,6 Sia-K^d targeted BM-DCs to induce/ expand Tregs from naïve TCR75 T cells.

Tregs were induced when BM-DCs were targeted with α2,6 Sia-K^d supporting a study by Perdicchio *et al.*, (2016). In fact, according to Perdicchio *et al.*, (2016), targeting Siglec $E^{-/-}$ BM-DCs with α2,6 Sia-OVA drastically reduced the proportions of induced $Foxp3^+$ Tregs in comparison to WT BM-DCs. This may imply that interactions between α2,6 Sia-K^d and Siglec E on our B6 BM-DCs contributes to the increase of $Foxp3^+$ Tregs, and this could be confirmed, as future work, by assessing Tregs induction potentials between Siglec $E^{-/-}$ BM-DCs and WT BM-DCs. Given that various Siglecs are present on DCs, it remains to be determined if any of the other Siglecs are involved in Treg induction.

B cell Siglecs are able to interact with α2,6- linked sialic acids and in turn impair BCR signalling by decreasing Ca^{2+} mobilization and limiting B cell activation (Hutzler *et al.*, 2014). Therefore, this chapter addressed whether these Siglecs could be targeted with α2,6 sialylated alloantigens to promote tolerance and identified that targeting these B cells with sialylated constructs did not affect T cell responses or induce/ expand Tregs as seen with Sia-K^d targeted BM-DCs. However, literature states that B cell Siglecs are well-defined as receptors for preventing autoimmunity, for example Jellusova *et al.*, (2010) demonstrated that double-deficient Siglec G and CD22 knockout murine B cells contributed to systemic autoimmunity. When these Siglecs were absent on B cells these authors noticed the development of B cell proliferation in response to TLR stimulation and an increase in IgG autoantibodies associated with glomerular damage in the kidneys (Jellusova *et al*. 2010). B cell associated Siglecs are known to be inhibitory making them potential receptors to promote tolerance, especially since these receptors can interact with sialic acids both in *cis* and *trans* (Meyer *et al*., 2018).

Siglecs are present on transitional B cells (Zhang *et al*., 2004), we addressed if targeting Siglecs on B cells can promote immunoregulatory T cell function, and whether transitional, MZ or FO B cells play a role. In the current study, binding of both K^d and α2,6 Sia-K^d was observed in T1, T2, MZ and FO B cell subsets. However, unlike α2,3 Sia-K^d, we identified that α2,6 Sia-K^d, binds more to T2 subsets as demonstrated by the increase of FITC MFI in comparison to K^d peptide. This finding is quite promising as it demonstrates that this sialylated

peptide has more binding potential to a subset of B cells which are known for promoting tolerance (Moreau *et al.*, 2014). Regardless of this interaction and similarly to α2,3 Sia-K^d targeting in Chapter 3, no T cell proliferation or Treg induction was observed.

In comparison to other studies, there are several distinctions in the methodology which may explain our findings. It was initially thought that targeting naïve B cells may not be sufficient to stimulate T cells proliferation, however, one study demonstrated that naïve B cells targeted with OVA, were able to stimulate OT-II $CD4^+$ T cells proliferation *in vitro* (Reichardt *et al.*, 2007). Their experiments consisted of targeting naïve B cells with OVA overnight and again 4 hours before use, followed by co-culture with OT-II T cells for a further 72 hours at a 1:1 B:T ratio (Reichardt *et al.*, 2007). What was notably interesting was that OT-II T cells cocultured with OVA-pulsed naïve B cells produced significant amounts of IL-2 and IFN-γ, whereas these cytokines were negligible in our experiments. However, significant differences between studies are the origin of the T cells for the co-cultures (OT-II versus TCR75) and the antigen (OVA whole protein versus K^d peptide). In 1995, one study showed that peptide antigens where more efficiently presented via DCs to naïve $CD4^+$ T cells rather that it's protein form, whereas proteins Ags acquired via B cells were more efficient at initiating an immune response in comparison to protein targeted DCs (Constant *et al.*, 1995[a]; Constant *et al.*, 1995[b]). Therefore, as a future experiment, we can target B cells with whole MHC monomers as opposed to peptide, so that the antigen processing mechanism is similar to a whole OVA protein.

In the aforementioned study, they observed an increase of IL-10 was found in supernatants containing B and T cells, which is in line with our findings from T cell co-cultured with K^d-pulsed B cells (Reichardt *et al.*, 2007). In our study, it was interesting to see that regardless for a lack of T cell proliferation or Treg induction, IL-10 was found in supernatants with K^d-pulsed B cells and TCR75 T cells. This raised the question as to whether the IL-10 is being released by B cells or the TCR75 T cells. This was addressed by Reichardt *et al.*, (2007), where they found that B cells were the actual source of IL-10 in the B/T supernatants, therefore this may provide some explanation to our IL-10 data.

Overall, targeting naïve B cells with OVA protein antigens can promote antigen-presentation and stimulate T cells proliferation. Therefore as future work, the B cell experiments in the study could be adapted to Reichardt *et al*. (2007) study, and be repeated by targeting B cells with whole MHC monomer protein (+/- sialylation) rather than a peptide, which is similar to the aforementioned study whole protein targeting strategy. This way, we will be able to establish whether targeting Siglecs on B cells can modulate indirect allorecognition, which was observed when BM-DCs were targeted.

Lastly, as mentioned in the previous Chapter 3, Siglec E expressing macrophages have been shown to limit TLR cytokine production following LPS stimulation (Boyd *et al*., 2009). It was interesting to see in their study following LPS activation, Siglec E receptors were up-regulated and inhibitory SHP-1 and SHP-2 were recruited to the receptor (Boyd *et al*., 2009). With respect to Boyd *et al*., (2009) observations for upregulation of Siglec E receptors following LPS stimulation, as a future experiment, it may also be worth targeting macrophages with sialylated alloantigen following LPS activation to determine whether activated macrophages with more Siglec E expression can become 'tolerogenic' and that proinflammatory cytokines such as IL-12 production, TNF-α and IFN-γ are dampened. Conversely, one study identified no differences in pro-inflammatory cytokines such as TNF-α release following LPS stimulation when WT versus Siglec $E^{-/-}$ macrophages were compared, further questioning whether Siglec E has a definitive role at controlling LPS stimulation (Nagala *et al*., 2018). The present study did not look at the levels of TNF-α following targeting, however it would be interesting to measure this cytokine so that comparisons can be made to the aforementioned studies. This study measured the levels of IL-12 and we noticed that macrophages that were treated with α2,6 Sia-K^d produced less IL-12 in comparison to the no peptide controls. Similarly to the macrophage data in Chapter 3, all peptide targeted BM-DMs stimulated with LPS produced less IL-12 in comparison to BM-DMs treated with LPS alone. However, unlike α2,3 Sia-K^d targeted BM-DMs where there was no significant difference in IL-12 production between +/- LPS groups, there was an increase of IL-12 production following LPS activation of BM-DMs treated with α2,6 Sia-K^d and LPS in comparison to the non-LPS treated counterpart. This suggests that α2,3 sialylated peptides may have an immunomodulatory effect in the presence

of LPS in comparison to α2,6 sialylated peptides. However, there is very little literature to support these findings other than Boyd *et al*., (2009) study. Nevertheless, there are significant differences to the aforementioned study and the current study such as their use of targeting Siglec E with a cross-linking Ab and their readouts such as TNF-α and IL-6, where we only assessed IL-12 (Boyd *et al*., 2009). Also, we cannot rule out the possibility that α2,6 Sia-K^d is binding to CD169 on macrophages, which is a Siglec that does not contain an ITIM, therefore limiting interaction with other inhibitory Siglecs. This is debatable as CD169 preferably binds to α2,3 linked sialic acids as opposed to α2,6 linked sialic acids (Pillai *et al*., 2012; Macauley *et al*., 2014).

Overall, targeting α2,6 Sia-K^d to Siglecs expressed by BM-DCs modulated indirect allorecognition *in vitro* in favour of impaired T cell proliferation and induction/expansion of Tregs. This was also seen when BM-DCs were targeted with α2,3/ α2,3L Sia-K^d. There were clear differences in peptide binding profiles to DCs, macrophages and B cells between the sialylated constructs, indicating that some of these constructs prefer to bind to specific Siglec receptors. However, in this chapter, targeting Siglecs on B cells and macrophages with α2,6 Sia-K^d did not promote an immunoregulatory function.

One of the aims of this project was to determine whether targeting different Siglecs on APCs by targeting with different sialylated alloantigens could promote an immunoregulatory environment that could potentially support allograft survival. As mentioned in Chapters 3-4, targeting BM-DCs *in vitro* with all the sialylated constructs demonstrated this by impairing $CD4^+$ T cell proliferation, effector cytokine production and distinctly induced/ expanded $Foxp3^+$ Tregs. We conclude that all the sialylated alloantigens used in this study may have the ability to instruct DCs to promote alloantigen-specific tolerance. What is yet to be confirmed is whether similar results can be obtained if BM-DCs or SPLN-DCs can targeted with all sialylated constructs at once to see whether this generates a more immunoregulatory environment, with a further increased proportion of Tregs, as opposed to targeting with one construct alone. In addition, it would also be necessary to determine whether Siglec-expressing BM-DMs can impact T cells proliferation and the development of Tregs as seen with BM-DCs.

CHAPTER 5:

TARGETING SIGLECS ON BATF3-DEPENDENT DCs PROLONGS SKIN TRANSPLANT SURVIVAL AND LEADS TO REDUCED ALLOANTIBODY RESPONSES

5.1 Introduction

Immunosuppressive drugs are effective at prolonging organ transplant survival on a short-term basis by reducing the incidences of acute rejection. However, this treatment fails to prevent chronic rejection and is associated with a number of side effects as outlined in the general introduction (Chapter 1). In order to promote graft survival without the use of ISD, several laboratories have opted to use cellular immunotherapeutic approaches to promote tolerance and reduce the incidences of chronic rejection. Some of these approaches include modifying donor (direct pathway) and recipient (indirect pathway) DCs to limit effector immune responses, the use of depletion antibodies and the induction/ expansion of Tregs (Morelli & Thomson., 2014).

Of relevance to this thesis is the literature looking at inhibiting the indirect allorecognition pathway, the pathway known to contribute to chronic rejection (Brennan *et al*., 2009), via manipulating DCs. In the context of allograft survival various methods have looked at manipulating recipient DCs or targeting endogenous DCs to induce tolerance. These have included treating transplant recipients with syngeneic/ autologous DCs that have been rendered 'tolerogenic' *ex vivo*, administering donor-alloantigen loaded recipient DCs or target endogenous DCs with alloantigens *in situ*. These treatments have proven to be successful in prolonging solid organ transplants in murine models (Tanriver *et al*., 2010; Divito *et al*., 2010; Morelli *et al*., 2014).

Peche *et al*., (2005) assessed the capacity of recipient-derived DCs to modulate allograft rejection in a rat model; they compared a non-adherent subpopulation of recipient derived BM-DCs with an adherent population of BM-DCs (Peche *et al*., 2005). They found that immature adherent BM-DCs induced significant heart allograft survival when introduced back into the recipient (Peche *et al*., 2005). Divito *et al*., (2010) and Morelli *et al*., (2014) both described that *ex vivo* expanded alloantigen pulsed-recipient DCs were short-lived when reintroduced into the host and that their immunomodulatory effects were dependent on the recipient endogenous DCs. These cells acquired and presented alloantigen from the injected DCs leading to deletion of alloreactive effector T cells and the promotion of $CD4^+$ $Foxp3^+$ Tregs, both of which were required for transplant

survival. However, maintaining the 'tolerogenic' state of *ex vivo*-generated DCs that are administered *in vivo* can be challenging and there are also issues with generating tolerogenic DCs on a large scale for clinical use such as the expense (Hu and Wan, 2010).

An alternative method to inhibit the indirect pathway is to target alloantigens to endogenous DCs. These cells are inactivated/quiescent and are present in the recipient SLOs as opposed to *ex-vivo* generated recipient DCs.

5.2 Aims

The aim of this chapter is to assess whether targeting sialylated alloantigen to Siglecs expressed on endogenous DCs promotes transplant tolerance by inhibiting the indirect pathway of allorecognition.

To achieve this, the following objectives were undertaken:
1. Determine whether Siglecs expressed by endogenous DCs bind sialylated K^d peptide following intravenous administration.
2. Assess whether targeting Siglecs on specific DC subsets promotes transplant tolerance.

The *in vivo* experiments in this chapter were adapted from a previous publication using an established skin transplant model (Honjo *et al*., 2000; Tanriver *et al*., 2010). Similarly, Tanriver *et al*., (2010) established that targeting 33D1-K^d constructs to DCIR2 receptors on endogenous DCs successfully prolonged MHC I mismatch skin allograft B6.K^d (B6 strain expressing H-2K^d transgene) survival in the absence of direct $CD8^+$ T cells.

This study has so far highlighted that targeting BM-DCs *in vitro* with sialylated alloantigen led to impaired indirect $CD4^+$ T cell allorecognition and the expansion/ induction of $CD4^+$ $Foxp3^+$ Tregs. In this chapter we sought to determine whether targeting Siglecs on DCs, *in vivo,* induced transplant tolerance. If successful this would support the use of administering sialylated alloantigens to target Siglec-expressing endogenous DCs for future clinical use in transplantation.

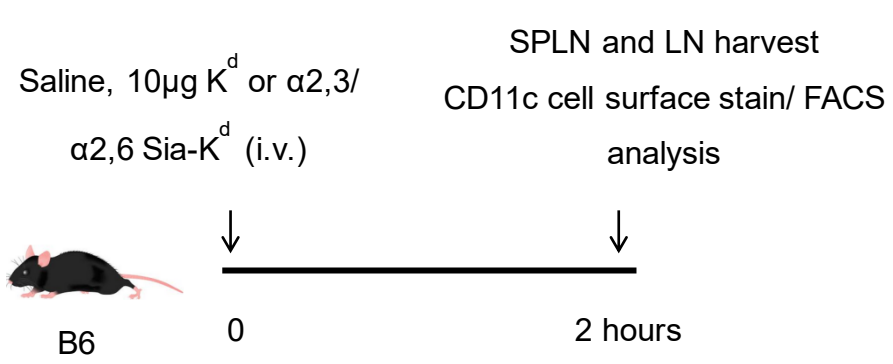
5.3 Results

5.3.1 Sialylated alloantigen peptides bind to endogenous DCs and B cells in the SLOs when administered intravenously

We have previously confirmed Siglec expression on both B6 derived splenic DCs and B cells as well as the ability of α2,3 Sia-K^d and α2,6 Sia-K^d peptides to bind to these cells *in vitro.* Before beginning the transplant experiments, we first determined whether the i.v. administered sialylated and non-sialylated K^d peptides are capable of binding to these cells in the SPLN and LNs, two organs that are essential for antigen presentation and immune cell interaction (Modino *et al.*, 1996). To assess alloantigen peptide binding *in vivo*, B6 mice were injected i.v. with 10µg of either K^d-FITC, α2,3 Sia-K^d-FITC, α2,6 Sia-K^d-FITC or saline as a negative control. SPLNs and LNs were harvested 2 hours after administration. This time point was selected to allow circulation of the peptides to reach the SLOs and was chosen based on based on previous studies (Mann *et al.*, 2017; Whitney *et al.*, 2011).

Splenocytes and LN cells were stained with either anti-CD11c antibodies to identify DCs or anti-B220 antibodies to identify B cells. Cells were then analysed using flow cytometry and peptide binding was determined by measuring the MFI of FITC expressed on these cells. The data suggests that following i.v. injection K^d, α2,3 Sia-K^d and α2,6 Sia-K^d peptides bind to DCs resident in the LNs, although the data is not statistically significant, a trend of increased FITC expression is observed [Fig. 5.1C (i)]. In addition, both sialylated and non-sialylated alloantigen peptides bind to B cells; however this again appears to be preferentially in LNs rather than in the SPLN [Fig. 5.2]. Therefore, it can be concluded that it is possible for the peptides to bind to DCs and B cells in the LNs *in vivo* following intravenous injection. It should be noted that the binding of α2,3L Sia-K^d *in vivo* could not be assessed as we did not have a fluorochrome-conjugated construct.

A


Saline, 10μg K^d or α2,3/
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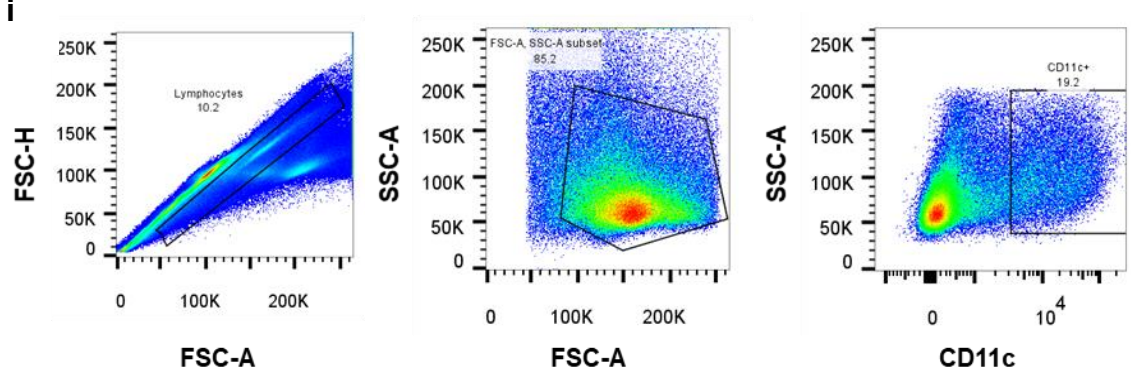
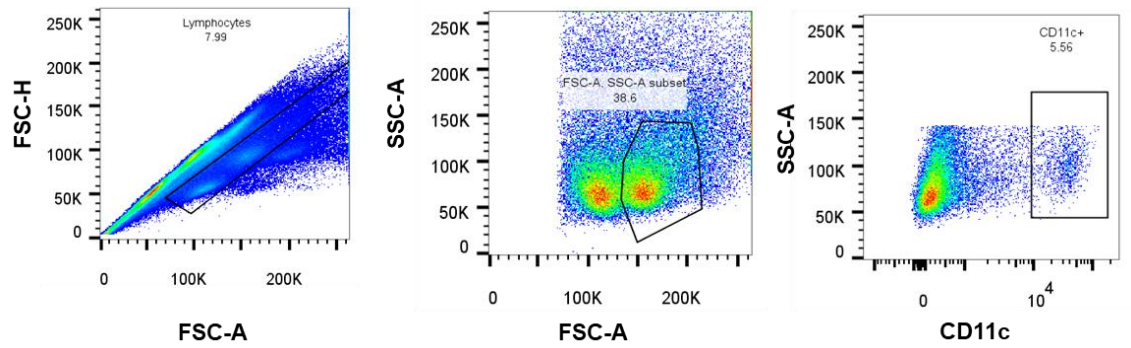
SPLN and LN harvest
CD11c cell surface stain/ FACS
analysis
B6
0
2 hours

B

i


Lymphocytes
10.2
FSC-H
FSC-A
FSC-A, SSC-A subset
85.2
SSC-A
FSC-A
CD11c+
19.2
SSC-A
CD11c

ii


Lymphocytes
7.99
FSC-H
FSC-A
FSC-A, SSC-A subset
38.6
SSC-A
FSC-A
CD11c+
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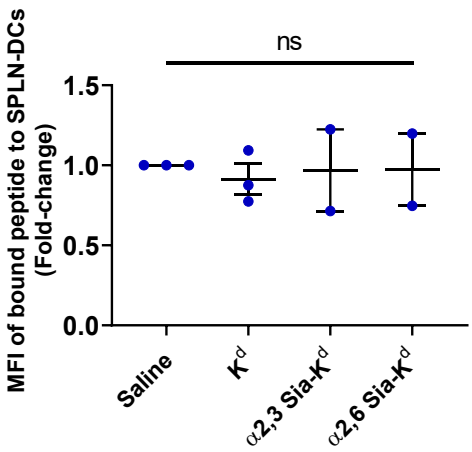
SSC-A
CD11c

C

i


ns
MFI of bound peptide to SPLN-DCs
(Fold-change)
Saline
K^d
α2,3 Sia-K^d
α2,6 Sia-K^d

ii

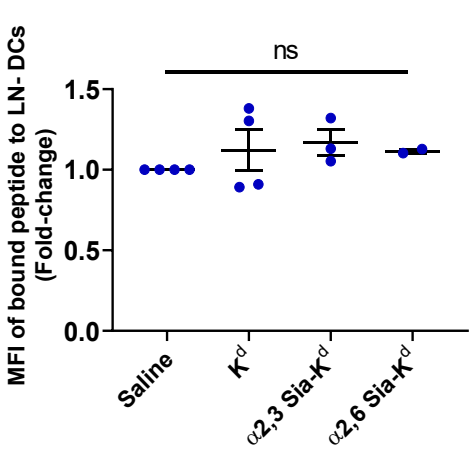
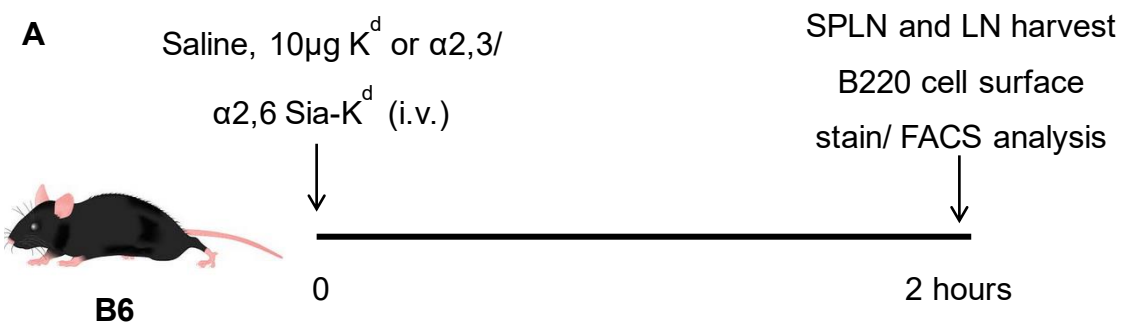
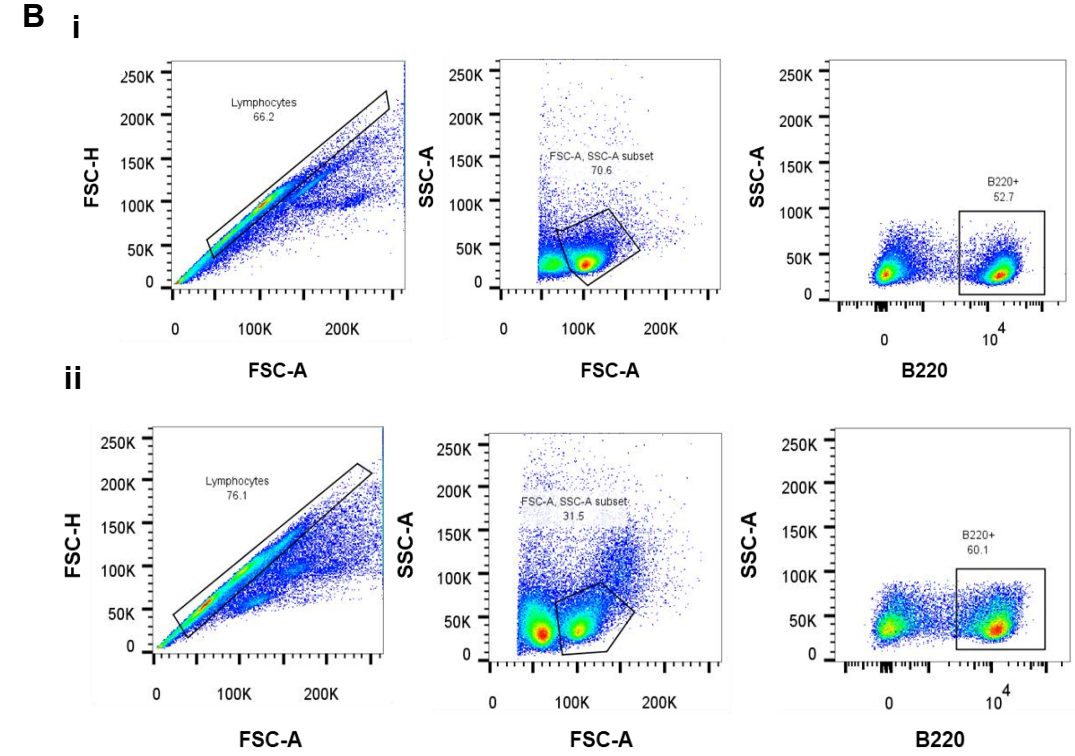

ns
MFI of bound peptide to LN- DCs
(Fold-change)
Saline
K^d
α2,3 Sia-K^d
α2,6 Sia-K^d

Figure. 5.1. Sialylated and unsialylated allopeptides bind to DCs in the lymph node following intravenous administration*. A. B6 mice (2-4 mice per group) received 10μg/200μl saline of either K^d –FITC, α2,3 Sia-K^d –FITC, α2,6 Sia-K^d –FITC or 200 μl saline (no peptide control) via i.v. injection. B. Two hours post injection mice were sacrificed; (i) spleens and (ii) LNs were harvested and DCs were identified by staining for CD11c expression. Doublets were initially excluded, followed by live cells gating based on forward scatter (FSC) and side scatter (SSC). The MFI of FITC expression on CD11c was analysed. C. MFI of FITC was expressed as fold-change against saline treated mice which was set to a value of 1. (i) saline, K^d-FITC, α2,3 Sia-K^d-FITC or α2,6 Sia-K^d-FITC peptide binding to SPLN-DCs. (ii) saline, K^d-FITC, α2,3 Sia-K^d-FITC or α2,6 Sia-K^d-FITC peptide binding to SPLN-DCs. Error bars demonstrate +/- SEM. Each dot is a representative of 1 mouse per condition. Statistical comparisons were performed using One-way ANOVA and Tukey's multiple comparisons. Statistical significance was expressed as follows; NS= p>0.05.*


A
Saline, 10µg K^d or α2,3/
α2,6 Sia-K^d (i.v.)
SPLN and LN harvest
B220 cell surface
stain/ FACS analysis
B6
0
2 hours


B
i
ii
Lymphocytes
66.2
FSC-A, SSC-A subset
70.6
B220+
52.7
Lymphocytes
76.1
FSC-A, SSC-A subset
31.5
B220+
60.1
FSC-H
SSC-A
FSC-A
B220

