AN INVESTIGATION INTO THE PHENOTYPE, FUNCTION AND IMMUNOMODULATORY PROPERTIES OF *IN VITRO* EXPANDED iNKT CELLS

CLAIRE MARY DEMPSEY

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College of Medical and Dental Sciences

University of Birmingham

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ABSTRACT

Invariant natural killer T (iNKT) cells are endowed with features of both innate and adaptive immunity. They are activated by the recognition of glycolipid agonists presented by CD1d which makes them excellent candidates for cellular therapies.

In order to investigate the ability of iNKT cells to suppress experimental acute Graft versus host disease (GVHD), iNKT cells were first expanded *in vitro,* which is likely to be required prior to their use as a cellular therapeutic. Interestingly, the expanded cells showed increased frequencies of IL-10, IL-13 and IL-17 producing cells and were found to robustly suppress alloreactive T cell proliferation *in vitro* compared to freshly isolated cells. However, in a model of acute GVHD induced by alloantigen-reactive TCR-transgenic T cells neither freshly isolated nor expanded iNKT cells suppressed GVHD, although some survival benefit was seen following the activation of host iNKT cells.

These data indicate that iNKT cells can be expanded *ex vivo*, that they can acquire different functional properties and that such cells robustly suppress alloreactive T cell proliferation *in vitro*. Therefore, further investigation into the suppressive behaviour of these cells is warranted despite a failure to suppress acute GVHD in the current study.

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Abbreviations

mAbs	**Monoclonal antibodies**
°C	**degrees Celsius**
7-AAD	**7-aminoactinomycin D**
ACAID	**Anterior Chamber Associated Immune Deviation**
AHR	**airway hyper-reactivity**
AIRE	**autoimmune regulator**
AML	**Acute myeloid leukaemia**
AP-1	**Activator protein 1**
APC	**antigen presenting cell**
ARG 1	**Arginase-1**
AT	**adoptively transferred**
B6	**C57BL/6**
BCL-2	**B-cell lymphoma 2**
BCL-xL	**B-cell lymphoma-extra large**
BCR	**B cell receptor**
BFA	**Brefeldin A**
BM	**bone marrow**
BMDCs	**bone marrow-derived dendritic cells**
BRDU	**Bromodeoxyuridine**
CAR	**Chimeric antigen receptor**
Cas9	**CRISPR associated protein 9**
CB6F1	**F1 cross of C57BL/6 and BALB/c**
CCR	**Chemokine receptor**
CD	**Cluster of differentiation**
CFSE	**carboxyfluorescein succinimidyl ester**
cGy.	**centigray**
CLRs	**C-type lectin like receptors**
CO_2	**carbon dioxide**
CRISPR	**Clustered Regularly Interspaced Short Palindromic Repeats**
cRPMI	**complete Roswell Park Memorial Institute Media**
CTLA-4	**cytotoxic T-lymphocyte-associated protein 4**
CTLs	**Cytotoxic T lymphocytes**
CXCL	**C-X-C motif ligand**
DAG	**diacylglycerol**
DAMPs	**Damage-associated molecular patterns**
DAPI	**4′,6-diamidino-2-phenylindole**

DCs	**Dendritic cells**
DN	**Double negative**
DNA	**Deoxyribonucleic acid**
EAE	**Experimental autoimmune encephalomyelitis**
EBV	**Epstein-Barr virus**
EDTA	**Ethylenediaminetetraacetic acid**
ELISAs	**enzyme-linked immunosorbent assays**
Erg	**ETS-related gene**
Exp	**expanded**
FACS	**Fluorescence-activated cell sorting**
FAS-L	**FAS-Ligand**
FCS	**Fetal calf serum**
FI	**Freshly isolated**
FKBP	**FKB506 binding protein**
FOXP3	**forkhead fox P3**
g	**Gravity**
GATA3	**GATA Binding Protein 3**
GFP	**green fluorescent protein**
GI	**gastrointestinal**
GM-CSF	**granulocyte monocyte stimulating factor**
GMP	**Good manufacturing practice**
GRAIL	**Gene related to anergy in lymphocytes**
GVHD	**Graft versus host disease**
GVL	**Graft versus leukaemia**
GVT	**Graft versus tumour**
Gy	**Gray**
HBSS	**Hanks' Balanced Salt solution**
HBV	**hepatitis B virus**
HEB	**E protein transcription factor**
HIV	**human immunodeficiency virus**
HLA	**human leukocyte antigen**
Hour	**h**
HSC	**Haematopoietic stem cell**
HSV	**herpes simplex virus**
i.p.	**intraperitoneal**
i.v.	**Intravenous**
IBD	**Inflammatory Bowel disease**
IC	**intracellular**
IDO	**Indoleamine 2,3-dioxygenase**
IFN	**Interferon**
IL	**Interleukin**
IL-10r	**IL-10 receptor**
IL-2r	**IL-2 receptor**

ILC3s	**Type 3 innate lymphoid cells**
IMPDH	**inhibitor of inosine monophosphate dehydrogenase**
iNKT	**invariant natural killer T**
iNKT cells	**invariant Natural Killer T cells**
iNOS	**inducible nitric oxide synthase**
IP_3	**inositol triphosphate**
IPEX syndrome	**immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome**
ITAM	**the immunoreceptor tyrosine-base motif**
iTreg	**induced (peripherally) regulatory T cells**
JAK	**Janus kinase**
Klf	**Kruppel-like factor**
KO	**knock-out**
LAG-3	**Lymphocyte activation gene 3**
LAT	**linker for activation of T cells**
LEF1	**Lymphoid Enhancer Factor 1**
LNs	**lymph nodes**
LPS	**lipopolysaccharide**
MACS	**Magnetic activated cellular sorting**
Mb	**megabase**
MCMV	**murine cytomegalovirus**
MCP	**Methyl-accepting chemotaxis protein**
MDSCs	**myeloid derived suppressor cells**
MFI	**Median Fluorescence intensity**
mHC	**Minor histocompatibility antigens**
MHC	**Major histocompatibility complex**
MIG	**Monokine induced by gamma interferon**
min	**minutes**
MIP	**Macrophage inflammatory protein**
MLN	**Mesenteric lymph node**
MS	**Multiple Sclerosis**
MSCs	**mesenchymal stem cells**
mTEC	**medullary thymic epithelial cells**
mTOR	**mammalian target of rapamycin**
NFκB	**nuclear factor kappa-light-chain-enhancer of activated B cells**
NK	**Natural killer**
NKR	**Natural killer receptor**
NKT cells	**Natural killer T cells**
NKTFH	**Follicular helper Natural Killer T**
NLRs	**Node like receptors**
NO	**nitric oxide**
NOD	**non-obese diabetic**
nTreg	**natural (thymically derived) Treg**

OCH	**2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-N-tetracosanoyl-2-amino-1,3,4-nonanetriol**
OCT	**optimum cutting temperature cryopreservation media**
PAMPs	**Pathogen associated molecular patterns**
PBS	**Phosphate-buffered saline**
PD1	**Programmed cell death protein 1**
PDL-1	**Programmed cell death protein ligand 1**
PIP_2	**phosphatidylinositol bisphophate**
PKC	**protein kinase c**
PLC-γ	**phospholipase C-γ**
PLZF	**promyelocytic leukaemia zinc finger**
PMA	**para-Methoxyamphetamine**
PRRs	**Pathogen recognition receptors**
r	**Recombinant**
r.t.	**room temperature**
RA	**Rheumatoid arthritis**
RAG	**lymphoid specific recombinase**
RLRs	**RIG-like receptors**
RNA	**Ribonucleic acid**
RORγ	**Retinoic acid receptor-related orphan receptor gamma**
RORγT	**RAR-related orphan receptor gamma thymus**
ROS	**reactive oxygen species**
RSS	**recombination signals sequence**
SAP	**signalling lymphocytic-activation molecule -associated protein**
sec	**seconds**
SI	**Small intestine**
siRNA	**Small interfering Ribonucleic acid**
SLAM	**signalling lymphocytic-activation molecule**
SLP-76	**SRC-homology 2 (SH2)-domain containing leukocyte protein 75kD**
STAT	**signal transducer of activation**
TALENs	**Transcription activator-like effector nucleases**
T-bet	**T-box transcription factor**
TBI	**total body irradiation**
TCD BM	**T cell depleted bone marrow**
TCF1	**T Cell Factor 1**
Tcon	**conventional T cells**
TCR	**t cell receptor**
TGF-β	**Transforming growth factor beta-1**
Th	**T helper**
TIGIT	**T cell immunoglobulin and immunoreceptor tyrosine-based inhibition motif domain**
TIM-3	**immunoglobulin and mucin domain 3**

TLRs	**Toll like receptors**
TNF	**Tumour necrosis factor**
Tr1	**type 1 regulatory T cells**
Treg	**Regulatory T cells**
VDJ	**variable, diversity and joining**
WT	**wild type**
ZAP-70	**PTK zeta chain-associated protein**
αGal	**alpha galactosylceramide**
β_2M	**Beta 2 microglobulin**

CHAPTER 1 INTRODUCTION

1.1. The Immune system

1.1.1. The innate Immune system

The immune system has evolved to protect organisms against infectious agents and maintain homeostasis. It is generally regarded to be made up of two arms; namely the innate immune system and the adaptive immune system. The innate immune system is the first line of defence against invading microbes. Pathogen recognition receptors (PRRs) are expressed by innate immune cells, such as macrophages and dendritic cells (DCs) and recognise conserved microbial antigens known as pathogen associated molecular patterns (PAMPs). In addition, some PRRs can be triggered by the products of cellular damage known as damage-associated molecular patterns (DAMPs), which are endogenous stress-induced self-molecules (1, 2).

There are several families of PRRs, such as Toll Like Receptors (TLRs), NOD-like receptors (NLRs), C-type lectin like receptors (CLRs) and RIG-I like receptors (RLRs)

(3). PRR activation leads to activation of downstream signalling molecules which initiate the innate immune response and leads to the upregulation of Major histocompatibility (MHC) molecules and co-stimulatory molecules on antigen-presenting cells (APCs). In response to their activation APCs also produce cytokines such as Interleukin (IL)-12 and IL- 18 and the cytokine milieu which they provide directs the differentiation of T cells. Once they have been activated by the binding of PAMPs to PRRs, the APCs are then regarded as mature, and have the capacity to not only present antigen but also to provide the co-stimulatory signals and cytokines that are necessary for a productive T cell response (2).

1.1.2. The Adaptive Immune system

The adaptive immune system is characterised by highly-specific antigen recognition, the generation of a huge repertoire of antigen receptors and the formation of memory cells that rapidly respond on secondary encounter of an antigen, resulting in a prophylactic response and faster clearance of antigen. The main cellular constituents of the adaptive immune system are T and B cells which bear receptors (T cell receptor (TCR) and B cell receptor (BCR)) that enable recognition of antigens in their native form or in the case of T cells as a peptide presented in the context of MHC (4). In contrast, following recognition of native antigen by the BCR, activated B cells develop into plasma cells which secrete antibodies with the same specificity as the BCR to, for example, neutralise the antigen, or enhance opsonisation of antigen-bearing cells (5). Conventional alpha beta (αβ) T cells can be divided into two broad categories Cluster of differentiation (CD)8^+ effector T cells and $CD4^+$ Helper T cells (Th cells). The main function of $CD8^+$ T cells is to kill pathogen-infected or cancer

cells (6). Th cells can be further divided into four main classes Th1, Th2, Th17 and regulatory T cells (Treg) (7).

1.1.3. Major histocompatibility complex (MHC)

In humans the MHC region includes a series of genes on chromosome 6p21.31, which spans 3.75 Megabase (Mb) and codes more than 120 genes (8, 9). Approximately 40% of these genes have a function in the immune system (9). Among these are the class I and class II Human leukocyte antigens (HLA). HLA class I antigens are divided into classical HLA class I (HLA-A, B, C) and non-classical (HLA-E, F, G) and HLA class II molecules (classical HLA DP, DQ and DR) and non-classical (HLA-DO and HLA-DM)) (10) (Table 1). As HLA mismatching is amongst the strongest risk factors for the development of both acute and chronic Graft versus host disease (GVHD), HLA matching has become a cornerstone in pre-transplant donor evaluation (11). While the norm in clinical practice is to HLA match donors and recipients where possible, minor histocompatibility complex (mHC) antigens may also lead to GVHD (12). mHC antigens are polymorphic peptides which may lead to recognition by T cells in the context of allogeneic transplantation and can therefore act as alloantigens.

In mice MHC molecules are called H-2 and are located on chromosome 17 (13). Similarly to humans they can be divided into class I (or MHCIa and MHCIb) and MHC class II (MHCIIa and MHCIIb) (14) (Table 1). MHC-Ia (classical-MHC class I) is made up of H-2D, H-2K and H-2L molecules and MHCIb (non-classical MHC class I) contains H-2Q, H-2M and H-2T molecules (14) (Table 1). MHC-IIa (classical MHC

class II) comprises H-2A (I-A) and H-2E (I-E), and finally MHC-IIb (non-classical MHC class II) contains H-2M and H-2O (14) (Table 1). MHC molecules in mice and humans are highly polymorphic and many laboratory strains of mice have a specific haplotype readily available (13).

Table 1 Mouse and Human MHC

MHC Class I	**MHC class II**	**MHC Class I**	**MHC class II**
Human HLA	Human HLA	Mouse H2	Mouse H2
Classical HLA- A, B, C	Classical HLA- DP, DQ, DR	H2Ia (Classical) H-2D, H-2K, H-2L	H2IIa (Classical) H-2A(I-A), H-2E(I-E)
Non-Classical HLA- E,F,G	Non-classical HLA- DO, DM	H2Ib (non-classical) H-2Q, H-2M and H-2T	H2IIb (non-classical) contains H-2M, H-2O.

The primary function of MHC molecules is to present peptide antigens to T cells following processing of the native protein. The process of antigen processing varies depending on whether it is to be loaded on MHC class I or class II molecules. MHC class I is expressed by all nucleated cells and primarily presents intracellular antigens to $CD8^+$ T cells. Some DCs can also present exogenously-derived antigen through the process of cross-presentation (15). MHC class II is constitutively expressed on professional APCs and additionally can be upregulated in response to Interferon (IFN)-γ (16, 17). MHC class II typically presents exogenously-acquired peptides to $CD4^+$T cells.

1.1.4. T cell development in the thymus

T cells originate in the bone marrow (BM), however their commitment to the T cell lineage occurs in the thymus. The process of variable, diversity and joining (VDJ) recombination is a process that involves the random splicing of different VDJ genes in order to generate a vast repertoire of TCR capable of recognising a panoply of antigens. The T cells then undergo an ordered selection process which favours the selection of potential useful TCRs, whilst avoiding the selection of potentially self-reactive TCRs. This enables the adaptive immune system to eradicate a vast array of pathogens whilst preventing the unwanted activation by self-reactive T cells and ensuing autoimmune disease.

Common lymphoid progenitor cells enter the thymus at the corticomedullary junction. Such immature thymocytes then migrate to the outer cortex where they undergo a series of well-established differentiation processes. At this point the thymocytes are committed to the T cell lineage but lack TCR and CD4 and CD8 expression and are referred to double negative (DN) (18-20). The DN thymocytes progress through 4 stages of differentiation from DN1 to DN4 (21).

β-chain rearrangement occurs between the DN2 and DN4 stages. The variable (V), diversity (D) and joining (J) gene segments are recombined to make a functional gene in the process of VDJ recombination. Specific sequence motifs called recombination signals sequence (RSS) flank gene segments that can be recombined. The products of the recombinase genes lymphoid specific recombinase (RAG)-1 and lymphoid specific recombinase RAG-2 form the lymphoid specific recombinase which bind to the RSS and bring together the specific gene segments that are to be recombined and cleave the Deoxyribonucleic acid (DNA) (22). The

individual gene segments to which the lymphoid specific recombinase binds are selected at random from a number of copies that are present at each gene locus and thus are responsible for the highly diverse TCR repertoire of conventional T cells.(23)

TCR-β recombination occurs during the DN2 and DN3 stages of thymocyte development (24, 25). First the Dβ-is recombined to Jβ and then Vβ is recombined to DβJ (26). A process called allelic exclusion occurs, which means that only one of two possible alleles is expressed at a time, meaning each individual cell only expresses one TCR-β chain (27). Following rearrangement of the TCR-β chain is expressed with a non-rearranged pre-Tα which is necessary for allelic exclusion (28-30).

TCR-α gene rearrangement occurs differently as approximately 30% of mature αβ T cells express two productive TCRα gene rearrangements (31). TCR-α gene segments recombines the V and J segments in a non-random manner (32, 33). Theoretically, there are 10^{15} possible TCR combinations in humans (34, 35), however this is 1000 fold higher than actual human T cell repertoires. The newly rearranged α-chain replaces the pre α-chain which leads to the cells being expressing αβTCR. Such cells are $CD4^{+}CD8^{+}$ double positive (DP).

DP thymocytes then undergo positive selection, in which cortical epithelial cells present self-peptide in the context of MHC class I and MHC class II molecules. The outcome of this interaction is dependent on the strength of the signal, too weak a signal leads to cell “death by neglect”, while too strong a signal leads to death via apoptosis (negative selection) (36, 37). This mechanism which is critical to central tolerance is a means by which strongly self-reactive T cells do not enter the periphery.

Thymocytes that recognise self-peptide present on MHC class I become committed $CD8^+$ T cells and thymocytes that recognise self-peptide presented on MHC class II become committed $CD4^+$ T cells (38). During negative selection, autoimmune regulator (AIRE) expressing medullary thymic epithelial cells (mTECs) and thymic DCs present tissue specific antigens which helps to negatively select T cells that would be autoreactive in the periphery (39-41). The medulla is also where factor forkhead box P3 (FOXP3) Treg are produced in response to antigens presented on mTEC (42). Thymocytes further mature for several days in the medulla before thymic egress occurs and the cells migrate to the periphery as fully functional mature thymocytes (43).

T cells undergo this lengthy process of T cell development in the thymus in which they are positively and negatively selected. However, notwithstanding this, mature T cells exhibit a high frequency of cross-reactivity or alloreactivity to foreign peptide-MHC complexes despite the fact that they have not previously encountered the MHC variant of antigen. This is a result of the inherent ability of T cells to recognise peptide MHC complexes (44, 45).

1.1.5. The T cell receptor (TCR) and T cell activation

T Cells are activated in the periphery to become functional mature T cells which can elicit damage during GVHD. This process is initiated by the presentation of antigen by MHC class I or MHC class II molecules on APCs. In order to be successfully activated T cells require three signals. The first signal is provided by the peptide in the context of an MHC molecule (46, 47). CD4 or CD8 molecules then stabilise the

complex (48). The second signal is provided by co-stimulatory molecules which are upregulated on APCs in response to infection or tissue injury (49-51). The best characterised interaction occurs between CD28 on the T cell and CD80 and CD86 on the APC (49-52).The third signal is provided by cytokines, the nature of which leads to the differentiation of Th cells into particular subsets, for example IL-12 promotes differentiation into Th1 cells (53). These external signals lead to the recruitment of intracellular effector molecules and adaptor proteins which lead to the activation, differentiation and proliferation of effector T cells.

1.1.6. TCR intracellular signalling

The term immunological synapse is used to refer to interface between the APC and T cells (54). In the case of GVHD the APC presents alloantigen. The interactions which occur at the immunological synapse lead to intracellular signalling leading to the activation and proliferation of T cells. The TCR receptor is composed of either an α and β chain or a γ and a δ chain (55, 56). The 2 TCR chains are bound together by disulphide bond subunits (55). The αβ TCR complex also includes a CD4 or CD8 co-receptor with the tyrosine kinase Lck (57, 58), as well as the CD3 complex which consists of two ε chains, a γ chain, δ chain and two ζ chains (59, 60). Each of the CD3 chains has at least one copy of the immunoreceptor tyrosine-base motif (ITAM) in the cytoplasmic domain (61-65). CD45 which contains a tyrosine phosphatase enzyme in its cytoplasmic domain is also involved (66).

The signalling is initiated by triggering of the TCR, leading to the activation of intracellular src family kinase enzymes such as fyn. Upon activation fyn kinase phosphorylates the ITAMS of the CD3 subunits, which can then act as binding sites for a second kinase PTK zeta chain-associated protein (ZAP-70) (67). ZAP-70 binds the phosphorylated zeta chain (67). In addition the co-receptor can bind to the same peptide MHC complex (58). This results in lck coming into close proximity with ZAP-70, which leads to lck phosphorylating and hence activating Zap70 (68). Upon activation ZAP-70 can bind to and activate a number of intracellular adaptor proteins. which in turn can activate other signalling pathways within the cell (69). ZAP-70 phosphorylates linker for activation of T cells (LAT) and SRC-homology 2 (SH2)-domain containing leukocyte protein 75kD (SLP-76) (69, 70). This leads to the activation of phospholipase C-γ (PLC-γ) and Ras (71-74).

PLC-γ cleaves phosphatidylinositol bisphophate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG), which act as second messengers required for intracellular $Ca2^+$ release and protein kinase c (PKC) activation (75). These second messengers lead to the activation of transcription factors Nuclear factor of activated T cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Activator protein-1 (AP-1), which translocate to the nucleus and promote the expression of genes required for proliferation, IL-2 production and T cell survival and differentiation (76, 77).

1.1.7. T cell subsets

T cells can be divided into two broad subsets based on the expression of CD4 and CD8 co-receptors (78). $CD4^+$ T cells can be further divided according to transcription factor and cytokine expression. Each subset of Th cells arises when naïve T cells encounter antigen in a certain local microenvironment in terms of the cytokine milieu and the availability of co-stimulatory molecules (2). This endows Th cells with the capacity to mount a response appropriate to the perceived threat. Th cells mainly elicit their effector functions through cytokine production.

Mossman and Coffman were the first to describe Th1 and Th2 cells according to cytokine production (79). Th1 cells primarily produce IFN-γ and express the cytokine T-box transcription factor (T-bet), while Th2 cells express GATA Binding Protein 3 (GATA3) and produce the signature cytokine IL-4 (80-82). Later cells that express RAR-related orphan receptor gamma thymus (RORγT) which predominantly produce IL-17 were discovered, which were subsequently termed Th17 cells (83, 84). This has culminated as a model in which proinflammatory Th cells are divided into the subsets based on cytokines and transcription factor expression. There is growing evidence for plasticity between the subsets, for example Th17 cells can develop the ability to produce IFN-γ (85, 86).

$CD8^+$ T cells are also known as cytotoxic T cells and they play an important role in killing virally infected cells and cancer cells (6). Upon recognition of cognate peptide presented by MHC class I, $CD8^+$ T cells undergo clonal expansion to increase the frequency of T cells specific for the target antigen (87). Activated $CD8^+$ T cells typically lyse target cells by inducing apoptosis by perforin and granzyme or through

FAS-FAS Ligand (FAS-L) interactions (88). Both $CD4^+$ and $CD8^+$ T cells contribute to the effector mechanisms that occur in GVHD**.**

1.1.8. Immune regulation and tolerance induction

As discussed previously, central tolerance takes place in the thymus and leads to the deletion of self-reactive T cells. However, this process is incomplete and therefore additional peripheral mechanisms are required to suppress autoreactive T cells in order to prevent autoimmune diseases. These include but are not limited to induction of anergy, autocrine feedback loops and suppression with regulatory cell populations such as Treg.

T cell anergy occurs *in vitro* upon the activation of T cells with their cognate antigen-MHC complex in the absence of co-stimulation (89). This leads to a state of active unresponsiveness where T cells do not respond to subsequent exposure to antigen even in the presence of co-stimulation (90-93). Anergic T cells have been shown to have an altered transcriptional programme with enhanced expression of genes such as Cbl-b and Gene related to anergy in lymphocytes (GRAIL) that target various signalling molecules used by TCR and co-stimulatory molecules for degradation by the proteasome via targeted ubiquitination (94, 95). There is also an immunological state commonly referred to as immunological ignorance in which self-reactive lymphocytes coexist with self-antigen without reacting. This is as a result of the antigen in question either being present at too low a concentration to induce a response or binding too weakly to induce a response or indeed being inaccessible to lymphocytes (4).

Inducing apoptosis in autoreactive cells has also been shown to be a mechanism of suppression employed against autoreactive and alloreactive T cells and this has been shown to occur in Experimental autoimmune encephalomyelitis (EAE; a mouse model for multiple sclerosis (MS)), a mouse model of oral tolerance and in transgenic mice capable of recognising the influenza virus hemagglutinin (HA) (96-98). In these cases apoptosis occurs as a result of repeat high dose stimulation with antigen, for example, repeat administration of myelin basic protein in EAE (96). Apoptosis as a result of a potent antigenic signal is thought to be a homeostatic mechanism that prevents the immune response causing collateral damage due to excessive inflammation caused by the unrestricted expansion of a particular clonotype or chronic antigen stimulation (99). Apoptosis can also be induced in the absence of co-stimulation, as co-stimulation leads to the production of anti-apoptotic genes such as B-cell lymphoma-extra large (BCL-XL) and enhances the production of growth factors such as IL-2, and their receptors (100).

Autoreactive T cells in the periphery can also be prevented from causing autoimmune disease by a process termed immune deviation. For example in the case of Anterior Chamber Associated Immune Deviation (ACAID) inhibition of activation and differentiation of T cells into Th1 cells occurs (101). Transforming growth factor beta-1 (TGF-β) produced by peritoneal exudate cells and acting on APCs, causes T cells to secrete IL-4 and not IFN-γ (101).