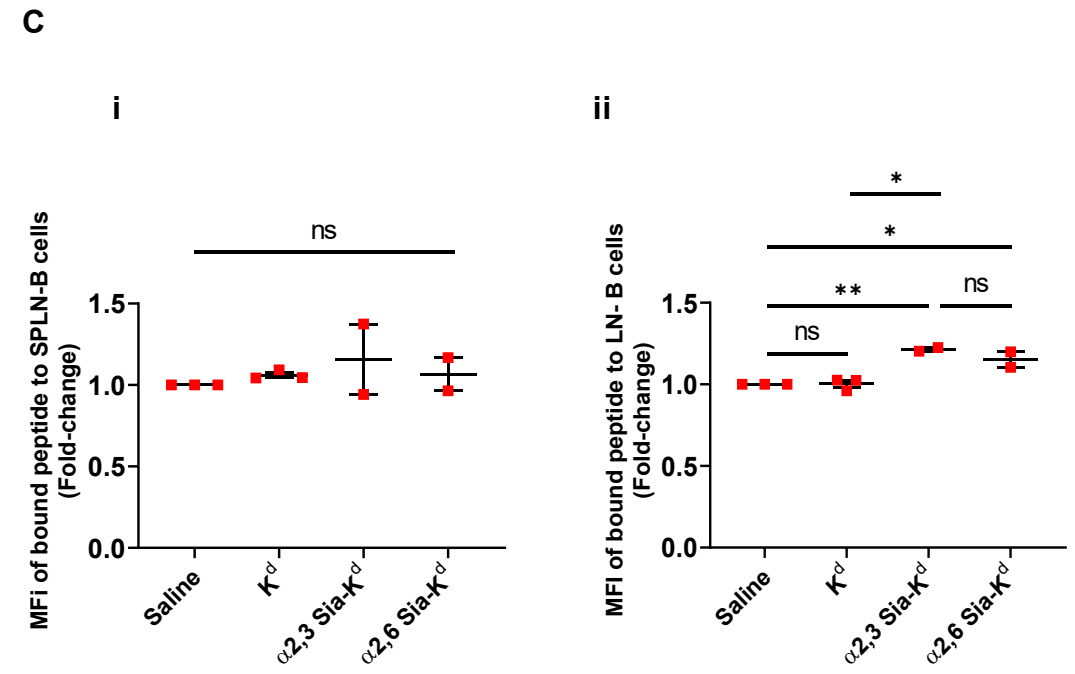

C
i
ii
MFi of bound peptide to SPLN-B cells
(Fold-change)
MFI of bound peptide to LN- B cells
(Fold-change)
ns
*
**
Saline
K^d
α2,3 Sia-K^d
α2,6 Sia-K^d

Figure 5.2. Sialylated alloantigen preferentially binds to Siglecs expressed on lymph node B cells following intravenous administration. *A. B6 mice (2-3 mice per group) received 10µg/200µl saline of either K^d –FITC, α2,3 Sia-K^d –FITC, α2,6 Sia-K^d –FITC or 200 µl saline (no peptide control) via i.v. injection. B. Two hours post injection mice were sacrificed; (i) spleens and (ii) LNs were harvested and B cells identified by stained for B220 expression. Doublets were initially excluded, followed by live cells gating based on forward scatter (FSC) and side scatter (SSC). The median fluorescence intensity (MFI) of FITC expression on B220 was analysed. C. MFI of FITC was expressed as fold-change against saline treated mice which was set to a value of 1. (i) saline, K^d-FITC, α2,3 Sia-K^d-FITC or α2,6 Sia-K^d-FITC peptide binding to SPLN-B cells. (ii) saline, K^d-FITC, α2,3 Sia-K^d-FITC or α2,6 Sia-K^d-FITC peptide binding to LN- B cells. Error bars demonstrate +/- SEM. Each dot is a representative of 1 mouse per condition. Statistical comparisons were performed using One-way ANOVA and Tukey's multiple comparisons. Statistical significance was expressed as follows; p<0.01**, p<0.05*. NS= p>0.05.*

5.3.2. Treating recipient B6 mice with Sia-alloantigens 10 days before a B6.K^d skin transplant did not prolong allograft survival

Having established that BM-DCs pulsed with Sia-K^d conjugates can impair indirect alloreactive $CD4^+$ T cell proliferation, cytokine release and induce/ expand $Foxp3^+$ Tregs *in vitro*, we investigated whether targeting these APCs *in vivo* prolonged allograft survival using an established skin transplant model designed to measure the indirect pathway of allorecognition (Tanriver *et al*., 2010). Here, B6 recipient mice were transplanted with skin taken from mice expressing native BALB/c- derived K^d MHC I molecule (B6.K^d donor mice) (Honjo *et al*., 2004). One of the mechanisms to assess whether the indirect pathway of allorecognition is being modified was to assess the levels of alloantibodies produced in response to donor alloantigens. Suave *et al*., (2004), identified that IgG alloantibodies are produced by B cells with the help of $CD4^+$ helper T cells with indirect allospecificity and the direct pathway is insufficient to generate these alloantibodies. Tanriver *et al*. (2010), assessed the levels of K^d-specific alloantibodies in recipient mice and found that mice treated with 33D1-K^d monomer to target DCIR2 subset of endogenous DCs had significantly

reduced alloantibodies in comparison to mice treated with saline alone. Therefore, in this chapter we will assess the levels of K^d-specific alloantibodies to determine whether sialylated alloantigen targeting impacts indirect allorecognition *in vivo* as it does *in vitro*.

To assess the efficacy of Siglec targeting *in vivo*, B6 mice were treated with 10 µg of either α2,3 Sia-K^d, or control peptide K^d 10 days before receiving a K^d skin transplant. Control mice received saline only. We reasoned that treating animals with the alloantigen peptides 10 days prior to transplant would allow sufficient time for antigen-specific Tregs to develop. For example, Kretschmer *et al*., (2005) administered one infusion of anti-DEC-205 fused with hemagglutinin peptide antigen for 14 days to allow the development of antigen-specific Tregs from adoptively transferred $CD4^+$ $CD25^-$ $Rag2^{-/-}$ 6.5 TCR-transgenic Thy-1.2^+ cells into Thy-1.1^+ BALB/c recipients. Perdicchio *et al*., (2016) however, administered sialylated antigen 7 days prior to OVA/poly(I:C)/anti-CD40 treatment, and also observed increase in Tregs with Sia-OVA treated mice. Therefore, it was decided to administer peptides on day 10, which approximately lies between 7-14 days and is also the same time-point Tanriver *et al*., (2010) adopted to administer K^d alloantigen in mice. In addition and similarly to Tanriver *et al*., (2010) study, mice were treated with anti-CD8 antibody on the day of transplant (day 0) and one day following to remove the $CD8^+$ T cells with direct allorecognition for K^d [Fig. 5.3 A-B].

Despite peptide and anti-CD8 treatment, no prolongation of K^d skin allograft survival was observed as compared to saline treated mice. The mean survival time (MST) of grafts for α2,3 Sia-K^d and K^d treated recipients was 13 days compared to 11.5 days for saline treated recipient mice [Fig 5.3A]. Although we did not observe allograft survival, we can conclude that our targeting regimen is not priming the animal as peptide and saline treated mice rejected with the same kinetics [Fig. 5.3].

Tanriver *et al*., (2010) established that contradictory to their *ex vivo* data, targeting a specific subset of DCs with K^d peptide coupled to a 33D1 antibody to B6 recipients 14 days before transplant did not prolong B6.K^d skin graft survival, but did observe significantly decreased K^d-specific alloantibodies in comparison

to untreated controls. As mentioned above, B cells which produce alloantibodies are primed by the $CD4^+$ T cells mediated indirect pathway of allorecognition, therefore a reduction of these alloantibodies is a hallmark for the inhibition of the indirect pathway (Suave *et al*., 2004). To determine whether our targeting regimen is suppressing the indirect alloresponse, serum was collected from transplanted recipients 4 weeks post-transplant and analysed for donor-specific (K^d) alloantibodies. As expected in saline treated mice, following transplantation, K^d-specific alloantibodies were observed which bound to B6.K^d splenocytes as opposed to B6 splenocytes [Fig. 5.3C]. Interestingly, there was a decrease in the development of alloantibodies in both K^d ($p=0.0004$) and α2,3 Sia-K^d ($p=0.003$) peptide treated recipients in comparison to the saline controls, however no significant difference was observed between the two peptide treated groups ($p=0.9$) [Fig. 5.3C]. These observations suggest that indirect $CD4^+$ T cells are being 'modified' once DCs are targeted with alloantigens prior to transplantation, as determined by the reduction of alloantibodies, however the rejection seen may be mediated by $CD8^+$ T cells directly recognising K^d on graft tissue following their transient removal. To assess this, we repeated the treatment regimen and transplant with prolonged infusions of anti-CD8 antibody, on days -1, 0, +7 and +14 post transplant. These time points were according to Tanriver *et al*., (2010) depletion regimen to ensure total depletion of $CD8^+$ T cells. However, despite this additional treatment no significant improvement in transplant survival was observed [Fig. 5.4].

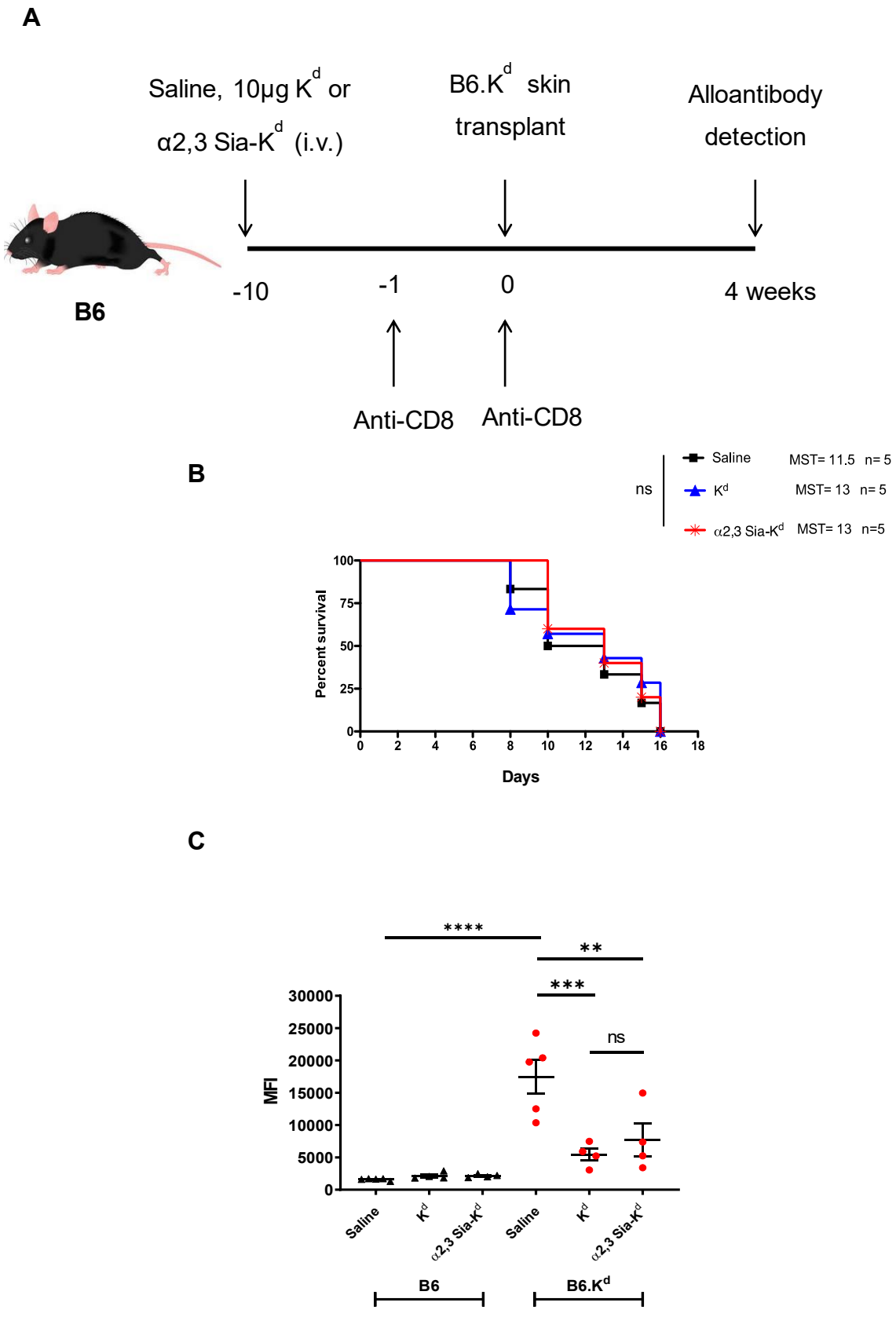


Figure 5.3. Graft survival was not prolonged when B6 mice were treated with α2,3 Sia-K^d 10 days before transplant, however alloantibodies were reduced. *A. Diagrammatic representation of the targeting and transplant*

*strategy. B6 mice (5 mice/group) received either 10µg/200µl saline of α2,3 Sia-K^d or K^d (i.v.). Control mice received 200µl saline i.v. only. One day following peptide treatment, mice received B6.K^d (K^d) skin transplant. Additionally mice received 250µg of anti-CD8 antibody (clone YTS169) (i.p.) on days -1 and 0. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. B. Data are shown as percentage of mice with surviving grafts (days) as demonstrated by MST (NS= p> 0.05 log-rank Mantel-Cox test). C. The development of K^d specific IgG Abs in serum derived from recipient mice was assessed against B6.K^d and B6 (control) target cells by flow cytometry 4 weeks following transplantation. Alloantibody for each individual mouse was detected using goat anti-mouse IgG-FITC and data is expressed as MFI of FITC binding to $CD3^+$ target T cells. Each dot represents one mouse and horizontal line between data points represents the mean MFI. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.0001****, p<0.001***, p<0.01**, NS= p>0.05.*

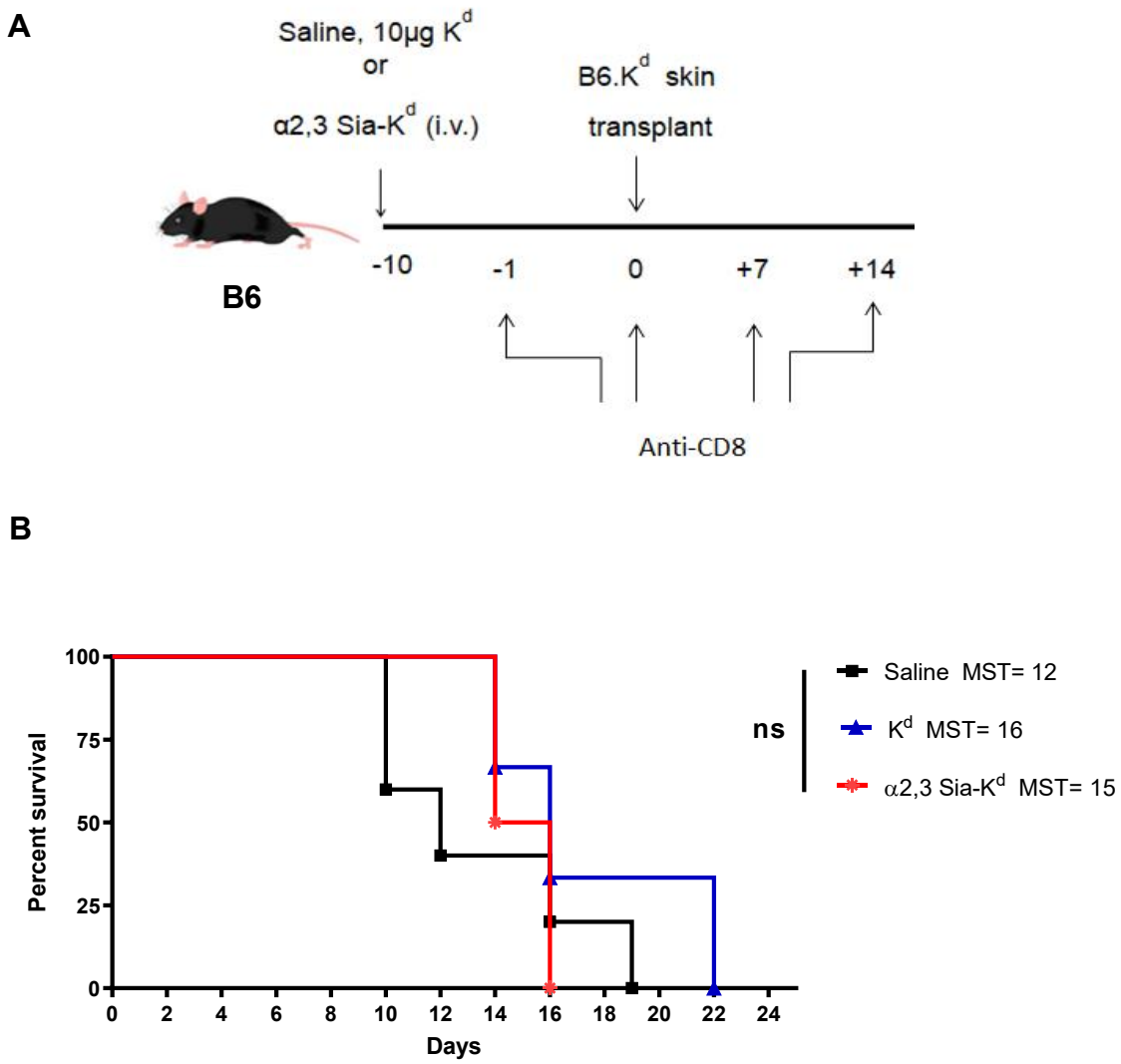


Figure 5.4. Graft survival was not prolonged when B6 mice were treated with α2,3 Sia-K^d 10 days before transplant, despite prolonged anti-CD8 treatment. *A. Diagrammatic representation of the targeting and transplant strategy. B6 mice (3-5 mice/group) received either 10µg/200µl saline of α2,3 Sia-K^d or K^d iv. Control mice received 200µl saline i.v. only. One day following peptide treatment, mice received B6.K^d (K^d) skin transplant. Additionally mice received 250µg of anti-CD8 antibody (clone YTS169) on days -1, 0, +7 and +14. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. B. Data are shown as percentage of mice with surviving grafts (days) as demonstrated by MST (*p< 0.05 log-rank Mantel- Cox test). Statistical significance was expressed as follows; NS= p> 0.05.*

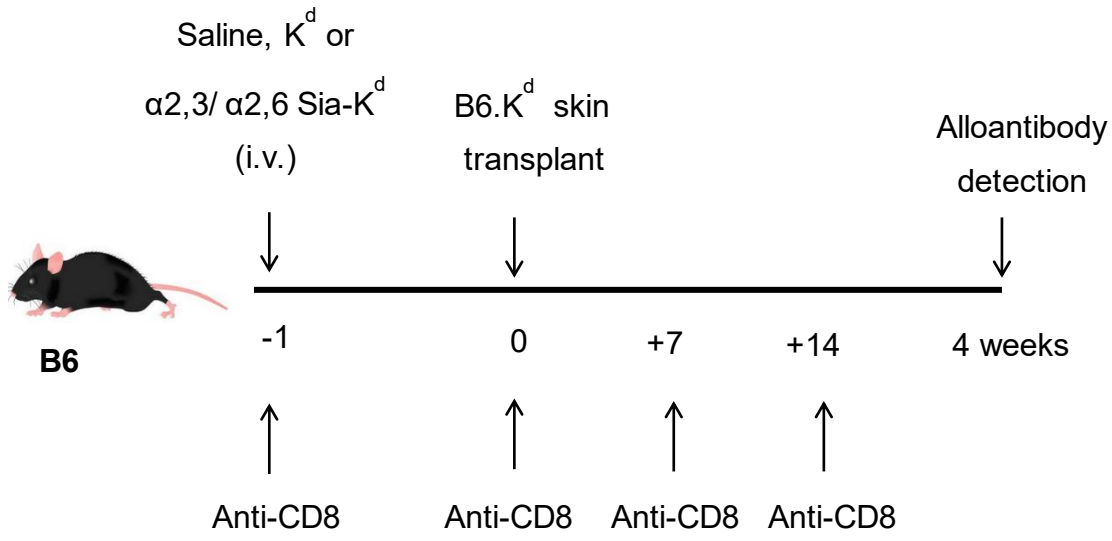
5.3.3. Targeting Siglecs in vivo with α2,3 Sia-K^d one day prior to a transplant prolonged allograft survival

As graft prolongation was not observed when alloantigens were administered 10 days prior to transplant, we decided to administer the peptide one day before as an alternative, especially since the half-life of these novel sialylated alloantigen peptides were not known. To this end B6 mice were administered with α2,3 Sia-K^d or α2,6 Sia-K^d as well as control K^d peptide 1 day before a B6.K^d skin transplant. Control mice received only saline. All mice additionally received weekly anti-CD8 treatment [Fig. 5.5A]. Skin transplant survival was monitored daily, and mice were bled 4 weeks post transplant to measure alloantibodies. B6 mice treated with α2,3 Sia-K^d had significant skin graft prolongation (MST: 16 days, p=0.002) compared to saline treated mice (MST: 11 days) and K^d treated mice (MST: 13 days) [Fig.5.5B]. In addition, there was no difference between K^d and saline treated mice [Fig. 5.5B]. In comparison to saline treated mice, this was not observed when the α2,6 Sia-K^d (MST: 13, p=0.6) was administered [Fig. 5.5B].

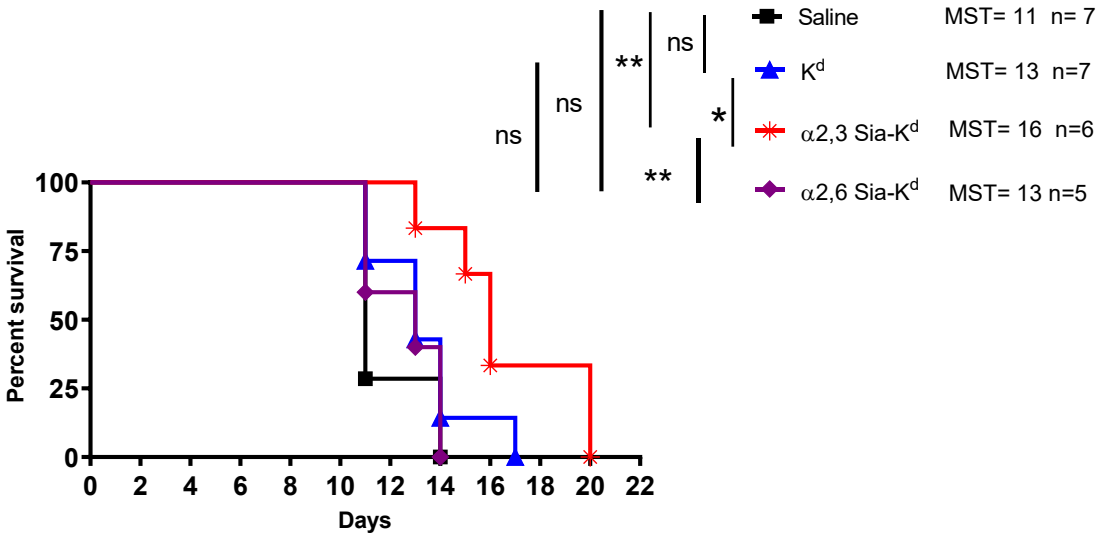
In addition, and as expected targeting B6 mice with K^d or sialylated alloantigens impaired CD4$^+$ indirect allorecognition pathway, leading to the reduction of K^d specific alloantibodies [Fig. 5.5C]. Overall, there was no significant difference in alloantibody production between the sialylated alloantigens, despite differences in graft survival. Although not statistically significant, there was a trend towards reduced alloantibodies with following sialylated alloantigen treatment in comparison to K^d.

Taken together, we concluded that targeting Siglecs with α2,3 Sia-K^d peptide one day before transplant, prolonged MHC I -mismatched skin grafts in the absence of CD8 by modifying the indirect pathway of allorecognition.

A


Saline, K^d or
α2,3/ α2,6 Sia-K^d
(i.v.)
B6.K^d skin
transplant
Alloantibody
detection
B6
-1
0
+7
+14
4 weeks
Anti-CD8
Anti-CD8
Anti-CD8
Anti-CD8

B


Saline MST= 11 n= 7
K^d MST= 13 n=7
α2,3 Sia-K^d MST= 16 n=6
α2,6 Sia-K^d MST= 13 n=5
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Percent survival
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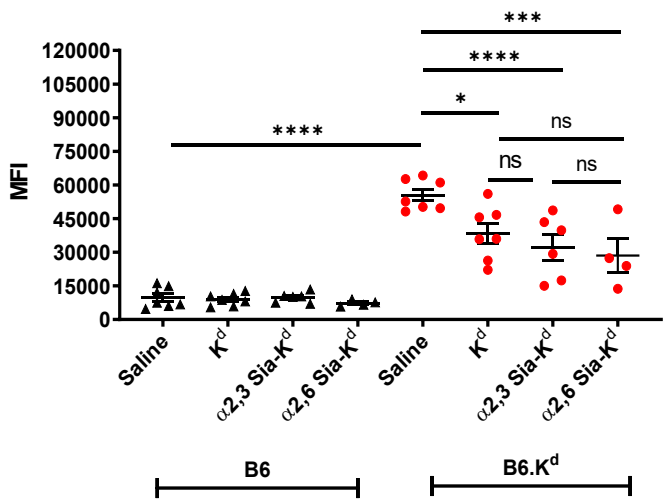
Days

C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α2,3 Sia-K^d
α2,6 Sia-K^d
Saline
K^d
α2,3 Sia-K^d
α2,6 Sia-K^d
B6
B6.K^d

Figure 5.5. Graft survival was prolonged when B6 mice were treated with α2,3 Sia-K^d one day before transplant*. A. Diagrammatic representation of the targeting and transplant strategy. B. B6 mice (5-7 mice/group) received either 10μg/ 200μl saline of α2,3 Sia-K^d or α2,6 Sia-K^d or K^d (i.v.). Control mice received 200μl saline i.v. only. One day following peptide treatment, mice received B6.K^d (K^d) skin transplant. Additionally mice received 250μg of anti-CD8 antibody (clone YTS169) on days -1, 0, +7 and +14. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. Data are shown as percentage of mice with surviving grafts (days). Mean survival time (MST) is shown. (*p> 0.05 (log-rank Mantel- Cox test)). C. The development of K^d specific IgG Abs in serum derived from recipient mice was assessed against B6.K^d and B6 (control) target T cells by flow cytometry 4 weeks following transplantation. Alloantibody for each individual mouse was detected using goat anti-mouse IgG-FITC and data is expressed as MFI of FITC binding to CD3$^+$ target T cells. Each dot represents one mouse and horizontal line between data points represents the mean MFI. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.0001****, p<0.001***, p<0.01**, p<0.05*, NS= p>0.05.*

5.3.4 Targeting recipient DCs with α2,3 Sia-K^d prolonged skin allograft survival in B6.Rag2$^{-/-}$ recipients

As it is observed that sialylated and unsialylated alloantigens bind to DCs as well as B cells *in vivo*, the question that arose was which of these APCs was driving the skin graft prolongation. To investigate whether skin graft prolongation is observed in recipient mice when B cells are absent but the DC subsets were present, we conducted our targeting experiments in Rag deficient mice, which lack T and B cells. Brennan *et al*., (2009) used an immunodeficient B6.Rag 2$^{-/-}$ model to assess the role of indirect CD4$^+$ T cell allorecognition in skin transplant rejection. These authors assessed rejection of BALB/c skin on B6.Rag$^{-/-}$ mice reconstituted with TCR75 CD4$^+$ T cells and noted that fully mismatched skin was rejected at approximately 10 days post transplant (Brennan *et al*., 2009). We modified this model slightly and chose to inject B6 rather than TCR75, derived CD4$^+$ T cells, as a slightly slower rejection was observed with B6 CD4$^+$ T cells [Appendix Fig. 6]. We reasoned that this would allow us a window of time to see differences between the treatments. When T cells are injected into an immunodeficient host such as Rag$^{-/-}$ mice, T cells are able to spontaneously undergo homeostatic proliferation, thereby increasing the T cell pool (Tanchot *et al*., 1997; Surh & Sprent, 2000). The concern was that mice that were administered with K^d alloantigen and TCR75 T cells followed by a BALB/c (WT mice that express H2-K^d MHC I) skin graft will have accelerated rejection due to the proliferation of K^d specificity of the T cells. We have previously shown in Chapter 3, increased T effector proliferation *in vitro* in response to K^d-pulsed DCs, therefore, we hypothesised that rejection would be too quick to measure. As a result a trial transplant experiment was conducted to compare BALB/c skin graft rejection between mice that were administered with TCR75 T cells (Brennan *et al*., 2009) versus B6 CD4$^+$ T cells. In comparison to B6.Rag2$^{-/-}$ mice receiving TCR75 T cells, a slightly slower pace of rejection, delayed by 2 days, was observed in mice that received B6 CD4$^+$ T cells, therefore, we opted to use the latter T cells [Appendix Fig. 6].

B6.Rag 2$^{-/-}$ mice were injected with saline,10μg K^d, α2,3 Sia-K^d, α2,3L Sia-K^d or α2,6 Sia-K^d peptides and 0.5 x 10^6 B6 CD4$^+$ T cells one day before receiving a BALB/c skin transplant [Fig 5.6A]. In comparison to saline treated mice (MST:

11 days), K^d peptide treatment significantly prolonged fully mismatched skin graft survival (MST: 13.5, p=0.02). However, this prolongation was significantly enhanced when recipient mice were treated with α2,3 Sia-K^d (MST: 25, p= 0.0005) but not α2,3L Sia-K^d or α2,6 Sia-K^d [Fig. 5.6B]. Overall, this data suggests that targeting Siglecs on DCs in mice devoid of B cells, with α2,3 Sia-alloantigens prolonged graft survival in B6.Rag $2^{-/-}$ mice reconstituted with B6 $CD4^+$ T cells, however this was not observed with the other sialylated peptides (α2,3L Sia-K^d or α2,6 Sia-K^d) [Fig. 5.6].

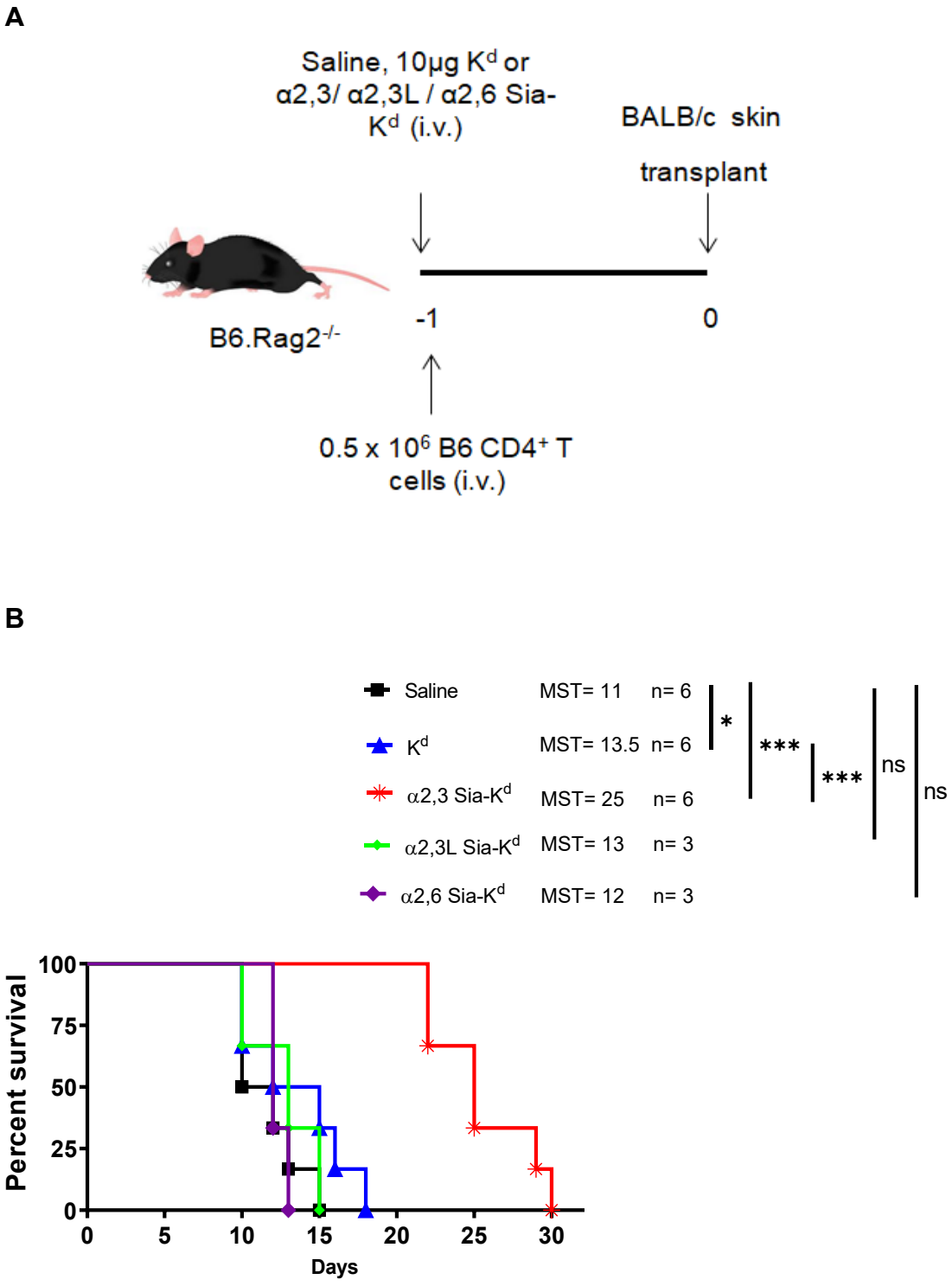


Figure 5.6. Allogeneic graft survival was prolonged in B6.Rag 2 $^{-/-}$ mice following α2,3 Sia-K^{d} treatment. *A. Diagrammatic representation of the targeting and transplant strategy. B. B6.Rag 2$^{-/-}$ mice (3-6 mice/ group) received 0.5 x 10^{6} B6 CD4^{+} T cells (i.v.) and either K^{d} 10µg/200µl saline or α2,3 Sia-K^{d} 10 µg/200µl saline, α2,3L Sia-K^{d} 10 µg/200µl saline or α2,6 Sia-K^{d} 10 µg/200µl saline (i.v.). Control mice received 200 µl saline i.v. only. One day following*

*peptide treatment, mice received BALB/c skin transplant. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. Data are shown as percentage of mice with surviving grafts (days). MST is shown. (*p> 0.05 (log-rank Mantel- Cox test). Statistical significance was expressed as follows; p<0.001***, p<0.05*, NS= p> 0.05.*

5.3.5. Targeting α2,3 Sia-K^d to recipient mice devoid of CD8α$^+$ DCs and CD103$^+$ DCs did not prolong transplant survival

The data so far suggests that targeting Siglec-expressing DCs, rather than B cells, with α2,3 Sia-K^d prolongs transplant survival. To further assess the contribution of endogenous DC subsets we repeated the experiments using B6.Batf3$^{-/-}$ knockout mice as transplant recipients. These mice lack the CD8α$^+$ and CD103$^+$ DCs, but have all the other DC subsets (eg; CD8α$^-$DCs, CD11b), macrophage and B cell compartments (Hildner *et al.*, 2008). Firstly, we confirmed the binding of the K^d and α2,3 Sia-K^d FITC peptides to B6.Batf3$^{-/-}$ splenic DCs *in vitro* [Fig. 5.7]. As expected α2,3 Sia-K^d and K^d peptides bound to CD11c$^+$ cells present in the B6.Batf3$^{-/-}$ spleen, which is consistent with our B6 data [Fig. 5.7].

Next, B6.Batf3$^{-/-}$ mice were administered with sialylated and non-sialylated K^d peptides either 1 or 10 days prior to a K^d skin transplant and rejection was measured [Fig. 5.8- 5.9]. In these experiments we tested only the α2,3 Sia-K^d peptide. Contrary to the B6 transplant recipients, α2,3 Sia-K^d treatment of B6.Batf3$^{-/-}$ mice 1 day before the transplant did not lead to graft prolongation with rejection times being comparable to mice treated with K^d or saline. The MST of the transplant in mice treated with saline or K^d peptide was 12 days whilst for α2,3 Sia-K^d treated individuals the MST was 10 days [Fig. 5.8B]. Similar observations were also made in B6.Batf3$^{-/-}$ mice treated with peptide alloantigens 10 days before transplant [Fig. 5.9B], suggesting that administering peptides at different time points has no impact on transplant outcome in these mice. Interestingly, B6.Batf3$^{-/-}$ mice treated with peptides had reduced alloantibodies, suggesting that the indirect pathway is still impaired but a reduction of alloantibodies is simply not enough to prolong allograft survival [Fig. 5.8- 5.9]. In summary, targeting Siglecs on either CD8α$^+$ DCs and/or

CD103^{+} DCs with α2,3 Sia-K^{d} may have led to prolonged allograft survival as seen in the B6 and B6.Rag 2$^{-/-}$ mice.

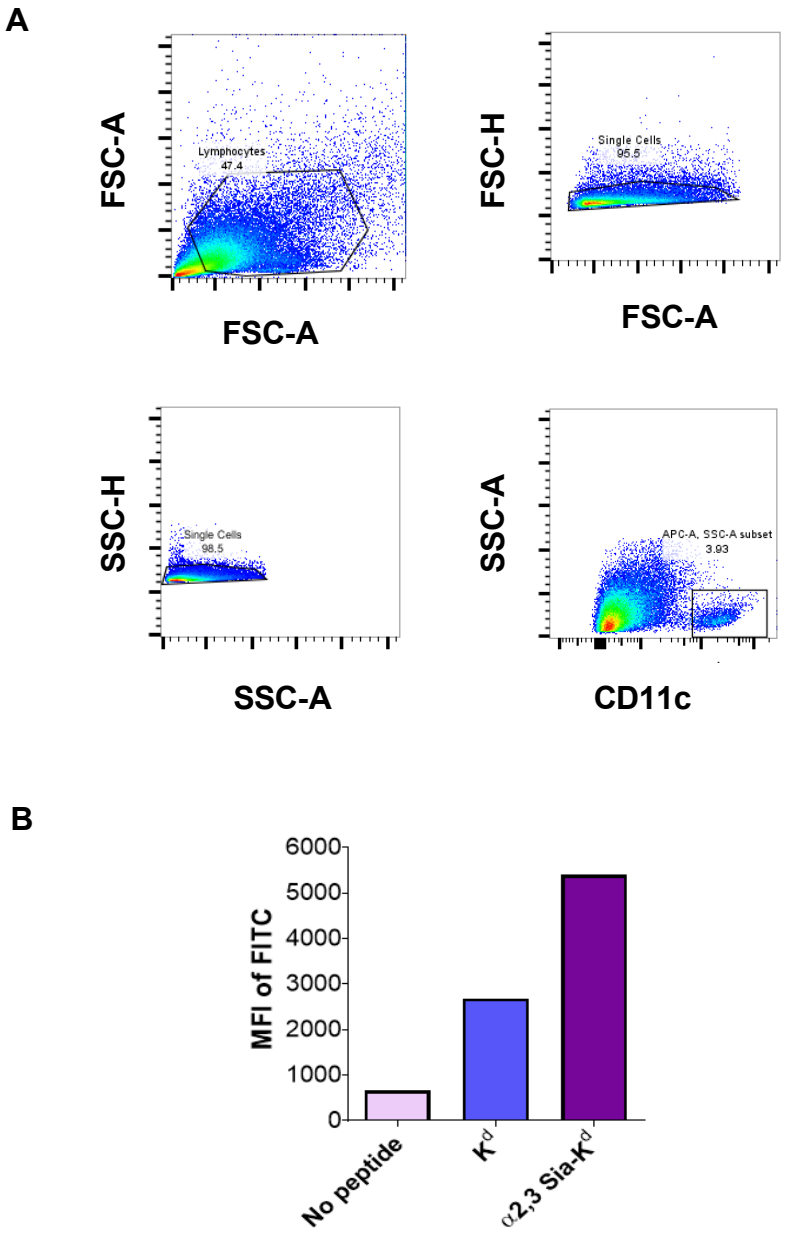


Figure 5.7. Alloantigen peptides bind to B6.Batf3$^{-/-}$ SPLN-DCs*. B6.Batf3 $^{-/-}$ SPLN-DCs were pulsed for 4 hours with 10 µg/ml peptide-FITC at 37°C incubation and analysed using flow cytometry. A. Live cells were gated on forward scatter (FSC), side scatter (SSC). B. MFI of peptide-FITC bound cells was assessed and each bar represents the MFI. Data represent one experiment.*

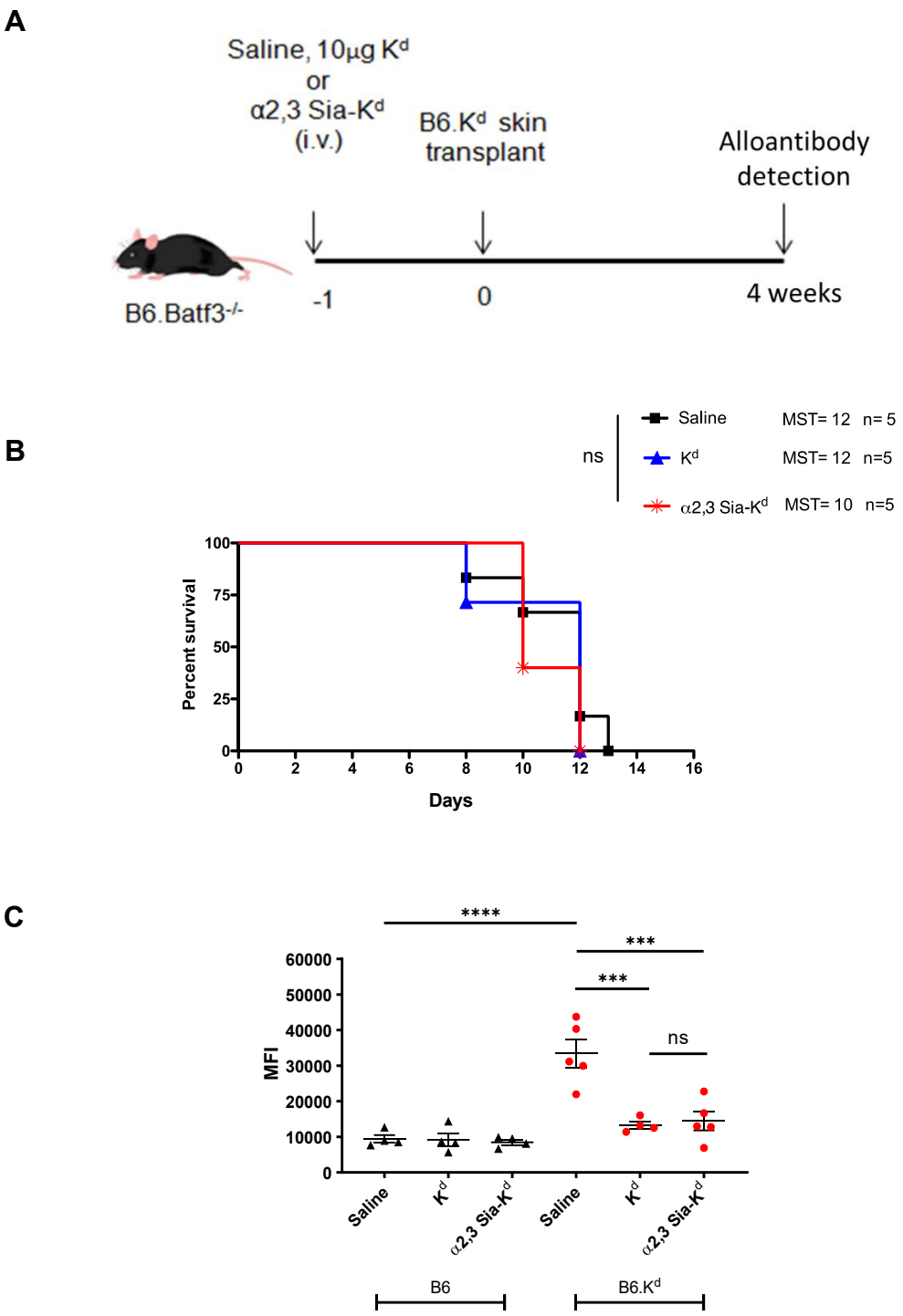
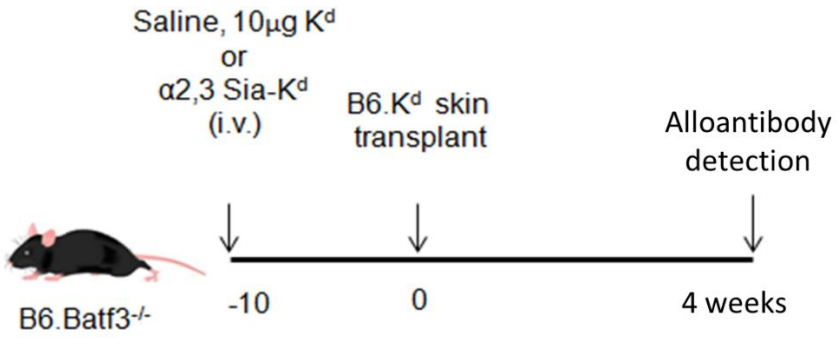


Figure 5.8. Targeting Siglecs expressed on B6. Batf3$^{-/-}$ DCs with α2,3 Sia-K^{d} does not prolong allograft survival. *A. Diagrammatic representation of the targeting and transplant strategy. B. B6.Batf3$^{-/-}$ mice (5 mice/group) received either 10µg/200µl saline of α2,3 Sia-K^{d} or K^{d} i.v. Control mice received 200µl saline i.v. only. One day following peptide treatment, mice received B6.K^{d} (K^{d}) skin transplant. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. Data are shown as percentage of mice with surviving grafts (days), MST is shown. (*p> 0.05 (log-rank Mantel- Cox test)). C.*

*The development of K^d specific IgG Abs in serum derived from recipient mice was assessed against B6.K^d and B6 (control) target T cells by flow cytometry 4 weeks following transplantation. Alloantibody for each individual mouse was detected using goat anti-mouse IgG-FITC and data is expressed as MFI of FITC binding to $CD3^+$ target T cells. Each dot represents one mouse and horizontal line between data points represents the mean MFI. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.0001****, p<0.001***, NS= p>0.05.*

A


Saline, 10µg Kd
or
α2,3 Sia-Kd
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B6.Kd skin
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B6.Batf3-/-
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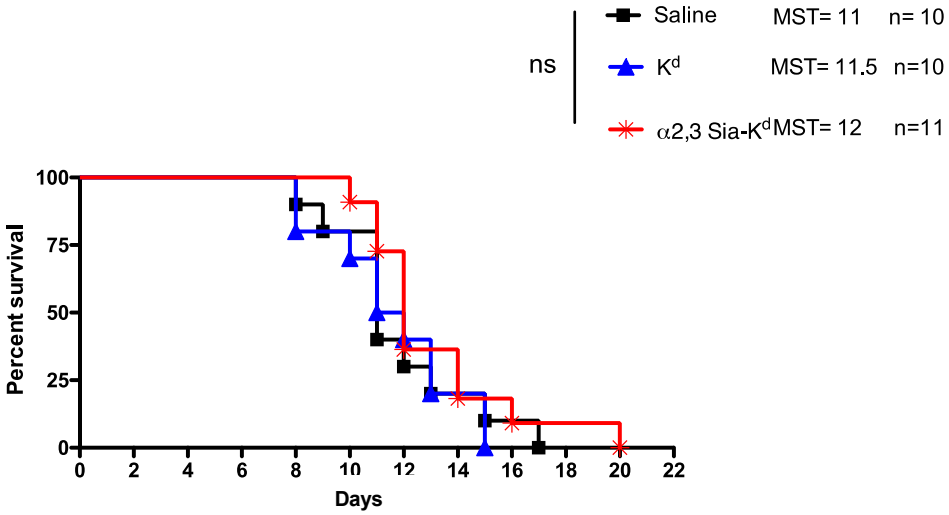
B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Percent survival
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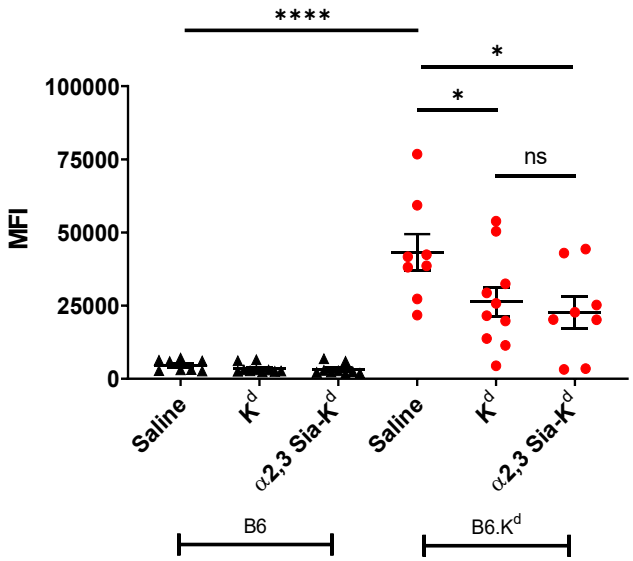
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B6
B6.Kd

Figure 5.9. Targeting Siglecs expressed on Batf3$^{-/-}$ DCs with α2,3 Sia-K^{d} does not prolong allograft survival. *A. Diagrammatic representation of the targeting and transplant strategy. B. B6.Batf3$^{-/-}$ mice (10-11 mice/ group) received either 10μg/200μl saline of α2,3 Sia-K^{d} or K^{d} i.v. Control mice received 200μl saline i.v. only. Ten days following peptide treatment, mice received B6.K^{d} (K^{d}) skin transplant. Mice were monitored daily and skin deemed rejected when 90% necrosis of donor skin was reached. Data are shown as percentage of mice with surviving grafts (days), MST is shown. (*p> 0.05 (log-rank Mantel-Cox test)). C. The development of K^{d} specific IgG Abs in serum derived from recipient mice was assessed against B6.K^{d} and B6 (control) target T cells by flow cytometry 4 weeks following transplantation. Alloantibody for each individual mouse was detected using goat anti-mouse IgG-FITC and data is expressed as MFI of FITC binding to CD3^{+} target T cells. Each dot represents one mouse and horizontal line between data points represents the mean MFI. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.001***, p<0.05*, NS= p> 0.05.*

5.4 Discussion

This chapter examined the possibility of promoting murine skin allograft survival by targeting DCs and B cells with sialylated alloantigens *in situ*. This chapter confirmed that K^d and α2,3 and α2,6 Sia-K^d peptides are capable of binding to lymph node-derived DCs, and B cells *in vivo* following intravenous administration. In addition, α2,3 Sia-K^d peptide targeting to B6. Batf3-dependent DC subsets, one day before a transplant, induced prolongation of skin graft survival in the absence of direct CD8 T cell responses. Moreover, both sialylated and non-sialylated alloantigens pre-treatment led to reduced alloantibodies in comparison to saline treated mice.

Siglec expression and peptide binding to splenic DC and B cells was confirmed *in vitro*, however, the *in vivo* was less clear-cut. This study initially identified that alloantigen peptides (sialylated or unsialylated) administered intravenously were capable of binding to splenic and lymph node DCs and B cells, however data was not statistically significant. As a future experiment, in order to refine this experiment and to perhaps allow better detection, harvesting the SPLN and LNs at a shorter time-point post peptide infusion such as 30 minutes (Mann *et al*., 2017), as opposed to 2 hours may be required. However, as we have observed a trend of increase in MFI of FITC in peptide-treated mice, we can suggest that peptides are binding to DCs and B cells in SLOs *in vivo*.

It has yet to be confirmed whether the sialylated peptides are binding to Siglecs present on APCs and whether they bind to particular Siglecs on APC subsets such Siglec G on $CD8\alpha^+$ DCs (Ding e*t al*., 2016), CD169 on M2 macrophages (Thornley *et al*., 2014) or Siglec E on T2 and MZ B cells (Zhang *et al*., 2004). It has yet to be investigated what other Siglecs are expressed on APC subsets and whether there is differential Siglec expression between APCs in LNs and SPLN. It was important to prove the presence of these peptides in SLOs such as the SPLN and LNs which are two of the essential organs where antigen presentation occurs and are also known for being the site for where cell trafficking and trapping of antigens from the bloodstream and peripheral tissues takes place, enabling them to interact with immune cells and initiate an adaptive immune response (Modino *et al*., 1996). It was especially important to confirm

the presence of these peptides in the lymph nodes as this is the site known for tolerance induction (Ochando *et al*., 2005; Dijke *et al*., 2008). During allorecognition, alloantigens derived from the graft can enter the SLOs via donor DCs, alloantigen-bound recipient DCs, exosomes carrying alloantigens or soluble antigens (Ng & Chalasani, 2010). However, there is an added complexity to transplantation; the pace of rejection can be dependent on the type of graft and the location of where the alloimmune response mainly occurs, the SPLN or the LNs. In a review by Ng and Chalasani (2010), they mention that the primary site for allorecognition for skin allografts are the LNs because once vascularisation occurs, the donor lymphocytes from the transplanted organs move via the lymphatics to the draining lymph nodes. The spleen also plays a role in rejection (particularly in heart allografts), however, Streilein and Wiesner (1977) found that splenectomised wild-type mice rejected skin grafts faster than mice with SPLNs, suggesting that the components of the spleen may have an immunoregulatory role, which remains to be determined.

One of our major findings was that targeting α2,3 sia-alloantigen to Siglecs on DCs resulted in transplant survival. Using B6. Batf3$^{-/-}$ mice we observed that engaging Siglecs on CD8α^{+} and CD103^{+} DC subsets (which require Batf3 transcription factor for their development) might have resulted in this outcome. CD8α^{+} DCs are primarily found in lymphoid tissues, whereas CD103^{+} DCs can be found in tissues such as the skin draining LNs, mesenteric LNs, lung and intestine (Edelson *et al*., 2010). Both these DC subsets are efficient for cross-presentation of antigens and CD8^{+} T cell immunity and are a source of IL-12 required for Th1 responses (Bedouj *et al*., 2009; Mashayekhi *et al*., 2011; Martinez- Lopez *et al*., 2015). In a murine transplant setting, one study reported that donor-derived CD103^{+} DCs present in donor skin grafts were responsible for allograft rejection, by carrying donor MHC molecules and stimulating direct allorecognition (Borges *et al*., 2018). On the contrary, these Batf3-dependent DCs have been shown to promote tolerance such as CD103^{+} DCs present in the gut are important cells involved in Treg induction leading to mucosal tolerance (Matteoli *et al*., 2010). In addition, targeting antigen to CD8α^{+} rather than CD8α^{-} DC subsets has been shown to promote antigen-specific T cell tolerance both *in vitro* and *in vivo,* however under proinflammatory conditions these CD8α^{+} DC were shown to promote Th1 responses (Manicassamy &

Pulendran 2011). As mentioned in the aforementioned review, one study identified that intraperitoneal administration of anti-DEC205 Ab conjugated to OVA antigen to mice (targeting DEC-205 $CD8\alpha^+$ DCs) induced $Foxp3^+$ cells from adoptively transferred $CD4^+$ $Foxp3^-$ cells (Yamazaki *et al.*, 2008). Therefore, this particular subset of DCs may be required to promote tolerance and additionally, the aforementioned study supports our findings where mice deficient of $CD8\alpha^+$ DCs are unable to prolong allograft survival when administered with sialylated alloantigen.