Treg play a major immunosuppressive function in the periphery. Treg were first discovered in 1972, when they were termed suppressor cells (102). Much controversy surrounded the existence of such cells (103) and it wasn't until 1995 that they were rediscovered by Sakaguchi *et al.* (104). Sakaguchi showed that $CD4^+$ T

cells which expressed the IL-2 receptor (IL-2r) α chain (CD25) had the capacity to prevent T cell mediated autoimmune diseases when adoptively transferred from naïve adult mice (104, 105). Treg function was later shown to be under the control of lineage specific transcription FOXP3 (106, 107). FOXP3 has been shown to be necessary for the conversion of naïve Th cells into Treg, and mutations in the *foxp3* gene results in defective thymic generation of Treg and importantly rampant autoimmune disease (106, 107). Scurfy mice lack functional $CD4^+FOXP3^+$ Treg and develop a lymphoproliferative disease which affects multiple organs especially the skin, lung and liver (108, 109). In addition, the human disease immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome occurs as a result of mutations in the FOXP3 gene and results in Treg dysfunction and severe autoimmunity (110, 111)

Treg are usually divided into two broad categories, those which are differentiate in the thymus derived (natural Treg (nTreg) or thymus-derived (tTreg)) and those that differentiate from naïve T cells in the periphery (inducible Treg (iTreg) or peripheral-derived (pTreg) (112, 113). nTreg develop in the thymus and required recognition of self-antigens on mTEC (114). On the other hand, iTreg are generated in the periphery following antigen-recognition in the presence of TGF-β (115-117). In addition to $FOXP3^+$ Treg it is worth noting that a number of other T cells with regulatory activity have been identified such as type 1 regulatory T cells (Tr1) cells which make IL-10 and Th3 cells that make TGF-β (118). Furthermore, $CD8^+$ regulatory T cells that produce IL-10 and TGF-β have also been identified (119, 120).

In addition to regulatory populations of T cells, innate cells can also be immunosuppressive. One such population is myeloid derived suppressor cells

(MDSCs). MDSCs were originally described as $CD11b^{+}Gr1^{+}$ in mice (121, 122). However the term now covers a heterogeneous population of cells, involving both monocytic and granulocytic cells, which have been extensively characterised in contributing to the suppressive microenvironment of tumours (123). While they can produce immunosuppressive cytokines such as IL-10 and TGF-β, in a pathological context they can also induce nitric oxide (NO) and reactive oxygen species (ROS) through inducible nitric oxide synthase (iNOS) and arginase-1 (ARG 1) respectively (118). Such molecules can induce apoptosis in alloreactive T cells (124). In addition they can mediate suppression indirectly by recruiting Treg as has been shown in models of heart transplant and GVHD (125, 126).

The Holy Grail in transplant research is to achieve operational tolerance. Operational tolerance is a state in which the immune system is unresponsive to the transplant while simultaneously maintaining the ability to mount an immune response against infection and cancer. It is important to understand the various forms of immune mediated suppression in order to design better therapies to combat GVHD in Haematopoietic stem cell (HSC) transplant.

1.2. Pathways of allorecognition

Mismatched MHC molecules are a significant risk factor for the occurrence of GVHD and transplant related mortality following BM or HSC transplantation (127). There are two ways in which T cells within BM grafts can recognise alloantigen in GVHD. The first is the direct antigen recognition in which donor T cells directly recognised recipient MHC-peptide complexes on recipient APCs (128, 129) (Figure 1.1). The second is indirect recognition of alloantigen whereby T cells recognise recipient derived peptides from MHC molecules that have been phagocytosed and processed and presented in the MHC of donor APCs (128, 129) (Figure 1.1). A third pathway, namely the semi-direct pathway has more recently been described in solid organ transplantation whereby donor cells may sequester intact recipient MHC+peptide complexes via either exosome uptake or trogocytosis (130, 131) (Figure 1.1).

The TCR has a propensity to bind MHC molecules (44, 45). While T cells undergo maturation in the thymus and hence are selected on their ability to form low avidity interactions with MHC+peptide complexes, a proportion of T cells exhibit cross-reactivity to other MHC alleles (132). In addition, memory T cells primed against a particular peptide presented by the MHC on which they were selected have also been shown to be able to respond to allo-MHC-peptides complexes presenting a different peptide (133, 134). Humans and mice usually have a high precursor frequency of alloreactive T cells of between 1 and 10% (135-138).

There are two models of peptide-MHC complex recognition to explain the cross-reactivity. The first is peptide-dominant binding in which the peptide presented in the allo-MHC is tightly bound in the TCR even if the interaction between the TCR and the MHC molecule is a weak interaction (139). The second is MHC dominant binding

when the MHC molecule provides a stronger interaction with the TCR and the peptide presented by the allo-MHC is a less important factor (139). Molecular mimicry has been suggested as the reason that T cells can recognise foreign MHC at all, although crystal structures have revealed that TCRs recognise self and allo-MHC by divergent mechanisms and thus cross reactivity can occur in the absence of molecular mimicry (140). At the heart of transplant rejection is the concept that it is mediated by T cells that recognise allogeneic MHC molecules.

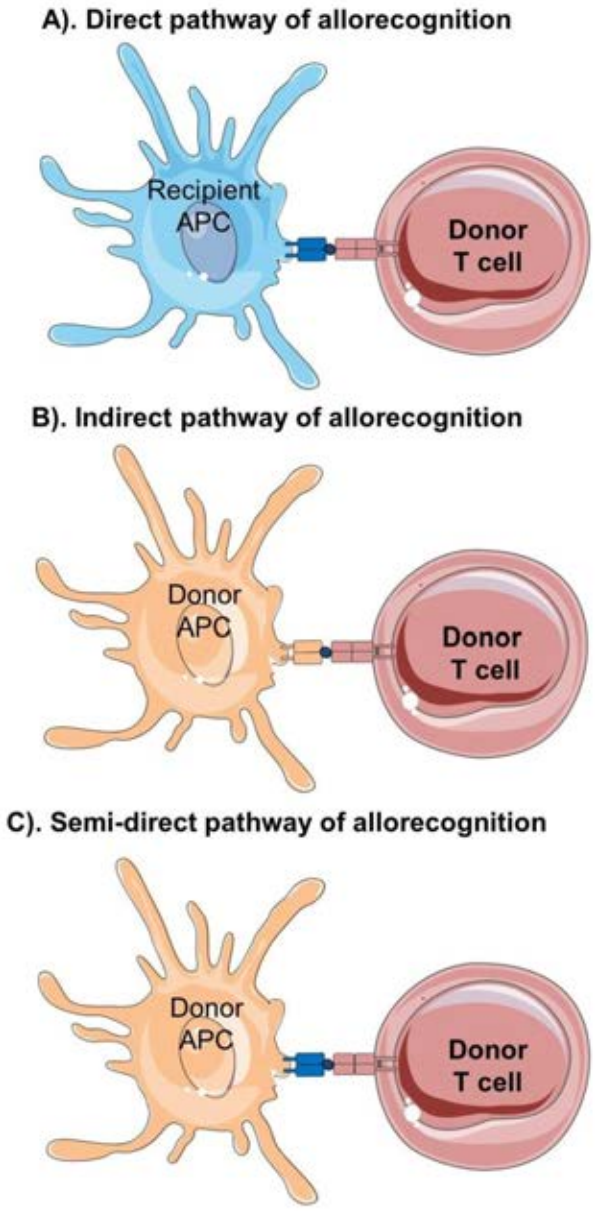

Figure 1.1 Pathways of MHC recognition following HSC transplantation There are three methods of recognition of alloantigen by alloreative T cells. A). Peptide can be recognised by direct methods in which donor T cells directly recognised MHC-peptide complexes on recipient APCs cells. B). Indirect recognition occurs when donor T cells recognised recipient derived peptides from MHC molecules that have been phagocytosed and processed and presented in the MHC of donor APCs. C). In semi-direct recognition donor cells obtain recipient MHC molecules via either exosome uptake or trogocytosis which are recognised by donor T cells.

1.3. Bone Marrow (BM) and Haematopoietic stem cell (HSC) transplantation

1.3.1. Bone marrow transplantation (BMT) as a curative treatment for leukaemia, lymphoma and myeloma

As outlined previously the immune system is essential to recognise and fight disease whilst maintaining homeostasis. Therefore it is not surprising that medical procedures that involve perturbing immune homeostasis have a profound effect and are strongly influenced by the immune system, as is the case with HSC transplantation. HSC transplantation is a curative treatment for leukaemia, lymphoma and myeloma. The first HSC transplant was performed in 1959 (141). Prior to HSC transplantation, the patient receives myeloablative conditioning generally in the form of lethal irradiation and chemotherapy. High dose irradiation leads to increased clearance of tumour cells, and additionally results in the loss of a patient's haematopoietic cells, and therefore the patient requires a HSC transplant to reconstitute the hematopoietic system.

HSCs are administered via intravenous (i.v.) infusion and are often from a genetically distinct donor. HSC can be derived from BM, peripheral blood or umbilical cord blood (142-144). As well as reconstituting the patient with a healthy haematopoietic system, the use of an allogeneic transplant has the added benefit of mediating anti-tumour effects (referred to as the graft versus leukaemia effect (GVL) or graft versus tumour (GVT) effect). These anti-tumour effects are mediated by contaminant donor T cells present within the HSC preparation, and are associated with decreased disease relapse (145-148). However, the same cells responsible for the GVT effect are also responsible for the induction of GVHD.

1.3.2. The immunobiology of Graft versus host disease (GVHD)

Acute GVHD is defined as GVHD which occurs in the first 100 days after transplantation, as opposed to chronic GVHD which is observed greater than 100 days post-transplant. It is a major cause of mortality and morbidity amongst the 30,000 recipients transplanted annually, worldwide.

1.3.2.1. Initiation

The classical description of GVHD begins with the activation of PRRs and the release of chemokines, microbial products and other PAMPs (Figure 1.2). This leads to the activation of innate immune cells, including APCs, upregulation of MHC molecules and production of proinflammatory cytokines such as IL-1, IL-6, and Tumour necrosis factor (TNF)-α (149, 150). Total body irradiation (TBI) also induces endothelial apoptosis in the gastrointestinal tract (GI) tract, which leads to epithelial cell damage, this also leads to the release of PAMPs such as lipopolysaccharide (LPS), which are usually sequestered away, to be released into circulation which further perpetuates the inflammatory response (151, 152). HSC transplants which contain monocytes and macrophages which are resistant to LPS but are otherwise normal have been shown to produce significantly less TNF-α, accompanied by reduced GVHD in the GI (153). Disruption of the intestinal epithelia can also lead to commensal bacteria translocating into deeper tissue where they can cause infection and septicaemia (154). Furthermore, the intensity of the conditioning regime positively correlates with the severity of GVHD further emphasising the impact of radiation induced damage (155, 156).

1.3.2.2. T cell activation

Activated recipient APCs present self-antigen to donor T cells which leads to activation of alloreactive donor T cells and subsequent release of additional proinflammatory cytokines, which contribute to the cytokine storm and further proliferation of the T cells (157) (Figure 1.2). Although direct presentation is critical in the case of a fully MHC mismatched transplant, mHC mismatched antigens can also occur where they are presented in the context of recipient MHC through the indirect pathway (157-159). In one model of GVHD, intestinal myofibroblasts have been shown to be responsible for the initiation of GVHD although it is generally accepted that DCs are the most potent APCs capable of initiating GVHD responses (160, 161). The inflammatory environment with elevated inflammatory cytokines, PAMPs, DAMPs and gut commensals can all activate DCs, causing them to exhibit a more mature phenotype capable of activating T cells.

The second signal of T cell activation, namely co-stimulation is also required to activate alloreactive T cells. Indeed co-stimulatory molecule blockade has proven to be an effective strategy to reduce GVHD in some models (162). In mouse models of GVHD the nature of the T cell response is dependent on the alloantigen presented. Alloantigen presented by MHC class I cells leads to a CD8 driven model, while alloantigen presented by MHC class II leads to a CD4 driven model (163).

In terms of the polarity of the Th response, Th1 cytokines are predominantly produced and are implicated in the pathology of GVHD. IFN-γ is a crucial cytokine in the initiation of GVHD and it is markedly elevated in mice with GVHD (154). IFN-γ can lead to the upregulation of MHC molecules, which leads to pathology in the skin and GI and a reduction in the amount of LPS which is required to induce NO

production by macrophages (164-167). It should be noted that Th1 cells are not the only cells with the capacity to produce IFN-γ. While Th1 have classically been shown to induce GVHD, Th17 cells also play a role. Until relatively recently, the role of Th17 cells was disputed. However a study by Yu *et al.* using T-bet$^{-/-}$ and RORγT$^{-/-}$ mice showed that while RORγT$^{-/-}$ mice had little impact on the systemic occurrence of GVHD, double knock-out (KO) mice produced less severe GVHD than T-bet$^{-/-}$ mice (168). The one drawback to this study is that the KO mice were not cell specific, and as such the gene was knocked out in all cells not just Th1 or Th17 cells respectively.

Chemokines and their cognate receptors play an important role in recruiting pathogenic T cells to the target tissues. A plethora of chemokines are known to be overexpressed in the target organs. Macrophage inflammatory protein (MIP)-1α and MIP-2 and C-X-C motif ligand (CXCL)-9 also known as Monokine induced gamma interferon (MIG) are overexpressed in the liver and spleen, while in the skin MIP-1α, MIP-2, Methyl-accepting chemotaxis protein 1 (MCP)-1 and MCP-3 are over expressed (169). Furthermore, there is an association between onset of liver GVHD and the recruitment of chemokine receptor (CCR)5^{+} T cells which are usually Th1 cells (170). In terms of the recruitment of CD4^{+} T cells and CD8^{+} T cells to the lungs, liver and spleen, MIP-1α is important for increasing the recruitment of CD8^{+} T cells, but not CD4^{+} T cells (169, 171).

1.3.2.3. Effector Phase

The activation of alloreactive T cells leads to further activation of innate cells which amplifies the cycle of inflammation. The end-stage result is organ damage with the skin, liver, lungs and gut being predominately targeted. The cellular effectors of GVHD are alloreactive T cells and Natural Killer (NK) cells (172). NK cells predominantly mediated their effect through the Fas-FasL and perforin and granzyme pathways but TNF-α and IFN-γ mediated cytotoxicity also plays a role (173). Perforin and granzyme mediated cytotoxicity is important in $CD8^+$ T cell driven GVHD, while in $CD4^+$ T cell mediated GVHD it appears to be less important than Fas/FasL mediated killing (173, 174). Furthermore, Fas-FasL mediated killing is thought to be more important in liver and skin damage (175).

The effector cytokines IL-1 and TNF-α are also thought to play a major role in the pathophysiology of GVHD. TNF-α has a pathogenic role in both humans and mice (176, 177). In addition to activating DCs and recruiting inflammatory cells TNF-α is an important effector molecule in skin and lymphoid tissues (176, 178). Furthermore lung damage could be prevented by administration of anti-TNF-α antibodies (179). IL-1 accumulates in the spleen and the skin during the effector phase (180). However, although anti-IL-1R blockade reduces mortality in animal models, unfortunately this has not translated to clinical trials (181-183).

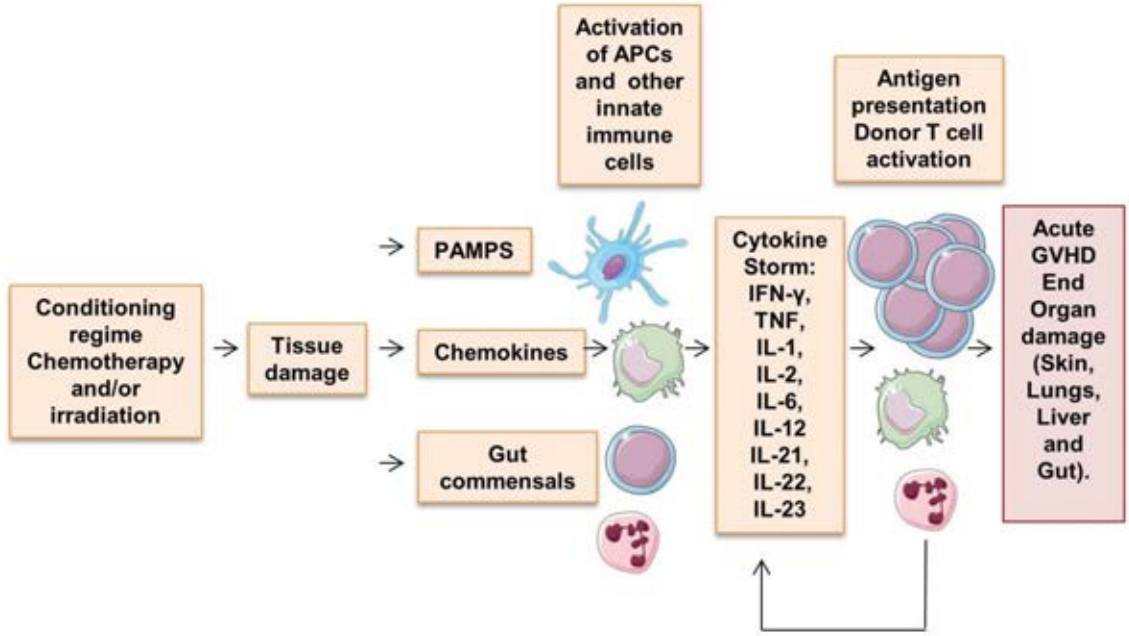

Figure 1.2 Initiation and maintenance of GVHD The process of GVHD is initiated by the conditioning regime, which usually consists of chemotherapy and irradiation. This causes tissue damage, which leads to the release of PAMPs, chemokines and gut commensals. This in turn leads to the activation of PRRs and hence the activation of innate immune cells including APCs. This results in the production of proinflammatory cytokines. The activated APCs can then go on to present host antigen to donor T cells. This results in alloactivation of the donor T cells. The donor T cells produce further cytokines and perpetuate the process. The end stage result is tissue damage to the skin, lungs, liver and gut. Adapted from Blazer *et. al.*, 2012, ***Nature Reviews Immunology* 12, 443-458.**

1.3.3. GVHD manifestations and clinical occurrence

Clinically, acute GVHD is classified from grade 0-IV according to number and severity of organ involvement (184). The first symptom to appear is generally skin GVHD, which is characterised by a maculopapular rash and often occurs contemporaneously with the engraftment of donor cells. The GI pathology occurs later and includes diarrhoea, nausea, vomiting, cramping and failure to thrive (185). Additionally, liver GVHD manifests as hyperbilirubinemia, which presents in the form of jaundice (186). Clinical GVHD is graded according the degree of severity from Grade I (mild) GVHD to Grade IV (very severe) GVHD according to the modified Seattle Glucksberg

criteria (187-190). The occurrence of GVHD is directly related to the degree of MHC mismatch (191). Disease prognosis is related to the severity of GVHD with 5-year survival of 25% for grade III and 5% for grade IV disease (192, 193).

1.3.4. Utilising the mechanisms of tolerance to self-antigens to prevent GVHD

Peripheral mechanisms of immune regulation and tolerance induction can be utilized to suppress GVHD. For example inducing apoptosis has been shown to be an effective way to suppress GVHD. Targeting heat shock protein 90 using Small interfering Ribonucleic acid (siRNA), and hence causing apoptosis of activated T cells has also been shown to be effective in preventing GVHD (194). Another effective way to prevent GVHD is by blocking co-stimulation, indeed it has been shown in a primate model of GVHD that blocking CD28 can control T cell activation, thus attenuating GVHD (195).

Treg have the ability to profoundly affect GVHD, indeed the balance of $CD4^+CD25^+$ Treg and inflammatory Th cells can determine the outcome of GVHD (196). Their ability to suppress GVHD has been shown to be partly dependent on IL-10 and partially on CD28 (196, 197). Adoptively transferred Treg have been shown to suppress GVHD (196, 198-203). The concern is that Treg may inhibit the anti-tumour effect of conventional T cells (Tcon). In some models of GVHD, Treg have been shown to suppress GVHD while maintaining the anti-tumour response (200-202). However, in non-GVHD mice following vaccination to a rare tumour antigen Treg have been shown to suppress the GVT effect (204, 205). The reason for this disparity has been postulated to relate to the relatively high precursor frequency of allospecific

T cells in many models of GVHD. In this context the expansion of cytotoxic T lymphocytes (CTLs) is less important for the anti-tumour effect. While in the tumour vaccination setting, the CTL precursor frequency is low and therefore expansion is important for CTLs to mediate their anti-tumour effect. Effectively this means that the maintenance of the anti-tumour effect in some models of GVHD is due to the suppressive effect of Treg inhibits proliferation, but not activation.

The encouraging results observed in mouse models have led to several clinical trials investigating the ability of Treg to suppress GVHD as a potential new therapy. The results of a phase 1 clinical trial have shown that early infusion of freshly isolated Treg followed by Tcon, prevented GVHD and improved immunity to infection and maintained the anti-tumour effect (206). Although this is only a phase 1 trial the results are promising. Furthermore, a clinical trial that is currently recruiting for a phase 1 trial plans to look at the potential of *ex vivo* expanded allo specific Treg cells to prevent GVHD (207).

1.3.5. Current Methods of GVHD prevention and treatment and mechanisms of action

According to the National Health Service (NHS) protocol, many patients who are at a high risk of developing GVHD are treated prophylactically with immunosuppressive drugs (187). The first line treatment upon development of GVHD usually includes systemic corticosteroids or calcineurin inhibitors (187). However, it has been reported that steroid refractory GVHD occurs in 50% of patients (208). Patients that develop steroid refractory GVHD have a mortality rate of as high as 95% (209). Second line therapies are dependent on the clinical presentation of GVHD and is highly

individualised based on the immune response of the patients (187). Table 2 shows some of the therapeutic strategies employed to treat GVHD and their mechanisms of action as further illustrated in Figure 1.3. The problem with many of these therapies is that as well as suppressing GVHD they also dampen down the anti-tumour response, which is required for progression free survival. Such therapies also increase susceptibility to infection (210).

Table 2 Current treatments for GVHD

Drug	**Main mechanism of action**	**References**
Steroids	Broad non-specific anti-inflammatory	(211)
Ciclosporin (aka Cyclosporine)	Inhibits calcineurin pathway	(212)
Tacrolimus	FKB506 binding protein (FKBP) thus inhibiting calcineurin	(213)
Light treatment (ECP)	During Extracorporeal photopheresis apoptosis is induced in lymphocytes which causes processing of these cells and the subsequent immune response is likely responsible for the protective effect	(214)
Monoclonal antibodies: a). Alemtuzumab (Campath) b). Infliximab (Remicade) c). Rituximab (Mabthera)	a). Anti-CD52, binds CD52 which is expressed on mature lymphocytes, targeting them for destruction b).Anti-TNF-α, block proinflammatory cytokineTNF-α c). Anti-CD20. Likely works through leading to the depletion of Programmed cell death protein ligand (PDL) 1^{hi} B cells and T follicular helper cells	(215) (216) (217)
Mammalian target of rapamycin (mTOR) inhibitors: a).Sirolimus (Rapamune) b).Everolimus	a, b). Bind to FKBP12 which inhibits mTOR signalling thus preventing IL-2 mediated signal transduction and hence arrested cell cycle progression	(218) (219)
Tyrosine kinase inhibitors	Inhibits the Janus kinase (JAK)/ signal transducer of activation (STAT) signalling pathway with is essential for lymphocyte function	(220)
Etanercept	TNF receptor II-Fc fusion protein, blocks proinflammatory cytokineTNF-α	(216)
Thaliodomide	Allows antigen specific suppressor cells to develop but inhibits precursor cytotoxic cell development	(221)
Mycophenolate mofetil	Inhibits inhibitor of inosine monophosphate dehydrogenase (IMPDH) an enzyme that is required for guanine synthesis which B and T cells are very dependent on	(222)
Pentostatin	Purine analogue, reduces DNA synthesis	(223)
Methotrexate	Antiproliferative	(224)

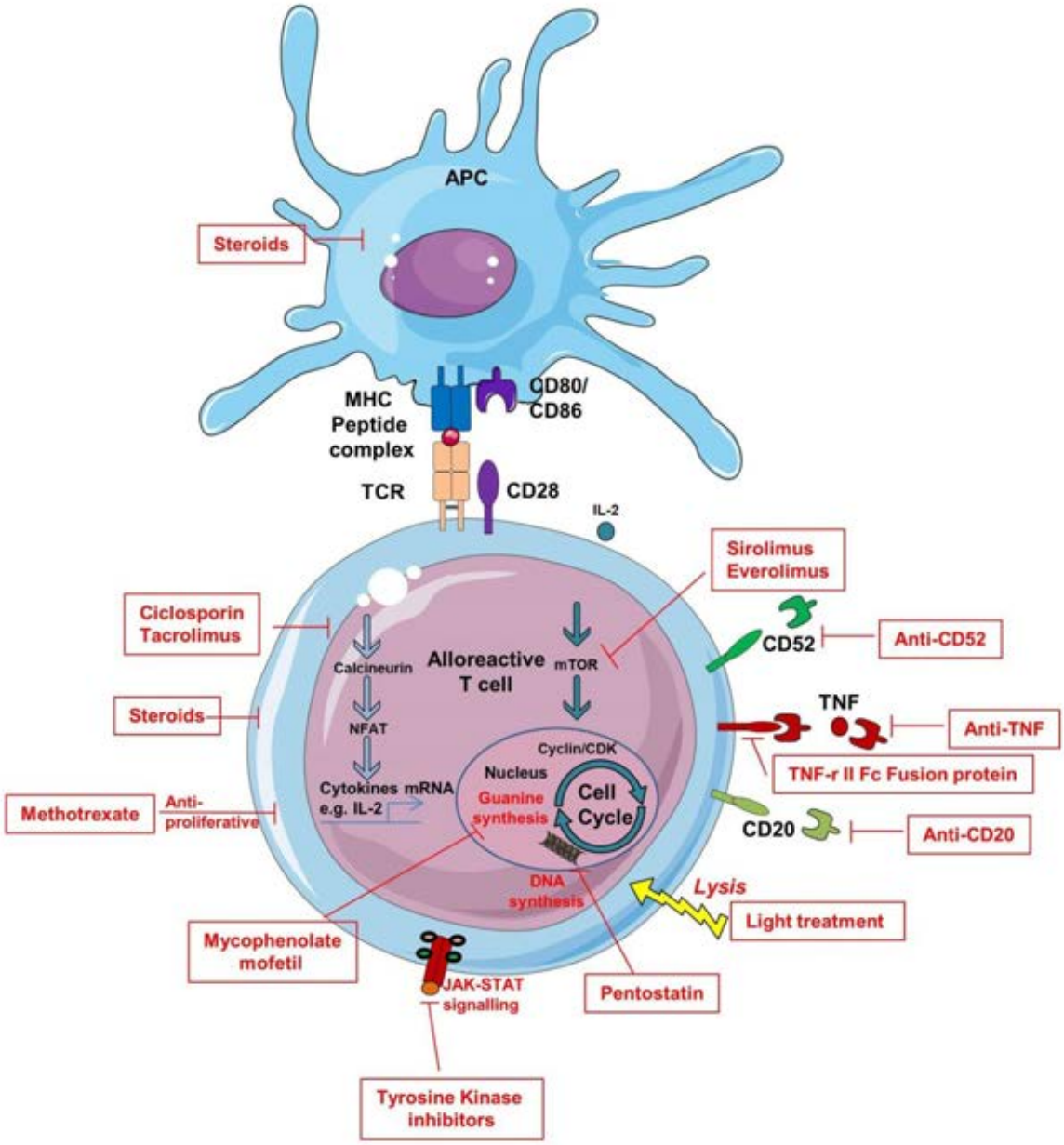

Figure 1.3 Current treatments for GVHD Current methods of treating GVHD are mainly non-specific and risk dampening the anti-tumour response. They can be broken down into several categories namely mTOR inhibitors, monoclonal antibodies, cell cycle inhibitors, tyrosine kinase inhibitors, anti-proliferative agents, broadly acting anti-inflammatory drugs, calcineurin inhibitors and other treatments such as light treatment. The exact mechanisms of action of these drugs are described in Table 2.