$CD8\alpha^-$ DC subsets on the other hand are more efficient at promoting $CD4^+$ T cell responses and also display multiple functions according to where they reside (Hasegawa & Matsumoto, 2018; Tanriver *et al.*, 2010). It was interesting to see that according to Tanriver *et al.*, (2010), where targeting CD8α- DCs alone by targeting 33D1 established long-term allograft skin survival, whereas we did not see this when B6.$Batf3^{-/-}$ mice (devoid of $CD8\alpha^+$ DC subset) were targeted. However, an important difference which may contribute to the contrasting findings is that the aforementioned study targeted B6 mice and established tolerance using a 33D1-K^d monomer as opposed to 33D1-K^d peptide (Tanriver *et al.*, 2010). They reasoned that targeting with a monomer as opposed to a peptide, will help to target a wider range of $CD4^+$ TCRs, rather than a single TCR epitope specific to a peptide, therefore as future work, sialylated MHC monomer may be used as an alternate targeting regimen (Tanriver *et al.* 2010).

Overall, various subsets of DCs when targeted independently can promote tolerance such as $CD4^+$ $CD8^-$ DCs ($DCIR2^+$ DCs) (Tanriver *et al.*, 2010) and DEC-205 DCs (Yamazaki *et al.*, 2008). $CD103^+$ DCs is another subset with the potential to promote tolerance when targeted. For example, Idoyaga *et al.*, (2013) showed that migratory $CD103^+$ DCs were able to generate MOG antigen-specific $CD4^+$ Tregs from adoptively transferred MOG-specific $CD4^+$ T cells *in vivo,* following s.c. administration of an anti-DEC monoclonal antibody engineered to conjugate to MOG. Although targeting these DC subsets has led to tolerance induction in other models, it is a possibility that in our model $CD103^+$ DCs as well as $CD4^+CD8^-$ DCs and/ or $CD11b^+$ DCs are required, perhaps through the generation of Tregs, which may lead to allograft survival in

our transplant model. We suggest a role for CD103$^+$ DCs, as B6 mice that were administered with anti-CD8 antibody, which depletes CD8 positive cells including CD8α$^+$ DCs (Smyth *et al*., 2013), had surviving allografts following sialylated K^d administration. On the contrary, we still cannot rule out the possibility of sialylated peptides binding to CD8α DCs$^+$, especially since we confirmed the presence of the peptides in the SPLN and LNs in this Chapter.

It has been demonstrated by Tanriver *et al*., (2010), that targeting a subset of endogenous murine DCs (DCIR2) with an MHC I monomer conjugated to a 33D1-crosslinking antibody promotes B6.K^d skin graft tolerance. In contrast they observed that targeting B6 WT mice with 33D1 antibody conjugated with a K^d monomer alone (without anti-CD8 depletion to deplete CD8$^+$ direct alloreactive cells) did not prolong allograft survival when administered 14 days prior to transplant but did lead to decreased alloantibodies. These findings are similar to what we observed where B6 mice were targeted with sialylated or non-sialylated peptide 10 days before the transplant did not improve transplant survival but did reduce alloantibodies. These alloantibodies can contribute to rejection through interactions such as with Fc gamma RIII on NK cells leading to antibody dependent cellular cytotoxicity, complement fixation forming antigen-antibody bound complexes to form foreign antigens recognised by APCs (Smith & Colvin, 2012). Importantly, these alloantibodies are known for being produced by B cells which have been activated via indirect allospecific CD4$^+$ T cells, therefore suggesting that similarly to our *in vitro* data, the T cell mediated indirect pathway of allorecognition is still being suppressed in our transplant models which is evident by the decrease in alloantibodies (Sauve *et al*., 2004; Steele *et al*., 1996).

Our results suggest that targeting DCs with α2,3 Sia-K^d rather than α2,3L Sia-K^d or α2,6 Sia-K^d can promote allograft survival. We reasoned that this may be due to the binding capacity of these constructs as we have seen in Chapters 3 and 4, where α2,3 Sia-K^d binding to BM-DCs was greater in comparison to K^d targeted BM-DCs, whereas there were no differences in binding between α2,6 Sia-K^d and K^d-binding to BM-DCs. Therefore, allograft survival may be due to α2,3 Sia-K^d binding to particular Siglecs on DCs that have an increased affinity to α2,3 sialic acids rather than α2,6 sialic acids. However in Chapter 3, no

significant differences in K^d and α2,3 Sia-K^d peptide binding was observed in SPLN-DCs which are endogenously found, therefore it is controversial to explain allograft survival based on peptide-binding to DCs *in vitro*. A review by Crocker *et al*., (2007), suggested that Siglec E has moderate binding to both α2,3 and α2,6 sialic acids, however Siglec F binds moderately to α2,3 sialic acids and interestingly, binds weakly to α2,6 sialic acids. Taking into account the data in this Chapter 5 and Chapter 3 where Siglec F was highly expressed on BM and SPLN-DCs than Siglec E and the review by Crocker *et al*., (2007); transplant survival may be due to interactions between α2,3 Sia-K^d and Siglec F and on to a lesser extent, Siglec E. Future investigations may include Siglec knockout mice which could help fully confirm the interaction of Siglec E/F and this sialylated peptide and their role in allograft survival.

The lack of transplant survival seen following administration of α2,6 Sia-K^d may be due to it interacting with Siglec E, suggesting that targeting this Siglec does not lead to transplant survival. This finding is in contrast to a study by Perdicchio *et al*., (2016) where they demonstrated that the interaction of Siglec E and α2,6- sialylated antigens induced $Foxp3^+$ Tregs *in vitro*. However, the obvious difference between the experiments is that the aforementioned experiment was carried out using Siglec $E^{-/-}$ BM-DCs *in vitro* and we tested our sialylated constructs in *an vivo* transplant model. In addition, their study did not compare directly α2,6 and α2,3 Sia-OVA/MOG constructs *in vivo*, therefore given our findings it is possible that α2,3 sialylated constructs may be better than α2,6 sialylated constructs at promoting immunosuppressive immune responses in an *in vivo* transplant model. Conversely, Perdicchio *et al*., (2016), did demonstrate the potential of targeting mice with α2,6 sialylated antigens to promote tolerance *in vivo,* in an autoimmune setting. They established that targeting endogenous DCs using α2,6 Sia-OVA *in vivo*, followed by OVA/ PolyI:C/ anti-CD40 sensitization, decreased proportions of T effectors and increased Tregs. Taken together they were able to demonstrate that targeting Siglec E can modulate DCs under pro-inflammatory conditions (Perdicchio *et al*., 2016).

The data in this chapter demonstrated that targeting Siglecs on B cells *in vivo* with sialylated alloantigens did not have a significant role in prolonging allograft

survival. In recipient mice lacking B cells which are B6.Rag2$^{-/-}$ mice reconstituted with B6 CD4^{+} T cells, α2,3 Sia-K^{d} pre-treatment prolonged allograft survival. Surprisingly, despite the inhibitory role of B cell Siglecs and their for preference for α2,6- linked sialic acids (demonstrated in Chapter 4), (Crocker *et al*., 2007; Pillai *et al*., 2012), targeting B6 mice with α2,6 Sia-K^{d} did not prolong allograft survival. Therefore, targeting B cell Siglecs *in situ* does not promote allograft survival.

The next question is, if targeting B cells does not prolong allograft survival by influencing them to become regulatory, how can the reduction of alloantibodies be explained? We observed that B6 and B6.Batf3$^{-/-}$ mice targeted with sialylated or non-sialylated peptide had reduced alloantibodies despite the different transplant survival outcome. These alloantibodies are produced by B cells which have been activated via indirect allospecific CD4^{+} T cells, therefore suggesting that similarly to our *in vitro* data, the T cell mediated indirect pathway of allorecognition is still being suppressed in our transplant models as evident by the decrease in alloantibodies (Sauve *et al*., 2004; Steele *et al*., 1996).

To date it has not been demonstrated that targeting B cell Siglecs with α2,6 sialic acids induces Tregs, but instead it has been shown that activation of BCR signalling is impaired. B cell Siglecs have ITIMs that recruit SHP-1 tyrosine phosphatases to inhibit BCR signalling (Meyer *et al*., 2018). One study found that targeting Siglec CD22 on B cells with an α2,6 Sia construct, that is also capable of binding to the BCR, was capable of impairing Ca^{2+} influx into the cytosol of B cells, thereby reducing B cell activation (Courtney *et al*., 2009). This study is described more in detail in Chapter 7. As we have seen that sialylated alloantigens bind to B cells in *vitro* and *in vivo*, it is a possibility that B cells are not being activated upon sialylated alloantigen, which is a consequence of B cell Siglec targeting that Meyer *et al*., (2018) and Courtney *et al*., (2009), mentioned. Therefore, a lack of B cell activation may explain a reduced generation of alloantibodies when B6 transplant recipient mice were administered with sialylated alloantigen. This conclusion is questionable given that K^{d} targeted mice also had reduced alloantibodies, therefore this reduction may be of consequence for T cell deletion which will be addressed in Chapter 6.

B cells have shown to be involved in allograft survival, particularly transitional B cells. Moreau *et al*., (2014) discovered that B cells transferred from tolerant mice who accepted MHC I mismatched skin allografts, were able to prolong allograft survival and significantly reduce production of alloantibodies against donor grafts. Additionally they identified that T2 and MZ B cells were amongst the tolerogenic B cell pool, with T2 B cells being able to suppress proliferation of T cells that have been activated with CD3/CD28 stimulation (Moreau *et al*., 2014). This would suggest that transitional B cells play a role in MHC I-mismatched allograft survival. We have demonstrated in Chapter 4, that α2,6 Sia-K^d preferentially bind to T2 subsets *in vitro* over K^d- pulsed B cells, demonstrating the potential of this construct to targeting tolerogenic B cells. To address whether B cells are truly required for allograft survival following sialylated alloantigen targeting, B6 WT mice which have all innate and adaptive lymphocyte compartments; were targeted with alloantigens. As expected, α2,3 Sia-K^d targeting still prolonged allograft survival, however, α2,6 Sia-K^d targeting did not, despite the presence of B cells. Therefore, targeting B cell Siglecs *in situ* does not promote allograft survival, and our *in vitro* data in Chapters 3 and 4 reflects this.

Overall, it has been demonstrated for the first time that MHC mismatch skin allograft survival and an inhibition of the $CD4^+$ T cell indirect allorecognition pathway can be achieved following sialylated alloantigen targeting. In this Chapter we did not address the mechanism behind the prolongation *in vivo*. However, literature focused on peptide-induced tolerance in models such has EAE, have found T cell anergy, Treg induction and impaired effector T cell proliferation (Maldono *et al*., 2015; Ghetts *et al*., 2012). Perdicchio *et al.,* (2016) showed that sialylated OVA antigen targeted to endogenous Siglec-expressing DCs *in vivo*, decreased T effector proportions and increase $Foxp3^+$ Tregs. Similar mechanisms could be at play following α2,3 Sia-K^d targeting and will be addressed in the next chapter.

CHAPTER 6: T CELL DELETION AND INCREASE OF $CD4^+$ $CD62L^+$ $FOXP3^+$ REGULATORY T CELLS CONTRIBUTE TO GRAFT PROLONGATION FOLLOWING α2,3 SIA-K^d ALLOANTIGEN TREATMENT

6.1 Introduction

This chapter focuses on investigating the underlying mechanisms for transplant survival following α2,3 sialylated alloantigen targeting to Siglecs expressed on DCs, as outlined in Chapter 5.

As described in Chapter 1, various mechanisms to induce tolerance exist such as T cell deletion and Treg induction/ expansion. Deletion can occur both in the thymus (central tolerance) and in the periphery (peripheral tolerance) as a means of promoting self-tolerance by removing self-reactive T cells. In a transplant setting, T cell deletion is a process whereby alloreactive T cells that have the potential to mature and elicit effector function against the transplanted tissue are killed. Mentioned in a review by Li *et al*. (2001), the proportions of direct alloreactive T cells responding to MHC-mismatched grafts, is greater in frequency than T cells responding to nominal antigens. Therefore, the increased frequency of MHC responsive alloreactive T cells contributes to transplant failure. One approach to deplete these alloreactive T cells is to target alloantigen to receptors on specific DCs *in vivo*. Tanriver *et al*., (2010) found that when DCs were targeted with K^d alloantigen *in vivo,* using an antibody to 33D1 conjugated to-K^d monomer, reduced numbers of adoptively transferred TCR75 T cells in comparison to mice treated with saline alone. This would suggest that alloantigen targeted to $CD8\alpha^-$ DCs *in vivo* promoted deletion of alloreactive specific T cells leading to murine skin graft prolongation (Tanriver *et al*., 2010), and one of the focuses of this chapter is to assess whether T cell deletion contributes to allograft survival following alloantigen targeting.

The presence of $CD4^+$ Tregs in a transplanted organ is associated with prolonged graft survival (Shaban *et al*., 2018; Yu *et al*., 2011; Shinoda *et al*., 2014). This has led to many laboratories designing protocols to achieve transplant tolerance by expanding these cells *ex vivo* and *in vivo*. For example, studies have found that the adoptive transfer of expanded recipient-derived Tregs is an effective approach to induce tolerance in mouse transplant models (Ratnasothy *et al*., 2019; Tsang *et al*., 2008). It is also known that Treg proportions can be increased *in vivo* and help prolong allograft survival as opposed to adoptively transferred Tregs. For example, Magee *et al*., (2019),

recently showed that by blocking Notch-1 signalling using anti-Notch-1 to block induction of endogenous naïve $CD4^+$ T cells to Th cells; $Foxp3^+$ Tregs proportions were increased and MHC-mismatched allograft survival was prolonged.

Tregs which are $CD62L^{high}$, have shown to be highly immunosuppressive (Ermann *et al.*, 2005; Fu *et al.*, 2004; Lange *et al.*, 2011). Ermann *et al.*, (2005), identified in a murine GVHD model, $CD62L^+$ Tregs were able to accumulate in recipient LNs and SPLNs and inhibit expansion of alloreactive T cells in contrast to the $CD62L^-$ Tregs. This would suggest that these $CD62L^+$ migratory Tregs have an advantage over their $CD62L^-$ counterparts as they have the ability to enter sites where alloantigen presentation by recipient DCs occurs such as the SPLN and LNs (Ermann *et al.*, 2005). Demonstrated in Chapters 3 and 4, *in vitro* targeting of BM-DCs with sialylated alloantigens consistently induced $CD4^+$ $Foxp3^+$ Tregs, therefore, this chapter will assess whether this outcome was observed *in vivo* in transplant recipients. The proportions of $CD62L^+$ Tregs in recipient mice will determine whether these particular Tregs are assisting in graft prolongation observed in Chapter 5.

6.2. Aim

To dissect the mechanisms of tolerance behind skin allograft survival following Siglec targeting with α2,3 alloantigen construct.

In order to address this aim, the following objectives were undertaken:
1. Assess whether *in vivo* deletion of alloreactive T cells, K^d-antigen specific $CD4^+$ T cells (TCR75 T cells), occurs in mice administered with α2,3 sialylated alloantigens.
2. Measure the proportions of $Foxp3^+$ Tregs in transplanted recipient mice and assessing their phenotype ($CD62L^+$).

In Chapters 3 and 4 we demonstrated that targeting BM-DCs with α2,3, α2,3L and α2,6 sialylated alloantigen *in vitro* impairs proliferation of TCR75 T cells and induced/expanded Tregs. Having demonstrated that targeting Siglecs using sialylated constructs impaired indirect allorecognition *in vitro*, the constructs

were tested in murine skin transplant models in Chapter 5. Only pre-treatment with the α2,3 sialylated construct induced transplant prolongation highlighting that *in vitro* and *in vivo* findings can differ. Some of the other significant findings in the aforementioned Chapter demonstrated that B6 WT and B6 $Rag^{-/-}$ mice treated with α2,3 Sia-K^d one day before transplant prolonged allograft survival, whereas B6.$Batf3^{-/-}$ mice (devoid of $CD8\alpha^+$ DCs and $CD103^+$ DCs) treated with the same peptide did not prolong transplant survival. This data suggested that targeting Siglecs on the aforementioned DCs with α2,3 Sia-antigens may contribute to prolonging the graft.

Despite these differences, the generation of alloantibodies produced by B cells that have been activated via the indirect pathway $CD4^+$ T cell help, were profoundly decreased in B6 WT and $Batf3^{-/-}$ mice in response to K^d and all Sia-K^d peptide treatments prior to transplantation (Suave *et al*., 2004). This would suggest that in both models, the indirect pathway of allorecognition is impaired; however the reduction of alloantibody production may not be a significant contributor to allograft survival in this study given the data in Chapter 5. Aside from the similarity of alloantibody generation between models and targeting regimens, there is a clear difference in immune response mediated by K^d and Sia-K^d which is demonstrated by the transplant data in Chapter 5. Therefore, this chapter will focus on whether other mechanisms such T cell deletion assist in transplant survival following α2,3 Sia-K^d targeting and the contribution of $CD62L^+$ $Foxp3^+$ Tregs.

In terms of clinical relevance, this chapter will provide some input into understanding the mechanisms of allograft survival following of targeting Siglec receptors on endogenous DC subsets *in situ*.

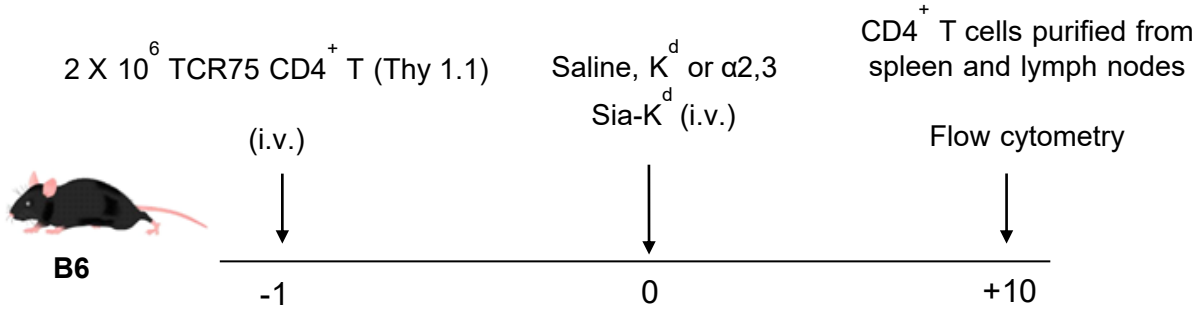
6.3. Results

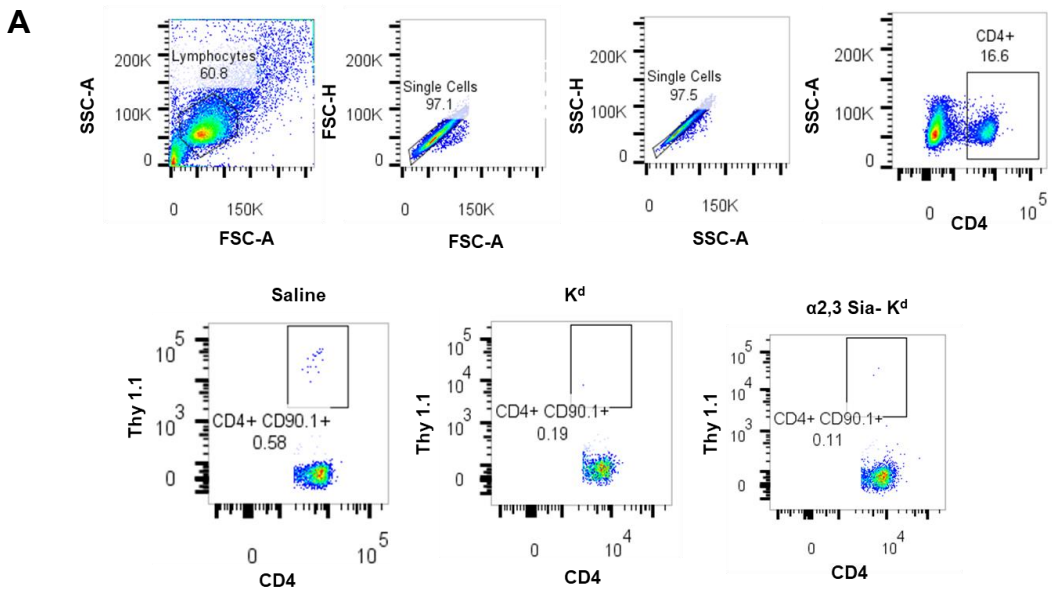
6.3.1 Indirect alloantigen-specific CD4$^+$ T cells are deleted following peptide targeting in vivo

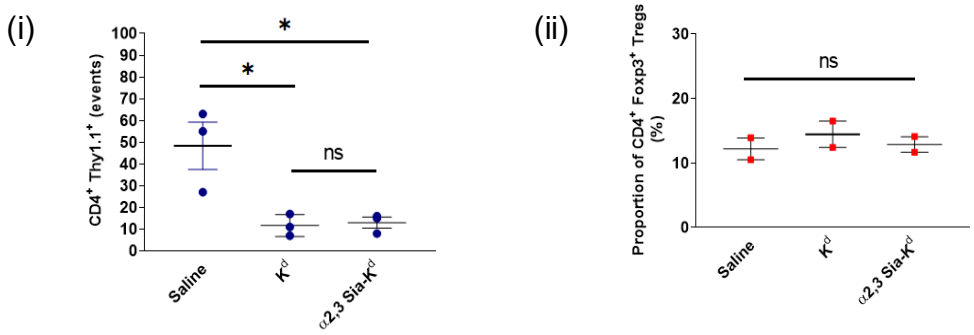
One of the main mechanisms that led to both central and peripheral tolerance is deletion of antigen specific T cells, which is possible to achieve in murine models by immunizing mice at a steady state with soluble antigen or peptide. Tanriver *et al.*, (2009), found that adoptively transferring TCR75 CD4$^+$ T cells into naïve recipient B6 mice followed by administration of 33D1 Ab- K^d peptide 24 hours later, resulted in TCR75 CD4$^+$ T cell deletion without altering the endogenous Treg pool (Tanriver *et al.*, 2009). Therefore, in the current study, we determined whether antigen-specific deletion is one of the mechanisms contributing to allogeneic skin transplant survival following α2,3 Sia-K^d treatment seen in Chapter 5. B6 and B6.Batf3$^{-/-}$ mice received 2 x 10^6 TCR75 CD4$^+$ T cells (Thy1.1) intravenously, 24 hours prior to 10μg/ml K^d or α2,3 Sia-K^d peptide administration. Control mice received T cells and saline only. Both the spleens and lymph nodes from these mice were harvested 10 days later and the presence of TCR75 CD4$^+$ T cells was assessed using an anti-Thy1.1 antibody. As expected, less Thy1.1$^+$ CD4$^+$ T cells were present in B6 mice treated with K^d in comparison to mice treated with saline only [Fig. 6.1B (i)]. In addition, α2,3 Sia-K^d alloantigen peptide treatment led to reduced TCR75 CD4$^+$ T cells. However, there were no significant differences in the number of Thy1$^+$ T cells between the sialylated and non-sialylated peptides (p=0.98) [Fig. 6.1B (i)]. Similar results were observed when B6.Batf3$^{-/-}$ mice were used [Fig. 6.1C (i)], demonstrating deletion of TCR75 T cells in response to α2,3 Sia-K^d (p=0.03) and K^d (p=0.02) in comparison to saline.

In line with the findings of Tanriver *et al.,* (2010), K^d treatment did not significantly alter the endogenous Treg pool in either of the mouse strains [Fig. 6.1]. In addition, treatment with α2,3 Sia-K^d in both mouse strains resulted in a similar finding. Therefore, antigen-specific deletion may be one of the mechanisms contributing to graft survival, or contributing to a reduction of alloantibodies by depleting the proportions of Th cells engaging with

alloantibody-producing B cells. However, as B6.Batf3$^{-/-}$ mice did not induce graft survival following targeting but were able to delete antigen-specific T cells, deletion may not be the sole mechanism for graft prolongation in this study.


2 X 10^6 TCR75 CD4^+ T (Thy 1.1)
(i.v.)
B6
or
B6.Batf3^-/-
Saline, K^d or α2,3 Sia-K^d (i.v.)
CD4^+ T cells purified from spleen and lymph nodes
Flow cytometry
-1
0
+10


A
Lymphocytes 60.8
Single Cells 97.1
Single Cells 97.5
CD4+ 16.6
SSC-A
FSC-A
FSC-H
SSC-H
CD4
Saline
Kd
α2,3 Sia- Kd
Thy 1.1
CD4+ CD90.1+ 0.58
CD4+ CD90.1+ 0.19
CD4+ CD90.1+ 0.11


B
(i)
(ii)
CD4^+ Thy1.1^+ (events)
Proportion of CD4^+ Foxp3^+ Tregs (%)
ns
Saline
Kd
α2,3 Sia-Kd

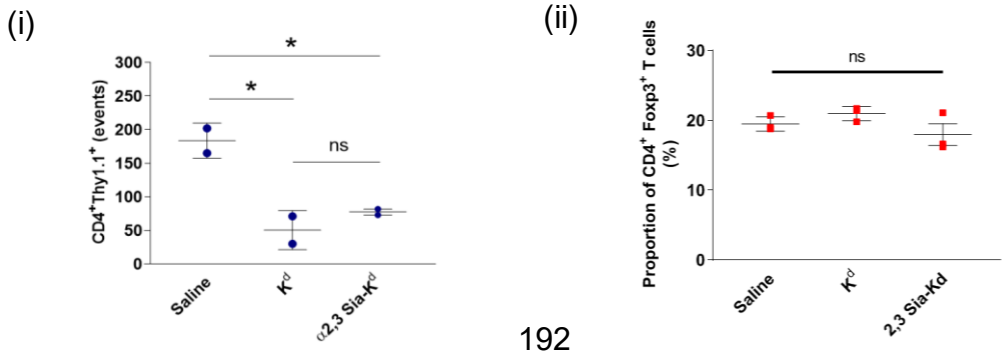
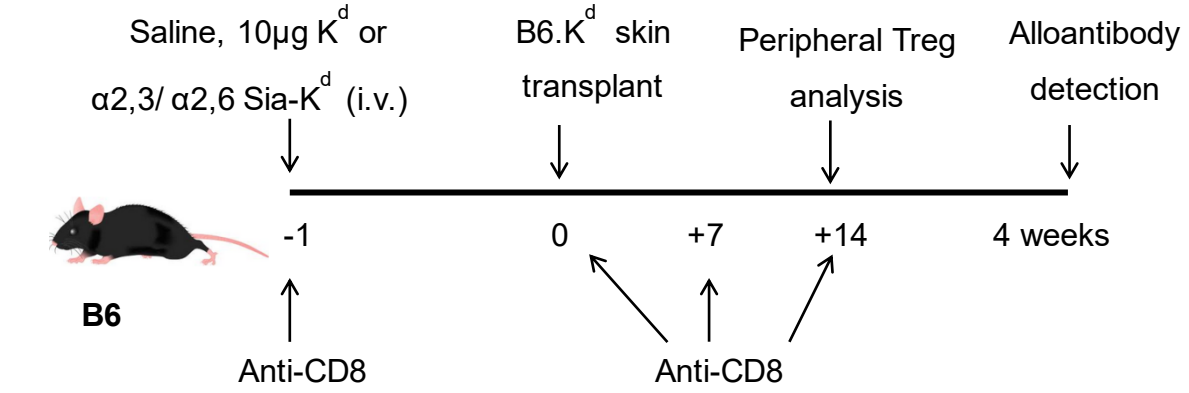

C
(i)
(ii)
CD4^+Thy1.1^+ (events)
Proportion of CD4^+ Foxp3^+ T cells (%)
ns
Saline
Kd
α2,3 Sia-Kd
2,3 Sia-Kd

Figure 6.1. CD4$^+$ T cells are deleted following peptide targeting, however, CD4$^+$ Foxp3$^+$ Tregs numbers remain unchanged*. B6 or B6.Batf3$^{-/-}$ mice were administered with 2 x 10^6 TCR75 T cells (Thy1.1) one day before mice received K^d 10µg/200µl saline or α2,3 Sia-K^d 10 µg/200µl saline, i.v. No peptide controls received 200 µl saline i.v. only. Ten days later spleens and lymph nodes were harvested and stained for CD4, Thy1.1 and Foxp3. A. Live cells were gated on forward scatter (FSC), side scatter (SSC) then CD4 versus Thy1.1 cells. B. (i). Number of cellular events of CD4$^+$ Thy1.1$^+$ T cells in B6 mice (n=3 mice per group). (ii). Percentage of CD4$^+$ Foxp3$^+$ Tregs in B6 mice (n=2 mice per group). C. (i). Number of cellular events of CD4$^+$ Thy1.1$^+$ T cells in B6.Batf3$^{-/-}$ mice (n=2 mice per group). (ii). Percentage of CD4$^+$ Foxp3$^+$ Tregs B6. Batf3$^{-/-}$ mice (n=2 mouse per group). Each data point represents one mouse. Error bars represent +/- SEM and horizontal line between data points represent mean. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p<0.05*, NS= p>0.05.*

6.3.2. α2,3 Sia-K^d administration increased proportions of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs

Ermann *et al.*, (2005), Fu *et al.*, (2004) and Lange *et al.*, (2011), have described that CD62L$^+$ Foxp3$^+$ Tregs are more suppressive than CD62L$^-$ Tregs. In order to determine whether CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs were contributing to graft prolongation observed in *Figure 5.5A*, B6 mice transplanted with B6.K^d skin grafts following administration of peptide alloantigens, K^d, α2,3 Sia-K^d and α2,6 Sia-K^d one day before transplant, were bled two weeks post transplant and CD4, CD62L and Foxp3 expression analysed via flow cytometry. An increased proportion of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs was observed in α2,3 Sia-K^d treated mice (mean= 4.01%) in comparison with mice treated with K^d (mean= 1.66%), saline (mean= 1.63%), which was not seen when the recipients mice were treated with α2,6 Sia-K^d (mean= 0.68%) [Fig.6.2-3]. In fact, it was observed that mice treated with K^d had increased ability of inducing/ expanding of CD4$^+$CD62L$^-$ Foxp3$^+$ Tregs [Fig. 6.3B]. As demonstrated in Chapter 5, B6 mice treated with α2,3 Sia-K^d, had significant graft survival, which may be due to an

increase of CD62L^{+} Tregs. Whereas, B6 mice treated with K^{d} and α2,6 Sia-K^{d} peptide did not have increased transplant survival nor CD62L^{+} Tregs, but interestingly had increased proportions of CD62L^{-} Tregs [Fig. 6.2 & Fig. 6.3 A]. This would suggest that targeting Siglecs on endogenous CD8α^{+} and CD103^{+} DCs with α2,3 Sia-K^{d} *in vivo* increases CD62L^{+} Tregs which may be a contributing factor to graft survival.


Saline, 10μg K^d or
α2,3/ α2,6 Sia-K^d (i.v.)
B6.K^d skin
transplant
Peripheral Treg
analysis
Alloantibody
detection
-1
0
+7
+14
4 weeks
B6
Anti-CD8
Anti-CD8

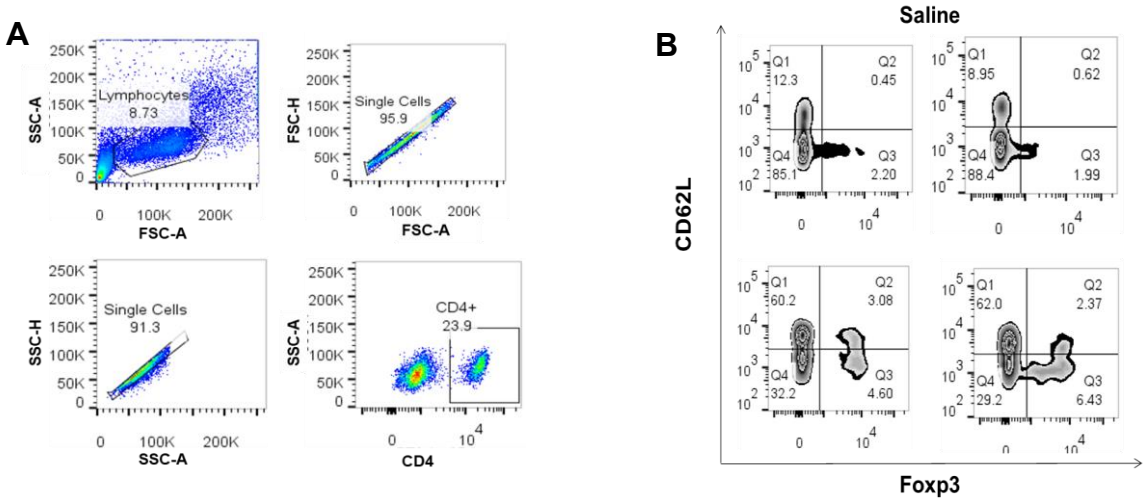

A
Lymphocytes
8.73
Single Cells
95.9
Single Cells
91.3
CD4+
23.9
SSC-A
FSC-A
FSC-H
SSC-H
CD4
B
Saline
CD62L
Foxp3
C
10μg K^d
CD62L
Foxp3
D
10μg α2,6 Sia-K^d
CD62L
Foxp3
E
10μg α2,3 Sia-K^d
CD62L
Foxp3

Figure 6.2. Gating strategy for peripheral CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs. *B6 mice (4-5 mice/group) received either 10μg/200μl saline of α2,3 Sia-K^d or α2,6 Sia-K^d or K^d i.v. Control mice received 200 μl saline i.v. only. One day following peptide treatment, mice received B6.K^d (K^d) skin transplant. Additionally mice received 250 μg anti-CD8 (clone YTS169) on days -1, 0, +7, +14 and were bled 14 days post-transplant to look for percentage Treg proportions by staining with CD4, CD62L and Foxp3. A. Live cells were gated on forward scatter (FSC), side scatter (SSC) followed by gating on CD4$^+$ T cells. Contour FACS plots indicate CD62L$^{+/-}$ vs. Foxp3$^{+/-}$ T cells. B. Control mice received 200 μl saline iv only. C. K^d 10 μg/200μl saline. D. α2,6 Sia-K^d 10μg/200μl saline or E. α2,3 Sia-K^d 10μg/200μl saline.*

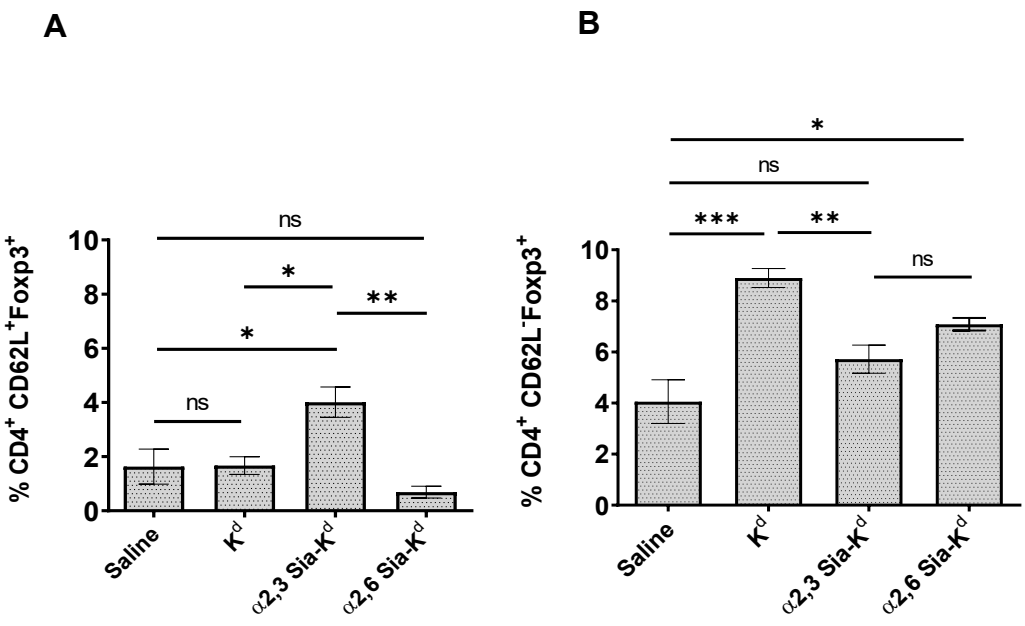


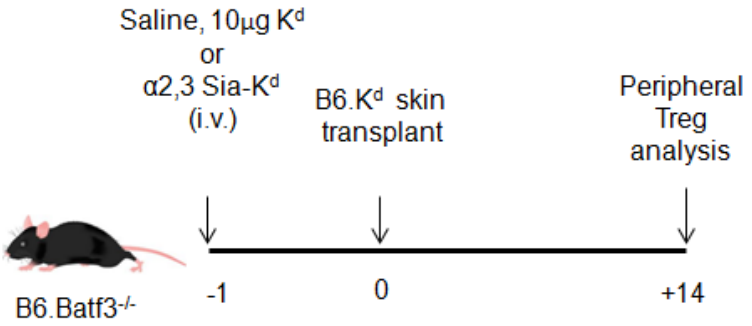
Figure 6.3. Targeting Siglecs on DCs in vivo with α2,3 Sia-K^d one day before transplant treatment leads to significant increase of peripheral CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs*. B6 mice (4-5 mice/group) received either α2,3 Sia-K^d 10μg/200μl saline or α2,6 Sia-K^d 10μg/200μl saline or K^d 10 μg/200μl saline (i.v.). Control mice received 200 μl saline (i.v.) only. One day following transplant, mice received B6.K^d (H2^b + K^d) skin transplant. Additionally mice received 250 μg anti-CD8 (clone YTS169) on days -1, 0, +7 and +14. Recipient mice were bled 14 days post-transplant and the percentage of Treg assessed by staining with CD4, CD62L and Foxp3. Live cells were gated on forward scatter (FSC), side scatter (SSC), followed by gating on CD4, CD62L and Foxp3 [Fig. 6.2]. A. Percentage of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs. B. Percentage of CD4$^+$ CD62L$^-$ Foxp3$^+$ Tregs. Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Error bars represent mean ± SEM and each bar represents 4-5 pooled mice. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*.NS = p>0.05.*

6.3.3. Targeting α2,3 Sia-K^d to Siglecs expressed on Batf3$^{-/-}$ DCs did not induce CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs

We observed that unlike B6 WT mice, B6.Batf3$^{-/-}$ recipients treated with α2,3 sialylated alloantigen one day before transplantation did not have prolonged B6.K^d skin survival. In order to determine whether CD62L Treg proportions

reflect transplant survival in these mice, B6.Batf3$^{-/-}$ recipients were bled 2 weeks post peptide and B6.K^{d} skin transplant and CD4, CD62L and Foxp3 were analysed using flow cytometry. Unlike B6 recipient mice, there was no significant increase in the percentage of CD62L^{+} Tregs observed over untreated mice (saline mean= 2.6%, K^{d} mean= 2.5%, α2,3 Sia-K^{d} mean= 4.38%) [Fig. 6.4 A and B]. Therefore, targeting Siglecs on CD8α^{-} CD11b^{+} DCs with α2,3 Sia-K^{d} does not result in an increase in CD62L^{+} Tregs numbers, suggesting that Siglecs expressed on Batf3-dependent DC subsets may be required for the prolongation seen via the induction/expansion of CD62L^{+} Tregs.

Overall, our data suggests that targeting Siglecs expressed on cDC1s, either resident CD8α^{+} or migratory CD103^{+} DCs with α2,3 Sia-K^{d} increased CD4^{+} CD62L^{+} Foxp3^{+} Tregs whilst targeting cDC2s, such as resident CD8α^{-} (CD4^{+}) CD11b^{+} with sialylated or unsialylated alloantigens leads to antigen specific T cell depletion.


Saline, 10μg Kd
or
α2,3 Sia-Kd
(i.v.)
B6.Kd skin
transplant
Peripheral
Treg
analysis
B6.Batf3-/-
-1
0
+14

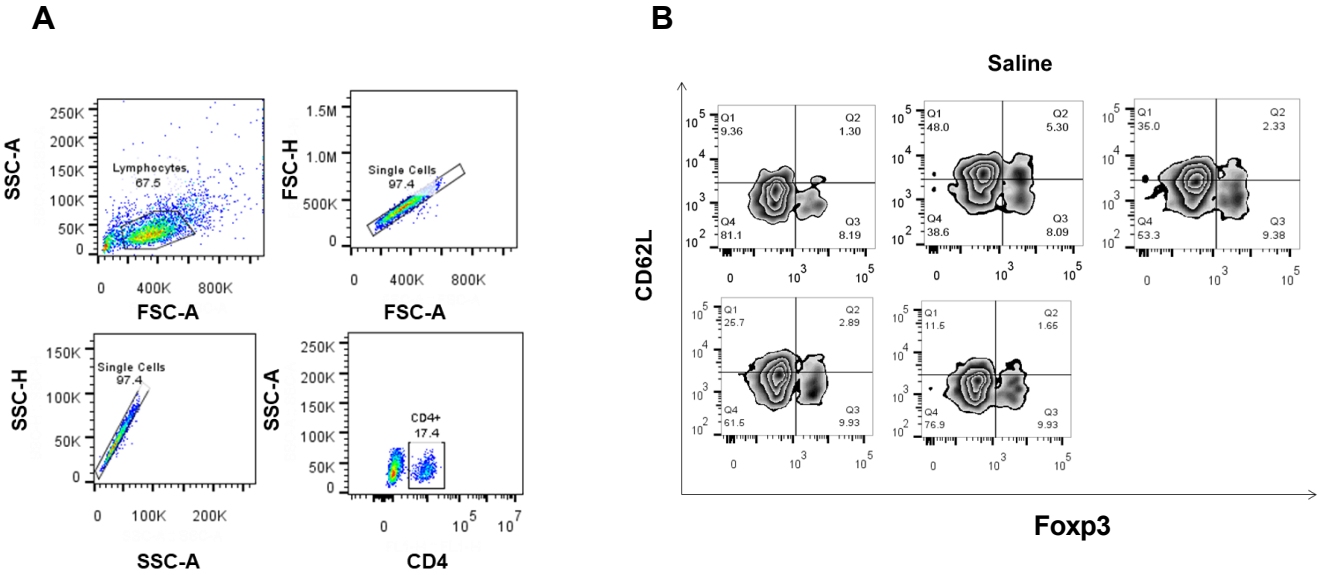

A
SSC-A
FSC-A
Lymphocytes
67.5
FSC-H
Single Cells
97.4
SSC-H
CD4+
17.4
CD4
B
Saline
CD62L
Foxp3
C
10μg Kd
CD62L
Foxp3
D
10μg α2,3 Sia-Kd
CD62L
Foxp3

Figure 6.4. Gating strategy for peripheral CD4^{+} CD62L^{+} Foxp3^{+} Tregs in B6.Batf3$^{-/-}$ mice. *B6.Batf3$^{-/-}$ mice (4-5 mice/group) received either 10µg/200µl saline of α2,3 Sia-K^{d} or α2,6 Sia-K^{d} or K^{d} (i.v.). Control mice received 200 µl saline (i.v.) only. One day following peptide treatment, mice received B6.K^{d} (K^{d}) skin transplant. Mice were bled 14 days post-transplant to look for percentage Treg proportions by staining with CD4, CD62L and Foxp3. A. Live cells were gated on forward scatter (FSC), side scatter (SSC) followed by gating on CD4^{+} T cells. Contour FACS plots indicate CD62L$^{+/-}$ vs. Foxp3$^{+/-}$ T cells. B. Control mice received 200 µl saline (i.v.) only. C. K^{d} 10 µg/200µl saline. D. α2,3 Sia-K^{d} 10µg/200µl saline.*

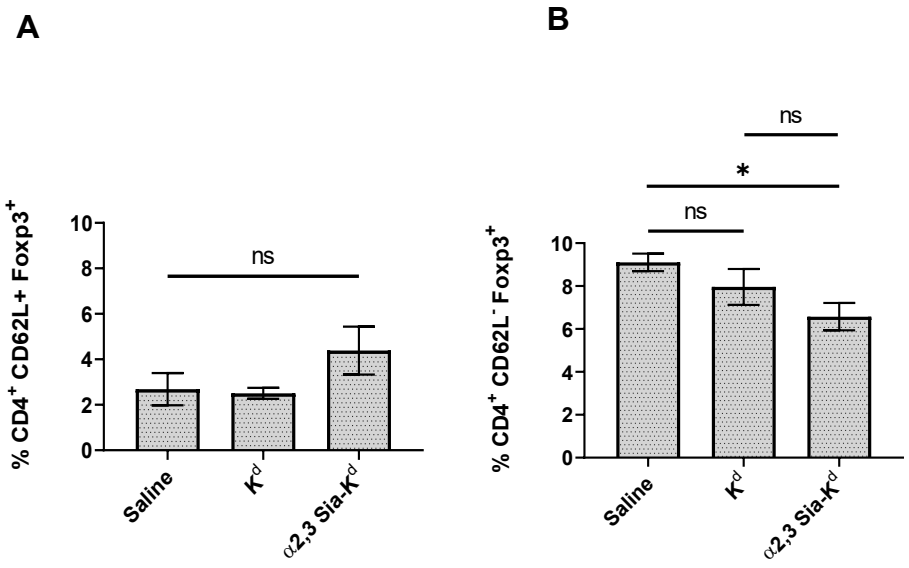


Figure 6.5. CD8α$^+$ and CD103$^+$ DCs may be required for the development of peripheral CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs following α2,3 Sia-K^d targeting and B6.K^d skin transplant. *Batf3$^{-/-}$ mice (3-5 mice/group) received either α2,3 Sia-K^d 10μg/200μl saline or K^d 10 μg/200μl saline (i.v.). Control mice received 200 μl saline (i.v.) only. One day following transplant, mice received B6.K^d (H2^b + K^d) skin transplant. Recipient mice were bled 14 days post-transplant to look for percentage Treg proportions by staining with CD4, CD62L and Foxp3. A. Percentage of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs (n=5). B. Percentage of CD4$^+$ CD62L$^-$ Foxp3$^+$ Tregs (n=5). Statistical comparisons performed using One-way ANOVA and Tukey's multiple comparisons test. Data shown is mean ± SEM. Statistical significance was expressed as follows; p<0.001***, p<0.01**, p<0.05*, ns= p>0.05.*

Table. 7. Summary of transplant survival following Sia-K^d targeting in recipient mice

Recipient mice	Graft survival with Sia-K^d peptide administration?	Additional therapy	Decreased alloantibodies?	Increase of Tregs over K^d injected?	CD4$^+$ T cell deletion?
B6	Yes (α2,3 Sia-K^d peptide treatment one day before skin graft)	Mice received 250 µg anti-CD8 (clone YTS169) (i.p.) on days -1, 0 ,+7 and +14.	Yes	Increase of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs.	Yes
B6.Rag2$^{-/-}$	Yes (α2,3 Sia-K^d peptide treatment one day before skin graft	Mice were adoptively transferred with 0.5 x 10^6 B6 CD4$^+$ T cells (i.v.) one day before skin graft.	-	-	-
B6.Batf3 $^{-/-}$	No graft survival with peptide administered one day before skin graft.	-	Yes	No significant difference in percentage of CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs.	Yes

6.4 Discussion

This chapter examined the underlying mechanisms contributing to allograft survival following targeting Siglec-expressing DCs with α2,3 Sia-K^d. This chapter identified that similarly to Tanriver *et al*., (2010), mice treated with alloantigens showed profound deletion of K^d-specific T cells with no deletion of $CD4^+$ $Foxp3^+$ Tregs. In fact recipient B6, but not B6.$Batf3^{-/-}$ mice, treated with α2,3 Sia-K^d prior to transplantation had a signifiant increase of $CD4^+$ $CD62L^+$ $Foxp3^+$ Tregs in peripheral blood. These preliminary observations suggests that targeting Siglecs on DCs subsets with α2,3 Sia alloantigens expands/induces $CD62L^+$ expressing Tregs which may contribute to the prolonging transplant survival seen in Chapter 5.

The expression of CD62L homing receptor on Tregs allows these Tregs to migrate to sites such as the LNs to initiate suppression, and these cells have been found to be more suppressive than their $CD62L^-$ counterparts, which could be the reason for graft prolongation following α2,3 Sia-K^d targeting (Ermann *et al*., 2005; Fu *et al*., 2004). The spleen and the LNs are two organs that are essential for the interaction between alloantigen acquired APCs such as DCs and priming of alloreactive T cells. Therefore, the migratory capacity of $CD62L^+$ Tregs to secondary lymphoid organs is pivotal for the inhibition of priming of naïve alloreactive T cells at these sites, as demonstrated by Ochando *et al*., (2005). These authors determined that administration of anti-CD62L antibody to cardiac allograft recipient mice exacerbated rejection due to impaired accumulation of $CD4^+$ $CD25^+$ Tregs in LNs, SPLN and blood. Siglecs targeted with α2,3 Sia-K^d *in situ* increased $CD62L^+$ Tregs in the blood (although we did not check the SPLN and LNs for these Tregs), however although graft survival was prolonged, we did not observe indefinite skin graft survival. This suggests that this targeting regimen may be a useful complimentary therapy to increase $CD62L^+$ Tregs or expand $CD62L^+$ Tregs from adoptively transferred Tregs, which can improve allograft survival, but additional strategies such as sialylating an MHC monomer instead of an MHC peptide in order to induce indefinite skin graft survival may be useful (Tanriver *et al*., 2010).

The importance of CD103$^+$ DCs for promoting tolerance was demonstrated by Bain *et al*., (2017), where reducing CD103$^+$ DCs decreased the proportion of inducible Tregs *in vivo* and *in vitro.* For example, one study found that murine mesenteric LN CD103$^+$ DCs have a role of converting naïve T cells into Foxp3$^+$ Tregs which was dependent on TGF-beta and retinoic acid (Coombes *et al*., 2007). These authors also described that CD103 and CCR7 expression on these migratory DCs were essential to migrate to the intestine and mediate control of induced-colitis (Coombes *et al*., 2007). In addition, expression of Indoleamine 2,3-dioxygenase (IDO), which is involved in tryptophan catabolism and in limiting T cell activation, by CD103$^+$ DCs was essential for the development of Foxp3$^+$ Tregs (Matteoli *et al*., 2010). However, CD103$^+$ DCs may also function in organs other than the intestine. In fact, Batf3-dependent dermal CD11b$^{low/-}$ Langerin$^+$ CD103$^+$ DCs present in the skin (Edelson *et al*., 2011) generates Tregs (Hasewgawa *et al*., 2018). In another study donor CD103$^+$ DCs migrate to host LNs from the donor skin allograft and drive the direct pathway of allorecognition leading to transplant rejection (Borges *et al*., 2018). Borges *et al*., (2018), identified that donor-derived CD103$^+$ DCs are the prime APCs carrying donor MHC II which migrate to recipient LNs and drive direct allorecognition and instigate murine skin graft rejection.

Interestingly, CD103$^+$ DCs have been shown to express Siglec F (Bain *et al*., 2017). It is therefore possibility that in our study, targeting Siglec F on migratory CD103$^+$ DCs (Bain *et al*., 2017) using α2,3 Sia-K^d may have induced the CD62L$^+$ Tregs seen in the blood of B6 mice. Idoyaga *et al*., (2013) showed that dermal CD103$^+$ DCs generated MOG antigen-specific CD4$^+$ Tregs *in vivo* following administration of an anti-CD103 monoclonal antibody (s.c.) engineered to express MOG. Their study aligns with our findings that targeting endogenous Batf3-dependent DCs induces Foxp3$^+$ Tregs, which may be a contributing factor for allograft survival as seen with α2,3 Sia-K^d targeting. What would be interesting to assess is the presence of sialylated peptides in non-lymphoid organs where CD103$^+$ DCs reside such as the lungs, skin and intestine (Edelson *et al*., 2010), especially since these DCs are migratory. That said, CD103$^+$ DCs do not limit their function to non-lymphoid organs; they have also be found in the skin draining lymph nodes at both a steady and inflamed

state, therefore in this study peptides present in the lymph nodes may be in the vicinity of $CD103^+$ DCs resulting in a possible interaction (Ginhoux *et al.*, 2007).

The next question is, do $CD8\alpha^+$ DCs play a role in prolongation following Siglec targeting? Depletion of $CD8^+$ cells with routine administration of anti-CD8 antibody has been used in various murine transplant models as a means of assessing the indirect pathways of allorecognition (Tanriver *et al.*, 2010). This treatment also depletes cross-presenting $CD8\alpha^+$ DCs (Smyth *et al.*, 2013). In our transplant experiments, anti-CD8 antibody treatment was given on the same day of peptide treatment, but, following peptide treatment, therefore, it is a possibility that sialylated alloantigens would have already bound to $CD8\alpha^+$ DCs before anti-CD8 was administered. It is questionable to rule out the possibility of sialylated peptides binding to CD8α DCs^+, especially since we confirmed the presence of the peptides in the SPLN and LNs in Chapter 5.

In both WT and B6.$Batf3^{-/-}$ mouse models, antigen-specific $CD4^+$ T cells were deleted following administration with either sialylated or unsialylated peptides, Therefore, antigen-specific T cell deletion may also be one of the mechanisms involved in the reduction of alloantibodies in our model, an observation very similar to that published by Tanriver *et al.*, (2010). A possible explanation is that i.v. injection of peptides contributed to thymic tolerance. Although we injected our peptides i.v., so that they reach the SPLN and LNs, we cannot rule out the possibility that they entered other organs within the body such as the thymus, although we have yet to confirm this. It is possible that the K^d allopeptides were presented via MHC II by thymic DCs to developing thymocytes. This theory is supported by one study by Garrovillo *et al.*, (1999) which suggested that acquired thymic tolerance and indirect allorecognition exists in the thymus, they first targeted immunodominant MHC I peptide to recipient rat BM-DCs *in vitro* and injected these cells via intrathymic injection which was then followed by a heart transplant (Garrovillo *et al.*, 1999). Donor type allografts were indefinitely accepted, whereas unrelated donor were acutely rejected, therefore inducing alloantigen specific tolerance (Garrovillo *et al.*, 1999). However, a significant difference between our study and the aforementioned study is that they injected antigen-pulsed BM-DCs intrathymically, whereas the current study administered peptides systemically. Therefore, the thymic tolerance theory in our study is

questionable, especially given the concentration of peptide used. Several studies have reported thymic tolerance via injecting high doses of peptides systemically, for example Liblau *et al*., (1996) demonstrated that 750µg, but not 75µg, of influenza virus hemagglutinin (HA peptide) administered intravenously induced thymic tolerance by depleting CD4 and CD8 thymocytes and inducing peripheral tolerance in the SLOs by antigen specific deletion and anergy. In addition repetitive intravenenous administration of 750µg HA peptide was also shown to induce depletion of CD4 and CD8 thymocytes in mice, whereas a single dose of this peptide did not induce long-lasting tolerance (Bercovici *et al*., 1999). As we only administered 10µg of peptide once per mouse, and not intrathymically, we suggest that thymic deletion may not be involved however we can substantiate this by isolating the thymus from K^d-FITC targeted mice and assessing binding on thymic DCs using flow cytometry. Altogether, we do not have experimental evidence to suggest thymic tolerance; nevertheless this can be addressed as future work with thymectomized mice.