1.3.6. Graft versus leukaemia effect

A caveat with the use of current therapies for GVHD is that as well as suppressing GVHD, they also inhibit the anti-tumour effect. Donor derived T lymphocytes are thought to be the main mediators of the GVT effect in both experimental models and in the clinic (145-148). The impact of the GVT effect in the context of haematological malignancies is clearly evident from the improved outcome of patients suffering from leukaemia and lymphomas which are generally incurable by means of standard chemotherapy and radiation alone. These patients show more robust remission when they receive a HSC transplant in addition to chemotherapy and/or irradiation. For example, patients with acute myeloid leukaemia (AML), have a long term prognosis of ~10% with conventional chemotherapy, compared to 30-50% following allogeneic stem cell transplantation (225).

1.3.7. Mouse models of GVHD

There are multiple mouse models of GVHD; some are fully MHC mismatched while others are driven by mHC mismatches. The severity and clinical signs of GVHD can vary depending on a variety of factors including but not limited to the age and sex of the mice, the number and type of T cells used to mediate GVHD (202, 226, 227), variations in environmental pathogens and gut microflora (which can be affected by the animal supplier and local animal facilities) and genetic differences between donor and recipient (228). In addition, GVHD can vary according to the dose of irradiation which influences the extent of tissue damage and therefore alloreactive T cell activation (229). Where there is a full MHC-mismatch, GVHD is induced due to differences in MHC class I and MHC class II (H2) molecules, which can be

recognised either directly (as intact recipient MHC + peptide complexes) or indirectly (following processing and presentation of recipient MHC molecules in the context of donor MHC). This leads to the activation of $CD8^+$ and $CD4^+$ T cells. Mouse models of GVHD will be discussed further in Chapter 3.

The use of such mouse models of GVHD has provided much key information on the mechanisms of GVHD and has enabled the efficacy of potential new therapies for GVHD to be tested. One exciting area of therapeutic potential is the use of immune cells to modulate the unwanted immune responses that lead to GVHD whilst sparing the GVT or GVL response. For example, as previously mentioned Treg were shown to suppress GVHD in a number of mouse models (196, 198-203). This has resulted in Treg being used in clinical trials for the treatment of GVHD (206, 207). Our own interest lies predominantly with another type of immune cell that has been demonstrated to have immunomodulatory properties; iNKT cells (230). It has previously been demonstrated that iNKT cells can act in an immunosuppressive manner following solid organ transplantation (231-233). Indeed, it has now been shown that iNKT cells decrease the risk and severity of GVHD in multiple mouse models (126, 234-238), whilst maintaining the GVT effect (126, 234, 238).

1.4. Natural Killer T cells; at the interface between innate and adaptive immunity

Natural Killer T (NKT) cells, are postulated to bridge innate and adaptive immunity. They are endowed with features of both the innate and adaptive immune system and they can facilitate and sculpt the subsequent adaptive response.

1.4.1. Invariant (i)NKT cells

Over the last 20 years the term NKT cells has evolved considerably and now encompasses any CD1d restricted T cell. NKT cells are further classified into subpopulations, namely type 1 NKT cells which express a semi-invariant TCR, type 2 NKT cells which express a more diverse TCR repertoire and finally NKT-like cells which is a broad term referring to MHC-restricted T cells which possess some or the properties of type 1 and type 2 NKT cells (Table 3). Type 1 or invariant (i) NKT cells are the most well characterised subset of NKT cells and are thought to be the main immunosuppressive subset in transplantation.

The process of TCR gene rearrangement takes place in the thymus, and while conventional T cells can form approximately 10^{15} possible variations of αβTCR, iNKT express a semi-invariant TCR Vα14Jα18 in mice and Vα24Jα18 in humans (239, 240). This is paired with a limited Vβ chain repertoire (Vβ2, Vβ7, Vβ8, Vβ8.2 or Vβ8.3 in mice and Vβ11 in humans) (241-245). iNKT cells are a rare population of cells, which vary in their frequency in different anatomical site. The exceptions are the the adipose tissue where they constitute 10-25% of T cells (or 2-8% of lymphocytes) in mice and the liver where they they make-up up to 50% of intrahepatic lymphocytes in mice (246-254). Despite their relative paucity they have been shown to be able to profoundly influence immunity as they are able to respond to the same glycolipid antigens due to the expression of the semi-invariant TCR.

Table 3 NKT cell subtypes

	Type 1 NKT	**Type 2 NKT**	**NKT-like**
Other names	Classical NKT Invariant NKT (iNKT)	Non-classical NKT Diverse NKT	NK1.1+ T cells CD3+ CD56+ T cells
Restriction	CD1d	CD1d	MHC, other?
α-Gal reactivity	**+**	**-**	**-**
TCR repertoire	Restricted: Vα14 iNKT (mouse) Vα24 iNKT (human)	Diverse	Diverse

1.4.2. Discovery

iNKT cells were first identified in several independent studies, which culminated in the designation of iNKT cells as a separate lineage of T lymphocytes (18, 255-259). Initially a small subset of DN thymocytes which were capable of differentiating into all other subsets were discovered (18). Subsequently it was reported that this population of $CD4^{-}CD8^{-}$ T cells in the thymi of mice favoured the use of Vβ8 (255, 256, 259). Shortly after this, Taniguchi *et al.* cloned an invariant TCR Vα14-Jα18 from keyhole limpet hemocyanin-specific suppressor T cell hybridomas (257, 258). Furthermore, around the same time the first allotypic monoclonal antibody (mAb) to the TCR was

developed, which as serendipity would have it, was specific for the Vβ8 TCR, which is over-represented among iNKT cells.

It was later noted that there was a subset of cells in the spleen and liver which expressed NK and T cell markers and moreover this was shown to be in the case in the Vβ8-biased thymocytes (260). Such cells were later shown to have a lineage relationship with Vβ8 iNKT cells in the liver (261). Finally, in 1994 this was all brought together by the observation of cells which express NK receptors and highly biased TCR expression and rapidly secrete cytokines and they were designated iNKT cells (239, 262). Around the same time human iNKT cells were also discovered (244, 263).

1.4.3. iNKT cell ligands

Following the discovery of iNKT cells the search began for the iNKT cell ligand. Initial studies assessed the selection of such cells in MHC class I and II deficient mice (264-266). These studies suggested that MHC class I or Beta 2 microglobulin (β_2M) was involved in ligand recognition. However, in mice lacking the transporter required for loading peptides on the class-I complexes during antigen processing, NKT cells developed normally (267). This suggested that MHC molecules other than MHC class I that used β_2M were potential restriction elements for these cells. Bendelac *et al.* provided robust functional evidence that CD1d was required to select and activate NKT cells (268). This was later proven when the CD1d$^{-/-}$ mouse was generated (269).

CD1d is a non-polymorphic MHC class-I like molecule which presents glycolipids to NKT cells and is highly conserved between humans and mice (270). However, it was

not yet clear whether CD1d alone was enough to activate NKT cells and this was not formally proven until Koezuka, Taniguchi and colleagues discovered that a synthetic version of α-galactosylceramide (αGal), which had originally been isolated from marine sponge (*Agelas mauritianus*), could activate iNKT cells in a CD1d dependant manner (271). αGal is now regarded as the prototypical agonist for iNKT cells. The discovery that αGal was an exogenous ligand for iNKT cells also brought about the development of the PBS-57 (an αGal analogue) tetramer which had a significant impact on the advancement of the field.

Later, many microbial ligands were discovered which were α-anomeric glycolipid antigens and could be recognised by iNKT cells (272, 273). However, the identification of the endogenous glycolipid responsible for selecting iNKT cells in the thymus proved difficult and controversial. Several weak self-reactive antigens were suggested (274, 275) and to further complicate things the *dogma* was that mammals could not synthesize α-anomeric glycolipid antigens. This lead to the search for a β-anomeric glycolipid and one study suggested that β-glucosylceramide (β-GluCer) was indeed the endogenous antigen (275). However, further research by Kain *et al.* showed that it was in fact contaminating α-anomers in the β-GluCer prep, which were causing the reactivity (276). Using a CD1d-α-GalCer antibody (L363) in functional iNKT experiments they showed that the iNKT cell reactivity was indeed related to CD1d presentation of an α-anomer.

The chemical nature of the glycolipids is also thought to sculpt the subsequent adaptive response through driving iNKT cells to produce different sets of cytokines. For example αGal leads to the production of predominantly Th1 type cytokines such as IFN-γ, whereas the glycolipid 2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-N-

tetracosanoyl-2-amino-1,3,4-nonanetriol (OCH) leads to preferential production of IL-4 and other Th2 type cytokines (271, 277, 278).

1.4.4. Glycolipid and CD1d structure and loading

In terms of structure, glycolipids consist of an oligosaccharide chain and a linked lipid component (Figure 1.4). αGal is glycosphingolipid, which is a glycolipid containing ceramide. A ceramide is composed of an amino alcohol, which contains a sphingosine (a carbon chain containing an amino alcohol) and a fatty acid. In αGal the sphingosine chain contains 18 carbons and the longer fatty acid chain is 26 carbons, while in contrast OCH has a 9 carbon sphingosine chain and a 24 carbon fatty acid chain and C20.2 has an 18 carbon sphingosine chain and a 20 carbon fatty acid chain (271, 277, 278) (Figure 1.4).

CD1d is encoded by the *CD1d genes* located on chromosome 1 in humans and chromosome 3 in mice (279-282). Structurally, they are similar to MHC class I as they consist of two heavy chains, non-covalently bound to a β_2M to require processing, however in order to interact with the TCR αGal requires removal of terminal sugars (283).

Self or exogenous glycolipids that require processing in order to be presented require localisation to the late endosome and lysosomal subcellular compartments (283, 284). The glycolipid enzymes (including α-galactosidase A) and lipases process the lipid or glycolipid antigens and a set of lipid transfer proteins load the processed antigen into CD1d (283, 284). CD1d has large antigen binding groove which is found between the α1 and α2 helices which lie on top of 6 β strands. X-ray crystallography

has shown that the lipid portion of αGal fits tightly into the binding groove creating a stable interaction (285, 286).

It has been suggested that there is a relationship between the length of the lipid chain and the biological activity of the glycolipid. The strength of the signal seems to be determined by its stability is the CD1d complex, which determines its cytokine profile. Glycolipids such as αGal with longer fatty acid chains are most effective at stimulating iNKT cells, suggesting that the long fatty acid chain may be required for the glycolipid to stably bind in the CD1d molecule (271). Furthermore, another study showed that the length and saturation of both alkyl chains both contribute to the stability of binding in the CD1d binding groove, which leads to altered binding affinity (287).

As previously mentioned, activation of iNKT cells by OCH has been shown to lead to a type 2 response. This has been suggested to be due to its shorter carbon chains leading to lower affinity binding than αGal (288). C20.2 also has a shorter fatty acid chain, which leads to a mainly type 2 response, the presence of a double bond at carbon 11 and carbon 14 in the C20.2 fatty acid chain has been shown to increase the potency of the C20.2 (278). It should be noted that while the stability of the glycolipids is considered to be an important factor responsible for the bias in cytokine production, the nature of the APCs presenting the glycolipid may also play a role in an *in vivo* setting, as may the site of lipid loading (289-291).

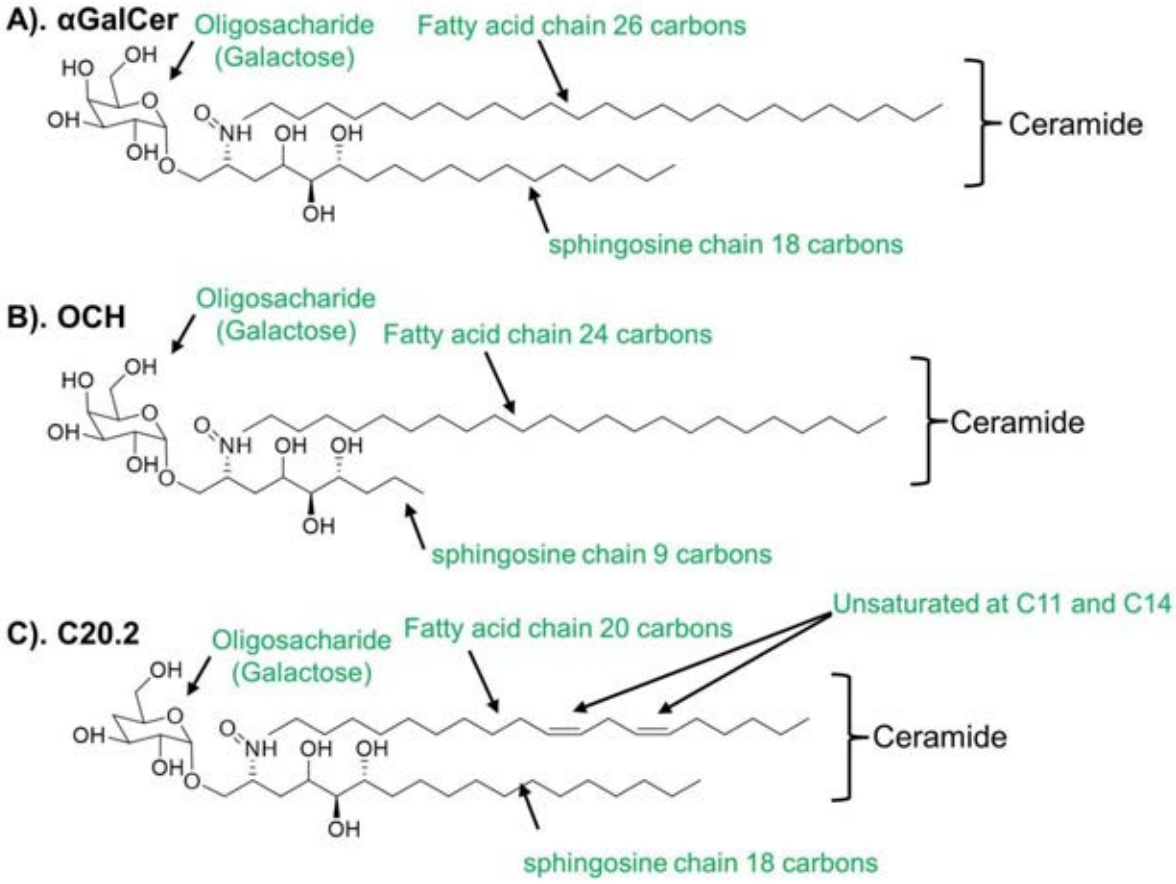

Figure 1.4 Glycolipid structures In terms of structure, glycolipids consist of an oligosaccharide and a linked lipid component A). αGal has a sphingosine chain contains 18 carbons and a longer fatty acid chain containing 26 carbons. B). OCH has a 9 carbon sphingosine chain and a 24 carbon fatty acid chain. C). C20.2 has an 18 carbon sphingosine chain and a 20 carbon fatty acyl chain.

1.4.5. iNKT cell development

Like conventional T cells, iNKT cell development takes place in the thymus. However, limited pairings of Vα genes and Vβ genes occur in the TCR. In mice Vα14-Jα18 is coupled with a limited Vβ repertoire (Vβ8, Vβ7, or Vβ2) (239-242), while in humans iNKT TCR are Vα24-Jα18/Vβ11 (239, 243-245). β-chain rearrangement occurs at the DN3 and DN4 stage, while α-chain rearrangement occurs during the immature single positive (ISP) stage and the DP stage (292, 293).

iNKT cells diverge from conventional αβ T cells at the DP stage of development (292). Positive selection is mediated by $CD4^+CD8^+$ thymocytes which present glycolipid antigens to iNKT cell precursors (268, 294). RAR-related orphan receptor gamma (RORγ) has been shown to be required for the generation of iNKT cells as it promotes the survival of immature iNKT cells through regulating the expression of anti-apoptotic protein BCL-xL and thus enabling successful T cell rearrangement to occur (292, 293, 295). Furthermore, E protein transcription factor (HEB) plays a role in promoting immature iNKT cell survival as it regulates both RORγT and BCL-xL (296).

In addition, the transcription factor c-Myb also plays a role in regulating the development of iNKT cells as it facilitates Vα14 to Jα rearrangement by supporting the long half-life of DP thymocytes (297-299). In the absence of c-Myb expression iNKT cells are not generated (299). Using Nur77 green fluorescent protein (GFP) mice (which is an immediate-early gene upregulated by TCR stimulation), iNKT cells have been shown to require a strong TCR signal during selection (300). However, iNKT cells also require an additional signal from signalling lymphocytic-activation molecule (SLAM)F receptors which are expressed on DP thymocytes (301). Further to its role supporting the half-life of DP thymocytes, c-Myb has been shown to promote the expression of CD1d and SLAM family members and the adapter molecule signalling lymphocytic-activation molecule-associated protein (SAP) (299, 301).

Subsequent to positive selection, precursor iNKT cells are referred to as stage 0 iNKT cells. They are CD1d-glycolipid tetramer positive, $CD44^-$, $CD24^+$, $CD69^+$ (302, 303). Historically, the model for development in C57BL/6 (B6) mice was linear, progressing from stage one to three based on CD44 and NK1.1 expression (302). More recently, the lineage diversification model has been proposed in which CD24 is downregulated and promyelocytic leukaemia zinc finger (PLZF) is upregulated (304). $PLZF^{high}$ cells then relocate from the cortex to the medulla, in a process likely to be mediated by CCR7 (305-308). Based on CD1d tetramer and immunofluorescence staining, the subsets of iNKT cells namely NKT1, NKT2 and NKT17 cells have been found to be predominantly located in the medulla (309).

NKT1 cells express T-bet and low levels of PLZF and produce IFN-γ when stimulated and low amounts of IL-4 (310). NKT2 cells are $PLZF^{high}$ and upon stimulation produce high amounts of IL-4 (310). NKT17 cells express intermediate levels of PLZF, RORγT and when stimulated produce IL-17 (310).

It has recently been reported that the TCR signal strength is responsible for the subtype of iNKT cells that is generated, with strong signalling leading to the promotion of NKT2 and NKT17 development (311). ETS-related gene (Erg) 1 and Erg 2 are transcription factors that are important at stage 0 for the further development of iNKT cells, as they are required for the induction of transcription factor PLZF (312). Erg2 expression can be influenced by the strength of the TCR signal, with decreased signalling effecting Erg-2 expression and hence the upregulation of PLZF (311).

Another transcription factor Kruppel-like factor 2 (KLF) 2 negatively regulates differentiation into NKT2 cells, while KLF13 a member of the same family of transcription factors plays the opposite role (313, 314). The transcription factor Hobit also plays a role in the differentiation of iNKT cells (315). Its highest expression occurs in $CD44^{high}$ $NK1.1^{+}$ iNKT cells which are mainly NKT1 cells, the number of which is significantly reduced in Hobit KO mice. This suggests that Hobit promotes the development or thymic retention of NKT1 cells (308). T Cell Factor 1 (TCF1) and Lymphoid Enhancer Factor 1 (LEF1) also play key roles in development. Deleting TCF1 led to defects in all three subsets of NKT cells, while LEF1 was necessary for survival and proliferation particularly during the NKT0 stage in which NKT cells numbers expand significantly (316, 317).

Cytokines are also important for iNKT effector cell differentiation. IL-15 production has been shown to be required for development in NKT1 cells (318). Similarly, TGB-β has been shown to be necessary for differentiation into NKT17 cells (319). IL-17RB the IL-25 receptor is expressed on both NKT2 and NKT17 cells and has been shown to be required for the production of αGal-induced IL-9, IL-10, IL-13 and IL-17 (320). The cytokines that mature iNKT cells produce on activation are diverse and help delineate the subsets of iNKT cells. It is these subsets that are partially responsible for the diverse roles that iNKT cells play in immunity.

1.4.6. iNKT cell activation

iNKT cells are activated during a plethora of infections and inflammatory conditions (321-325). Under physiological conditions, TCR signals and cytokine signals are required for iNKT cell activation (Figure 1.5). Unlike conventional T cells which recognise processed peptide antigens presented by MHC molecules, iNKT cells recognise lipid antigens and antigen processing is not an absolute requirement. Two models of iNKT cells activation have been proposed; the first involves a dominant TCR signal provided by a high affinity lipid binding to the TCR and to a lesser extent APC-derived cytokine, the second comprises dominant cytokine mediated signals along with a weak TCR-CD1d-lipid interaction (Figure 1.5).

There is also evidence that iNKT cells can be activated independently of TCR signalling in some models of infection (321, 326). Furthermore, solely innate activation is also thought to be possible through natural killer receptors (NKRs). Crosslinking NKR NK1.1 with an antibody can directly activate iNKT cells and NKR NKG2D ligation can also activate iNKT cells in the absence of lipid antigens (327, 328). In contrast, inhibitory NKRs may negatively regulate activation (329).

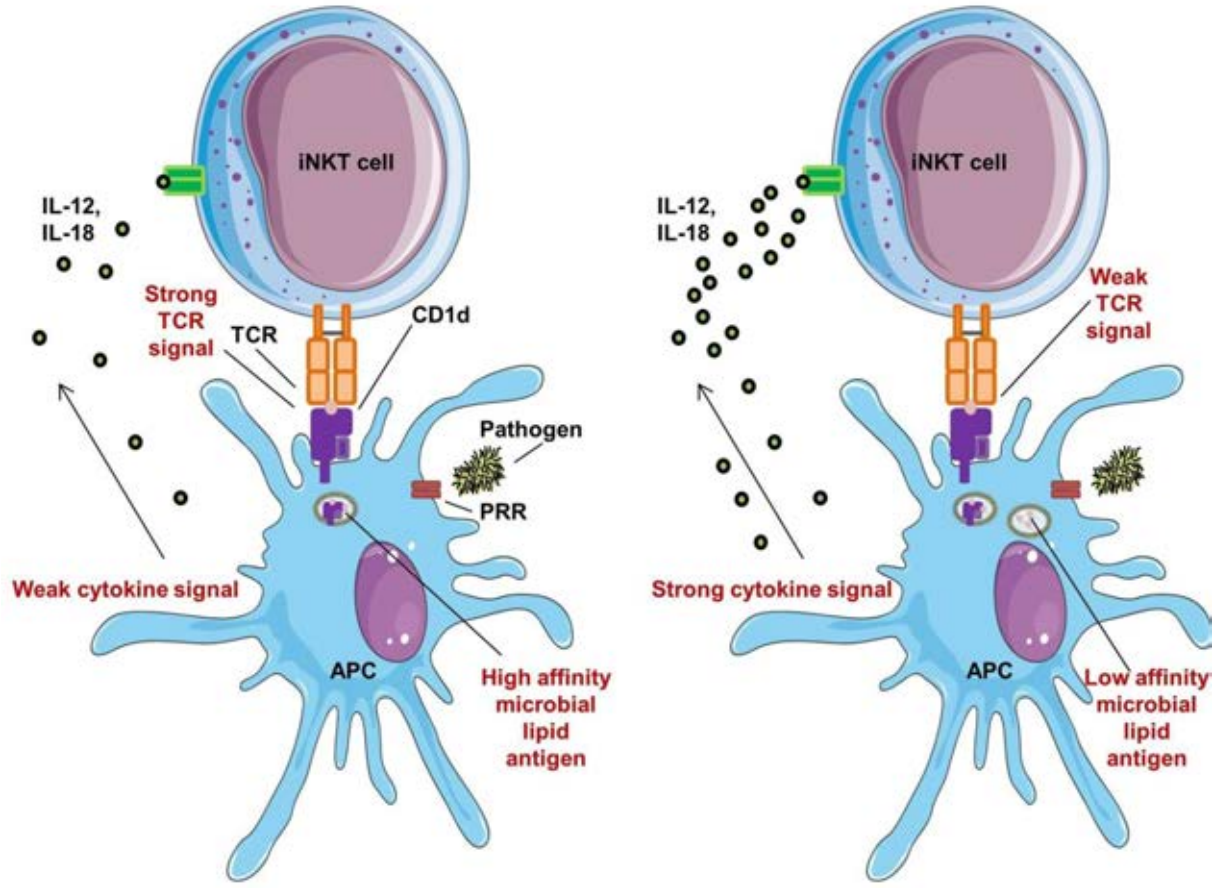

Figure 1.5 Models of iNKT cells activation Two models of iNKT cell activation have been proposed. The first involves a dominant T cell receptor signal provided by a high affinity lipid binding to the TCR and to a lesser extent APC-derived cytokine. The second comprises a predominantly cytokine mediated signals along with a weak TCR-lipid interaction.. Adapted from Brennan *et al*., 2013, ***Nature Reviews Immunology*** **13, 101-117.**

1.4.7. iNKT cell subtypes

Rapid production of cytokines upon activation is a hallmark of iNKT cells. In B6 mice the majority of iNKT cells in the liver and spleen are described as Th1-like (or NKT1) cells, like Th1 cells they express T-bet and produce mainly IFN-γ but they can also produce low amounts of IL-4 (310, 320, 330, 331) (Figure 1.6). NKT1 cells can be either $CD4^+$ or $CD4^-$ and can be $NK1.1^+$, or $NK1.1^-$ (320). iNKT cells are most frequent in the liver where the can constitute up to 50% of intrahepatic lymphocytes (249-254).

Conversely, Th2-like NKT cells (NKT2 cells) produce large amounts of IL-4 as well as IL-9, IL-10 and IL-13 (310, 332, 333) (Figure 1.6). Thymic NKT cells predominantly produce IL-4 when activated with thymic DCs (334). However, the outcomes of iNKT cell activation are strongly influenced by the context in which the iNKT cells are activated. When thymic iNKT cells are co-cultured with APCs from the spleen in the presence of αGal, there is a significant increase in IFN-γ expression, compared to when they are co-cultured with APCs from the thymus (334).

Yet another subset of iNKT cells analogous to $CD4^+$ T cells are Th17-like iNKT cells (or NKT17 cells), which are most commonly contained within the $CD4^-NK1.1^-$ subpopulation (335). Like Th17 cells they express the transcription factor RORγT, CCR6 and primarily produce IL-17 (Figure 1.6).

More recently a regulatory subset of iNKT cells has been identified, which have been named regulatory iNKT cells (or NKT10 cells; Figure 1.6) (336, 337). These cells are enriched in the adipose tissue and produce IL-10 and IL-2. While other iNKT cells express the transcription factor PLZF, NKT10 cells express E4BP4, which is required for their IL-10 production (Figure 1.6), NKT10 cells can also be generated in response to repeated activation with αGal. (336, 337). Follicular helper NKT (NKTFH) are another subset of iNKT cells and are analogous to T follicular helper cells in that they have a role in promoting affinity maturation and antibodies in the germinal centres of spleens (338, 339).

In humans the subsets of iNKT cells are not definitively defined. However, $CD4^-CD8^-$ iNKT cells and $CD8^+$ iNKT cells produce IFN-γ are exhibit cytotoxic function when activated and are thought to be similar to NKT1 cells (340, 341). Understanding the

subtypes of iNKT cells, at what point they diverge and identifying lineage specific markers is essential for unravelling the contrasting roles of iNKT cells in the immune response. Furthermore, it is of fundamental importance if iNKT cells are to be isolated and manipulated for cell based therapies, such as in the treatment of GVHD.

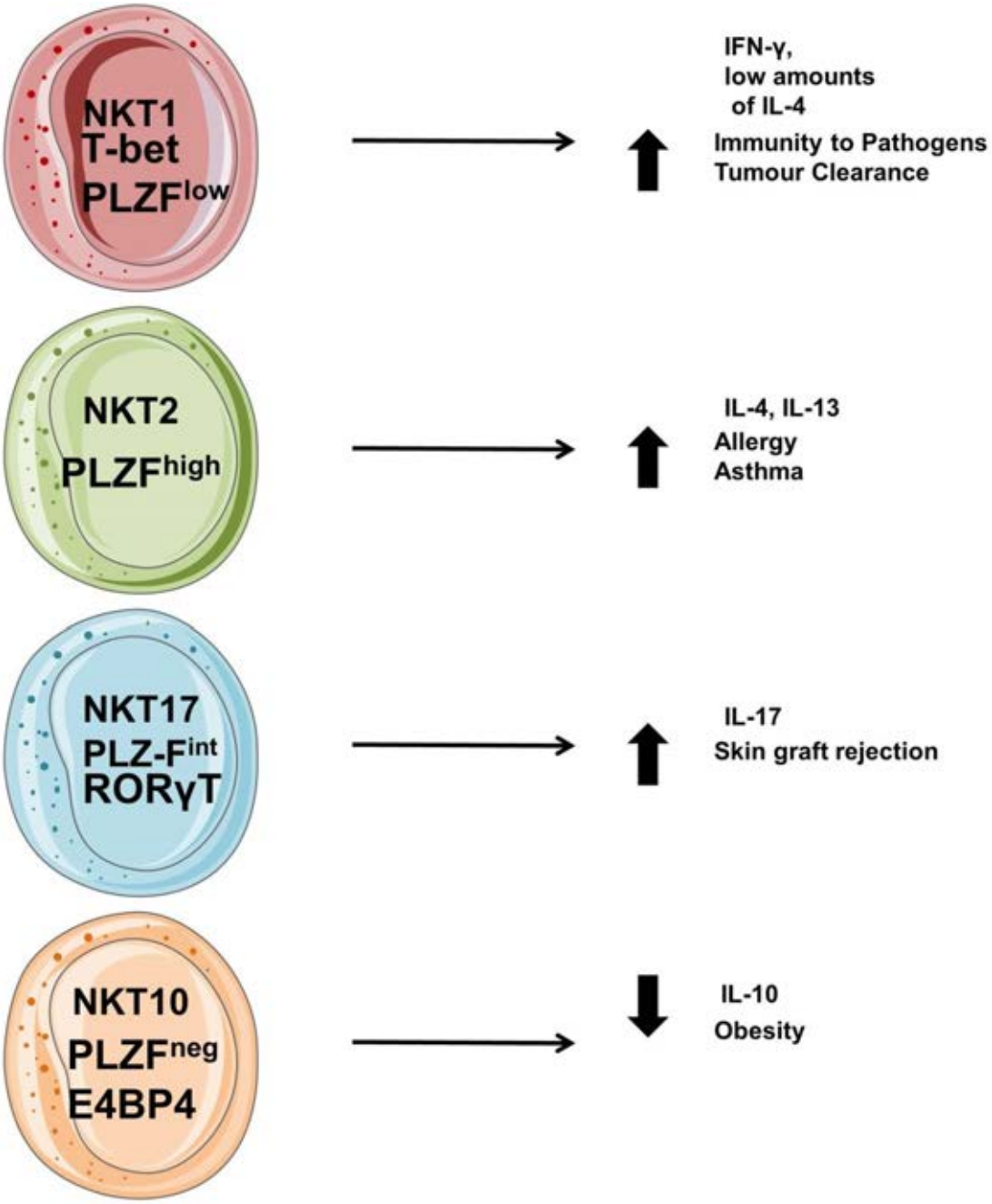

Figure 1.6 iNKT cell subtypes iNKT cells can be divided into subtypes based on their transcription factor and cytokine expression NKT1 cells express T-bet and low levels of PLZF and produce IFN-γ when stimulated and low amounts of IL-4. They are associated with anti-viral and anti-tumour immunity. NKT2 cells are PLZF^{high} and upon stimulation produce high amounts of IL.-4 and IL-13. NKT2 cells are increased in asthma. NKT17 cells express intermediate levels of PLZF, RORγT and when stimulated produce IL-17. They are increased in skin graft rejection. NKT10 cells express E4BP4 and produce IL-10.and are decreased in obesity.

1.4.8. iNKT cells in disease

iNKT cells have been shown to be involved in a wide variety of immune responses showing both protective and pathogenic roles in disease due to their ability to act in both a proinflammatory and anti-inflammatory manner. Moreover, iNKT cells are not a homogenous population and their frequencies vary depending on the anatomical site in which they are located as well as the local microenvironment and cytokine milieu. Furthermore, the bidirectional activation between DCs and iNKT cells contributes to the sculpting of iNKT cells responses.

1.4.8.1. iNKT cells in viral infection

In the context of viral infection iNKT cells have been shown to possess both protective and pathogenic roles depending on the virus involved. iNKT cell deficient mice show exacerbated phenotypes in infections such as herpes simplex virus type (HSV)-1 and HSV-2 (342-344). In addition, studies of patients with defects in iNKT cell development have shown that iNKT cells are protective in Epstein-Barr virus (EBV) in humans (345-347). Furthermore, treatment with αGal leads to enhanced viral immunity in a number of infections including human immunodeficiency virus (HIV), hepatitis B virus (HBV) and murine cytomegalovirus (MCMV) (348-350). These effects are often not mediated directly by iNKT cells as is the case with MCMV in which NK cells mediate the effect through perforin and IFN-γ (350).

However, iNKT cells have been shown to contribute to pathology in some viral infection for example they promote chronic lung disease in mice infected with Sendai virus (351). Despite the absence of a known microbial glycolipid some viruses including HSV-1 possess strategies to evade the immune system via disruption of

CD1d-mediated antigen presentation, suggesting that iNKT cells may be protective following CD1d-glycolipid activation in some viral infections (352). iNKT cells have also been shown to play an important role in promoting B cell immunity to viruses through the production of IL-4 at the interfollicular areas of LNs which allows for B cells to seed germinal centre reactions (353).

While iNKT cells can play antithetic roles in viral infection, it is not clear whether this is driven by the primary site of infection, the lifecycle of the virus, the mode of activation or a combination of factors.

1.4.8.2. iNKT cells in bacterial infection

iNKT cells have primarily been mostly studied in the context of intracellular bacteria. They have been shown to be protective in a variety of infections including *mycobacterium tuberculosis*, *Leishmania major* and *Streptococcus pneumoniae* (354-356). For example, the transfer of wild type (WT) or Jα18$^{-/-}$ splenocytes to mice that received sub-lethal irradiation and infection with *mycobacterium tuberculosis* resulted in a reduction in the bacterial burden in mice that received WT splenocytes compared to KO cells, suggesting a role iNKT cells in the clearance of the bacteria (354).

1.4.8.3. iNKT cells in fungal infection

iNKT cells can play a role in protection against fungal infections such as *Cryptococus neoformans* and *Aspergillus fumigatus*. In *C.neoformans* infection iNKT cells were shown to accumulate in the lungs in the early stages of infection and Jα18$^{-/-}$ mice show a reduced Th1 response and a delay in clearance of the pathogen (357). Similarly CD1dKO mice poorly control *A.fumigatus*, infection (358).

1.4.8.4. iNKT cells in protozoan infection

iNKT cells generally play a protective role in the context of protozoan infection. For example lipids extracted from mouse parasites can actually be loaded onto CD1d molecules and used to stimulate iNKT cells (359). iNKT cells have also been shown to be important for control of parasitic replication *in vivo* (360). However, when the immune response goes unchecked this can lead to collateral damage, as is the case in *Visceral leishmaniasis* in which iNKT cells lead to a chronic dermal complication called Post-kala-azar dermal leismaniasis (361, 362). There are also some cases in which iNKT cells play a pathogenic role in protozoan infection such is the case with *Toxoplasma gondii.* In *Toxoplasma gondii* iNKT cells are thought to produce IL-4, and suppress the production of heat shock protein by γδ T cells, which is required for protective immunity to the disease (363).

1.4.8.5. iNKT cells in autoimmune diseases

iNKT cells have been shown to have contradictory roles in autoimmune disease. However, this can be partially explained by the use of different models, the dose of glycolipid, the timing of glycolipid administration, and the route by which it is delivered as well as the stage of the disease in question (364-366). In general low numbers of peripheral blood iNKT cells have been observed in autoimmune diseases such as rheumatoid arthritis (RA), Grave's disease and type 1 diabetes (367-369).

One example of an autoimmune disease in which iNKT cells have been shown to play both protective and pathogenic roles is MS. MS is a T cell mediated autoimmune disease of the central nervous system which leads to progressive paralysis. In relapsing remitting MS, which is associated with period of latency and relapse of the disease, there were decreased peripheral blood iNKT cells, which seems to correlate with onset of relapse (370). Remission periods meanwhile have been associated with Th2 biased NKT cells (371). Similarly in mice, SJL/J mice which are particularly susceptible to EAE, have a defect in Vβ8, the most common β-chain used in iNKT cells, and adoptively transferring $NK1.1^+$, $DX5^+$ T cells (which contain a high frequency of iNKT cells) to such mice confers protection and recovery from EAE (372). Additionally, overexpression of the Vα14-Jα18 TCR chain in non-obese diabetic (NOD) mice leads to reduced susceptibility to EAE (373).

αGal administration has been observed to be protective in some models of EAE, whilst in other models its administration has been reported to exacerbate disease (364-366). Typically protection from disease was associated with a Th2-biased response (364-366). The aforementioned studies used cytokine blockade and cytokine KO mice to inform on iNKT cells function and it was shown that IL-4 and IL-

10 are involved in protection from EAE, although one study found IFN-γ attenuated disease (364-366). Interestingly, OCH which is known to bias towards a type 2 immune response was more protective than αGal and this protective effect was mediated by IL-4 (277). However, another model found no difference between the two glycolipids (374).

Taken together these studies show that the role of iNKT cells in autoimmune disease is by no means fully understood and is clearly context dependent, and suggests that iNKT cells can play paradoxical roles even within the same disease.

1.4.8.6. iNKT cells in allergy

iNKT cells particularly NKT2 cells have been observed in allergic diseases. By far the best characterisation of iNKT cells in allergic disease exists for asthma. Asthma is a common long-term multifaceted and heterogeneous lung disease characterised by inflammation, bronchospasms and airway hyper-reactivity (AHR). The most common form of asthma is allergic asthma and type-2 cytokines are crucial for disease development, leading to the hypothesis that iNKT cells can promote AHR (375).

A study in which iNKT cells were isolated from the lungs and bronchiolar lavage fluids of patients suffering from asthma showed that iNKT cells were the dominant T cells in the lung and solely produce NKT2 cytokines (376). Two later studies supported the hypothesis that iNKT cells are important for the development of asthma as they also observed increased numbers of iNKT cells in the lungs of patients with asthma (377, 378). However, one study showed that patients with severe asthma have higher numbers of iNKT cells compared to those with mild

asthma (379). Furthermore, it has been suggested that iNKT cells have a modulatory rather than a causative role in asthma (380, 381). It is possible that these discrepancies are due to different causative agents or the severity of disease. (379)

Mouse studies have shown that Jα281$^{-/-}$ mice, which lack iNKT cells, do not develop AHR in response to stimulation (332). Furthermore, adoptive transfer of iNKT cells from WT but not IL-4$^{-/-}$ or IL-13$^{-/-}$ mice leads to the establishment of AHR (332). Conversely, skewing the immune response in asthma towards a Th1 dominant response has been shown to prevent asthma is several models. For example, administering αGal during the sensitization phase of allergic asthma suppressed the allergic type-2 response by means of IFN-γ production (382). Furthermore, adoptive transfer of BMDCs loaded with αGal has been shown to prevent the development of an allergic response in the lung via iNKT cell derived IFN-γ (383). Together these studies show that while iNKT cells may not initiate asthma, they are at least a key player in the progression of the disease through their production of NKT2 type cytokines such as IL-4 and IL-13, while iNKT cell derived IFN-γ appears to play a protective role in the context of asthma.

1.4.8.7. iNKT cells in cancer

iNKT cells have a well-established role in anti-tumour immunity (384-387). In mice treated with αGal iNKT cells have been found to be essential for NKT mediated anti-tumour immunity (385, 387, 388). However, it should be noted that the Jα18$^{-/-}$ mice that were used for many of these studies had an inability to produce TCR Jα regions past Jα19 which may have impacted on the conventional T cell repertoire in addition

to preventing development of iNKT cells (385). Induction of tumours or transfer of tumour cells lines into mice deficient in iNKT cells lead to an increase in the occurrence of tumours compared to WT mice and this anti-tumour effect was found to be mediated by IFN-γ (389).

In humans and mouse models iNKT cells can mediate their effects directly by killing CD1d expressing tumour cells, as can occur in many forms of cancer including AML and acute lymphoblastic leukaemia, myelomas, myeloid leukaemias and prostate cancer (390-393). Alternatively, iNKT cells can be activated by other CD1d expressing cells, such as tumour-associated macrophages (394).

As well as activating iNKT cells via αGal, iNKT cells can be adoptively transferred in order to exploit their natural anti-tumour activity (395). Indeed, adoptive transfer of iNKT cells which have been activated with IL-12 has been shown to lead to the prevention of hepatic metastasis of B6 melanoma (395). Studies, of the anti-tumour properties of iNKT cells have led to a phase 1 clinical trial in which expanded iNKT cells are being investigated as a potential therapy for advanced melanoma which was found to be feasible and safe and the iNKT cells produced IFN-γ (396). The activation with αGal has also been investigated as a potential therapy for patients with solid tumours in phase 1 clinical trials and was deemed to be safe (397).

1.4.8.8. iNKT cells in obesity

In obesity adipose tissue can make up 50% of body mass. iNKT cells have been shown to be enriched in the adipose tissue in both humans and mice (249, 398). The environment in the adipose tissue in obesity is proinflammatory, and adipose tissue

iNKT cells can produce copious amounts of IL-10 (337). Lynch *et al.* showed that adipose tissue iNKT cells are depleted in obese patients compared to lean controls, as well as in diet-induced models of obesity (249, 398). This was confirmed by two other labs (246, 248). iNKT cell numbers fall in mice on high fat diets in a similar manner that occurs in human obesity and adoptively transferring WT iNKT cells into $J\alpha18^{-/-}$ or $CD1d^{-/-}$ mice leads to increased weight loss in obese mice (249). Furthermore, administration of αGal leads to rapid weight loss and reversal of glucose and insulin sensitivity in obese mice (248, 249). The protective role of iNKT cells is thought to be mediated through the production of regulatory cytokines such as IL-4 and IL-10 and their subsequent effect on the frequency and phenotype of macrophages (248, 249). Such cytokines promote M2 macrophages which suppress inflammation and indirectly influence adipose function leading to increased weight loss and improved fatty liver and insulin resistance (248, 249).

1.4.8.9. iNKT cells in solid organ transplantation

Mouse models have shown that the kinetics of organ rejection are not altered in the absence of iNKT cells in fully-mismatched skin, cardiac or islet (placed under the kidney capsule) allografts (399-401). However, this may be due to the high frequency of alloreactive T cells overshadowing any contribution of iNKT cells. Indeed, iNKT cells have been found to infiltrate skin and heart allograft prior to rejection and expanded numbers of iNKT cells are present in the peripheral lymphoid tissue post transplantation (402-404).

In certain contexts iNKT cells have been shown to be required for the facilitating the induction of tolerance. iNKT cells have been shown to be required for graft acceptance in cardiac transplant models in which tolerance is induced by blocking either co-stimulatory molecules, integrins or co-receptors (232, 399, 404). Furthermore, adoptive transfer of iNKT cells into iNKT cell deficient cardiac or skin transplant recipients being treated with such antibodies restores tolerance. This has been shown to be dependent on IFN-γ, IL-10 and/or CXCL16 (232, 399, 403, 404). iNKT cells have are also required for the induction of tolerance to corneal allografts (233, 405).

However, iNKT cells can also play a role in promoting allograft rejection in transplantation as is the case in skin-graft transplantation (231). Islet transplanted into the liver via the portal vein has also been shown to promote rejection by activating iNKT cells and downstream triggering IFN-γ production by Gr-1^{+}CD11b^{+} cells (400, 406).

The role of iNKT cells in transplantation may be influenced by whether or not the transplanted organ or tissue is directly vascularised or not. Experimental skin transplants drain to the local lymph nodes (LNs) in mice where there is predominance of IL-17 secreting iNKT cells (407). Indeed, IL-17 produced by iNKT cells post skin transplantation has been shown to promote graft rejection (231). Furthermore, iNKT cells can be manipulated in order to promote allograft acceptance, multiple injections of αGal have been shown to promote skin graft survival (403).

1.4.8.10. iNKT cells in BM transplantation

A decreased incidence of acute stage II-IV GVHD has been observed patients that receive above the median dose of iNKT cells contaminants in their bone marrow transplant (BMT) (408). In addition, an increased number of iNKT cells in the graft have been show to correlate with improved GVHD and progression free survival (409). Recovery of iNKT cells post-transplant has been shown to be associated with reduced non-relapse mortality without a risk of relapse and improvement in the overall survival rate (410). Furthermore, recent studies in mice have shown that adoptive transfer of iNKT cells can protect from GVHD in mice, which will be discussed in detail in chapter 6 (126, 234-238).

1.4.8.11. iNKT cell summary

iNKT cells are not a homogenous population of cells therefore they can be involved in dichotomous responses to immune perturbation. The same subtype of iNKT cells can be protective in one setting but not another, even within the same disease. Despite the regulatory effect of iNKT cells in the context of some forms of transplantation, they can have pathogenic roles in many contexts as outlined above. Other examples of pathogenic roles are in pregnancy wherein iNKT cells have been shown to induce preterm birth and early to mid-gestation pregnancy loss and in sterile hepatic injury they initiate hepatic reperfusion injury (411, 412). The role of iNKT cells is very much dependent on the local microenvironment, site of infection and or injury, stage of disease, dose of glycolipid (if any) and timing and route of administration all contributing to the nature of the response. Furthermore, discrete

differences between animal models of the same disease or between mice at different animal facilities may influence the nature of the iNKT cell response.

1.5. Thesis hypothesis and aims

1.5.1. Hypothesis

Subsets of iNKT cells have the potential to be used as cellular therapies for the treatment of GVHD.

1.5.2. Global Aims

Cell based therapies are an emerging area of therapeutics with the potential to treat a plethora of immune diseases by either enhancing or dampening down the immune response, depending on the nature of the disease being treated. Of particular interest to our lab is the ability of cellular therapies to promote immune regulation in the context of transplantation. iNKT cells possess the ability to facilitate and sculpt the adaptive immune response. They have been shown to be capable of suppressing GVHD in several models. We hypothesise that in order for this therapy to be clinically translatable the iNKT cells would require expansion and characterisation. The aims of this project were to:

1. Establish a mouse model of GVHD in which we can track alloreactive transgenic TEa T cells *in vitro*.
2. Characterise the phenotype and function of freshly isolated and expanded splenic iNKT cells
3. Investigate the immunomodulatory ability of freshly isolated and expanded iNKT cells *in vitro*
4. Investigate the immunomodulatory ability of freshly isolated and expanded iNKT cells in the established mouse model of GVHD

CHAPTER 2 MATERIALS AND METHODS

2.1. Mice

Mice were either bred in house or sourced from Charles River and were between 8 and 12 weeks at the time of first procedure (Appendix I). Mice from external sources were housed for a minimum of one week prior to the initiation of experiments under SPF conditions at the Biomedical Services Unit and the University of Birmingham. Animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 under the appropriate project and personal licence (I14963778). On average 5 mice were housed per cage under alternating 12 hours (h) light/dark cycles at 23ºC. Mice were killed by cervical dislocation and all organs and tissues were removed by dissection under sterile conditions.

2.2. Cell isolations

2.2.1. Isolation and culture of cells

Spleens were mashed through a cell strainer (70 µm pore size) (Fisher), with complete Roswell Park Memorial Institute Medium (cRPMI) (Appendix II). Cells were centrifuged at 400 gravity (g) at 4 degrees Celsius (°C), for 5 minutes (min) and following this 1 ml of 1x red blood cell lysis buffer (BD) was added. The reaction was neutralised after ~1 min using cRPMI (Appendix II).

2.2.2. Isolation of lymph node (LN) cells

Fat was dissected from LNs prior to being mashed through a cell strainer (70 µm pore size), with cRPMI.

2.2.3. Isolation of leukocytes from Liver

Livers were mashed through a cell strainer (70 µm pore size), with cRPMI (Appendix II). Cells were centrifuged at 400 g, for 5 min and resuspended in 10 ml cRPMI. Each sample was then divided in two, and each half was layered on top of 7mL of room temperature (r.t.) Optiprep (1.09 g/ml) (Sigma Aldrich). The cells were centrifuged at 1000 g (with no break), for 25 min, at r.t. Cells were removed from the interface that formed as a result of centrifugation and washed and resuspended in Fluorescence-activated cell sorting (FACS) buffer (Appendix II) for FACS staining.

2.2.4. Isolation of leukocytes from Small intestine (SI)

Small intestine (SI) (end of the stomach to the cecum), was removed by dissection and placed in a petri dish containing ice cold CaMg free Hanks' Balanced Salt

solution (HBSS) (Sigma Aldrich) containing 2% Fetal calf serum (FCS) (Sigma Aldrich) HBSS 2% FCS (Sigma Aldrich) (Appendix II). The mesenteric fat and Peyer's patches were carefully removed on tissue soaked in ice cold HBSS 2% FCS. The SI was sliced longitudinally opening it up and the contents removed by agitating it in the HBSS 2% FCS. Following this, the SI was cut into ~2 mm sections and placed in a 50 mL tube (Sarstedt) containing HBSS 2% FCS and shaken vigorously for 20 seconds (sec) before being placed on ice.

The SI was filtered through mesh supported in a funnel, and placed in a 50 mL tube containing 20 mL HBSS with 2 mM Ethylenediaminetetraacetic acid (EDTA) (Corning) (Appendix II) and shaken vigorously for 20 sec. The SI was then incubated for 20 min in a shaker at the maximum speed at 37°C, which strips the epithelium of the SI. The SI was filtered again, placed in 20 mL HBSS with 2 mM EDTA and shaken vigorously for 20 sec and returned to the shaker for a further 20 mins. The SI was then filtered and shaken vigorously with HBSS (EDTA free) twice, before being placed in 15 ml of pre-warmed cRPMI containing collagenase VIII (1 mg/ml) (Sigma Aldrich), and shaken manually for 10 sec before being placed in the incubated shaker (Multitron Standard (Infors HT)). After 10 min the SI was removed and shaken vigorously for 10 sec and then returned to the incubator for a further 5 min. The SI was then placed on ice, to stop the reaction and filtered through a 100 µm followed by a 70 µm strainer and centrifuged and resuspended in FACS buffer.