It is possible that targeting Siglecs F with Sia-K^d inhibited allorecognition by modifying DCs leading to peripheral tolerance, via a combination of peripheral deletion and an increase in $CD4^+$ $CD62L^+$ $Foxp3^+$ Tregs. This is in contrast to K^d peptide administration of which only induced peripheral deletion, which was not enough to significantly prolong allograft survival. Overall, two tolerance inducing mechanisms could be contributing to allograft survival following Siglec interaction with α2,3 sialylated alloantigen, which are induction/ expansion of $CD4^+$ $CD62L^+$ $Foxp3^+$ Tregs and antigen-specific deletion. Other tolerance inducing mechanisms such as anergy could also be at play, but this could not be addressed.

CHAPTER 7:
FINAL DISCUSSION & FUTURE WORK

7.1. Final discussion

7.1.1 Can sialylated alloantigen targeting to APCs inhibit indirect allorecognition?

Chronic transplant rejection remains a persistent obstacle for long-term transplant survival. As previously mentioned, ISD can be administered prior or post-transplant and are effective at prolonging the survival of the transplant on a short-term basis. However, these treatments fail to prevent chronic mediated rejection, instead, there is an increase of opportunistic infections and malignancies (Sampaio *et al*., 2012; Munoz-Price *et al*., 2004; Razonable *et al*., 2013). In order to promote graft survival without the use of ISD, immunotherapeutic approaches targeting specific immune cell interactions that contribute to chronic allograft rejection are being investigated.

One of the pathways known to contribute to chronic rejection is the indirect pathway of allorecognition (Stanford *et al*., 2003; Hornick *et al*., 2000). Immune cells that are known to contribute to indirect allorecognition are DCs and B cells that can contribute to initiating this pathway. Upon recognition and processing of alloantigens by recipient DCs/ B cells, alloantigens are presented to recipient T cells. These T cells can then instruct follicular B cells via CD40/ CD40L interaction to differentiate into alloantibody producing B cells or memory B cells (Steele *et al*., 1996). It is also established that DCs have the ability to present antigen which are in its native state to B cells to influence humoral immune responses to T-independent antigens, via receptors such as Fc receptor, FcgRIIB on DCs (Bergtold *et al*., 2005; Wykes *et al*., 1998; Qi *et al.,* 2006). Qi *et al*., (2006) identified that DCs that had acquired antigen, could present this to B cells in LNs and using imaging studies they discovered B cells that captured the antigen, were activated and migrated towards the T-B border to initiate immune response. Therefore, not only can DCs present antigen to T cells, then can also present antigens to B cells. In addition to DCs, macrophages are also known to contribute to rejection, for example by releasing proinflammatory cytokines, eg: TNF- α and IFN- γ, which were shown to cause transplanted tissue fibrosis and vascular injury (Mitchell, 2009).

As mentioned previously in this thesis, DCs, B cells and macrophages share a group of inhibitory receptors, known as Siglecs and various studies have demonstrated the role of these receptors in inhibiting immune cell interactions (Pillai *et al*., 2012). Inhibitory Siglecs are expressed on both human and murine cells where 14 human and 9 murine Siglecs have been identified (Bornhöfft *et al*., 2018). To date, it has not been established whether targeting Siglec receptors on APCs can modify the indirect pathway of allorecognition and induce transplantation tolerance. Therefore, this thesis investigated targeting Siglecs on recipient DCs, B cells and macrophages using a sialylated MHC I alloantigen peptide (an established alloantigen used to assess indirect allorecognition *in vitro* and *in vivo*) (Tanriver *et al*., 2010) to modify the indirect pathway of allorecognition and to promote organ transplant survival. The overall goal of this study was to determine whether targeting Siglec expressing antigen presenting cells using sialylated MHC I K^d alloantigen peptide could modify indirect allorecognition to favour skin allograft survival.

The present study's objectives and key findings were:

1. To test the possibility of improving skin transplant survival by targeting Siglec-expressing cells using Sia-alloantigen (K^d) peptide as model alloantigens.

Findings: Siglec -expressing BM-DCs, but not B cells targeted with Sia-K^d alloantigens *in vitro* had impaired abilities to promote $CD4^+$ T cell proliferation however, they induced/expanded $CD4^+$ $Foxp3^+$ Tregs [Fig. 7.1A-B]. Targeting BM-DMs with α2,3 sialylated alloantigens decreased IL-12 in the presence of LPS [Fig. 7.1C].

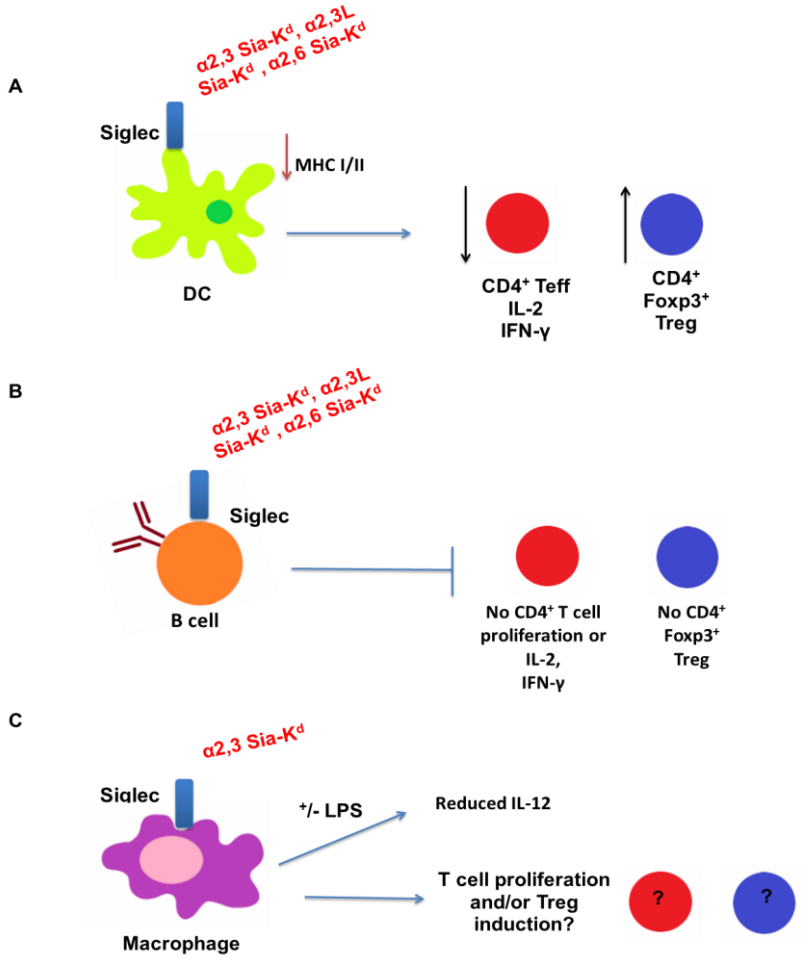


Figure. 7.1. Graphical representation for the outcome from targeting DCs, B cells and macrophages with Sia-K^d in vitro. *A. Targeting BM-DCs in vitro with α2,3 Sia-K^d impaired MHC I and MHC II expression and inhibited TCR75 T cell proliferation and IFN-γ and IL-2 production. BM-DCs targeted with all*

sialylated alloantigens induced/ expanded CD4$^+$ Foxp3$^+$ Tregs from naïve TCR75 T cells. B. B6 B cells targeted with alloantigens did not stimulate TCR75 CD4$^+$ T cell proliferation or induce/ expand CD4$^+$ Foxp3$^+$ Tregs. C. Targeting B6 BM-DMs with α2,3 Sia-K^d impairs IL-12 production even in the presence of LPS.

2. To identify whether specific DC subsets, B cells or macrophages are targeted by sialylated alloantigen and whether this contributes to transplantation outcome.

Findings: Skin allograft survival occurred following pre-treatment with α2,3 Sia-K^d [Fig. 7.2 A-B]. Siglec-expressing B cells may not be required for allograft survival following sialylated alloantigen targeting, however DCs were required [Fig. 7.2]. Targeting Siglecs on Batf3-dependent DCs with α2,3 Sia-K^d prolongs allograft transplant survival and reduced alloantibodies [Fig. 7.2].

3. If improved transplant outcome occurs, to assess what are the mechanisms of 'tolerance' following targeting recipient APCs with sialylated alloantigen.

Findings: *In vivo* targeting of Sia-K^d alloantigen to DCs leads to T cells with indirect specificity deletion, without deleting the Treg pool. Mice with prolonging allograft following α2,3 Sia-K^d targeting have increased proportions CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs in peripheral blood [Fig. 7.2B].

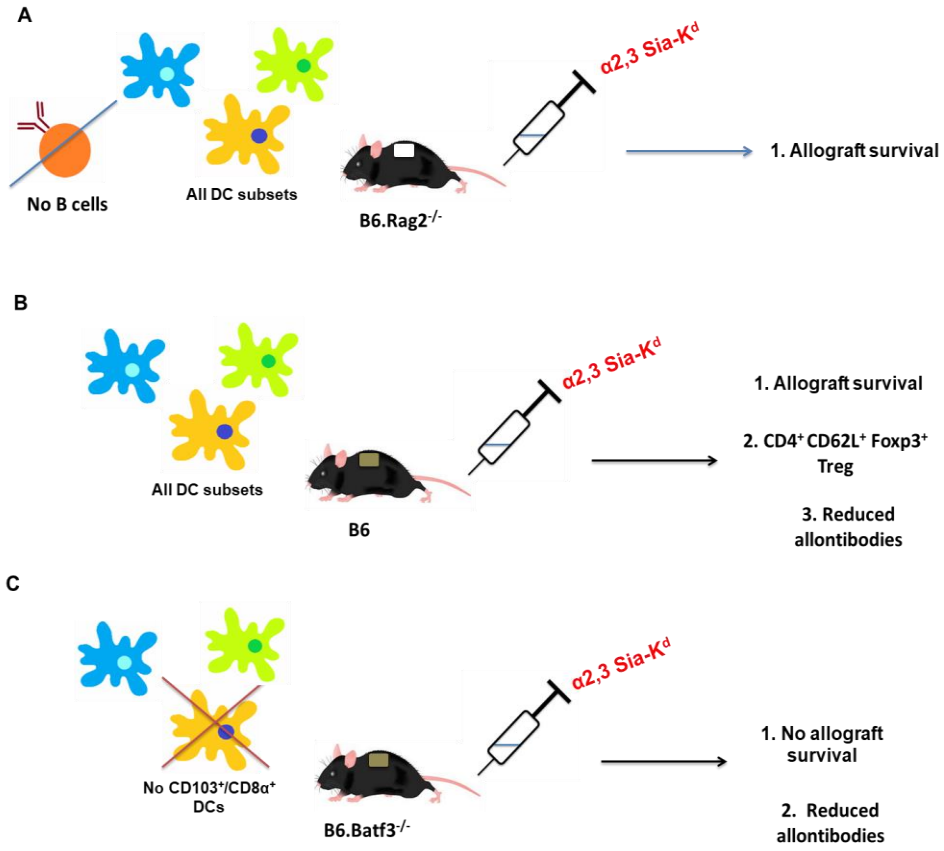


Figure. 7.2. Graphical representation for the transplant outcome following α2,3 Sia-K^d targeting in vivo. *A.B6.Rag2$^{-/-}$ mice devoid of B cells and reconstituted with B6 CD4$^+$ T cells have prolonging skin graft survival following α2,3 Sia-K^d targeting. B. B6 WT mice, that consists of all DC subsets and administered with α2,3 Sia-K^d and anti-CD8 had increased allograft survival, CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs in peripheral blood and reduced alloantibodies. C. Batf3- dependent CD8α$^+$ and CD103$^+$ DCs are required for allograft survival following α2,3 Sia-K^d targeting.*

Chapters 3 and 4 highlight the ability of sialylated alloantigen targeted BM-DCs to promote alloantigen-specific tolerance *in vitro*. As mentioned in Perdicchio *et al.*, (2016) study, α2,6 sialylated antigen interaction with Siglec E on BM-DCs promoted induction of Tregs *in vitro*. Perdicchio *et al.*, (2016), did not publish data on the effect of α2,3 Sia-OVA on Siglec E$^{-/-}$ BM-DCs or whether their constructs are interacting with Siglec F, however as suggested by Crocker *et al.*, (2007), this linkage of sialic acids bind to Siglec E and F, therefore α2,3 Sia-K^d may be binding to Siglec E and/or Siglec F to mediate their

immunoregulatory effects. However, in order to fully confirm this, the binding of our constructs need to be tested on Siglec E and F knockout DCs.

We have confirmed *in vitro* that both α2,3 and α2,6 sialylated K^d constructs bind to BM-DCs, however there is preferential binding for α2,3 Sia-K^d compared to α2,6 Sia-K^d. As mentioned in Chapter 5, the differences in binding may reflect the type of Siglecs these constructs are binding to. It is known that Siglec E has moderate binding to α2,3 and α2,6 sialic acids, however Siglec F binds moderately to α2,3 sialic acids and interestingly, binds weakly to α2,6 sialic acids (Crocker *et al.*, 2007). Therefore, as Siglec F is highly expressed on BM-DCs and SPLN DCs it is possible that this resulted in the increased binding of α2,3 Sia-K^d observed. Whereas α2,6 sialylated K^d binds to Siglec E on BM-DCs, which is not as highly expressed as Siglec F. Currently, there are very few publications demonstrating Siglec F expression and functional role of this receptor on BM-DCs. Siglec F expression is mostly associated with macrophages, namely present in the lung and eosinophils (Feng & Mao, 2012; Kiwamoto *et al.*, 2014), though it has been reported that Siglec F is expressed on BM-DCs and BM-DMs (which we have confirmed), however the authors did not present this data in their publication but did mention this in their discussion (Tetano *et al.*, 2007). We cannot rule out the possibility of sialylated alloantigen binding to MHC as well as Siglecs, however to fully confirm the binding profiles, *in vitro* peptide binding experiments can be repeated on both B6 WT DCs and MHC I/II DCs derived from MHC KO mice.

The next question raised is whether Siglecs have the ability to present sialylated alloantigens. Tateno *et al.*, (2007) demonstrated that Siglec F can mediate endocytosis of anti-Siglec F antibody and sialylated ligands such as bacterium *Neisseria meningitides.* They discovered that the ITIM of Siglec F mediated endocytosis which is dependent on ADP-ribosylation factor 6 (ARF6) (Tateno *et al.*, 2007), similarly, peptide-MHC II complexes that enter DCs also require $ARF6^+$ tubular endosomes (Walseng *et al.*, 2008). To further support that sialylated alloantigens are involved in antigen-presentation, Perdicchio *et al.,* (2016), investigated whether native and sialylated OVA protein antigens had different intracellular routes that could impact MHC presentation and T cell activation (Perdicchio *et al.,* 2016). It was revealed that sialylation did not alter

the intracellular route within the DC, in fact, both native and sialylated antigen had higher colocalization with lysosomal (LAMP-1) compartment rather than the endosomal (EEA-1) (Perdicchio *et al.,* 2016). By observing the intracellular route of the antigens, they found that both Sia-OVA and OVA protein were present within the same compartments where MHC II-peptide complexes are formed, thus confirming that irrespective of sialylation, antigens are presented via self MHC II to $CD4^+$ T cells (Perdicchio *et al*., 2016). In order to confirm that Sia-K^d binding and the intracellular fate of the conjugates is also not affected, peptide binding and intracellular routing of Sia-K^d-FITC could be assessed using confocal microscopy.

One significant difference between the current study and Perdicchio *et al*., (2016) study is that the aforementioned study targeted BM-DCs with a sialylated whole OVA protein as well as MOG peptide, whereas we are targeted DCs with a sialylated MHC-derived peptide. Their study did confirm that both sialylated proteins and sialylated peptide antigens promoted tolerance *in vitro* and *in vivo*, however they did not confirm the intracellular routing of sialylated MOG peptide antigen which has yet to be investigated. A particular difference between protein and peptide antigens could be a slightly enhanced and quicker immune response with peptides as opposed to whole proteins. In 2013, Rosalia *et al* (2013), identified that in comparison to whole OVA proteins, OVA peptides constituting of 24 amino acids were more efficiently and quickly processed by murine DCs and presented via MHC molecules I/II to T cells. Therefore, when considering sialylated alloantigens as a potential therapy, it may be worth testing sialylated whole MHC monomer to decipher whether this enhances the impaired alloreactive immune responses observed with peptide. Sialylated alloantigens targeting may be useful for human therapy, especially since human leukocytes also express inhibitory Siglecs and studies have suggested a role of these receptors in inhibiting effector immune responses.

The human homologue of Siglec F is Siglec 8, both of which are known to be expressed on eosinophils and impair effector functions of eosinophils in allergic inflammation (Gao *et al*., 2010; Kiwamoto *et al*., 2014). The question is; given that these Siglecs function similarly on eosinophils in humans and mice, does Siglec F have similar inhibitory mechanisms to Siglec 8 in humans and can

targeting this Siglec in humans prevent effector alloreactivity? It is known that Siglec F is expressed on eosinophils, but we have confirmed their expression on murine BM-DCs and SPLN-DCs, and it would be interesting to know whether Siglec 8, which expression is established on eosinophils, is expressed on human monocyte-derived DCs and has a similar inhibitory function. Unlike murine Siglec F which can bind to α2,3 sialylated ligands, Siglec 8 only binds strongly with sulphated ligands such as Sialyl-Lewis X which comprises a combination of sialic acid, fucose and an N-acetyllactosamine (Kiwamoto *et al*., 2012). Therefore, when targeting sialylated alloantigen to humans, different glycans attached to the alloantigen should be taken into consideration.

Perdicchio *et al*., suggested that sialylated antigen loaded DCs mediate their tolerogenic effects via contact-dependent mechanisms, not via soluble mediators. They addressed this using Transwell chambers, where OVA-pulsed DCs and naïve OT-II T cells were placed on the lower Transwell and were separated from Sia-OVA pulsed DCs or OVA pulsed DCs, which were placed on the top well of the Transwell. They identified no difference in IFN-γ production of OT-II $CD4^+$ T cells when either OVA or Sia-OVA pulsed DCs were placed on the top Transwell, suggesting that tolerogenic effects were not influenced by soluble mediators released by Sia-OVA pulsed DCs, but instead are mediated via contact-dependent mechanisms (Perdicchio *et al*., 2016). Several surface molecules such as MHC, PD-L1 and ICOS-L were assessed in Sia-alloantigen and Siglec contact dependent immune modification.

Markers such as PD-L1 were increased on murine BM-DCs that were made tolerogenic using agents such Dexamethasone and Minocycline (antibiotic used to generate TolDCs) (Lee *et al*., 2017), and interaction of PD-L1 on DCs and PD-1 on T cells resulted in down-regulation of T cell activation (Brown *et al*., 2002). Their study recognised that blockade of PD-L1 on monocyte-derived DCs enhanced $CD4^+$ T cell proliferation in MLRs *in vitro* (Brown *et al*., 2002). *In vivo*, Sander *et al*., (2005) found that blocking PD-L1 ligand accelerated skin graft rejection in an MHC II-mismatched transplant model; in fact PD-L1 blockage enhanced T cell proliferation. PD-L1 expression on BM-DCs treated with sialylated and non-sialylated alloantigen targeted BM-DCs remained unchanged, suggesting that the inhibition in T cell proliferation may not be

mediated by PD-L1 or Siglec signalling does not lead to upregulation of PD-L1. The latter conclusion remains inconclusive, given that human macrophages expressing Siglec 9 and once engaged with hypersialylated tumours, can up-regulate PD-L1 expression in order to evade host effector immune responses (Beatson *et al.*, 2016). Therefore as future work, it would be interesting to assess PD-L1 expression on BM-DMs following Sia-K^d engagement.

ICOS-L present on DCs interacts with ICOS expressing $CD4^+$ T cells and in turn promotes IL-10 production. A study by Tuettenberg *et al.*, (2009), found that T cell anergy and IL-10 secretion were impaired when ICOS-L and ICOS interactions were blocked in DC/T cell co-cultures. Moreover, in patients with ICOS-deficient $CD4^+$ T cells, peripheral tolerance was not observed and these patients' T cells were refactory to anergy induction (Tuettenberg *et al.*, 2009). Similarly to PD-L1, ICOS-L expression was not modified following Siglec engagement. It remains to be determined whether Sia-K^d treated DCs have increased expression of molecules previously shown to contribute to a 'tolerogenic' phenotype which could polarise $CD4^+$ T cells to a suppressive phenotype, or a state of T cell anergy such as ILTs (Chang *et al.*, 2002), or induce apoptosis of T cells such as Fas-L (Marin *et al.*, 2018) given that T cell deletion was observed in our *in vivo* experiments. It would also be interesting to determine whether Sia-K^d targeted DCs impair T cell immune responses by altering their metabolic activity such as through tryptophan catabolism via IDO (Xie *et al.*, 2015), or perhaps Tregs are induced/ expanded by DCs as a result of production of anti-inflammatory cytokines such as TGF-β (Zheng *et al.*, 2007).

MHC I and II are expressed sub-optimally on tolerogenic DCs (Marin *et al.*, 2018). An interesting finding was the reduction of MHC I and II molecules on the surface of BM-DCs following α2,3 Sia-K^d targeting. The low expression of MHC I and MHC II molecules suggested that α2,3 Sia-K^d targeting may influence DCs to have a limited capacity to present antigen leading to reduced T cell activation. It has yet to be determined whether α2,3L Sia-K^d or α2,6 Sia-K^d targeted BM-DCs show similar results.

Investigations into MHC expression following Siglec interaction has not been fully elucidated *in vivo*, however it is established that Siglec G expressed on SPLN- DCs may have a role in impairing MHC I-mediated antigen-presenting ability. One study demonstrated in CD8α$^+$ DCs that upon pathogenic antigen engulfment, Siglec-G expressed within the phagosome inhibited NOX2 activation and ROS production as a result of ITIM-SHP1 recruitment, and created a highly acidic environment within the phagosome (Ding *et al*., 2016). This led to excessive degradation of the antigen and inhibition of peptide-MHC I complex formation resulting in attenuated cross-presentation and CD8$^+$ T cell activation (Ding *et al*., 2016). However, the aforementioned study did not report any significant differences in MHC I (H-2K^b) expression on murine splenic CD8α$^+$ DCs between Siglec G$^+$ and Siglec KO mice following the administered of *L. monocytogenes* that secrete OVA protein. Their study supports the idea that Siglec G is expressed within DC phagosomes on CD8α$^+$ DCs and is involved in impairing MHC I:peptide interactions and CD8$^+$ T cell immunity. Ding *et al*., (2016) did not see any difference in OT-II CD4$^+$ T cell proliferation *in vitro* when Siglec G$^{+/+}$ and Siglec G$^{-/-}$ CD8α SPLN-DCs were pulsed with OVA protein, suggesting that Siglec G may have a role inhibiting CTLs responses rather than CD4$^+$ T cell responses. Given the relationship between Siglec G and MHC I, it may be worth investigating CD8$^+$ T cell immune responses following sialylated alloantigen targeting.

Siglec G is also established as a negative regulator of DAMP release (Toubai *et al*., 2014), which are released from the physical trauma caused by the skin transplant (Benichou *et al*., 2011). The inhibitory role of Siglec G may provide some suggestion as to why B6.Batf3$^{-/-}$ mice which lack Siglec G-expressing CD8α$^+$ DCs do not prolong allograft survival following α2,3 Sia-K^d targeting.

Considering B6.Batf3$^{-/-}$ mice did not consist of a significant increase of these CD4$^+$ CD62L$^+$ Foxp3$^+$ Tregs, these finding may suggest that CD8α$^+$ DCs are required for the development of peripherally induced Foxp3$^+$ Tregs. One study identified that these DCs specifically express B- and T-lymphocyte attenuator (BTLA) which is a receptor that binds specifically to herpes virus entry mediator (HVEM) resulting in up-regulation of CD5 expression on T cells (Jones *et al*., 2016). Following engagement of BTLA and HVEM, CD5 expression is

increased, resulting in induction of Tregs. In fact, their study found that by blocking BTLA on CD8α^{+} DCs using an anti-BTLA, Treg induction was significant impaired (Jones *et al*., 2016). Therefore, targeting CD8α^{+} DCs and possibly Siglecs on these DCs using sialylated alloantigens may be required for Treg induction/ expansion, which was not observed in the B6.Batf3$^{-/-}$ transplanted mice.

Similarly to Siglec 9 on human macrophages, mouse Siglecs also regulate inflammatory cytokines release in response to proinflammatory stimulus, such as LPS (Ando *et al*., 2008). It has already been established that α2,3-linked sialic acids that coat bacterium *Group B Streptococcus* (GBS), are able to interact with murine Siglec E on macrophages (Chang *et al*., 2014). Their study demonstrated that Siglec E KO mice injected with GBS had increased IL-12 transcript levels in comparison to WT mice (Chang *et al*., 2014). They were not able to detect any proinflammatory cytokines in mice 48 hours post infection, however, using RT-PCR increased levels of IL-12 mRNA was detected in the lungs of Siglec E KO mice (Chang *et al*., 2014). It was interesting to observe that out of all the sialylated constructs in the current study, α2,3-Sia-K^{d} targeted BM-DMs were the only cells with no significant increase of IL-12 in response to LPS which was not observed with BM-DCs, thus highlighting that Siglec engagement on different cell types may result in different immunosuppressive responses. Overall, sialylated alloantigen targeted macrophages may have an ability to limit IL-12 cytokines secretion and in turn may prevent allograft damage (de Paiva *et al*., 2009). It remains to be determined whether targeting macrophages with sialylated alloantigens impairs T cell proliferation and promote Tregs induction, but as we have confirmed Siglec expression, peptide binding and dampened proinflammatory IL-12 cytokine production, they may prove to be promising targets.

Recipient B cells have been shown to act as APCs by presenting alloantigens to indirect alloreactive T cells and contributing to transplant rejection (Shiu *et al*. 2015). However, in one clinical trial renal graft rejection was exacerbated when patients received the B cell depleting monoclonal antibody Rituximab, suggesting that B cells also have a regulatory role and can limit alloreactivity (Jackson *et al*., 2015). CD22 and Siglec G are two B cell associated Siglecs

with ITIMs that recruit SHP-1 tyrosine phosphatases leading to inhibition of BCR signalling (Meyer *et al*., 2018). One study managed to assess the outcome of targeting CD22 using a sialylated antigen. In this study the authors targeted this receptor using a polymeric antigen that consists of a CD22 ligand (α2,6 Sia) and a BCR-binding antigen (Courtney *et al*., 2009). The antigen used was 2,4-dinitrophenyl (DNP) that can interact with DNP-specific BCRs (Courtney *et al*., 2009). They discovered that targeting B cells with a DNP construct stimulated rapid Ca^{2+} influx into the cytosol of B cells, thereby signifying B cell activation. On the contrary targeting B cells with DNP/CD22L resulted in little to no Ca^{2+} release (Courtney *et al*., 2009). These results suggested that targeting Siglec CD22 and the BCR with a sialylated antigen impairs B cell activation (Courtney *et al*., 2009). Therefore as future work, it would be worth measuring calcium influx levels of K^d and Sia-K^d targeted B cells, to determine whether B cell Siglec and Sia-K^d interaction impairs B cell activation. In addition, instead of targeting total population of B cells, our study may be modified to target antigen-specific B cells *in vitro* similarly to Courtney *et al*., (2009), to confirm whether B cells are truly altered by sialylated alloantigen targeting- this may be one of the flaws in our study.