2.2.5. Isolation of bone marrow (BM) cells

Bones from the femora and tibiae of mice were removed under sterile dissection. BM was flushed from the bone cavities of the femora and tibiae with cRPMI, using a 25 gauge needle (Terumo). Cells were centrifuged at 400 g, for 5 min and following this 1 ml of 1x red blood cell lysis buffer (BD) was added, followed by 10 ml of cRPMI.

2.3. Cell enrichment by dynabeads, Magnetic Activated Cell Sorting (MACS®) and cell sorting

2.3.1. Magnetic labelling and depletion of non-T cells using dynabeads

Cells were suspended in 200 µl of Magnetic activated cellular sorting (MACS) buffer (Appendix II) per $5x10^7$ cells. Subsequently, 100 µl of FCS (Gibco, Life technologies) and 50 µl CD4 enrichment cocktail (Life technologies), were added per $5x10^7$ cells. The CD4 depletion cocktail contained non-T cell antibodies (cocktail contains biotin-conjugated antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, Ter-119 and TCRγ/δ), which were used to deplete non-T cells by means of negative selection. The cells were incubated for 20 min, at 4°C. Cells were subsequently washed with 10 ml MACS buffer and resuspended in 4 ml of buffer. The 4 ml cell suspension was then added to washed dynabeads (invitrogen) (250 µl of beads per $5x10^7$ cells) and placed on a rotator for 20 min at 4°C before being placed into the DynaMag magnet stand (Life technologies). The supernatant was removed into a new tube; this fraction contained enriched $CD4^+$ cells. Cell purity was determined using flow cytometry, mean frequency post sort 87%±0.08% (Appendix V).

2.3.2. Magnetic labelling and enrichment iNKT cells using Manual MACS® Separator

The enriched $CD4^+$ cells were washed with MACS buffer and resuspended in 200 µl of buffer per $7.5x10^7$ cells, with 5 µl of Phosphate-buffered saline (PBS) 57-CD1d-APC tetramer (1/40 dilution of 1.3-1.5 mg/ml; NIH Tetramer Core Facility) per $7.5x10^7$ cells, and incubated for 30 min, at 4°C. Cells were then washed with 10 ml of buffer and resusupended in 80 µl MACS buffer per $1x10^7$ cells with 10 µl anti-APC beads (MACS®) per $1x10^7$ cells and incubated for 20 min at 4°C. Cells were then washed with 10 ml of MACS buffer, and resuspended in 1 ml of MACS buffer. The MACS column was placed in the Manual MACS® Separator and primed by washing with 3 ml of MACS buffer. The 1 ml cell suspension was then added to the primed column, and washed through by washing 3 times, with 3 ml of MACS buffer. The column was then removed from the magnet, 5 ml of MACS buffer were added to the column and the cells were eluted into a 15 ml tube using the plunger supplied with the column. The column purification was repeated in order to gain a purer sample. Mean frequency post sort 85%±11% (Appendix V).

2.4. Preparation of glycolipids

Prior to either the pulsing of BMDCs with αGal (Abcam/from Gurdyal S. Besra, School of Biosciences, University of Birmingham), or injection of αGal or C20.2 (from Gurdyal S. Besra, School of Biosciences, University of Birmingham) glycolipids were sonicated for 5 min using the ultrawave (Wolf Labs). Following this the glycolipid was heated for 5 min at 40°C. Following this glycolipids were either added to the BMDC culture, or diluted in PBS (Appendix II) for intraperitoneal (i.p.) injection.

2.5. iNKT cell sorting

Isolation of specific iNKT cells from reporter mice was required in both *in vitro* and *in vivo* experiments, namely RORγT negative cells from RORγT reporter mice and IFN-γ positive cells from IFN-γ reporter mice. In order to sort IFN-γ positive iNKT cells the mice first required injection with αGal. αGal was prepared as described in section 2.4 Mice were given i.p. αGal (2 µg) before being sacrificed 48 h later, and splenocytes were isolated. In the case of sorting both RORγT negative and IFN-γ positive cells, cells were first enriched using micro-bead sorting as described in section 2.3. Following this cells were stained on ice for 30 mins. To sort RORγT negative cells from RORγT reporter mice, cells were stained on ice for 30 min, (for CD5 and PBS57 tetramer). Cells were then sorted on expression of CD5 and PBS57 tetramer (iNKT) cells and GFP (negative cells sorted) using either the FACSAria Fusion (BD) or the Mo-Flow Astrios (Beckman). To sort IFN-γ positive cells from IFN-γ reporter mice, cells were stained on ice for 30 min, (for CD5 and PBS57 tetramer) and following this GFP positive (IFN-γ postive) iNKT cells were purified. For WT B6 mice Mean frequency post sort 85%±0%, for IFN-γ positive iNKT cells mean frequency post sort 94%±6%, for RORγT negative iNKT cells Mean frequency post sort 87%±12% (Appendix V).

2.6. Microarray

IFN-γ reporter mice were injected i.p. with αGal or PBS-vehicle control and mice were sacrificed and spleens harvested after 48 h. iNKT cells were isolated as described in sections 2.3 and further sorted on expression of IFN-γ as described in

section 2.5 Ribonucleic acid (RNA) was then isolated from iNKT cells using a RNA micro kit (Qiagen). Cells were spun at 800 g for 5 min at 4°C and supernatant was removed and 350µl RLT (lysis buffer) containing β-mercaotoethanol (10ul per 1ml RLT) was added to each sample. Samples were added to RNA shredder column with collection tube which were spun at 20,000 g for 2 min at r.t. 350 µl of 70% ethanol was added to the flow through and samples were added to RNeasy Min Elute Spin column with collection tube. Samples were spun at 8000 g for 15 sec at r.t. To wash the column, 350 µl of RW1 was added to each column. Samples were spun at 8000 g for 15 sec at r.t. The DNAse solution (DNAse-I 10 µl and RDD buffer 70 µl) was added to each membrane on column and left at r.t. for 15 min to eliminate the genomic DNA. To wash the column, 350 µl of RW1 were added to the column. Samples were spun at 8000 g for 15 sec at r.t. Samples were placed into a new collection tube and 500 µl of RPE were added to each sample for further wash. Samples were spun at 8000 g for 15 sec. 500µl of 80% ethanol were added to each sample. Samples were spun at 8000 g at r.t. for 2 min and following this placed in a new collection tube. Samples were spun at 20,000 g at r.t. for 5 min to eliminate the residual ethanol on membrane in column. Samples were placed in new collection tubes and spun twice with 15 µl of RNase Free water at 20,000 g at r.t. for 5 min to elute the RNA. RNA purity (Ratio of 260/280, 260/230) and yield were determined using the thermos Scientific Nanodrop2000c spectrophotometer (Thermo Scientific). Purified RNA was sent to Aros applied biotechnologies who conducted the further RNA quality check (RNA pico chip) using a Bioanalyzer (Agilent Technologies) and the microarray (Affymetrics (GeneChip Mouse Transcriptome Assay 1.0 (MTA 1.0).

Microarray analysis was performed using Transcriptome Analysis Console (Affymetrix).

2.7. Bone marrow dendritic cell (BMDC) culture

BM cells were isolated as described in section 2.2.5. Isolated BM cells were centrifuged and resuspended at a concentration of $1x10^6$ cells/ml, with a final concentration of 10 ng/ml granulocyte monocyte stimulating factor (GM-CSF) (R&D Systems) and 1 ng/ml IL-4 (Peprotech), and plated in a 24 well plate (Costar) with 1 ml of cell suspension per well. Cells were cultured in carbon dioxide (CO_2) incubator (Sanyo incubator (5% CO_2)) for 1 week (Appendix V).

2.8. In *vitro* expansion of iNKT cells

2.8.1. In *vitro* expansion of iNKT cells with αGal pulsed BMDCs

This protocol was based on the method described by Chiba *et el.* (413). BM cells were cultured as described in section 2.7. αGal (100 ng/ml) was added to the culture on day 6 and cells were harvested on day 7. The αGal pulsed BMDCs were washed with medium and irradiated at 3000Rad (~30 Gray (Gy.)) using the CIS bio international IBL 437c, and $2x10^5$ BMDCs were plated with $2x10^6$ purified iNKT cells in a flat bottomed 24 well plate in iNKT cell medium (Appendix II), in the presence of IL-2 and IL-7 (both 10 ng/ml final concentration). The iNKT cells were restimulated with irradiated αGal pulsed BMDCs on day 8 and fresh cytokines were added as required. Cells were harvested on day 14. Average fold expansion was 2.7. Estimated average purity at end of expansion was 71±12%.

2.8.2. *In vitro* expansion of iNKT cells with plate bound anti-CD3 and soluble anti-CD28

This method was based on the protocol described by Govindarajan *et al.* (414). A flat bottomed 96-well plate (Sarstedt) was coated with purified anti-CD3 (3 µg/ml) in carbonate bicarbonate buffer (Sigma Aldrich), and incubated for 1 h at 37°C. The plate was washed twice with PBS and once with cRPMI. Purified iNKT cells (which were purified as described in section 2.3), at a concentration of $5x10^5$/mL (or $1x10^5$ per well) were plated in cRPMI containing IL-2 (10 ng/ml), IL-12 (1 ng/ml) and soluble anti-mouse CD28 (5 µg/ml). Cells were incubated for 2 days at 37°C in a CO_2 incubator (BB15 Thermo Scientific). After 2 days cells were moved to fresh uncoated wells. In later experiments, cells were counted on day 2, and re-plated at $5x10^5$/mL. On day 4, cells were re-plated at a concentration of $5x10^5$/mL in cRPMI containing IL-7 (5 ng/ml). Cells were monitored closely and plated with fresh IL-7 in cRPMI when required. On day 8, cells were restimulated with anti-CD3 and anti-CD28, and moved to fresh wells on day 10. On day 11 cells were re-plated in cRPMI containing IL-7 (5 ng/ml) and monitored closely and plated with fresh IL-7 in cRPMI when required. Cells were harvested and counted on day 14, for use in *in vitro* and *in vivo* experiments. Estimated average purity at end of expansion was 74±11%. Average fold expansion was ~1.6 when calculated from day 0, but ~7 when calculated from day 2.

2.9. Flow Cytometry

When cytokine staining was required, cells were stimulated with para-Methoxyamphetamine (PMA) (Sigma Aldrich) (final concentration 500 ng/ml) and ionomycin (final concentration 500 ng/ml) (Sigma Aldrich) and incubated at 37°C for 1h. After 1h, 1 µl of Brefeldin A (BFA) (e biosciences) (working concentration 3.0 µg/ml), and 1 µl of monensin (2 µM) (ebioscience), were added to the culture and the cells were cultured for a further 4h. Cells were then transferred to FACS tubes (Alpha Labs) and washed by adding 1 mL of FACS buffer, at 400 g for 5 min. Supernatants were removed and cells were incubation with live/dead dye (Zombie dye (biolegend) in PBS or 7-AAD (ebioscience) in FACS buffer for 10 min at r.t. Following this cells werewashed with FACS buffer and stained with anti-CD16/32 (Biolegend) in FACS buffer (final concentration 1 µg/ml) for 10 min at r.t. and then washed twice with 2 ml of FACS buffer.

2.9.1. Controls

In order ensure the correct compensation was applied to each experiment, single colour staining was carried out, using cells and compensation was adjusted accordingly. Single colour staining was also used in order to determine the appropriate voltages used. The relevant antibody was added to each compensation control (Appendix) Isotype controls (Appendix III) were used for intracellular (IC) and intranuclear stains, which enabled the correct gating strategy to be applied. Fluorescence minus one (FMOs) were used for surface markers that are known to be expressed at low levels. Where cell number permitted, unloaded tetramer (Appendix III) (NIH Tetramer Core Facility) was used in order to gate iNKT cells.

2.9.2. Surface Staining

The relevant antibodies (Appendix III) were diluted in FACS buffer, and 50 µl were added to each sample. The samples were incubated for 30 min at 4°C and subsequently washed twice with 1ml FACS buffer.

2.9.3. Intracellular cytokine (IC) staining

When intracellular staining was required 250 µl of IC fixation buffer (eBioscience) (or FOXP3 fixation buffer when intra-nuclear stains were required (eBioscience)), were added to the cells, and incubated for 20 min at 4ºC, and then washed twice with 1 ml pf permeabilization buffer. The IC antibodies (Appendix III) were then diluted in IC permeabilization buffer (eBioscience) (or FOXP3 fixation buffer for intra-nuclear antibodies (eBioscience), and 50 µl were added to each sample. Cells were 16 incubated for 30 min at 4ºC and then washed twice with permeabilization buffer. Finally, cells were resuspended in 300 µl of FACs buffer, filtered before acquisition.

2.9.4. Alternative stimulation methods

A 48 well plate was coated with carbonate bicarbonate buffer containing 10 µg/mL anti-CD3 at 37°C for 1 hr. The plate was washed twice with PBS and once with cRPMI. iNKT cells were purified as described in section 2.3, and purified cells were then plated in cRPMI containing 1 µg/ml anti-CD28, 500 µl were added to each coated well with a maximum concentration of $6x10^6$ per well. Alternatively, cells were plated with BMDCs which had been pulsed with αGal 12 h previously, such that the number of BMDCs was a tenth of the number of the iNKT cells. Cells were incubated in a CO_2 incubator at 37°C, after 2 hr BFA (working concentration 3.0 µg/ml), and 1 µl of monensin (2 µM) and cells were incubated for a further 4 h. After 6 h cells were collected for flow cytometry.

2.9.5. Stimulation of iNKT cells to examine the modulation of surface maker expression

A 96 well plate was coated with carbonate bicarbonate buffer containing 10 µg/mL anti-CD3 at 37°C for 1 hr. The plate was washed twice with PBS and once with cRPMI. iNKT cells were purified as described in section 2.3, and purified cells were then plated in media containing 1 µg/ml anti-CD28, $5x10^3$ were plated per well. Alternatively cells were plated in uncoated wells, or plated with BMDCs which had been pulsed with αGal 12 h previously or unpulsed BMDCs. The number of BMDCs ($5x10^3$/well) was such that the number of BMDCs was a tenth of the number of the iNKT cells ($5x10^4$/well). Cells were incubated in a CO_2 incubator at 37°C. After 6 h cells were collected for flow cytometry.

2.10. Cell counting

Cells numbers for flow cytometry experiments were determined using beads (Spherotech inc.), ($1x10^4$ particles/10 µl). Cell numbers for the purpose of tissue culture cell were enumerated using a light-microscope and dead cells were excluded using trypan blue.

2.11. Confocal microscopy

2.11.1. Sectioning tissues for confocal microscopy

Following confirmation of cardiac death, organs were promptly isolated, frozen using dry ice or liquid nitrogen and stored at -80ºC. In order to section tissues, organs were mounted on optimum cutting temperature cryopreservation media (OCT) (Tissue Tek), frozen with cryospray (Bright) and cut in 7 µm sections using a cryostat (Bright).

2.11.2. Staining

Sections were blocked in anti-CD16/32 (Biolegend), diluted in PBS with 1% BSA (final concentration 1 µg/ml) for 10 min at r.t. Primary antibodies were diluted in PBS with 1% BSA (Appendix IV), 100 µl were added to each section and the sections were incubated for 1 hr at r.t. Slides were washed in PBS and secondary antibodies were diluted in PBS with 1% BSA (Appendix IV) and left to incubate for 1 hr at r.t. Slides were washed and then submerged in 4′,6-diamidino-2-phenylindole (DAPI) diluted in PBS(25 µg/ml) for 30 sec. Slides were washed again and antifade (Cell signalling technology) was applied to each section. Slides were covered with coverslips, sealed and analysed using the Zeiss 510 Meta confocal microscope.

2.12. *In vitro* TEa T cell suppression assay

$CD4^+$ TEa T cells were isolated as in section 2.3.1. BMDCs were removed from culture washed with RPMI and irradiated at 3000Rad (~30Gy 753 sec). The isolated TEa T cells were washed 3 times with 10 ml FCS free medium resuspended in FCS free RPMI with Carboxyfluorescein succinimidyl ester (CFSE) (final conc. 2.5 μM/ml) (eBioscience), and incubated for 10 min at 37ºC for 10 min in the dark. To neutralise the reaction, 10 ml of cRPMI were added to the suspension. αGal pulsed BMDCs were plated in a round bottomed 96 well plate at a concentration of $2.5x10^4$/ml ($5x10^3$/well), with TEa T cells at a concentration of $2.5x10^5$/ml ($5x10^4$/well) (10X the number of BMDCs). Purified freshly isolated (FI) or Expanded (Exp) iNKT cells were added at a ratio of 1:1 with the TEa T cells, and additional at ratios of 1:0.5 (TEa T cells:Exp iNKT cells),1:0.25,1:0.0125 and 1:0.1) were tested on some occasions. In addition to the basic assay as described above, this assay was also performed in the presence of anti-IL-10 receptor (IL-10r) (10 μg/ml), anti-IFN-γ (10μg/ml), anti-Programmed cell death protein 1 (PD1) (10μg/ml), anti-PDL1 (5 μg/ml) and anti-PDL2 (5 μg/ml) and with excess IL-2 (10ng/ml). The cells were cultured for 4 days and the harvested for flow cytometry.

In some assays TEa T cells were stimulated with anti-CD3 and anti-CD28. A 96 well plate was coated with carbonate bicarbonate buffer containing 5 μg/mL anti-CD3 at 37°C for 1 hr. The purified labelled TEa T cells were then plated in cRPMI containing anti-CD28 (2ug/ml) in the presence and absence of Exp iNKT cells and cultured for 4 days, before being harvested for flow cytometry.

2.13. Establishing a mouse model of GVHD

2.13.1. BM T cell depletion

BM was flushed from W.T. B6 mice and the BM cells suspended in 200 µl of MACS buffer (Appendix II), per $5x10^7$ cells, along with FCS (100 µl per $5x10^7$) and Thy1.2 antibody (10 µl per $5x10^7$ cells) (Biolegend). The cells were incubated for 20 min, at 4°C and then washed and resuspended in 4 ml of MACS buffer. The 4 ml cell suspension was then added to washed dynabeads (250 µl of beads per $5x10^7$ cells; Life Technologies). The cell and bead suspension was then placed on a rotator for 20 min at 4°C and then onto a DynaMag magnet (Life Technologies). The supernatant was then removed into a new tube; this fraction contained the T cell depleted BM (TCD BM). Mean frequency post sort of contaminating $CD4^+$ T cells 0.4±0.7%. $CD8^+$ T cells 0.2±0.1% (Appendix V).

2.13.2. BM transplant

CB6F1 recipient mice were given Baytril (Bayer) (0.16 mg/ml) in their water for 1 week prior to BMT and for 14 days post-transplant. On Day 0, such mice were lethally irradiated with 2 doses of TBI, (2x550 centigray (cGy)), 4 h apart. Following irradiation, $5x10^6$ B6 TDBM cells were i.v. injected via the tail vein.

2.13.3. Cell isolations and TEa T cell and i.v. injection

TEa T cells were isolated from the spleen as in section 2.3.1 and re-adjusted such that 200 µl contained $1x10^4$ TEa T cells. TEa T cells were i.v. injected via dorsal or ventral tail vein ($1x10^4$ cells/mouse). Mice were closely monitored for clinical signs of weight loss (Appendix I), and weighed daily.

2.13.4. GVHD manipulation

In order to investigate the ability of FI and Exp iNKT cells to suppress GVHD iNKT cells were injected i.v. Where FI iNKT cells were injected cells were isolated as described in section 2.3. Such cells were then injected i.v. on day 2, concomitantly with the TEa T cells, $1x10^5$ FI iNKT cells were injected. When Exp iNKT cells were injected cells were expanded for 14 days. On day 2, $1x10^5$ FI iNKT cells were injected per mouse, concomitantly with the TEa T cells. In some experiments glycolipids were used to activated iNKT cells. Glycolipids were prepared as described in section 2.4. Single or multiple doses of αGal (2 µg) or C20.2 (5 µg) were injected i.p. starting on day 2, as described in the individual experiments.

2.13.5. GVHD scoring and mortality criteria

Mice were weighed daily and monitored for clinical signs of GVHD (Appendix I).Mice were sacrificed prior to reaching reached 20% weight loss and/or if the animals' clinical score was likely to exceed that stipulated within a moderate severity limit.

2.14. Histology

Organs were dissected from mice and liver and SI (short 5 mm sections), were fixed in formaldehyde 4% aqueous solution buffered (formalin 10%) (VWR chemicals). Tissues were processed by Royal Orthopaedic Hospital NHS Foundation Trust histology service. Briefly tissue was embedded in wax and sliced using a cryostat and sliced into 3 µm sections. Tissues were stained with haematoxylin and eosin. Photomicrographs were taken using ZeissAxio Scan.Z1 slide scanner (for the liver)

and using the Leica DM4000 B LED (for the SI). The magnification was x20 for the liver and x40 for the SI. Tissue was analysed for clinical signs of damage with the assistance of experts (Dr. Mona Elshafie, Consultant Histopathologist Queen Elizabeth Hospital and Dr. Scott Davies, University of Birmingham, Liver Labs). In the SI apoptotic bodies were signs of SI GVHD. In the liver bile duct damage, fibrotic tracts and perivascular infiltrates were sings of liver damage.

2.15. Statistical analysis

Student's T test (unpaired; two-tailed) was used to determine the significance between two sets of related data. One-way ANOVA was used to compare 2 or more sets of data. Weight loss and clinical scores were compared with two-way ANOVA with post-hoc Bonferroni comparison (or Tukey comparison when comparing 5 or more groups). Survival curves were analysed using a log-rank test. Normality of data was investigated using the D'Agostino-Pearson omnibus normality test to choose whether a parametric or non-parametric test should be performed. Statistics were performed using GraphPad Prism 6 (GraphPad Software). P values <0.05 were interpreted as statistically significant. Data are presented as mean ± standard deviation unless otherwise stated.

2.16. Software

Flow cytometry data was analysed using FlowJo (Treestar). Microarray analysis was performed using Transcriptome Analysis Console (Affymetrix). Statistics were carried out using Graphpad Prism 6 (Graph Pad Software, La Jolla California USA). Glycolipid structures were drawn using ChemDraw (online version). Modification of files from Servier Medical Art (licensed under a Creative Common Attribution 3.0 Generic License) was used in some figures.

CHAPTER 3 ESTABLISHING AN *IN VIVO* MODEL OF GVHD

3.1. Introduction

GVHD is a disease that develops following HSC transplant as a result of contaminating donor T cells in the transplant recognising alloantigen leading to tissue destruction in the skin, lungs, liver and GI tract. Acute GVHD occurs in the first 100 days after HSC transplantation and it is a major cause of mortality and morbidity in the 30,000 people who receive transplants worldwide every year. Numerous mouse models of acute GVHD have been developed with the aim of understanding the process of disease initiation and progression, in addition to possible methods of treatment. Indeed, much of what is known about the initiation of GVHD comes from studying mouse models. This research stemmed from the seminal murine studies of Korngold and Sprent, who first discovered that alloreactive T cells are the main mediators of GVHD (415, 416).

While effector T cells are essential for disease in all models of GVHD the nature of the T cells and their interactions with recipient and host cells varies. Models of GVHD can be either $CD4^+$ T cell driven, $CD8^+$ T cells driven or both. Some models are fully MHC mismatched, while others depend on mHC disparities. Where there is a full MHC-mismatch, GVHD is induced due to differences in MHC class I and MHC class II (H2) molecules, which can be recognised either directly (as intact recipient MHC + peptide complexes) or indirectly (following processing and presentation of recipient alloantigens by donor MHC). This results in the activation of both $CD8^+$ and $CD4^+$ T cells. In humans, high-resolution DNA typing is used to match transplants, which are major histocompatibility matched and usually only differ at mHCs, and as such mHC-mismatched models represent clinical transplantation more closely.

The first step in inducing GVHD in mice is generally TBI, although chemotherapy with cyclophosphamide, fludarabine, and busulfan may also form part of the conditioning regime (417). The irradiation dose required for lethal irradiation varies according to mouse strain. B6 mice are generally more resistant than BALB/c mice and F1 progeny are generally more resistant than their parents (418). The conditioning regime initiates GVHD by causing damage to the host tissue and release of cytokines such as IL-1β and TNF-α which contribute to what is commonly referred to as the cytokine storm (149, 172, 419). In addition, PAMPs, DAMPs, chemokines and gut commensals are released (420). This leads to the activation of APCs (158, 160). The conditioning regime can be particularly damaging to the GI tract, where the release of LPS and other immunostimulatory microbial products can further perpetuate the cycle of inflammation (151, 153, 172, 421). Indeed the severity of GVHD can be correlated with the intensity of the treatment regime in both humans and mice (151,

154-156). Models which use irradiation doses of 1100 cGy are delivered in fractionated doses to decrease gut toxicity (163). Human conditioning regimes usually consist of chemotherapy with or without irradiation, and irradiation is usually fractionated.

BM ablation provides a niche for the donor BM to engraft and reconstitute the immune system. This is reflective of what happens clinically when a patient is reconstituted with a healthy immune system, although HSCs in humans are now most commonly derived from mobilised stem cell products. Generally mice are given TCD BM, in order that alloreactive T cells can be added back in a controlled manner. This allows the GVHD-causing T cells to be added back at the time point, phenotype and number relevant to the biological question. Upon transplantation T cells traffic to the secondary lymphoid organs within 24 h (422, 423). This is thought to be critical for GVHD initiation. In the context of GVHD T cells produce further proinflammatory cytokines amplifying the cycle of inflammation.

The release of cytokines such as IFN-γ leads to the activation and recruitment of additional effector cells such as NK cells, B cells, neutrophils and monocytes resulting in further T cell proliferation in an antigen independent manner (157, 419). Additionally, proinflammatory macorphages produce TNF-α, IL-1β and NO (153, 167). Donor T cells exhibit a recently activated phenotype ($CD62L^{low}CD44^{high}$) and by day 3 have upregulated the gut homing receptor α4β7 and migrate to the GI tract where they are activated in an antigen specific manner when they encounter alloantigen presented by myofibroblasts and epithelial cells within the GI tract (423, 424). The initiation of gut GVHD precedes cell infiltration into the skin (423). The end result is that activated effector T cells traffic to all GVHD target organs, namely the GI

tract, skin, lung and liver, causing organ damage. Such damage is mediated through perforin and granzymes, TNF-α and Fas-FasL interactions (173, 425-428). Additionally Th17 cells may play a role in the pathophysiology of GVHD, although such cells have been shown to be sufficient but not an absolute requirement to cause GVHD (429).

The severity and clinical signs of GVHD can vary depending on multiple factors such as the dose and type of T cells used to mediate GVHD (202, 226, 227), the age and sex of the mice, variations in environmental pathogens and gut microflora (which can be influenced by animal supplier and local animal facilities) and genetic disparity between donor and recipient (228). In addition, as previously mentioned, GVHD can vary according to the dose of irradiation which influences the extent of tissue damage and therefore alloreactive T cell activation (229).

While mouse models can be very useful in understanding GVHD, it is important to be aware of the caveats associated with such models and the disparities between humans and mice. Factors such as the proportions of effector cells, differing condition regimes, differing sources of haematopoietic cells and differences in gut microflora between species all have the ability to influence the initiation and progression of the disease.

Despite these caveats, much of what we know about GVHD was discovered from mice, and they continue to provide critical information that allows for significant innovations to reach the clinic. A recent example of this is the use of hypomethylating agents such as decitabine and azacitidine which can peripherally convert $CD4^+$ T cells into $FOXP3^+$ Treg which can abrogate GVHD in mice (430). This has led to

stage 1/2 clinical trials. Mouse models provide us with tools which allow us to dissect and manipulate GVHD in a way that is not possible in humans. For example, some mouse models of GVHD make use of transgenic and knockout mice as either donors or recipients. This can be extremely useful in enabling mechanistic dissection. *In vivo* imaging is also possible using CFSE, Bromodeoxyuridine (BRDU) or luciferase transgenic cells. In addition, the GVT effect can be investigated using transplantable tumour cell lines. Furthermore, newly developed humanised mouse models provide us with a system in which human T cell mediated GVHD can be studied and manipulated *in vivo*.

It is evident that despite extensive research traditional therapies are less than perfect as at least half of patients develop some form of GVHD. An exciting area of therapeutic potential is the utilisation of immune cells to regulate the unwanted immune responses that result in GVHD whilst sparing the GVT or GVL response. For example, Treg have been shown to suppress GVHD in a number of mouse models (196, 202, 203) which has resulted in Treg being tested in clinical trials for the treatment of GVHD (206, 431). Our own interest lies predominantly with another type of immune cell that has been demonstrated to have immunomodulatory properties; the iNKT cell (230). This interest in iNKT cells as a therapy for GVHD originated in work on the immunosuppressive behaviour of iNKT cells following solid organ transplantation (231-233). Indeed, it has now been shown that iNKT cells decrease the risk and severity of GVHD in some mouse models (238, 408), whilst maintaining the GVT effect (234, 238).

3.2. Aims and hypothesis

To enable us to investigate the ability of iNKT cells to influence GVHD we aimed to set up a model utilising alloreactive TCR-transgenic T cells. This model involves using $CD4^+$ T cells isolated from the TEa line of mice (B6 background; $H2^b$), which specifically recognises a 17-mer peptide of the BALB/c H2IEα-chain presented in the context of $H2IA^b$ (i.e., presented by B6 MHC class II via the indirect pathway) (432). CB6F1 mice (F1 progeny of B6 and BALB/c) endogenously produce and present the H2IEα peptide in the context of $H2IA^b$ enabling recognition by TEa T cells (Figure 3.1). CB6F1 mice are CD45.2, whereas the GVHD-causing TEa T cells are CD45.1 which enables identification of TEa T cells when injected into CB6F1 mice when combined with staining for the TEa TCR Vα chain (Vα2). TEa T cells have previously been shown to be capable of inducing GVHD (433).

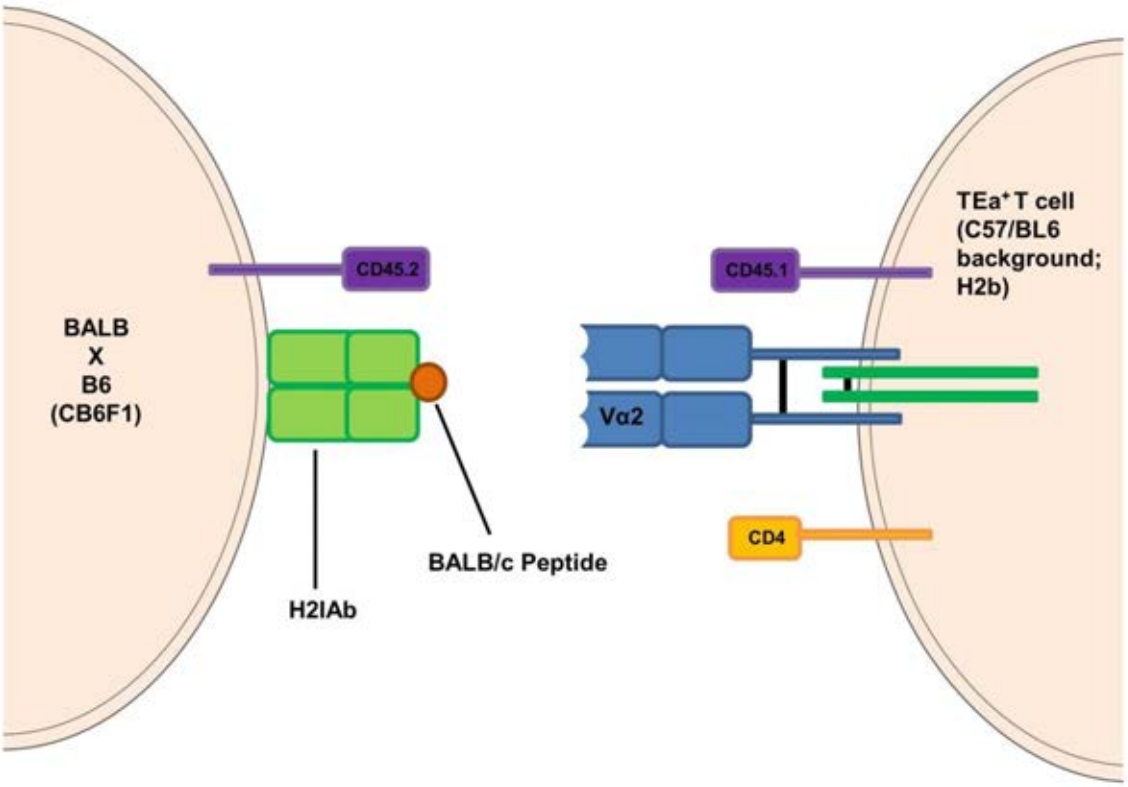

Figure 3.1 Recognition of MHC-peptide complex by TEa T cells Transgenic $CD4^+$ TEa T cells (from B6 background mice; $H2^b$), specifically recognise a 17-mer peptide of the BALB/c H2IEα-chain presented in the context of $H2IA^b$ (i.e. presented by B6 MHC class II via the indirect pathway). CB6F1 mice (F1 progeny of B6 and BALB/c) present this BALB peptides to TEa T cells, causing them to be alloactivated, proliferate and mediate GVHD. TEa T cells express CD45.1, whereas the host cells are $CD45.2^+$. Additionally, they express a Vα2 TCR which further aids their identification.

3.3. Results

3.3.1. TEa T cells induce weight loss, increased clinical signs of GVHD and death following transfer to lethally irradiated CB6F1 mice

We first set-out to establish a model of GVHD where the GVHD-causing alloreactive T cell response could be followed *in vivo*. The model would then be employed to investigate the ability of iNKT cells to suppress GVHD. To this end, CB6F1 mice were lethally irradiated (1100 cGy split-dose) and were given $5x10^6$ B6 TCD BM i.v. on day 0. On day 2, CB6F1 mice received adoptive transfer of $5x10^4$ TEa T cells i.v. and such mice were weighed daily and monitored for the development of clinical signs indicative of the development of GVHD.

Mice that received TCD BM and $5x10^4$ TEa T cells demonstrated significant weight loss compared to control mice that received TCD BM transplant only (Figure 3.2A). This loss of weight correlated with increased clinical scores which suggest that TEa T cells can induce GVHD in this model (Figure 3.2B). 2 out of 3 mice that received TEa T cells were sacrificed 10 days after transplant due to the loss of 20% body weight whereas all control mice survived with no significant weight loss or clinical signs of GVHD.

To assess whether the weight loss observed in mice that received TEa T cells was related to the transfer of such cells, we investigated if TEa T cells were detectable in secondary lymphoid organs at the time of the appearance of symptoms of GVHD. The number of leukocytes and TEa T cells in the spleen and Mesenteric lymph node (MLN) of such mice were evaluated (Figure 3.2). There was an increase in the total number of leukocytes in the MLN compared to BM only controls (Figure 3.2G) and TEa T cells were detectable in the spleen and MLN of diseased mice (Figure 3.2F,

D). This suggests that that the induction of GVHD symptoms was caused by an anti-recipient immune response that required TEa T cells.

The kinetic of GVHD shown in Figure 3.2 was very acute which may impact any attempt to prevent this form of GVHD. Therefore, in order to slow the onset of the disease and increase the window in which it would be possible to manipulate GVHD in future experiments, the experiment was repeated with lower doses of TEa T cells. To this end, CB6F1 mice were lethally irradiated and given $5x10^6$ TCD BM i.v. on day 0 as well as $2.5x10^4$ or $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. Mice were weighed daily and monitored for clinical signs of GVHD. Mice were sacrificed prior to 20% weight loss and/or if the animals' clinical score exceeded that stipulated within the moderate severity limit.

Mice that received either $2.5x10^4$ or $1x10^4$ TEa T cells experienced significant weight loss starting on day 9 compared to controls (Figure 3.3A). Furthermore, by day 10 the mice that had received $2.5x10^4$ had lost significantly more weight than mice injected with $1x10^4$ TEa T cells. Similarly, mice that received $2.5x10^4$ and $1x10^4$ TEa T cells (Figure 3.3B) showed clinical signs of GVHD on day 9 and 10, which correlated with decreased survival compared to controls (Figure 3.3C). However, the survival of the mice that had received $1x10^4$ TEa T cells was only extended by 1 day compared to those that received $2.5x10^4$ (Figure 3.3C).

Given that using $1x10^4$ TEa T cells only increased survival by one day we examined the ability of $5x10^3$, $2.5x10^3$ and $1x10^3$ TEa T cells to induce GVHD. While all groups except those that received $1x10^3$ TEa T cells led to significant weight loss (Figure 3.4A) and all groups induced clinical signs of GVHD (Figure 3.4B), the optimum survival that still resulted in complete penetrance was $1x10^4$ TEa T cells (Figure

3.4C). Therefore, we decided to proceed with a dose of $1x10^4$ TEa T cells in all future experiments.

3.3.2. The onset of the symptoms of GVHD correlates with the presence of TEa T cells in CB6F1 recipients and an increase in proinflammatory cells

In order to examine the various immune cells that participate in the induction of GVHD in this model, leukocytes were isolated from the spleens and MLN of mice that received $2.5x10^4$ or $1x10^4$ or no TEa T cells and analysed by flow cytometry.

An examination of absolute cell numbers revealed that there was a significant increase in the total number of leukocytes in the spleen and MLN in mice that received $2.5x10^4$ and an increase in total leukocyte numbers in the MLN of the mice that received $1x10^4$ or $2.5x10^4$ TEa T cells (Figure 3.5A and B). Similarly, TEa T cells were detectable in the spleen of both experimental groups compared to control mice (Figure 3.5C). There was no difference in myeloid cells (MC) ($Gr1^+CD11b^+$) or $Ly6C^+$cells (monocytes (Mo)) and $Ly6G^+$ cells (polymorphonuclear (PMN) leukocytes) in both the MLN (Figure 3.5D) and spleen (Figure 3.5E). The number of DCs was increased in both experimental groups in the MLN (Figure 3.5F) and spleen (Figure 3.5G), which is in line with observations that DCs are important for GVHD initiation (434). Interestingly, B cells were increased in the MLN (with $1x10^4$ TEa T cells) but not the spleen (Figure 3.5H and I). Additionally, there was no increase in the number of NK or iNKT cells (Figure 3.5C).

The changes in immune cell populations during GVHD were further investigated in terms of relative cell frequencies in a given lymphoid compartment. An examination of cells by flow cytometry showed a significant increase in the frequency of TEa T cells and myeloid cells (Mo and PMN). This is expected as alloreactive T cells in the spleen are known to be associated with the onset of GVHD and neutrophils have been shown to increase in frequency in the spleen in GVHD (435).There was a decrease in the frequency of NK cells, DCs, iNKT cells and B cells in the spleen in mice i.v. injected with $1x10^4$ TEa T cells, compared to controls (Figure 3.6A). As DCs were decreased in absolute number, the decrease in the frequency of DCs must be due to the expansion of other populations. Furthermore, the majority of myeloid cells were Mo in the group that received $1x10^4$ TEa T cells compared to in the control group, in which there was no difference between the frequency of Mo and PMN (Figure 3.6A). In contrast in the MLN, there were decreased frequencies of B cells, myeloid cells both Mo and PMN (Figure 3.6B) in group that received $1x10^4$ TEa T cells compared to in the control group. Furthermore, in contrast to what was observed in the spleen, the majority myeloid cells were Mo in the control group (Figure 3.6B).

Taken together, these findings show that in addition to TEa T cells being detectable in the spleen and MLN (as shown in the previous experiment), the onset of symptoms of GVHD is also associated with an expansion of DCs which are responsible for presenting alloantigen to TEa T cells. Perturbations in other immune cell populations such as an increase in the frequency of MC in the spleen also occur which is in line with what is expected in inflammation. However, MC were decreased in frequency in the MLN in mice that received TEa T cells, suggesting that as GVHD

is a complex multi-organ disease the immune response is not always uniform at all anatomical locations at disease onset.

To assess whether the TEa T cells and myeloid cells observed in the spleen by flow cytometry were also detectable by confocal microscopy spleens from mice that received $1x10^4$ TEa T cells or no TEa T cells were examined. Sections were stained for CD45.1, which is expressed by TEa T cells only, and Gr1, which is expressed by myeloid cells. TEa T cells could be detected in the spleens of mice that received $1x10^4$ TEa T cells (Figure 3.7B) cells but not controls (Figure 3.7A). The TEa T cells in the mice that received $1x10^4$ TEa T cells appeared to be clustered in what may be a germinal centre with $Gr1^+$ cells located in the surrounding tissue (Figure 3.7D). This confirmed that TEa T cells are detectable in the spleen as was observed from the flow cytometry analysis.

3.3.3. Examination of different immune cells present 2 weeks post BM transplant

In order to investigate the balance of donor and recipient cells are present two weeks post-transplant, and hence what cells might be available to interact with AT iNKT cells, $CD45.1^+$ TCD BM was transferred into lethally irradiation CB6F1 recipients. Immune cells were isolated from the spleen on day 14 and analysed by flow cytometry.

An examination of immune cell frequency in the spleen revealed a decrease in iNKT cells in chimeras compared to CB6F1 WT controls (Figure 3.8A). In addition, there were more recipient iNKT cells than donor iNKT cells in terms of frequency,

suggesting a radioresistant population of iNKT cells (Figure 3.8A). The overall frequency of recipient NK cells was also reduced in the chimeras compared to WT controls (Figure 3.8B), as was $CD4^+$ and $CD8^+$ T cell frequencies (Figure 3.8D). In contrast, there was an increase in overall frequency and donor frequency of $Gr1^+$ cells (Figure 3.8C).

3.3.4. GVHD caused by TEa T cells involves recognition of alloantigen and is not a product of homeostatic expansion

To ensure that GVHD in this model was caused by recognition of alloantigen and not due to the homeostatic activation and expansion of the TEa T cells, $1x10^4$ TEa T cells were injected into either CB6F1 ($H2^{b/d}$) or B6 ($H2^b$; syngeneic) recipients, both of whom had received TBI and TCD BM. Mice were culled on day 8 and the activation and expansion of TEa T cells in different tissues analysed. TEa T cells were found to have increased in frequency and number in the spleen and MLN of CB6F1 recipients compared to being barely detectable in syngeneic B6 mice (Figure 3.9A-D). In addition, the TEa T cell frequency and number also dramatically increased in 2 GVHD target tissues namely, the SI and Liver (frequency only) compared to syngeneic controls (Figure 3.9E-G). A cohort of B6 (syngeneic) mice that received TCD BM and TEa T cells were followed for 50 days post BM transplantation (data not shown). No symptoms of GVHD were recorded, demonstrating that in the absence of alloantigen TEa T cells do not induce GVHD. Together these data demonstrate that TEa only expand and infiltrate GVHD target tissues when the recipient expresses the cognate alloantigen.

3.3.5. Expansion of alloreactive TEa T cells in CB6F1 recipients occurs concomitantly with the expansion of other inflammatory cells such as MC and DCs, as well as perturbations in other populations, in a location dependent manner

Mice that had received TCD BM and TEa T cells were culled on day 8, prior to the expected earliest onset of disease, to enable all mice to be compared at the same time point, and in order that we could investigate which cells were contributing to GVHD. An examination of immune cell frequency and absolute numbers showed an increase in total $CD4^+$ T cells and donor BM derived $CD4^+$ T cells in the spleen and MLN of mice with GVHD (Figure 3.10A-D). In addition there was an increase in total $CD4^+$ T cell frequency and donor $CD4^+$ T cell frequency in the SI and Liver (Figure 3.10E, G). Furthermore, the predominant population (in terms of frequency) of $CD4^+$ T cells was host derived in all experiment groups in the MLN, SI and liver as well as the group that received BM only in the spleen (Figure 3.10 A, C, E, G). These observations are consistent with previous studies that have shown that host residual radio resistant T cells can be identified post BMT (436-439).

In contrast, the only difference in B cells observed between mice that received TEa T cells or BMT only was that the frequency of donor cells in the MLN was decreased in the GVHD group compared to BMT controls (Figure 3.11C) and a decrease in the number of B cells in the SI in both the donor and host compartments in GVHD mice (Figure 3.11F). The observed decreases in B cells are in keeping with previous studies in humans and mice which suggest that B cell generation from B cell progenitors in the BM is delayed in GVHD (440-442). In the spleen, the dominant population in both experimental groups was recipient derived (Figure 3.11A, B), while in the MLN there was only an increase in recipient cells in the group that received BM

only (Figure 3.11C, D). Similarly, in the SI the dominant population was recipient derived in the disease group in terms of frequency (Figure 3.11E), whereas in terms of numbers the recipient population only dominated in the group that received BM only (Figure 3.11F). Collectively, these data suggest that day 8 is too early a time point for B cells to have reconstituted from the BM.

There was no difference in NK cells between the GVHD and control group in the spleen in terms of cell number and in the Liver in terms of cell frequency (Figure 3.12 A, B, G). NK cells were predominantly of donor origin in the spleen and liver in the mice which were in the disease group and additionally NK cells were mainly donor cells in the control mice in the liver (Figure 3.12 A, B, G) cells could not be convincingly detected in the MLN and SI. The tendency towards NK cells being predominantly of donor origin in mice that have GVHD may be due to the inflammatory conditions of the disease contributing to NK cell expansion.

Donor iNKT cells were increased in the mice that received TEa T cells in the spleen compared to BMT controls in terms of frequency and absolute number (Figure 3.12C, D) and there was an decrease in the overall iNKT cell frequency in the liver of GVHD mice compared to BM controls, as well as a decrease in recipient derived cells in disease (Figure 3.12G). However, iNKT cells numbers and frequencies were similar in all groups in the SI (Figure 3.12E, F) and were not detectable in the MLN. The increase of iNKT cells in the spleen in mice with GVHD may be due to the inflammatory conditions leading to production of inflammatory cytokines leading the maintenance and proliferation of the donor iNKT cells. It is possible that at a later time point iNKT cells would also be observed to expand in other anatomical locations.

In terms of the provenance of the iNKT cells, recipient cells were the dominant population in both experimental groups in the spleen (Figure 3.12C, D), which was also true regarding the frequencies in the liver (Figure 3.12G). In contrast in the SI, the only difference observed was an increase in recipient iNKT cells in the GVHD group. The dominance of recipient iNKT cells observed is in keeping with the observation that radioresistant iNKT cells remain post-BMT, due to higher levels of anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) (443).

Upon examination of MC in the MLN we observed a decrease in the frequency of MC in disease (Figure 3.13C), while in the SI there was an increase in total MC frequency and in total and recipient PMN frequency in disease (Figure 3.13E). (444, 445). Regarding the provenance of the inflammatory myeloid cells in the spleen, MLN, SI and Liver recipient cells dominated in the Mo and PMN compartments in all locations (Figure 3.13A, C, E, G).This indicates that this time point is too early to observe full reconstitution by donor cells. Furthermore in terms of numbers, there was an increase in the number of recipient PMN and Mo compared to donor cells in the spleens of both experimental groups (Figure 3.13B) and an increase in the number of recipient PMN in both groups in the SI (Figure 3.13F), and neither subset dominated in the MLN (Figure 3.13D). In addition, in the group that received TEa T cells there is a higher frequency of PMN than Mo in the MLN (Figure 3.13C) and a higher frequency and number of PMN than Mo in the SI (Figure 3.13E).

Mice with GVHD were found to have an increased frequency of donor DCs in the spleen compared to BMT controls (Figure 3.14A), however this was not observed in the MLN, where there was an increase in the number of recipient DCs in disease mice compared to controls (Figure 3.14C). In contrast, a significant increase in the

frequency and number of such cells was seen in non-disease CB6F1 mice, as well as recipient derived DCs in the SI (Figure 3.14E, F). The lack of expansion of donor DCs may suggest that the time point was too early to see an increase in donor DCs. Further examination revealed that in terms of frequency, in both experimental groups recipient DCs were the dominant population compared to donor cells in all locations (Figure 3.14A, C, E, G). This is in line with what was observed with other innate cells such as Mo, again suggesting that the innate compartment is not fully reconstituted by day 8.

Collectively, these data demonstrate that although there was significant increase in TEa T cells, the donor cells had not fully engrafted by day 8. Furthermore, these data make evident the immunological context into which we planned to transfer iNKT cells in later experiments, which may have an influence on their response.

3.3.6. Signs of damage can be observed in the liver and SI of mice with GVHD

In order to investigate if the increased weight loss and clinical scores and expansion of TEa T cells and other inflammatory cells correlated with tissue damage in target organs of GVHD we harvested livers and SI samples to investigate if histological signs of GVHD were present. Liver lobes and small segments of the SI were harvested on day 8 just before the onset of disease and investigated using histopathlogy. In the SI, apoptotic crypt cells were identified in the SI of CB6F1 mice that received B6 TCD BM and TEa T cells, but not in BM controls (Figure 3.15A, B). Apoptotic crypt cells are a known sign of intestinal GVHD (446). As this was an early

time point, we did not expect to see extensive crypt damage, to investigate the occurrence of crypt damage it would be necessary to look after disease onset.

In the liver bile duct damage and fibrotic tracts were seen in CB6F1 mice that received B6 TCD BM and TEa T cells, but not in BM controls. These features are signs of liver GVHD (3.15C) (446). Furthermore, perivascular infiltrates, a known sign of liver GVHD, were also observed in mice that had ongoing GVHD (Figure 3.15D)(446). These histopathological signs of GVHD corroborate our previous findings regarding clinical scores, weight loss and infiltration of the SI and liver with TEa T cells.

3.4. Discussion

We have established a robust model of GVHD involving weight loss, clinical symptoms and damage to the liver and GI tract. Zhang *et al.* previously showed that $5x10^4$ TEa T cells can induce lethal GVHD (433). Therefore, in the initial pilot study $5x10^4$ TEa T cells were administered (Figure 3.2). The mice required sacrifice due to weight loss and or clinical symptoms consistent with GVHD on day 10. The expansion of TEa T cells in the spleen and MLN of mice with GVHD was easily detectible using flow cytometry (Figure 3.2F, H). TEa T cells were also identified in the spleen from mice that received 1 $x10^4$ TEa T cells as well as by confocal microscopy in the spleen (Figures 3.5, 3.6 and 3.7). In terms of timing, the results were broadly consistent with what has previously been reported in the literature; disease onset was on day 20 for Zhang *et al.* and Day 14 for Koyama *et al.* (424, 433).

However we felt that the rapid disease progression may hamper the ability of iNKT cells to suppress GVHD, but we found that lower doses also induced GVHD with a similar kinetic (Figures 3.3 and 3.4). One factor that might be responsible for such aggressive GVHD was that purified TEa T cells were injected without any accessory cells such as Treg that may harbour immunomodulatory potential and slow the course of the disease. Treg would be present as contaminants in most T cell mediated models of GVHD. This was also the case in other labs that use this model, and therefore this model may be reflective of an extremely rapid form of acute GVHD (424, 433). Furthermore, it is the very fact that TEa T cells do not have the caveat of contaminating regulatory cells or indeed non-alloreactive cells that we chose this model. Indeed, this model provides us with the ability to stringently test the capacity

of iNKT cells to suppress GVHD, and assign those properties to iNKT cells specifically.