Unlike DCs, sialylated alloantigen targeting to Siglecs on B cells did not induce $CD4^+$ $Foxp3^+$ Tregs nor was there T cell proliferation with unsialylated K^d peptide. This does not come as a surprise as recently, a study demonstrated that B cells require activation using anti-CD40 antibodies prior to T cell co-culture to increase CD80/86 expression and support T cell activation (Rossetti *et al*., 2018). As B cells in our study were not stimulated prior to co-culture, this may account for the lack of T cell activation observed. However, this still leaves the question why K^d-treated B cells produce cytokines that support the maintenance Tregs but Sia-K^d treated B cells did not unanswered. It is a possibility that targeting Siglecs on B cells does not induce Tregs, but rather modulates B cell activation only.

The definitive answer for why targeting Siglecs on DCs is better at prolonging graft survival than targeting Siglecs on B cells requires more investigation. It has been consistent that targeting DCs with α2,3 sialylated alloantigens promoted tolerogenic immune responses in comparison to targeted B cells as

demonstrated by induced Tregs *in vitro* and prolonged transplant survival in B6 transplanted mice and B6.Rag$^{-/-}$ mice (devoid of B cells). Therefore, it is still up for debate whether sialylated alloantigen targeting to B cells helps to promote tolerance. However, human B cell Siglec CD22 has become a promising target to modify autoimmune diseases. Humanized anti-CD22 antibody, Epratuzumab, is currently in-use in clinical trials for the purpose of down-regulating BCR engagement thereby inhibiting B cells activation and possibly reducing incidences of autoimmune disease (Sieger *et al*., 2012). As mentioned earlier, transitional B cells were amongst the population of B cells thought to prolong MHC-I mismatched skin graft survival (Moreau *et al*., 2014) thereby demonstrating the importance of their presence for tolerance. However, Epratuzumab has been shown to deplete some populations of transitional and naïve B cells (Dorner *et al*., 2016; Chu *et al*., 2018). This brings the question as to whether engaging CD22 Siglecs with sialylated alloantigens may be depleting transitional B cells which are required for allograft survival, especially as we have confirmed sialylated alloantigen binding to transitional subsets and no allograft survival when B6 WT mice were targeted with α2,6 Sia-K^{d}.

We have also seen graft prolongation in B6 mice in response to α2,3 Sia-K^{d} treatment in comparison to saline, K^{d}, α2,3L Sia-K^{d} and α2,6 Sia-K^{d} treatment, despite this, grafts were eventually rejected and did not induce indefinite skin graft survival. It may be a possibility that another pathway, known as the semi-direct pathway of allorecognition may be contributing to the rejection of the grafts. Unlike, the indirect pathway where alloantigen peptides derived from the donor MHC are presented via recipient DCs, the semi-direct pathways entails the transfer of the entire MHC molecules to recipient DCs. Therefore, recipient DCs are able to stimulate indirect CD4^{+} T cell responses via self-MHC II presenting donor alloantigens as well as direct CD8^{+} T cells responses via donor MHC I. As we have used B6.K^{d} donor skin with an MHC I transgene that expresses H-2K^{d}, it would have been a possibility that direct allorecognition would have mediated eventual rejection, particularly in the WT models.

To further assess the contribution of targeting Sia-alloantigen to DCs and/or macrophages, B6 mice expressing DTR under the CD11c promoter could be used. Injection of DT into these mice leads to the transient loss of all CD11c^{+}

DCs and/or macrophages (van Blijswijk *et al*., 2013). Mice would receive 100ng of DT prior to and after administration of Sia-K^d or PBS (untreated control). Following the targeting regimen, mice would receive a K^d expressing skin transplant and rejection measured as previously described. The results from these experiments will inform us about the level of contribution these Siglecs-expressing $CD11c^+$ APCs have on transplant tolerance. A disadvantage to CD11c.DTR mice, is that multiple infusions of Diptheria toxin for the long-term depletion of DCs, has proven to be fatal to mice due to cardiac complications (Männ *et al*., 2016) as unexpected DTR expression on non-immune cells have been reported (Zaft *et al*., 2005). To overcome this, mixed irradiation chimeras where WT mice would be reconstituted with bone marrow from CD11c.DTR mice, thus generating a chimera can be used (van Blijswijk *et al*., 2014).

Given our *in vitro* data, where all sialylated constructs induced Tregs and impaired TCR75 T cells proliferation, it was surprising that unlike α2,3 Sia-K^d, graft prolongation was not observed following α2,6 Sia-K^d treatment. This finding is contradictory to Perdicchio *et al*., (2016), where they demonstrated targeting endogenous DCs with α2,6 Sia-OVA one week before sensitization with OVA/poly(I:C)/anti-CD40, showed marked increase of $CD4^+$ $Foxp3^+$ Tregs and a reduction of effector CD4 and CD8 T cells. Some of the major differences between their study and this thesis are the models used and the doses of antigen targeted *in situ*. We injected 10μg α2,6 Sia-K^d sialylated alloantigen, whereas Perdicchio *et al*., (2016) targeted DCs *in situ* with a higher dose 50μg Sia-OVA protein, therefore in relation to α2,6 Sia-K^d, a higher dose may be required to mediate an immunoregulatory immune response and possibly increase the proportions of $CD4^+$ $Foxp3^+$ Tregs, which was observed following α2,6 Sia-OVA targeting. The aforementioned study used OVA protein and two 'adjuvants' poly(I:C) and anti-CD40 antibody to assess the regulatory role of α2,6 Sia-OVA, whereas we assessed the regulatory role of a K^d peptide in the absence of these reagents. It can be argued that the immune responses are significantly different between these two treatments, as the placement of donor skin onto a recipient mouse can trigger multiple inflammatory immune reactions, such as physical trauma impacted by the surgical process where DAMPS are released, and a sudden exchange of donor and recipient lymphocytes upon vascularisation of the graft, stimulates cytokine release and activation of CD4

and CD8 T cells (Benichou *et al*., 2011). Poly (I:C) on the other hand is an adjuvant that can mimic a PAMP so that it can interact with TLR 3 expressed on DCs (Apostolico *et al*., 2019). Poly (I:C) replicates the immune system reaction to a viral infection and stimulates T cell activation (Martins *et al*., 2014). In fact mice, treated with an antibody targeted to DEC-205 plus Poly (I:C) led to a stronger effector immune response as opposed to mice treated with the antibody alone, thus demonstrating the potential of Poly (I:C) as an adjuvant (Apostolico *et al*., 2019). Although both Poly (I:C) treatment and skin transplant stimulate a proinflammatory immune response, the discrepancies in the models used may contribute to the difference in findings following α2,6 Sia antigen/ alloantigen targeting *in vivo*.

Antigen-specific deletion is a known mechanism to promote tolerance by immunizing mice at a steady state with soluble antigen. In relation, we have found that B6 and B6.Batf3$^{-/-}$ mice treated with native K^{d} peptide or sialylated K^{d} alloantigens promoted deletion of adoptively transferred TCR75 CD4^{+} T cells, in comparison to mice treated with no peptide, without deletion or expansion of endogenous Tregs. Our findings with K^{d} complement that of Tanriver *et al*., (2010). Therefore, antigen-specific deletion may be one of the mechanisms partially contributing to graft survival following alloantigen targeting by reducing the pool of alloreactive T cells, although we have yet to confirm how this deletion is caused. The marked decrease of alloantibodies, may be accounted for alloreactive T cell deletion, however, this has yet to be confirmed (Steele *et al*., 1996).

In order to achieve transplant tolerance, a targeting regimen should have the ability to induce tolerance that is specific to the donor Ags/ allograft as opposed to being broad immunosuppressive towards non-transplant related antigens, such as pathogenic or viral antigens that require an effector immune response for their elimination. Although we noticed that K^{d} alloantigen specific TCR75 CD4^{+} T cells were deleted following peptide treatments, we did not assess non-alloantigen specific CD4^{+} T cell deletion. Perdicchio *et al*., (2016), assessed whether targeting DCs with sialylated OVA protein were suppressive towards non-antigen specific T cells. They identified that DCs loaded with Sia-OVA and unsialylated MOG peptide were unable to induce Foxp3^{+} Tregs from MOG-

specific $CD4^+$ T cells, additionally, Tregs were only induced when OVA-specific OT-II $CD4^+$ T cells were introduced into the MLR (Perdicchio *et al*., 2016). Thus demonstrating that sialylated antigen targeted DCs mediate antigen-specific tolerance (Perdicchio *et al*., 2016). Therefore as future work, we could assess whether Sia-K^d treated DCs induce antigen-specific tolerance or are broadly immunosuppressive, inhibiting non-K^d specific T cells. This can be tested by loading DCs with sialylated K^d and unsialylated MOG peptide, followed by co-culture with MOG-specific 2D2 $CD4^+$ T cells in an MLR.

7.1.2 Not all sialylated alloantigens mediate allograft survival

We have so far demonstrated that DCs targeted with the all the sialylated constructs (α2,3 Sia-K^d, α2,3L Sia-K^d, α2,6 Sia-K^d) induced Tregs and impaired T cell proliferation *in vitro*. *In vivo*, T cell deletion also occurred when mice were treated with these constructs without deleting the Treg pool. Despite this, only α2,3 Sia-K^d treatment prolonged allograft survival and α2,3L Sia-K^d (longer-sialylated-glycan-glycopeptide) and α2,6 Sia-K^d targeting did not prolong allograft survival. Due to the α2,3 sialic acid linkage on both α2,3L Sia-K^d and α2,3 Sia-K^d, we expected the former to prolong allograft survival as well. However, as we did not have a fluorochrome-conjugated α2,3L Sia-K^d construct, we could not confirm binding of this peptide to APCs, we could also question whether the modification of the sialic acid linkage may have modified the α2,3L Sia-K^d property to hit Siglecs *in vivo* and thereby not being as effective at prolonging transplant survival. To this end, we can suggest that this longer α2,3L Sia-K^d is not as effective as the shorter α2,3 Sia-K^d, at improving allograft survival.

7.1.3 Targeting with K^d monomer instead of K^d peptide

K^d is an immunodominant peptide derived from the entire MHC I K^d molecule; therefore, the T cells that specifically respond to this K^d peptide have a TCR that recognises this single peptide only. Tanriver *et al.,* (2010), identified that targeting with the K^d peptide did not induce indefinite skin transplant tolerance unlike targeting recipient's with a K^d monomer (entire MHC I H-$2K^d$), where indefinite skin graft survival was observed. Therefore as future work, it would be

interesting to determine whether the K^d monomer could somehow be encapsulated within in a sialylated barrier (e.g. liposomes or nanoparticles) (Ali *et al.,* 2018) and administered to recipients (Spence *et al*., 2015). This could target more alloreactive T cells as an entire MHC monomer would consists of multiple epitopes that can be recognized by multiple TCRs, thus a much larger pool of alloreactive T cells.

The short course of graft survival may also raise the question as to whether the dose and number of infusions were sufficient enough to induce lasting tolerance. We have demonstrated that targeting peptide 10 days before transplant did not promote transplant survival in comparison to targeting mice 1 day before. As future work, assessing different doses of peptide and targeting at different time-points could be used to determine the optimal targeting regimen. For example in Tanriver *et al*., (2010) study, they trialled various doses of 33D1-K^d monomer such as 2µg and 20µg, where the latter proved to be more efficient at T cell deletion and was therefore used in their *in vivo* skin transplant experiments. One significant difference between the current and aforementioned study is their monomer was administered 14 days prior to transplant whereas we administered peptide 10 days and 1 day prior to transplant, where the latter was more successful in prolonging allograft survival. So, it would appear that 14 days may not be efficient for promoting allograft survival using sialylated alloantigen. One thing to consider is the dose of sialylated alloantigen that is administered. We opted for 10ug of peptide, which was the same dose used in some of Tanriver *et al*., (2010) *in vivo* experiments which demonstrated alloreactive non-Treg T cell deletion with 33D1-K^d peptide. Conversely, Perdicchio *et al*., (2016) opted for a higher dose of 50ug of sialylated OVA protein, followed by priming mice 7 days post infusion, therefore a higher dose of sialylated K^d peptide can also be assessed as future work.

7.1.4 Can sialylated alloantigen targeting to DCs be applicable to human transplant survival therapy?

Majority of the clinical trials focused on TolDCs comprise of administration of DCs as opposed to *in situ* targeting. The safety of administering autologous antigen-loaded TolDCs was tested in 2001 by Dhodapkar *et al*. where they administered immature DCs to healthy volunteers subcutaneously, resulting in no signs of toxicity or autoimmunity. A single dose of 2×10^6 monocyte-derived immature DCs treated with influenza matrix peptide (MP) and keyhole limpet hemocyanin (KLH) generated significant reduction of MP-specific IFN-γ producing T cells, with little difference in the total population of effector T cells, suggesting that this tolerance regimen was antigen specific (Dhodapkar *et al*., 2001). This was one of a few studies that paved the way for the use of antigen-pulsed human TolDCs in generating transplant tolerance. A pilot study in 2012 investigated the effects of targeting autologous immature monocyte-derived DCs pulsed with myelin peptide antigens as means of a prospective immunotherapy for relapsing-remitting multiple sclerosis patients (Raïch-Regué *et al*., 2012). Impaired proliferation and a reduction of proinflammatory cytokines of myelin-specific T cells which were enriched from multiple sclerosis patients, and co-cultured with myelin peptide pulsed autologous TolDCs was observed, suggesting this to be potential TolDC therapy to treat this autoimmune disease (Raïch-Regué *et al*., 2012). A significant difference between the current study and the trials by Raïch-Regué *et al*., (2012) and Dhodapkar *et al*., (2001), is their method of pulsing DCs, prior to administration, whereas we have targeted DCs *in situ*. Despite this, an important similarity between the studies was observed; reduced antigen-specific effector T cell responses after treatment, therefore suggesting that targeting antigen-loaded DCs or targeting DCs *in situ* are effective tolerance induction strategies.

Overall, there is potential of using sialylated alloantigens for promoting tolerance in human trials. As a potential targeting regimen, DCs may be targeted with sialylated alloantigens and then administered to recipients or sialylated alloantigens can be administered *in situ* to target endogenous DCs. Perdicchio *et al*., (2016) demonstrated in murine models that targeting sialylated alloantigens *in vitro* to autologous DCs promoted tolerance when injected into

recipients. We did not attempt to pulse autologous DCs with the constructs and administer these to transplant mice as it has been demonstrated by Smyth *et al*., (2013), that K^d-pulsed autologous DCs did not prolong allograft survival. Although targeting K^d-pulsed autologous DCs did not improve transplant survival in mice, this regimen may prove to be more efficient in the human recipients, as highlighted in *section 1.4.1.2* (pg. 27).

Despite successes in human clinical trials, there are several disadvantages to creating TolDCs *in vitro* as opposed to targeting DCs *in situ* (as mentioned in Chapter 1). However, an important consideration to take into account when targeting endogenous DCs, is the immune status of the recipient and whether the DCs within the recipient are immature or have been activated by a pre-existing condition. For example, targeting alloantigens to a transplant recipient who may be experiencing diseases which can cause the recipient to remain in an activated state may exacerbate rejection as targeting matured/activated DCs can promote an effector immune response. However, studies have discovered that Siglec E on DCs and macrophages dampened TLR signalling and proinflammatory cytokine production as a result of NF-*κ*B attenuation in the presence of LPS stimuli (Wu *et al*., 2016; Boyd *et al*., 2009). Interestingly, in a human setting, Siglec 9 expressed on immature monocyte-derived DCs that were targeted with artificial glycopolymers linked with α2,3 or α2,6 sialic acid, were shown to reduce IL-12 cytokine levels even in the presence of LPS. Therefore, targeting Siglecs on murine as well as human -DCs may regulate immune responses, even under proinflammatory stimuli that has the potential to lead to activation of DCs (Ohta *et al*., 2010).

Another issue is whether antigens need to be sialylated in order to promote tolerance, or whether administration of peptide antigen and sialic acid separately can mediate a better or similar effect. Perdicchio *et al*., 2016 confirmed that DCs were only tolerogenic when targeted with Sia-OVA as opposed to being targeted with sialic acids and OVA separately. Hence, in order to mediate tolerance by Siglec targeting, sialic acids would be need to be conjugated to the antigen in question.

As with human clinical trials (Moreau *et al.*, 2012), more preclinical work will need to be carried out to establish the optimum targeting procedure, such as dose of Sia-antigen, number of infusions, time-point in which the antigen (whole antigen or peptide antigen) is administered, route of administration and whether targeting autologous DCs with sialylated alloantigens is more efficient that targeting endogenous DCs. It will also need to be established whether targeting a sialylated peptide derived from the donor MHC I would be better at promoting tolerance rather than a sialylated whole MHC I monomer especially since Tanriver *et al.,* (2010), identified that targeting recipient mice the K^d peptide did not induce indefinite skin graft survival unlike targeting with an MHC I K^d monomer.

We have shown that α2,3 Sia-K^d treatment prolonged allograft survival, but does not induce indefinite skin graft prolongation. This targeting regimen may be useful as a combination therapy with other regimens such as rapamycin to stabilize Foxp3 expression on Tregs (Battaglia *et al.*, 2006; Singh *et al.*, 2012) or administration of K^d alloantigen- specific Tregs and low-dose IL-2 (Kulachelvy *et al.*, 2019) to support their growth and expansion rather than a stand-alone therapy.

Although mouse models have shown promise in investigating Treg therapy in transplantation *in vivo*, one disadvantage is the immune disparity in species between rodents and humans, for example humans consist of memory T cells which have the potential to become alloreactive, whereas mice which are housed in specific pathogen and viral free isolators do not contain such cells (Kenney *et al.*, 2016). In order to overcome this, humanised mouse models have been used as a preclinical tool; in short these immunodeficient mice are engrafted/ reconstituted with human cells so that they consist of a functional human immune system. As an example, human myeloid DCs generated from PBMCs, were made tolerogenic by transducing these cells with an adenovirus encoding IL-10 (Coates *et al.*, 2001). Once the these DCs were introduced into a humanized NOD-scid chimeric mouse model that was adoptively transferred with allogeneic mononuclear cells, and engrafted with human skin, incidences of graft rejection was reduced with reduced damage to the skin graft (Coates *et al.*, 2001). Overall, humanized mouse models can be used to establish if

targeting human APCs with sialylated alloantigen can promote tolerance. This is something which can potentially be addressed prior to human clinical trials.

Similarly to mice, humans also have different DCs subsets (Collin & Bigley, 2018). $CD141^+$ DCs are a human DC subset which are also known to express Siglecs (mentioned in a review by Crespo *et al.*, 2013), which can be derived from human blood as well as skin, these cells have similar cross-presentation capacity as murine $CD8\alpha^+$ DCs (Chu *et al.*, 2012). These DCs have been described as tolerogenic due to their ability to secrete IL-10, induce T cell hyporesponsiveness and induce Tregs (Chu *et al.*, 2012). In order to assess the immunoregulatory capacity of these DCs in an *in vivo* humanized GVHD mouse model, $CD141^+$ monocyte-derived DCs treated with vitamin D3 were administered to NSG mice reconstituted with human PBMCs (Chu *et al.*, 2012). The authors identified significant graft survival in mice treated with vitamin D3 $CD141^{hi}$ DCs as opposed to mice treated with vitamin D3 $CD141^{dim}$ DCs, suggesting the potential role of these human DC subsets to promote tolerance (Chu *et al.*, 2012). Therefore, it has been established that certain human subsets can be targeted to promote tolerance, however whether these subsets express Siglecs or whether human Siglecs on DCs [Table. 4] can be targeted with sialylated alloantigens to modulate these DCs has yet to be elucidated.

Although human Siglecs have demonstrated inhibitory functions [Table 4], targeting particular Siglecs on human DCs should be approached with caution. In 2014, it was established that human Siglec 7 is expressed on a particular subset of monocyte-derived DCs known as CD1 DCs (Kawasaki *et al.*, 2014). Although Siglec 7 has shown to be inhibitory for sialylated pathogen interaction (Crocker *et al.*, 2007), the authors of the 2014 study demonstrated otherwise. Delivery of mycobacterium antigens which was encapsulated within a liposome coated with lipid- ligand for Siglec 7 (Rillahan *et al.*, 2013), to CD1 DCs induced rapid T cell activation and was proposed as an effective vaccination against bacterial infection (Kawasaki *et al.*, 2014). In addition, Siglec 7 on human monocytes has also been shown to induce proinflammatory immune response following targeting with sialic acid-free pathogens (Varchetta *et al.*, 2012). Therefore, as an immunotherapeutic approach, further investigation into which specific human Siglecs should be targeted to promote tolerance will need to be

done and whether particular Siglecs should be blocked to avoid cross-reactivity of sialylated alloantigens with potential activatory Siglecs (Kawasaki *et al.*, 2014).

Another precaution to take note of when extending the current study to human subjects, is that inhibitory Siglecs are expressed on a variety of immune cells (Crocker *et al.*, 2012), which can be advantageous considering transplant rejection is mediated by numerous immune cells. One human study highlighted Siglec 9 expression on $CD8^+$ effector T cells (Haas *et al.*, 2019). They identified that following engagement of sialic acid ligands present on tumours were able to engage with Siglec 9 expressing CTLs and in turn functionally inhibit them (Haas *et al.*, 2019). Therefore, targeting on different immune cells including APCs may prove to be an effective graft survival strategy; however this approach requires much further investigation.

7.2 Final conclusion

Overall, this thesis has demonstrated for the first time that targeting sialylated alloantigen to Siglec-expressing recipient antigen presenting cells, particularly DCs (and possibly a particular DC subset $CD103^+$ and/or $CD8\alpha^+$), may represent a novel mechanism to regulate allorecognition in an antigen-specific manner [Fig. 7.1]. As described in Table 3, the studies by Ettinger *et al.*, (2012) and Tanriver *et al.*, (2010) demonstrate the potential of targeting a particular subset of DCs by targeting either receptor DEC205 or DCIR2, respectively, to promote allograft survival. We have demonstrated that multiple receptors (Siglecs) that may have been targeted by sialylated alloantigen also promote allograft survival. Given that we have identified allograft survival in WT mice as opposed to B6.Batf3$^{-/-}$ mice following α2,3 Sia-K^d targeting, it may be that targeting Siglecs on subsets $CD8\alpha^+$ and/or $CD103^+$ DCs are required and that targeting Siglecs on other DC subsets using sialylated alloantigen may not have a significant role. However, it has yet to be confirmed which Siglec and DC subset is definitely targeted.

Given that allograft survival was established in a B6.Rag$^{-/-}$ mouse model in the absence of B cells following α2,3 Sia-K^d targeting, Siglec expressing B cells

may not play a significant role in mediating allograft survival. In addition to DCs, sialylated alloantigen targeted macrophages may have also contributed to allograft survival as we have observed Siglec expression and peptide binding *in vitro*.

The current study provides an insight into the possibility of targeting Siglec-expressing DCs using sialylated alloantigens to promote allograft survival. Given our findings in mouse, targeting Siglecs in humans to promote allograft survival may be advantageous given their abundance on human DCs [Table 4], and expression on DC subsets such Siglec 6 on AXL^+ pre-DCs, a newly identified DC subset, thought to be efficient at stimulating T cell activation (Vallani e*t al.*, 2017; Collin & Bigley, 2018). The findings in this thesis could contribute to immunotherapeutic strategies to combat chronic rejection by targeting Ag-specific indirect immune responses within the transplant recipient without the use of prolonged immunosuppressive therapy. This thesis has overall suggested that targeting Siglec expressing APCs using sialylated alloantigens can promote murine skin allograft survival.

CHAPTER 8:

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A

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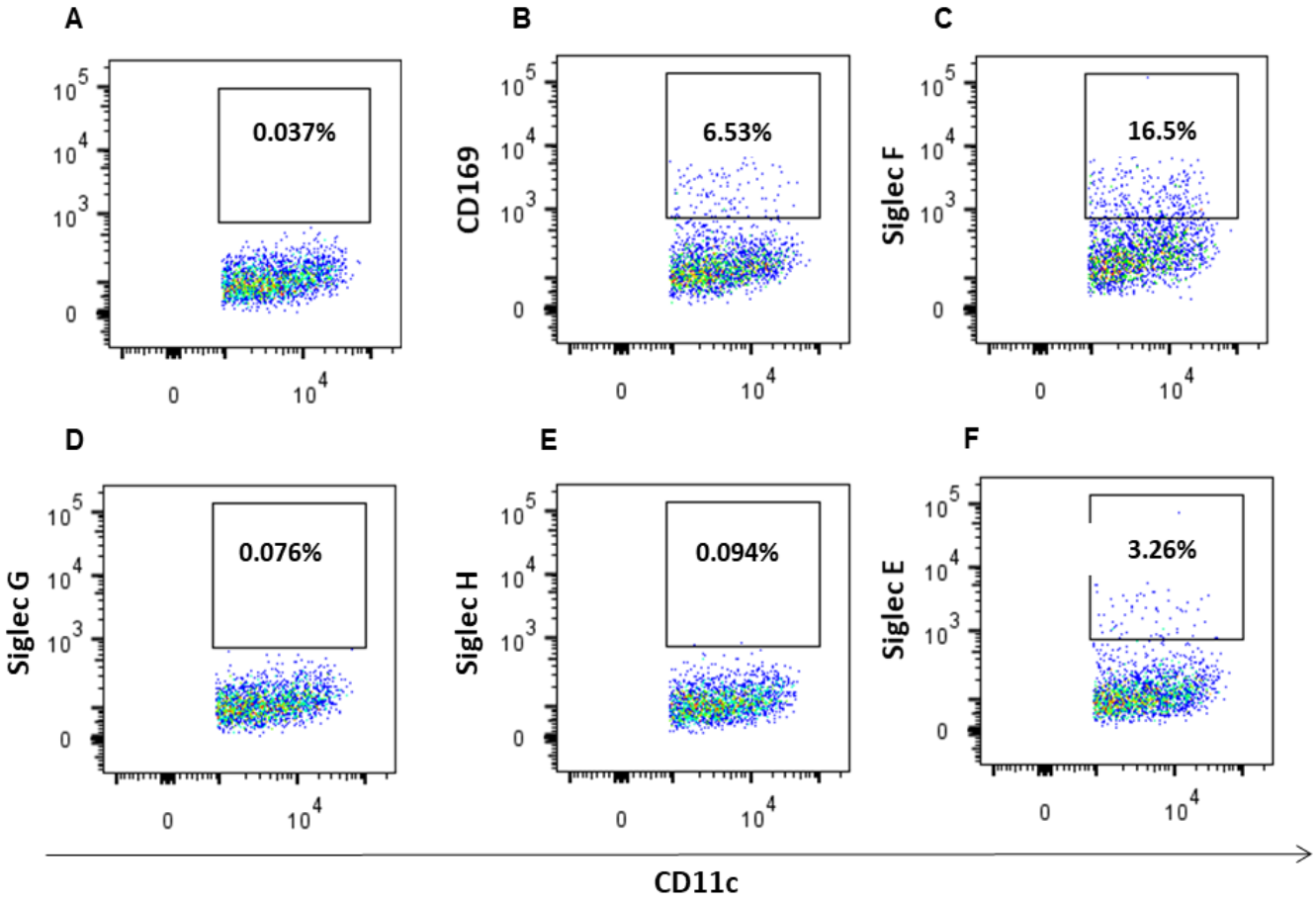
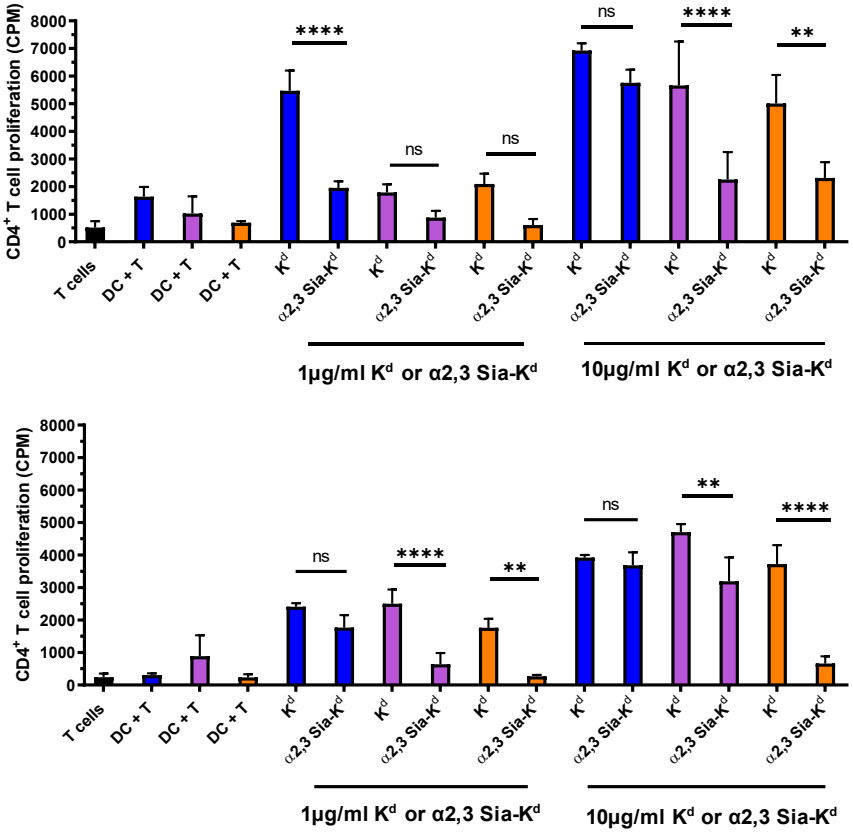
CHAPTER 9:

APPENDICES



Appendix figure 1. Siglecs are expressed on B6 BM-DCs. *For Siglec expression, day 6 or 7 B6 BM-DCs were stained using anti-mouse CD11c APC/PE and either anti-mouse Siglec E- PE, Siglec F- PE, Siglec G- PE, Siglec H-PE and CD169-PE. Live cells were gated on FSC, SSC. A. Percentage of BM-DCs expressing CD11c. B. Percentage of BM-DCs expressing Siglec CD169. C. Percentage of BM-DCs expressing Siglec F. D. Percentage of BM-DCs expressing Siglec G. E. Percentage of BM-DCs expressing Siglec H. F. Percentage of BM-DCs expressing Siglec E. Data is a representative of one experiment.*

A


CD4+ T cell proliferation (CPM)
8000
7000
6000
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T cells
DC + T
DC + T
DC + T
Kd
α2,3 Sia-Kd
1μg/ml Kd or α2,3 Sia-Kd
10μg/ml Kd or α2,3 Sia-Kd

CD4+ T cell proliferation (CPM)
8000
7000
6000
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4000
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2000
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ns
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T cells
DC + T
DC + T
DC + T
Kd
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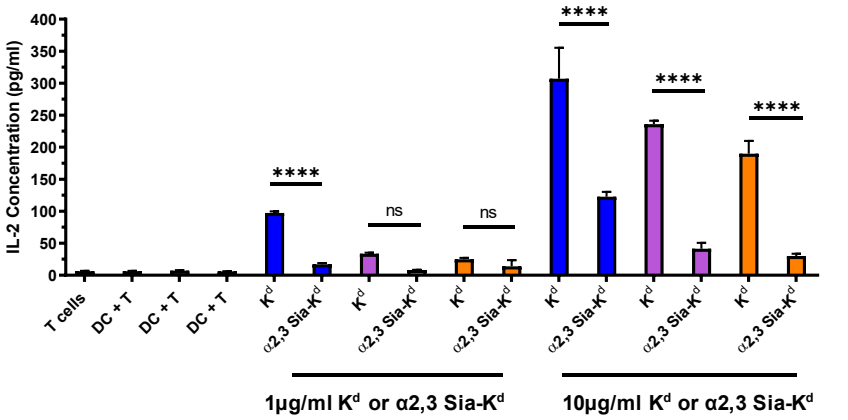
1μg/ml Kd or α2,3 Sia-Kd
10μg/ml Kd or α2,3 Sia-Kd

B


IL-2 Concentration (pg/ml)
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250
200
150
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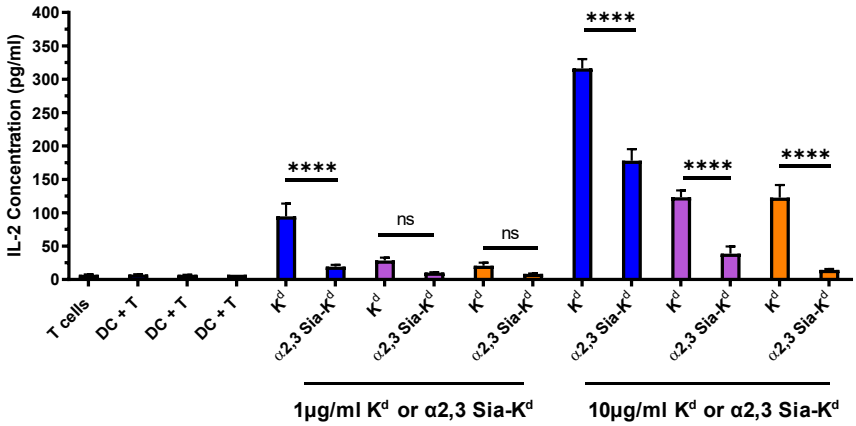
T cells
DC + T
DC + T
DC + T
Kd
α2,3 Sia-Kd
1μg/ml Kd or α2,3 Sia-Kd
10μg/ml Kd or α2,3 Sia-Kd


IL-2 Concentration (pg/ml)
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300
250
200
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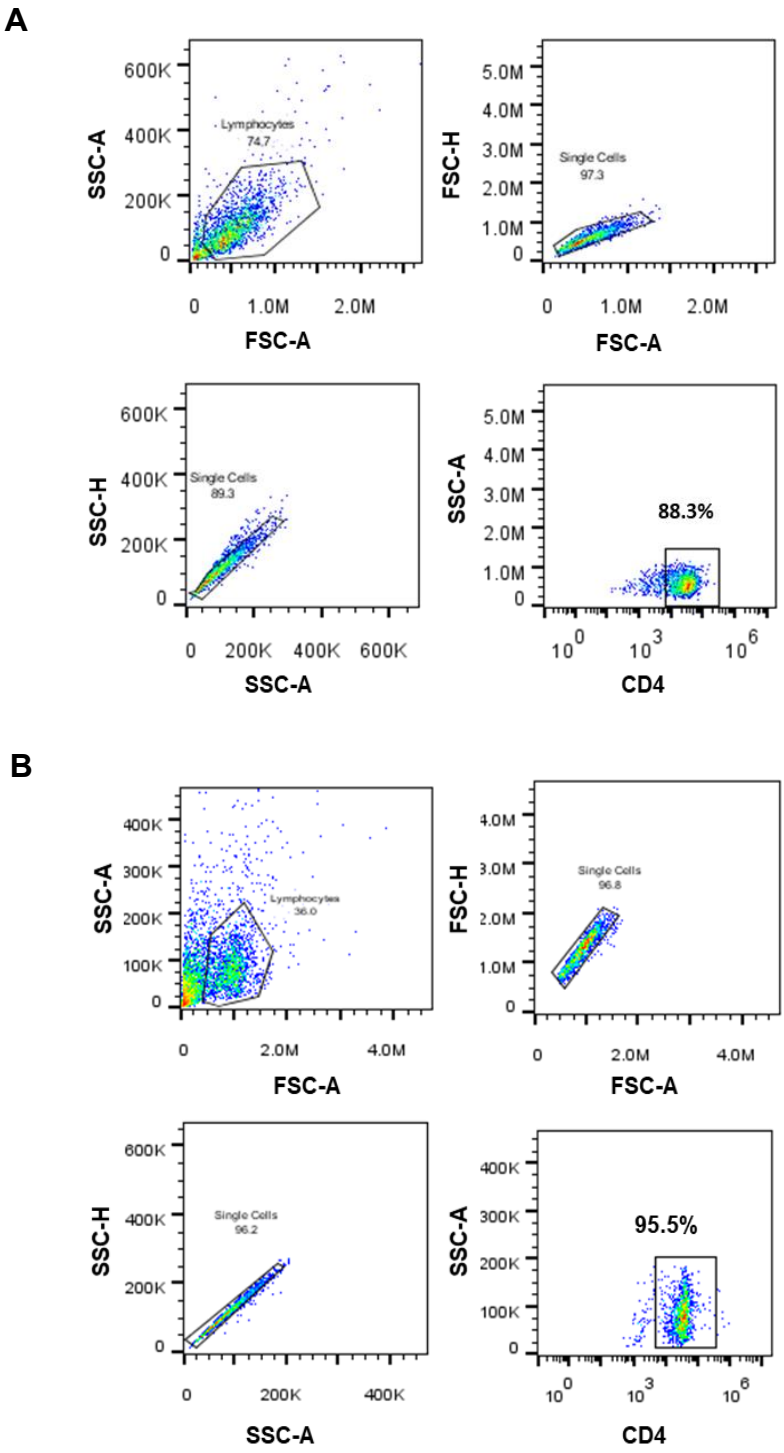
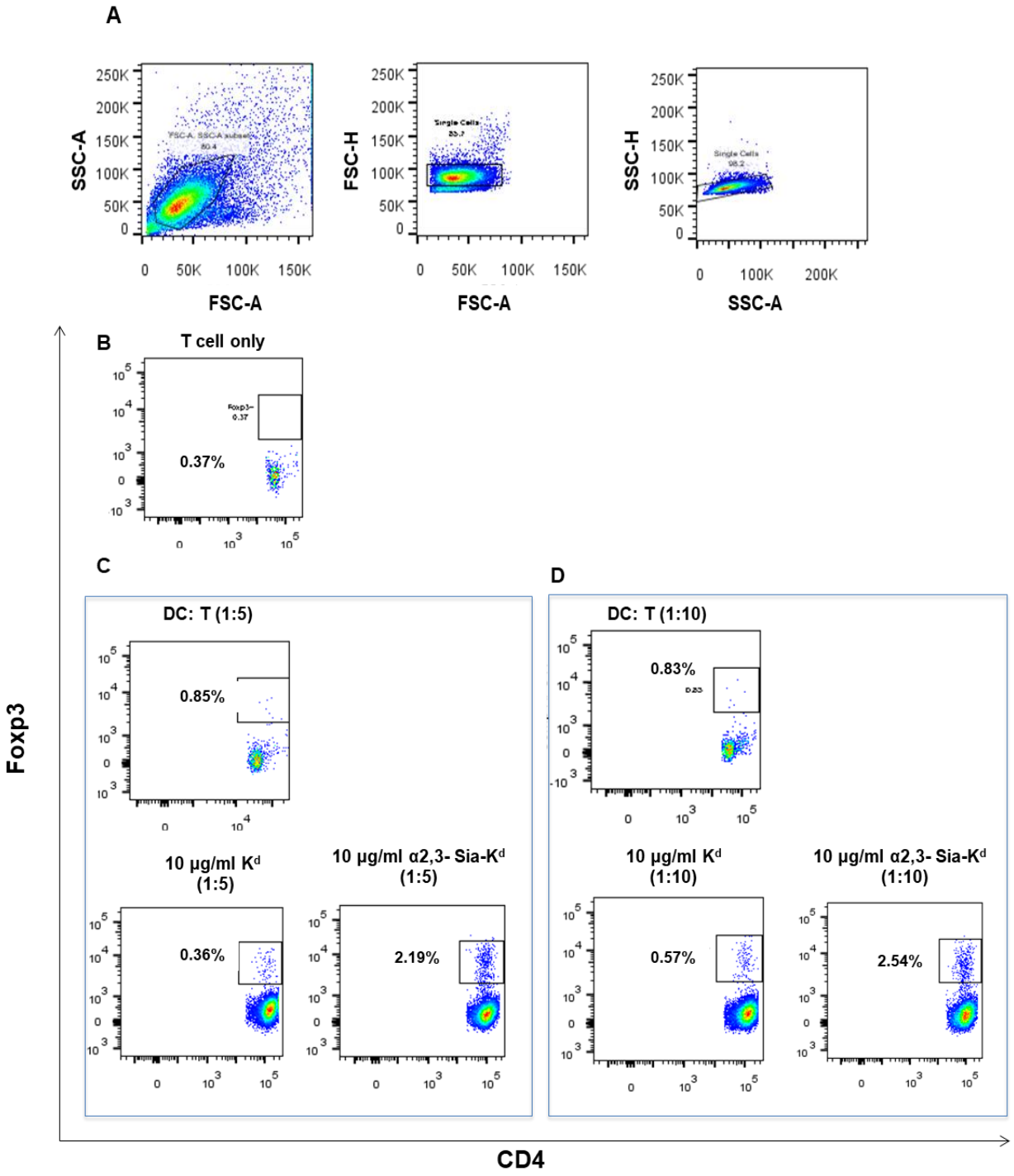
DC + T
DC + T
DC + T
Kd
α2,3 Sia-Kd
1μg/ml Kd or α2,3 Sia-Kd
10μg/ml Kd or α2,3 Sia-Kd

Appendix figure 2. Targeting BM-DC Siglecs with Sia-K^d leads to suppressed proliferation of TCR75 CD4$^+$ T cells and IL-2 in vitro. *B6 BM-DCs were pulsed with 1 or 10µg/ ml concentrations of peptide and co-cultured with CD4$^+$ T cells from TCR 75 Rag $^{-/-}$ mice at a 1:1, 1:5, 1:10 DC: T ratio. A. T cell proliferation was assessed on day 3 following the addition of ^{3}H thymidine for the last 18hrs of the culture. Proliferation is expressed as counts per minute (CPM) +/- SD. Data represents 2 experiments out of 3 performed. Each bar represents a technical triplicate +/-SD and statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test. B. IL-2 cytokine present in culture supernatants was determined using an IL-2 sandwich ELISA. Data represents 2 experiments out of 3 performed. Each bar represents a technical triplicate +/-SD and statistical comparisons were made using One-way ANOVA and Tukey's multiple comparisons test. Statistical significance was expressed as follows; p< 0.0001****, p<0.001***, p<0.01**, p<0.05*, NS= >0.05.*

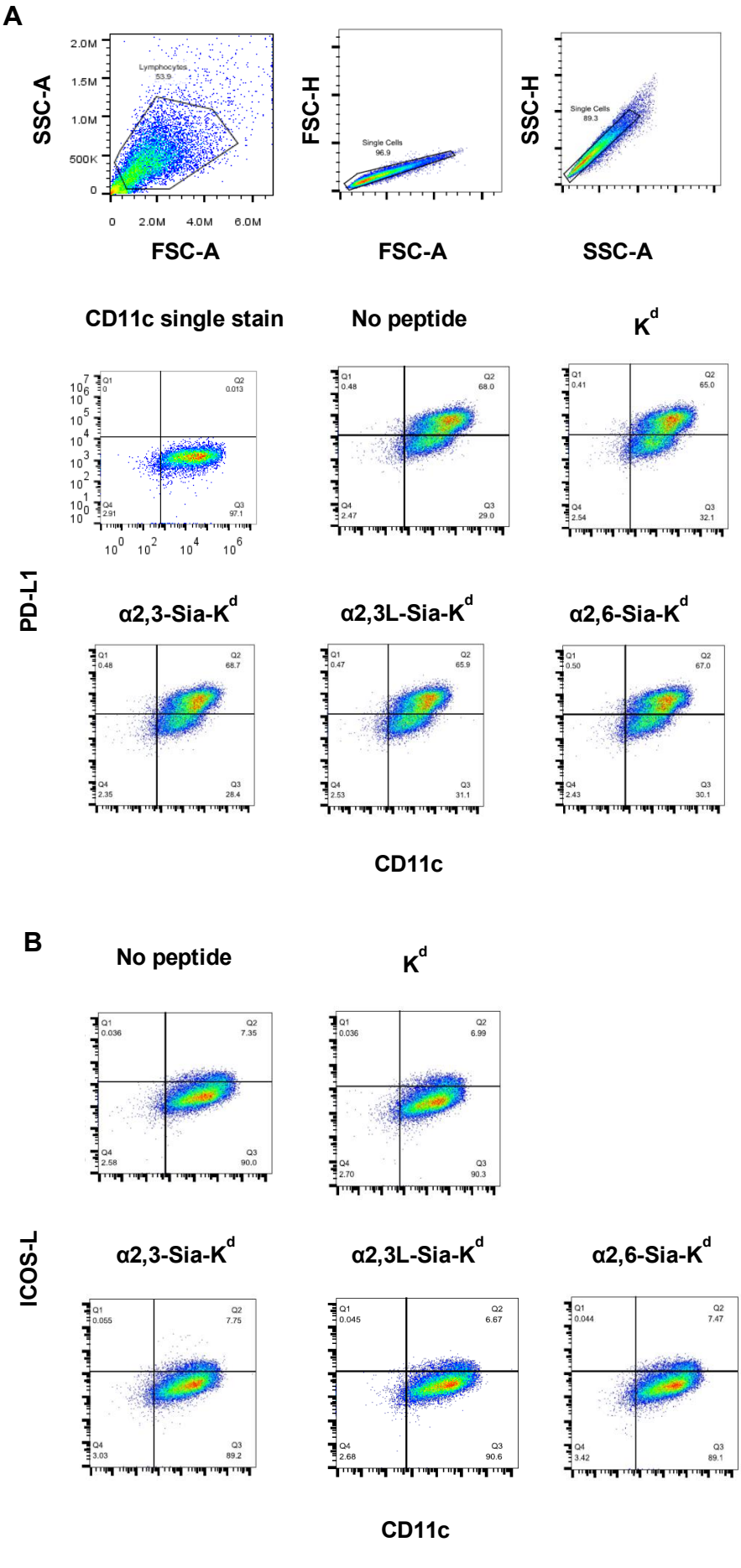


Appendix figure 3. Gating strategy for T cell proliferation assays. *B6 DCs or B cells were pulsed with 10μg/ml peptide and cocultured with CFSE labelled TCR75 CD4$^+$ T cells at 1:10 ratio. After 3 days, cells surface stained with CD4 antibody, followed by analysis of CFSE proliferation. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on CD4$^+$ T cells that were CFSE$^+$. A. Gating strategy for DC/T co-cultures. B. Gating strategy for B/T co-cultures.*

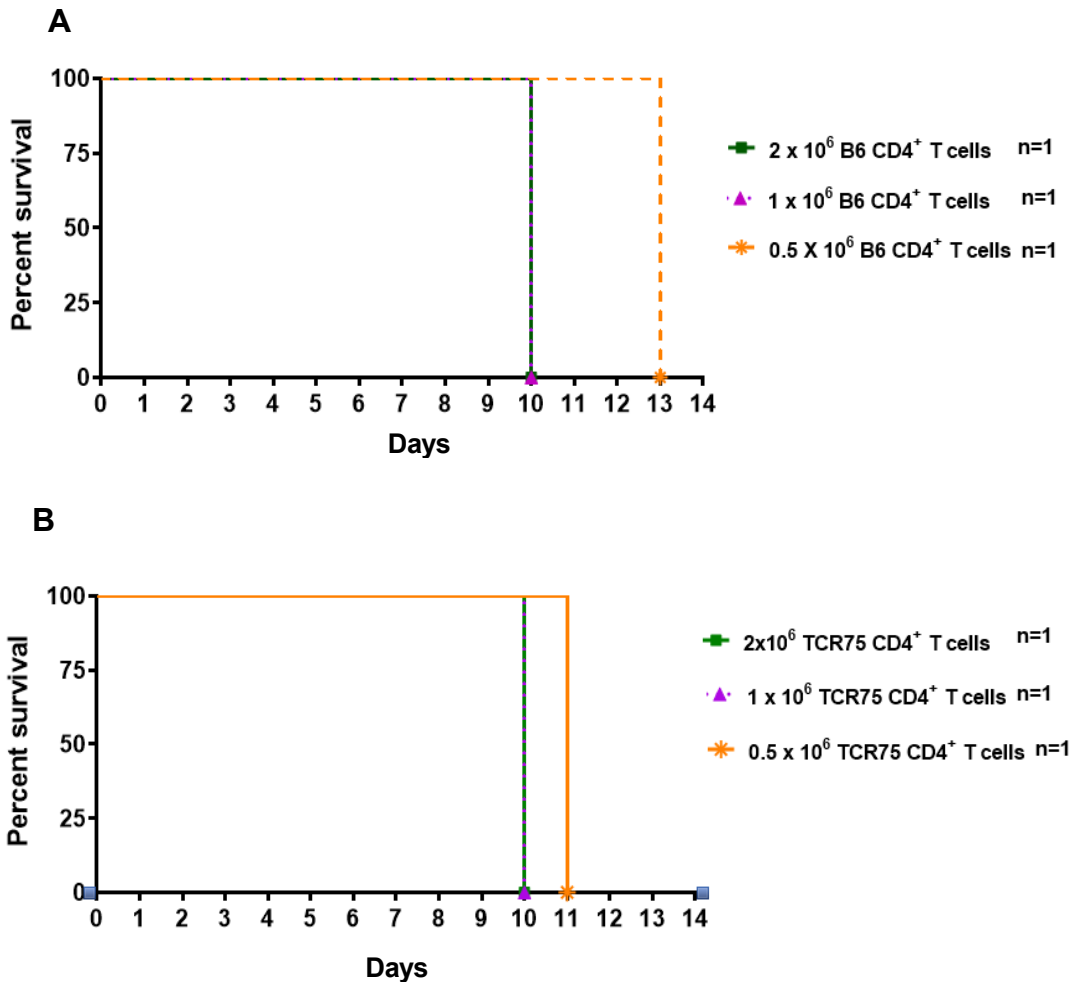


Appendix Figure 4. Gating strategy and titration experiments for in vitro Treg induction. *B6 BM-DCs were pulsed with 10µg/ml peptide and cocultured with TCR75 CD4$^+$ T cells at 1:5 or 1:10 ratio with subsequent addition of 5U IL-2 at day 0. After 3 days, expression of Foxp3 was measured by intracellular staining and subsequent flow cytometry. A. Cells were gated on live cells (FSC vs. SSC); doublets were excluded followed by gating on CD4$^+$ Foxp3$^+$ cells. B.T*

cell only control. C. Treg induction for 1:5 DC: T ratio. D. Treg induction for 1:10 DC: T ratio. Data is a representative for one experiment.

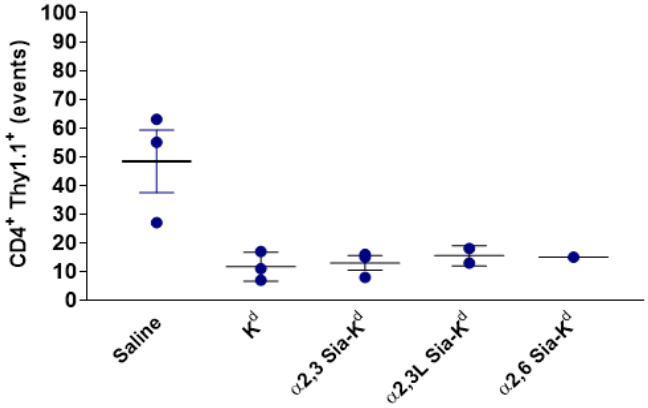

A
SSC-A
FSC-A
Lymphocytes
53.9
FSC-H
FSC-A
Single Cells
96.9
SSC-H
SSC-A
Single Cells
89.3
CD11c single stain
No peptide
K^d
PD-L1
α2,3-Sia-K^d
α2,3L-Sia-K^d
α2,6-Sia-K^d
CD11c
B
No peptide
K^d
ICOS-L
α2,3-Sia-K^d
α2,3L-Sia-K^d
α2,6-Sia-K^d
CD11c

Appendix Figure 5. Gating strategy for ICOS-L and PD-L1 expression on BM-DCs. *B6 derived BM-DCs targeted with no peptide, K^d or α2,6 Sia-K^d were pulsed for 4 hours with 10 µg/ml peptide at 37°C incubation and analysed using flow cytometry. Cells were gated on live cells (FSC vs. SSC), doublets were excluded followed by gating on $CD11c^+$ DCs. A. $CD11c^+$ $PD\text{-}L1^+$ BM-DCs. B. $CD11c^+$ $ICOS\text{-}L^+$ BM-DCs.*

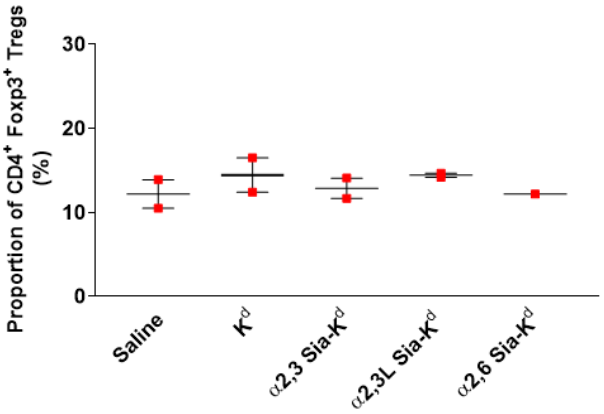


Appendix figure 6. Trial transplant experiment on B6.Rag2$^{-/-}$ mice to compare graft survival following B6 CD4^{+} T cell or CD4^{+} TCR75 T cell reconstitution. *A. (1 mouse/ group) B6.Rag2$^{-/-}$ mice received either 2x10^{5}, 1 x10^{5} or 0.5x10^{5} B6 CD4^{+} T cells (i.v.) 1 day before BALB/c skin transplant. Mice were monitored daily and skin was deemed rejected when >90% of donor skin had undergone necrosis. B. B6.Rag2$^{-/-}$ mice (1 mouse/ group) received either 2x10^{5}, 1 x10^{5} or 0.5x10^{5} TCR75 CD4^{+} T cells (i.v.) 1 day before BALB/c skin transplant. Mice were monitored daily and skin was deemed rejected when >90% of donor skin had undergone necrosis.*

A



B



Appendix Figure 7. Indirect alloantigen-specific CD4⁺ T cells are deleted following peptide targeting, without deleting CD4⁺ Foxp3⁺ Tregs in vivo. *B6 mice were administered with 2 x 10^6 TCR75 T cells (Thy1.1) 1 day before mice received K^d 10µg/200µl saline, α2,3 Sia-K^d 10 µg/200µl saline, α2,3L Sia-K^d 10 µg/200µl saline, or α2,6 Sia-K^d 10 µg/200µl saline i.v. No peptide controls received 200µl saline i.v. only. Ten days later spleens and lymph nodes were harvested and stained for CD4, Thy1.1 and Foxp3. Live cells were gated on forward scatter (FSC), side scatter (SSC), CD4 versus Thy1.1 cells. (A). Number of cellular events of Thy1.1⁺ T cells within CD4⁺ gate following saline (n=3 mice in group), K^d (n=3 mice in group), α2,3 Sia-K^d (n=3 mice in group), α2,3L Sia-K^d (n=2 mice in group), and α2,6 Sia-K^d (n=1 mice in group) targeting in B6 mice. (B). Percentage of CD4⁺ Foxp3⁺ Tregs following saline (n=2 mice in group), K^d (n=2 mice in group), α2,3 Sia-K^d (n=2 mice in group), α2,3L Sia-K^d (n=2 mice in group), and α2,6 Sia-K^d (n=1 mice in group) targeting in B6 mice. Horizontal line between data points represent the mean and error bars represent +/- SEM.*