Another possible factor which may have led to rapid death by GVHD in this model is that we are utilising TCR-transgenic T cells which recognise a specific alloantigen, rather than polyclonal T cells which are used in most models. However, even when we titrated the TEa T cells down to numbers as low as $1x10^3$ (Figure 3.4) there was a limited impact on disease suggesting that lowering the numbers of such cells does not significantly impact the kinetics of the disease.

Another possible explanationis is that excessive inflammation caused by the irradiation may cause GVHD to progress rapidly. All experiments conducted consisted of 2 doses of 550 cGy as this is what has been reported in the literature to result in lethal irradiation in CB6F1 mice (424). However, it is important to note that since variation in environmental pathogens due to different animal providers and facilities can affect GVHD it might be possible to slow progression by decreasing the irradiation dose (167). However, since UK regulations prohibit us testing to see if lower doses can still lethally irradiate a mouse, we decided to progress with the known and generally used lethal dose for CB6F1 mice.

Disease onset occurs concomitantly with the detection of TEa T cells in the spleen and MLN (Figure 3.2) and we have also analysed GVHD mice just prior to disease onset and detected the expansion of TEa T cells in the spleen and MLN as well as in two target organs, namely the SI and the liver (Figure 3.9). This data argue that GVHD results from a genuine alloresponse in this model and is not merely a product

of homeostatic expansion of the TEa T cells as TEa T cells did not expand or cause disease in syngeneic controls (Figure 3.9, and data not shown).

The decrease in the frequency of MC observed in the MLN may be due to them being located elsewhere at this time point, indeed neutrophils have been shown to infiltrate the intestine as a result of damage from irradiation and migrate to the MLN at a later point where they act as APCs (444). Indeed, there was an increase in total MC frequency and in total and recipient PMN frequency in the SI of mice that received TEa T cells on day 8 (Figure 3.13E). This observation is in line with what is expected as one would expect to see infiltration of immune cells during an inflammatory response more specifically during GVHD. Furthermore, in the group that received TEa T cells there is a higher frequency of PMN than Mo in the MLN (Figure 3.13C) and there was a higher frequency and number of PMN than Mo in the SI (Figure 3.13E). PMN are predominantly made up of neutrophils which are recruited to the intestinal during GVHD initiation when intestinal bacteria release due to loss of barrier integrity leads to their recruitment (445). Such cells then go on to contribute to tissue damage using ROS. In addition these cells are predominantly of donor origin which is in line with what is seen in the literature (445). Perhaps if we had looked in the SI after disease onset this increase would also have occurred in terms of absolute numbers. After disease onset, there was an increase in the frequency of MC (both MC and Mo) (Figure 3.6) in the spleen of diseased mice compared to controls which is not observed at Day 8. This is expected as the response is thought to affect the intestine first and then then become more wide-spread (151, 153, 172, 421).

Another interesting observation is that $CD4^+$ T cells are predominantly of recipient origin on day 8 (Figure 3.10). This is as expected as it is well known that there is a residual population of T cells following lethal irradiation (436-439). Furthermore, there is an increase in the frequency of donor BM derived $CD4^+$ T cells in the spleens and MLN of mice with GVHD compared to syngeneic controls, perhaps indicative of TEa T cell derived IL-2 causing such cells to expand.

It is also interesting to note that there was no change in the frequency of DCs in the spleen or MLN at day 8 (Figure 3.14A, C), whereas there was a decrease in the frequency of DCs in the spleen of GVHD mice at disease onset (Figure 3.7A). This is likely due to timing as one would expect an increase in inflammatory mediators after GVHD onset. Further examining the DCs at the day 8 time point, in the spleen we observed an increased frequency of donor DCs in the spleen compared to BMT controls (Figure 3.14A). In contrast the MLN, where there was no change in frequency between GVHD and controls, however an increase in the number of recipient DCs was observed in diseased mice (Figure 3.14B). Interestingly, a significant increase in the frequency of such cells was seen in non-disease CB6F1 mice, as well as recipient derived DCs in the SI (Figure 3.14E). This decrease in DCs in the SI in disease may be due to the killing of donor DCs by TEa T cells in the SI, however, this is unlikely due to the fact that $CD4^+$ T cells have poor cytotoxic function. A more likely hypothesis is that this observation is due to a perturbation of immune trafficking causing DCs to be directed to the MLN which is the priming LN for the SI and indeed there is an increase in the number of DCs in the MLN (Figure 3.14B).

It is interesting to note that there is a radioresistant population of iNKT cells present in BM chimeras (Figures 3.8 and 3.12) and that population is increased in the spleen in GVHD compared to control mice as well as being the dominant population of iNKT cells in terms of numbers in the spleen and SI of GVHD mice (Figure 3.12C, D, F). This is in line with what has been shown in humans (447).This means that activating AT iNKT cells in future experiments, will also activate these residual cells and unlike the donor cells this cannot be circumvented by using KO BM.

We also observed histological sings of damage in the SI, namely apoptotic crypt cells, which are a classic sign of gut GVHD (Figure 3.15) (448). In addition we observed liver cirrhosis, bile duct disruption and inflammatory infiltrates which are all signs of liver GVHD (Figure 3.15) (448). The degree of damage was not severe, however as the samples were taken just prior to disease onset in order to compare organs harvested contemporaneous and not according to survival. It is probably that if we examined mice at the height of disease the damage would be more marked. In addition, it would be interesting to observe the presence of GVHD associated cytokine such as IL-1β and TNF-α using RTqPCR in the future.

In conclusion we have established a model of GVHD in which we can track allogeneic transgenic TEa T cells *in vivo*. These cells induce weight loss and clinical symptoms of disease, which appear concomitantly with the expansion of TEa T cells and GVHD associated perturbations in other immune populations. In addition, TEa T cells are easily identifiable in both lymphoid and non-lymphoid tissue where they expand following alloantigen recognition and facilitate damage in at least two target organs, namely the gut and liver. Therefore, this model provides us with a critical tool to investigate the ability of iNKT cells to influence GVHD *in vivo*.

3.5. Figures

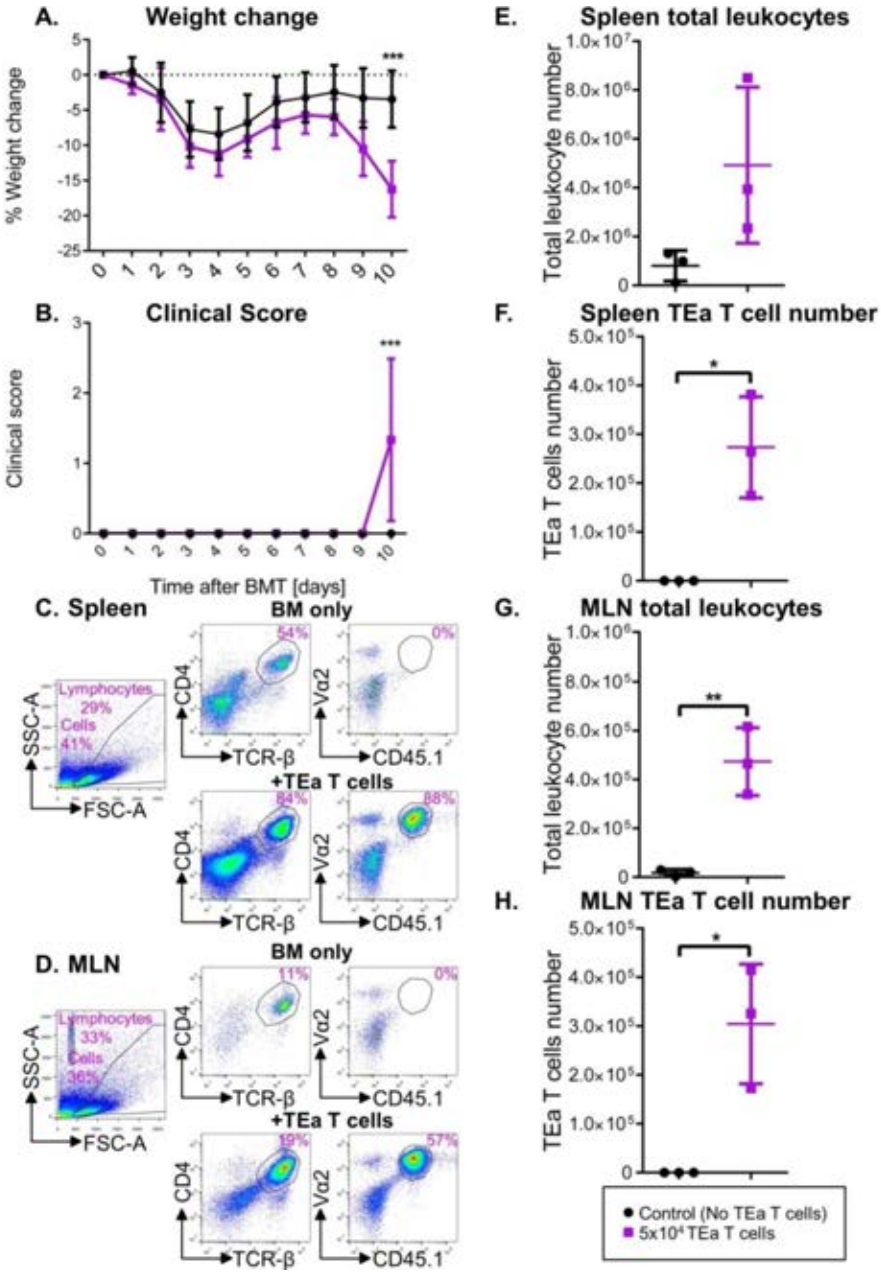

Figure 3.2 The onset of GVHD like symptoms occurs concomitantly with TEa T cell expansion in the spleen and MLN CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. On day 2, $5x10^4$ TEa T cells were administered via i.v. injection. Control mice received irradiation and BM transplant only. Mice were assessed for signs consistent with GVHD by monitoring (A) weight and (B) clinical symptoms. Mice were sacrificed prior to 20% weight loss and spleen and MLN derived leukocytes analysed by flow cytometry. Results are expressed as absolute numbers of leukocytes gated on (C, D, E, G) live cells and (C, D , F, H) absolute number of TEa T cells (gated on lymphocytes, live cells, TCR-β and CD4 and CD45.1 and Vα2 in the spleen and the MLN). Results are analysed using repeated measure two-way ANOVA with post hoc Tukey's comparison for weight loss and clinical scores, and unpaired t-tests were used for cell numbers. Data shown are mean ± SD (n=3 mice). *p<0.05, **p<0.01, versus control. See Appendix V for gating strategy.

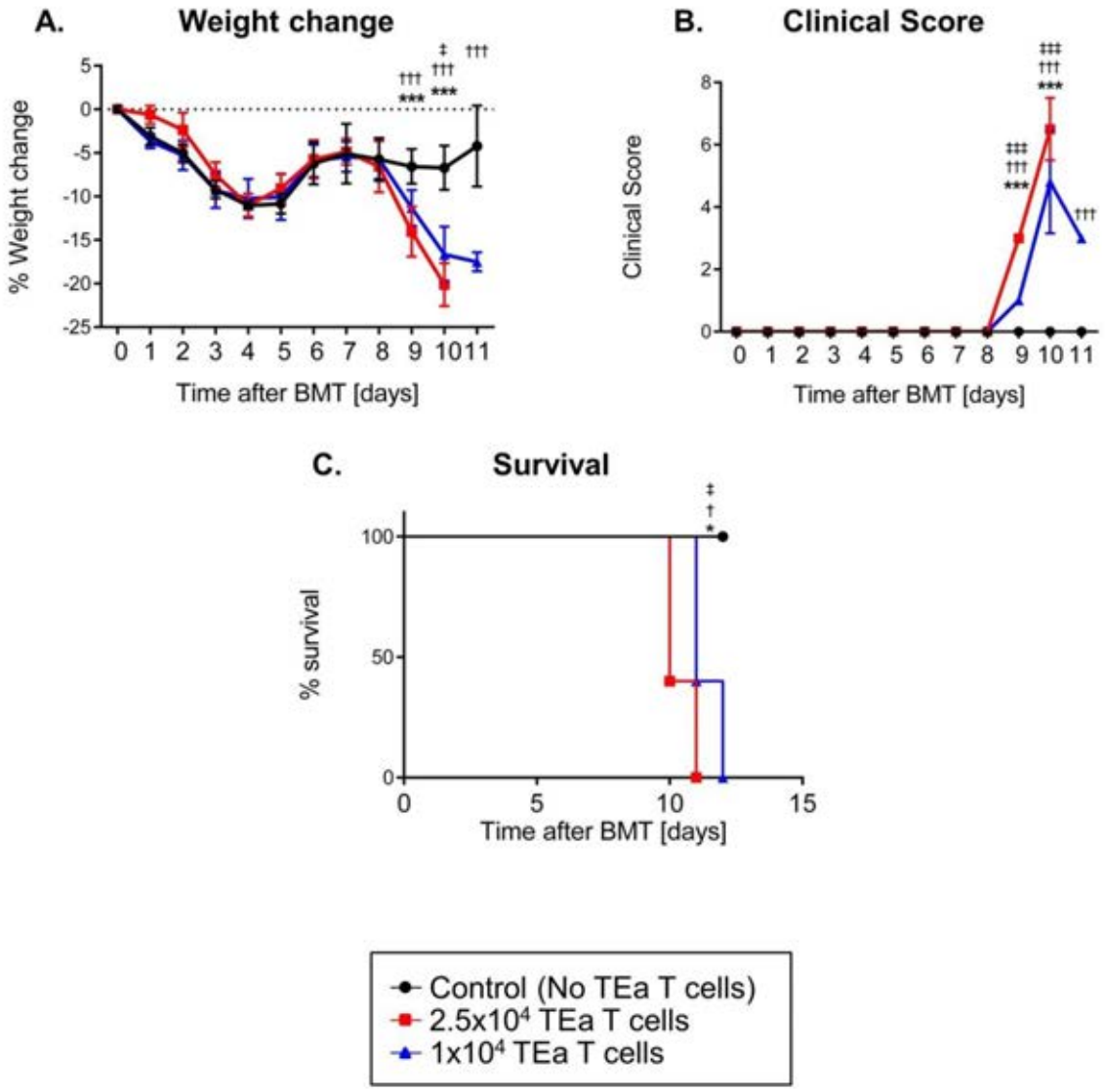

Figure 3.3 TEa T cells induce weight loss, increased clinical signs of GVHD and death CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received $2.5x10^4$ or $1x10^4$ or no TEa T cells via i.v. injection, on day 2. Mice were assessed for GVHD by monitoring (A) weight and (B) clinical scores consistent with GVHD. (C) Survival curve, mice were sacrificed before they exceeded 20% weight loss. Data shown are expressed as mean ± SD (n=5 mice per group). Results are analysed using repeated measure two-way ANOVA with post hoc Tukey's comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<005, **p<0.01, ***p<0.001.
*=$1x10^4$TEa T cells vs. BM only
†=$2.5x10^4$ TEa T cells vs. BM only.
‡ $1x10^4$TEa T cells vs 2.5x104 TEa T cells.

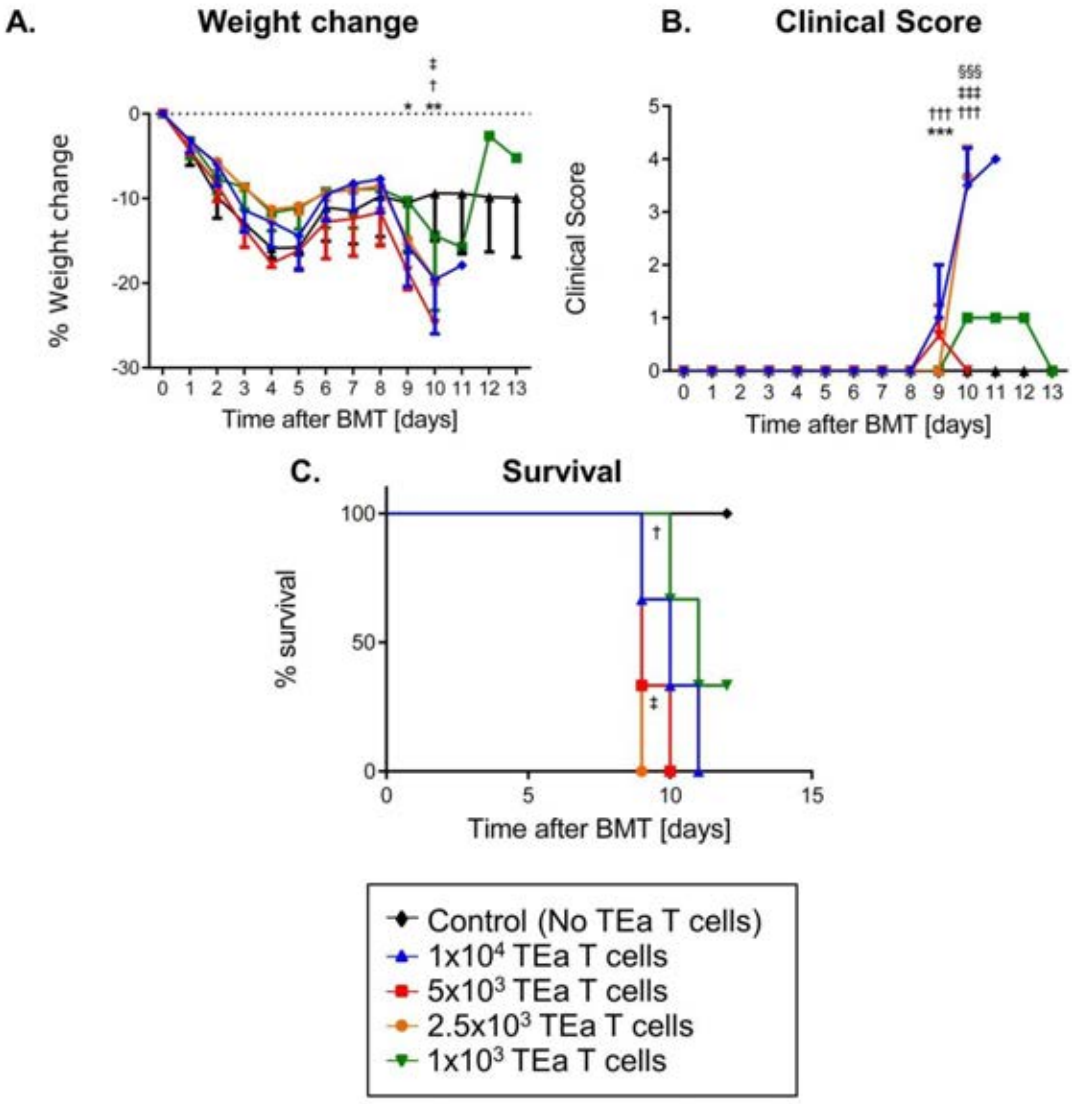

Figure 3.4 There is no significant change in weight loss, clinical score or survival with doses as low as 1x10^3 TEa T cells CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received 1x10^4, 5x10^3, 2.5x10^3, 1x10^3 or no TEa T cells via i.v. injection, on day 2. (A) Weight curves of mice show no significant difference as in the case with (B) clinical scores and (C) survival. Results are presented as the mean frequency of cells ± SD (n=2-3 mice). Results are analysed using repeated measure two-way ANOVA with post hoc Tukey's comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival.*p<0.05, **p<0.01 ***p<0.001, versus control.
*= 5x10^3 vs. no TEa T cells
†= 1x10^4 vs. no TEa T cells ,
‡= 2.5x10^3 vs. no TEa T cells,
§= 1x10^3 vs. no TEa T cells.

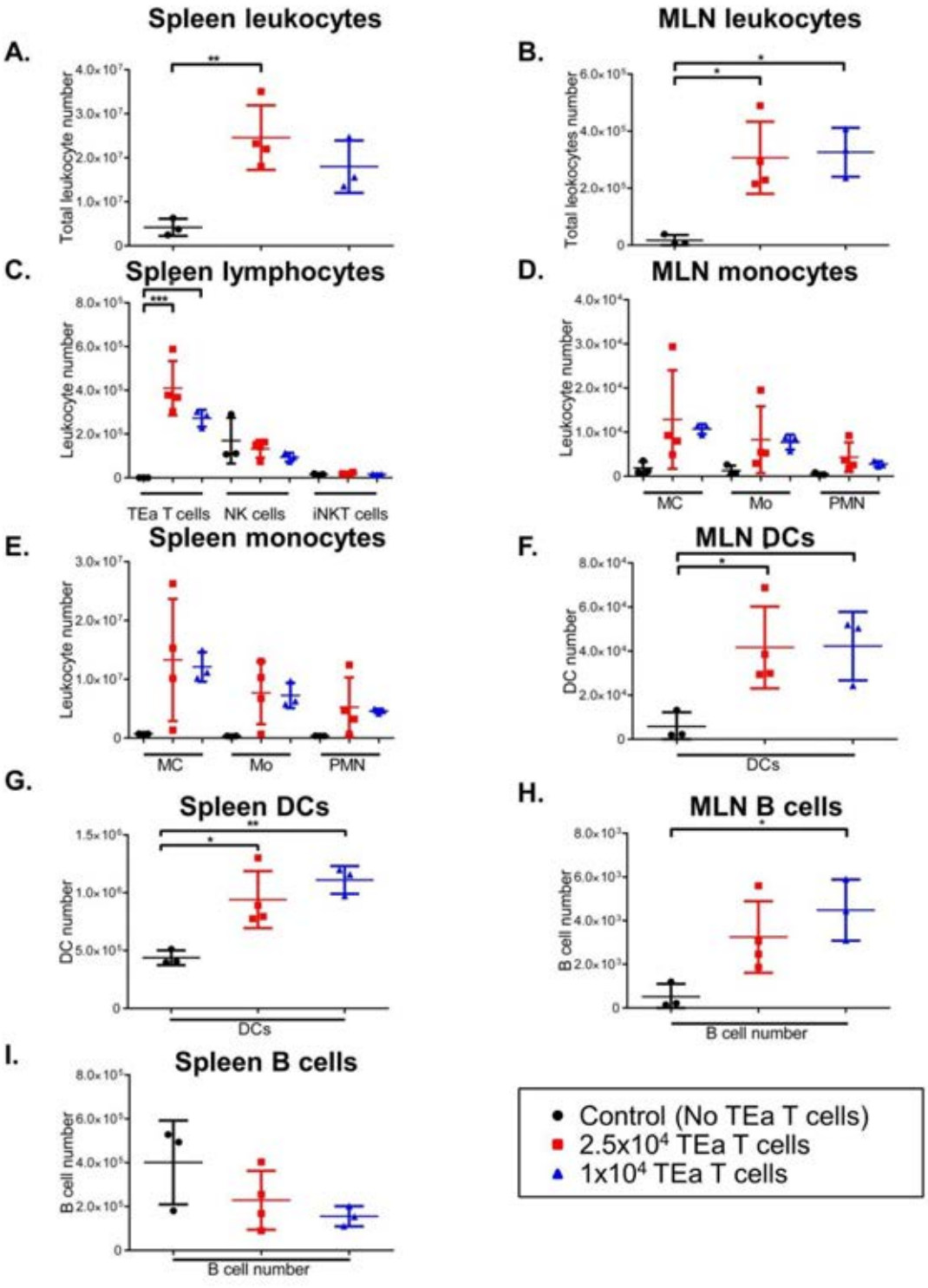
A.
Spleen leukocytes
Total leukocyte number
B.
MLN leukocytes
Total leokocytes number
C.
Spleen lymphocytes
Leukocyte number
TEa T cells
NK cells
iNKT cells
D.
MLN monocytes
Leukocyte number
MC
Mo
PMN
E.
Spleen monocytes
Leukocyte number
MC
Mo
PMN
F.
MLN DCs
DC number
DCs
G.
Spleen DCs
DC number
DCs
H.
MLN B cells
B cell number
B cell number
I.
Spleen B cells
B cell number
B cell number
Control (No TEa T cells)
2.5x10⁴ TEa T cells
1x10⁴ TEa T cells

Figure 3.5 GVHD correlates with the presence of TEa T cells and expanded numbers of DCs and B cells in GVHD CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received $2.5x10^4$ or $1x10^4$ or no TEa T cells via i.v. injection, on day 2. (A, B) Live leukocytes, were subdivided into (C) TEa T cells (TCR-β^+, $CD4^+$, $CD45.1^+V\alpha2^+$), NK cells ($NK1.1^+$TCR-β^-) and iNKT (TCR-β^+PBS57 CD1d-$tetramer^+$) cells in the spleen and myeloid cells (MC) ($Gr1^+CD11b^+$ cells), monocytes (Mo) ($Gr1^+$ $CD11b^+Ly6C^+$) and polymorphonuclear (PMN) leukocytes ($Gr1^+$ $CD11b^+Ly6G^+$) in (D) the MLN and (E) the spleen, DCs (MHC class II^+CD11C^+) in (F) the MLN and (G) the spleen and finally B cells ($CD19^+$) in (H) the MLN and (I) the spleen. Results are analysed using one-way ANOVAs. Results are presented as the mean number of cells ± SD (n=3-4 mice). *p<0.05, **p<0.01 ***p<0.001. See Appendix V for gating strategy.

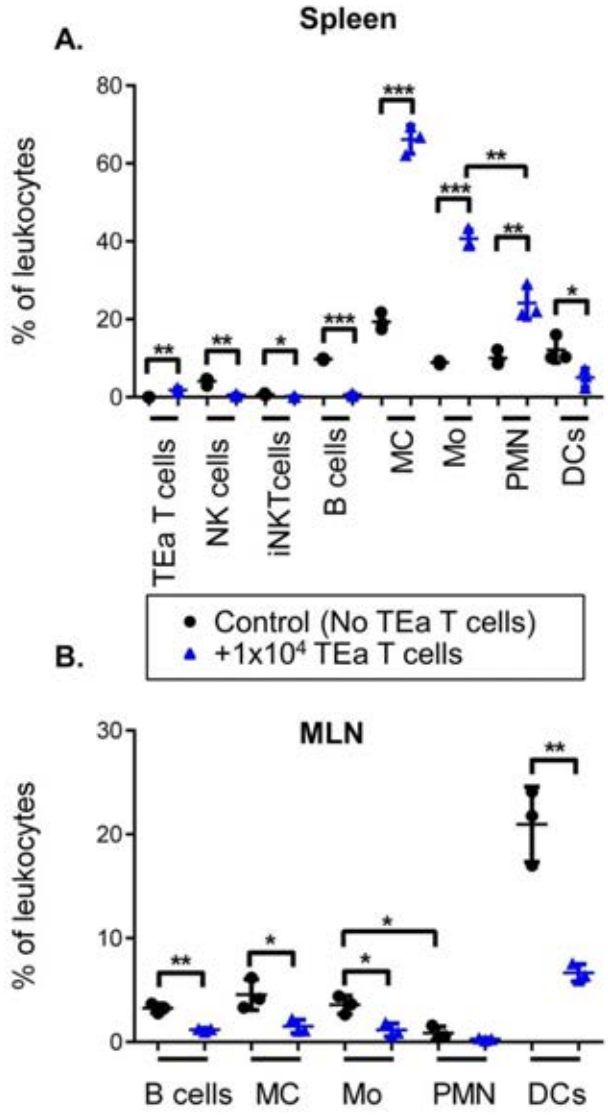

Figure 3.6 The presence of TEa T cells correlates with an increase in the frequency of inflammatory monocytes cells in the spleen in GVHD and there is a decreased frequency of B cells and inflammatory monocytes in the MLN in GVHD CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received $1x10^4$ or no TEa T cells via i.v. injection, on day 2. (A, B) Cell frequencies mice i.v. injected with $1x10^4$ compared to controls or no TEa T cells. Live leukocytes were subdivided into TEa T cells (TCR-β,CD4, CD45.1$^+$ and Vα2$^+$), NK cells (NK1.1$^+$TCR-β$^-$) and iNKT (TCR-β$^+$PBS57 CD1d-tetramer$^+$) cells in (A) the spleen. B cells (CD19$^+$), myeloid cells (MC) (Gr1$^+$ CD11b$^+$), monocytes (Gr1$^+$ CD11b$^+$Ly6C$^+$), polymorphonuclear (PMN) leukocytes (Gr1$^+$ CD11b$^+$Ly6G$^+$) and DCs in (A) the spleen, and (B) the MLN. Results are analysed using unpaired t-tests. Results are presented as the mean frequency of cells ± SD (n=3-4 mice).*p<0.05, **p<0.01 ***p<0.001. See Appendix V for gating strategy.

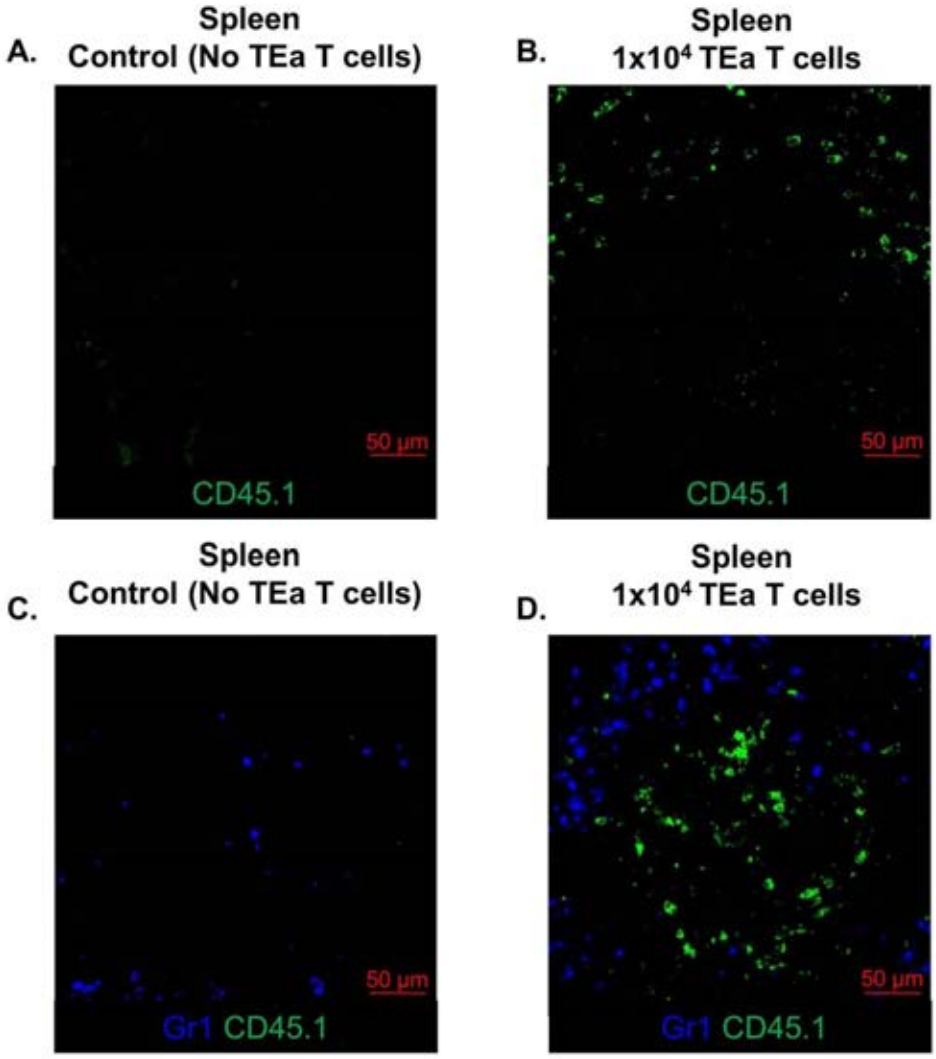

Figure 3.7 TEa T cells and Gr1$^+$ cells can be detected in the spleens of mice with GVHD CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice did or did not receive 1x10^4 TEa T cells via i.v. injection, on day 2. Spleens were sectioned and stained for CD45.1 (Green), Gr1 (Blue) and DAPI (not shown). Images showing CD45.1 in (A) Control mouse spleen (B) Mouse with 1x10^4 TEa T cells mice. Images showing CD45.1 and Gr1 in (C) Control mouse spleen and (D) Mouse with 1x10^4 TEa T cells mice, with suspected germinal centre. Images are representative images showing spleens n=3 mice per group.

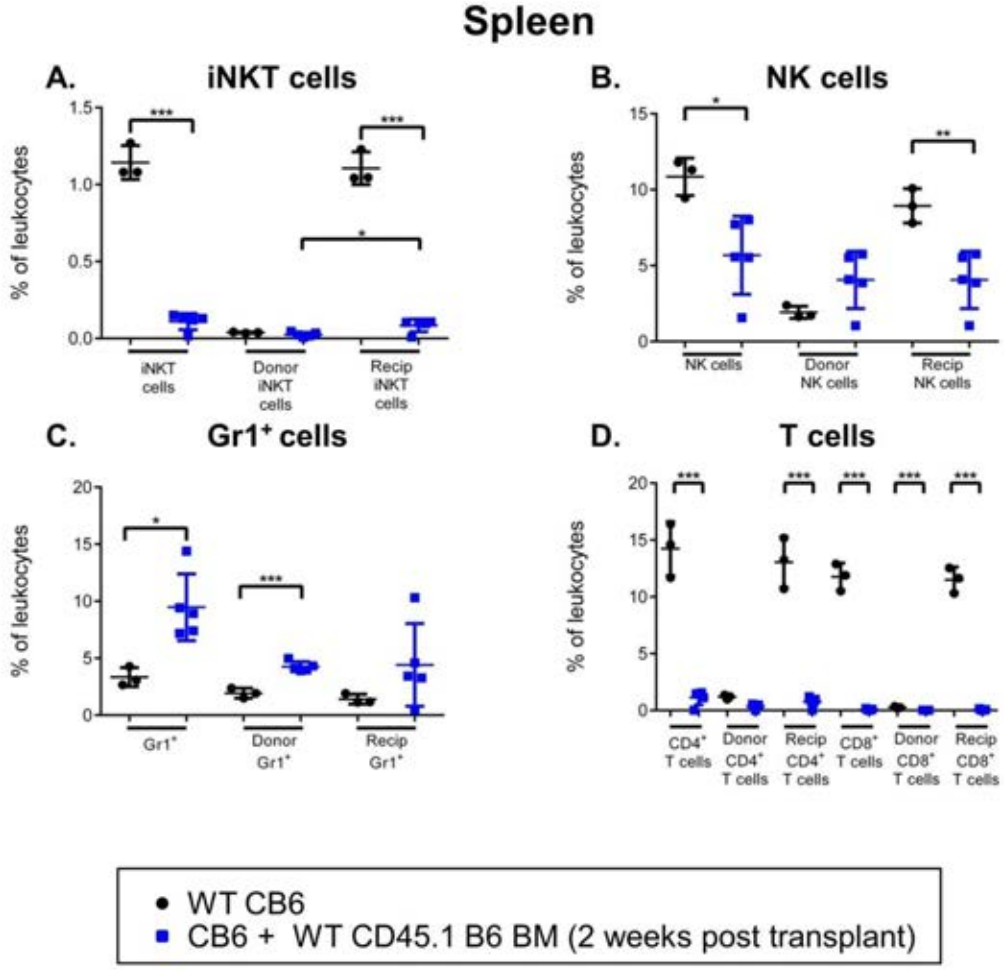

Figure 3.8 iNKT frequencies decrease post BM transplant, however a population of host residual radioresistant iNKT cells can be identified along with an increase in Gr1$^+$ cells and a decrease in NK cells and T cells 2-weeks post BMT CB6F1 mice were lethally irradiated and given 5x10^6 TCD CD45.1 B6 BM cells i.v. on day 0. Mice were harvested on Day 14 to assess the extent of reconstitution. Live cells were pre-gated on lymphocytes, live/dead and CD45 expression and further divided into (A) iNKT cells (TCR-β$^+$PBS57 CD1d-tetramer$^+$), (B) NK cells (NK1.1$^+$TCR-β$^-$) (C) Gr1$^+$ (Gr1$^+$), (D) CD4$^+$ T cells (TCR-β$^+$, CD4$^+$) and CD8$^+$T cells (TCR-β$^+$, CD8$^+$). Donor cells were identified by CD45.1 expression as determined by a CD45.1 B6 control. *p<0.05, **p<0.01 ***p<0.001. Results are analysed using unpaired t-tests. Results are presented as mean ± SD, n=5 transplanted mice versus 3 WT controls. See Appendix V for gating strategy.

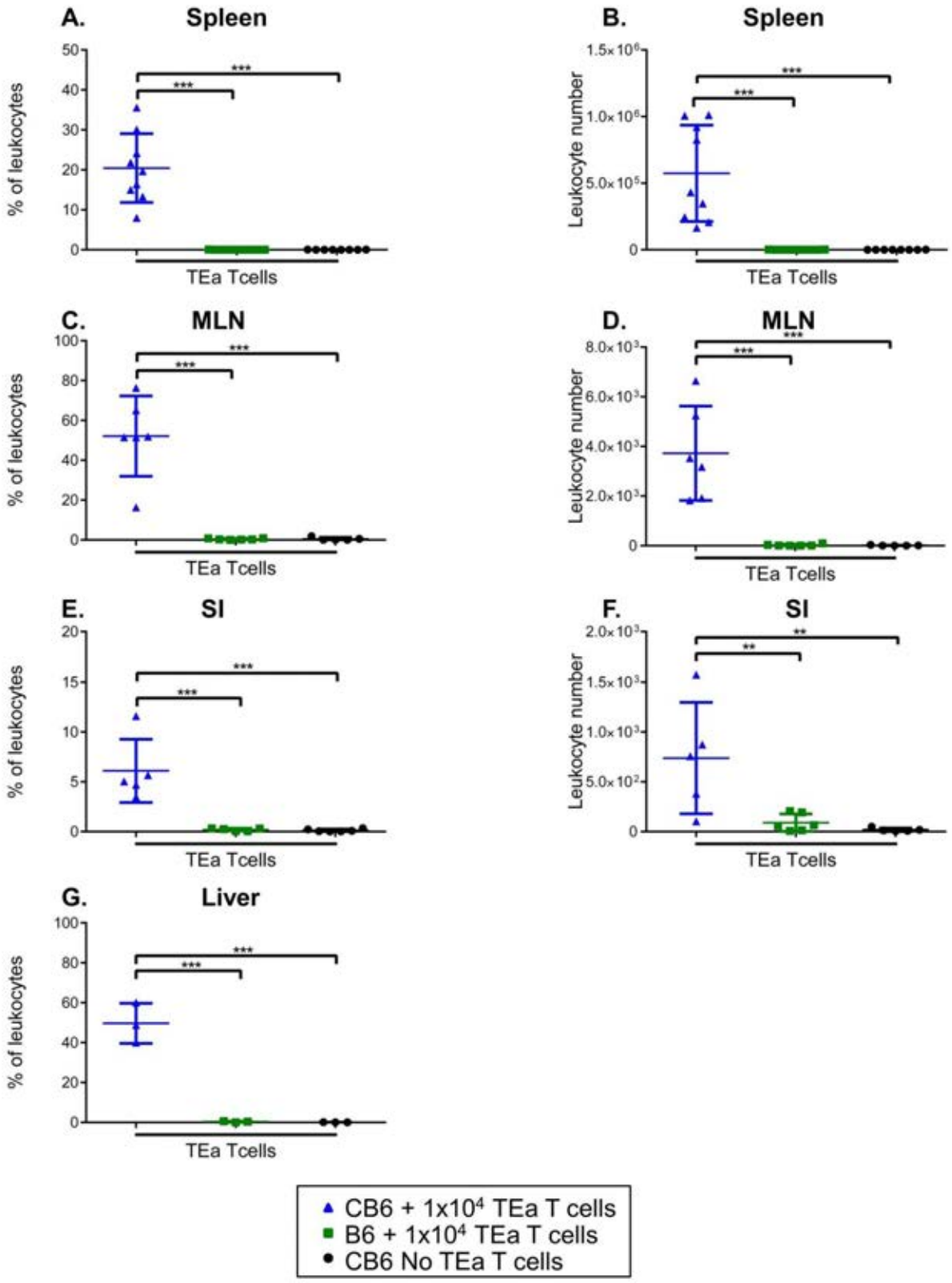
A.
Spleen
% of leukocytes
50
40
30
20
10
0
***
***
TEa Tcells
B.
Spleen
Leukocyte number
1.5x10^6
1.0x10^6
5.0x10^5
0
***
***
TEa Tcells
C.
MLN
% of leukocytes
100
80
60
40
20
0
***
***
TEa Tcells
D.
MLN
Leukocyte number
8.0x10^3
6.0x10^3
4.0x10^3
2.0x10^3
0
***
***
TEa Tcells
E.
SI
% of leukocytes
20
15
10
5
0
***
***
TEa Tcells
F.
SI
Leukocyte number
2.0x10^3
1.5x10^3
1.0x10^3
5.0x10^2
0
**
**
TEa Tcells
G.
Liver
% of leukocytes
100
80
60
40
20
0
***
***
TEa Tcells
CB6 + 1x10^4 TEa T cells
B6 + 1x10^4 TEa T cells
CB6 No TEa T cells

Figure 3.9 GVHD is caused by recognition of alloantigen by TEa T cells, not as a result of homeostatic expansion of such cells CB6F1 mice were lethally irradiated and given $5x10^6$ T cell deplete TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. These were also compared to syngeneic controls (B6 mice which received CD45.1 B6 BM on Day 0 and TEa T cells on day 2). Mice were harvested on Day 8, just before the onset of disease. Live lymphocytes were gated on CD45 expression and further divided into TEa T cells (TCR-β+$CD4^+CD45.1^+$ $V\alpha2^+$), in the (A, B) spleen, (C, D), MLN (E, F), SI and (G) Liver. Results are analysed using one-way ANOVAs with multiple comparisons. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, MLN and SI n=5-6 mice per group, Liver n=3 mice per group. See Appendix V for gating strategy.

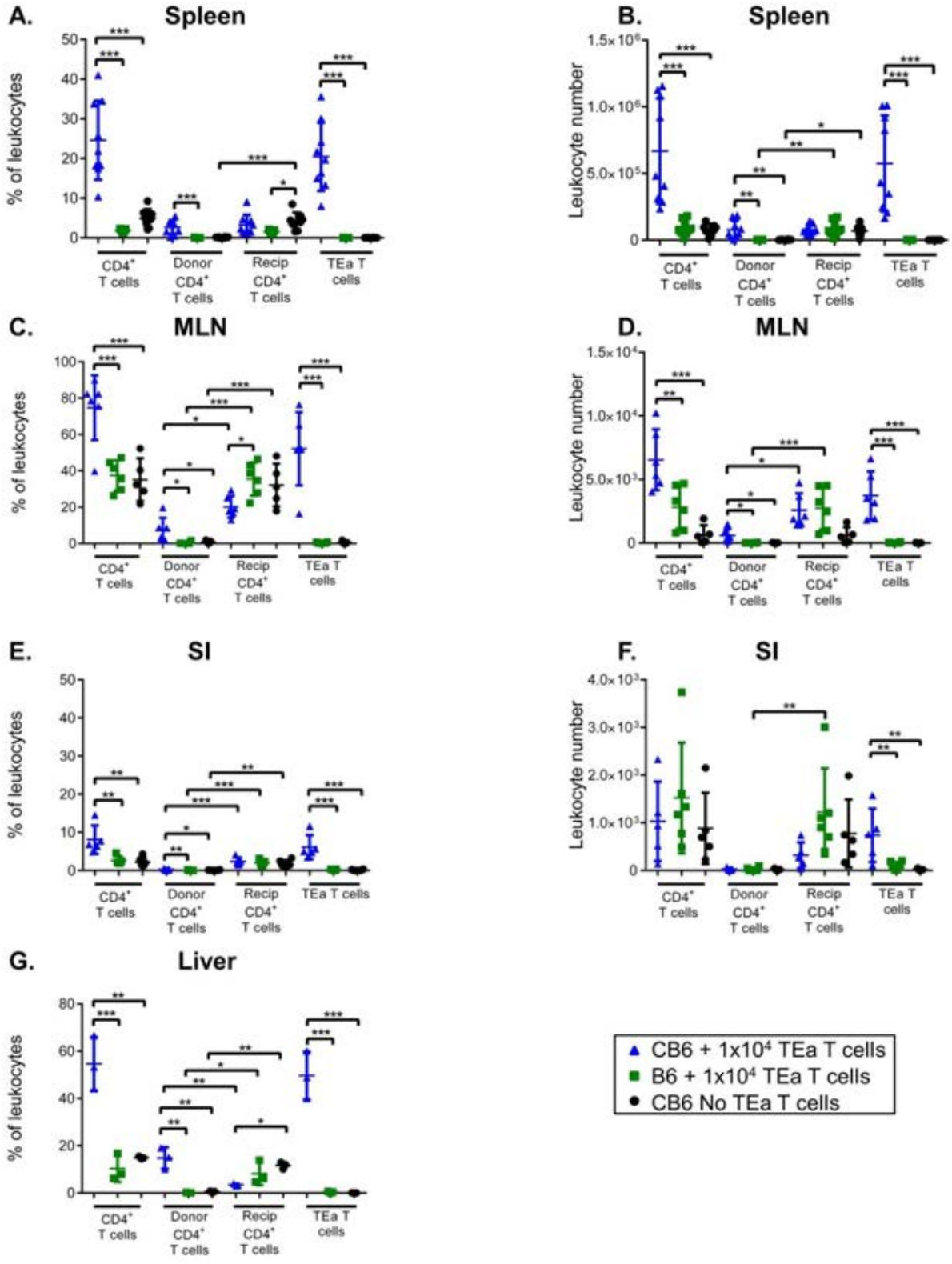
A.
Spleen
% of leukocytes
B.
Spleen
Leukocyte number
C.
MLN
% of leukocytes
D.
MLN
Leukocyte number
E.
SI
% of leukocytes
F.
SI
Leukocyte number
G.
Liver
% of leukocytes
CD4+ T cells
Donor CD4+ T cells
Recip CD4+ T cells
TEa T cells
CB6 + 1x10⁴ TEa T cells
B6 + 1x10⁴ TEa T cells
CB6 No TEa T cells

Figure 3.10 There is an increase in the frequency of $CD4^+$ T cells and in some locations the numbers of $CD4^+$ T cells in CB6F1 mice that receive TEa T cells compared to syngeneic and BM controls, and this increase is dominated by recipient cells at day 8 CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. These were also compared to syngeneic controls (B6 mice which received CD45.1 B6 BM on Day 0 and TEa T cells on day 2). Mice were harvested on Day 8, just before the onset of disease. and further divided into $CD4^+$ T cells (TCR-β^+, $CD4^+$), those which were either $CD45.1^+$(donor), $CD45.2^+$ (recipient) derived or TEa T cells ($CD45.1^+CD45.2^+$) in the (A, B) spleen (C, D), MLN (E, F), SI and (G) Liver. Results are analysed using one-way ANOVA with multiple comparisons. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, MLN and SI n=5-6 mice per group, Liver n=3 mice per group. See Appendix V for gating strategy.

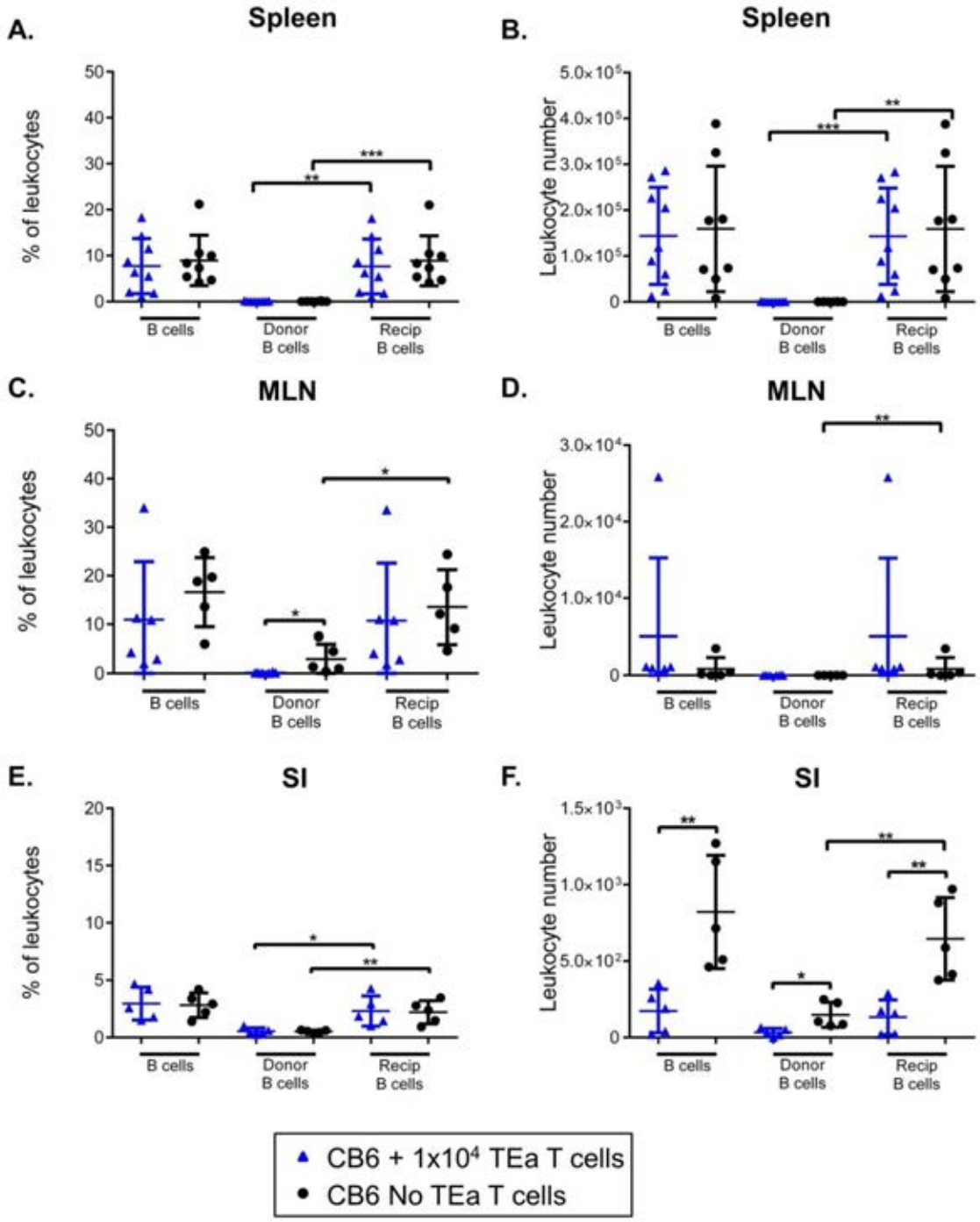
A.
Spleen
% of leukocytes
B.
Spleen
Leukocyte number
C.
MLN
% of leukocytes
D.
MLN
Leukocyte number
E.
SI
% of leukocytes
F.
SI
Leukocyte number
B cells
Donor B cells
Recip B cells
CB6 + 1x10⁴ TEa T cells
CB6 No TEa T cells

Figure 3.11 B cells were predominantly of recipient origin and the frequency of donor cells in the MLN was decreased in the GVHD group compared to BMT controls and there was a decrease in the number of cells in the SI in both the donor and host compartments of such mice consistent with B cells showing delayed reconstitution in GVHD CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. Mice were harvested on Day 8, just before the onset of disease. Live lymphocytes were further divided into B cells ($B220^+$) those which were either $CD45.1^+$(donor), $CD45.2^+$ (recipient) derived in the (A, B) spleen, (C, D) MLN and (E, F) SI. Results are analysed using unpaired t-tests. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, MLN and SI n=5-6 mice per group. See Appendix V for gating strategy.

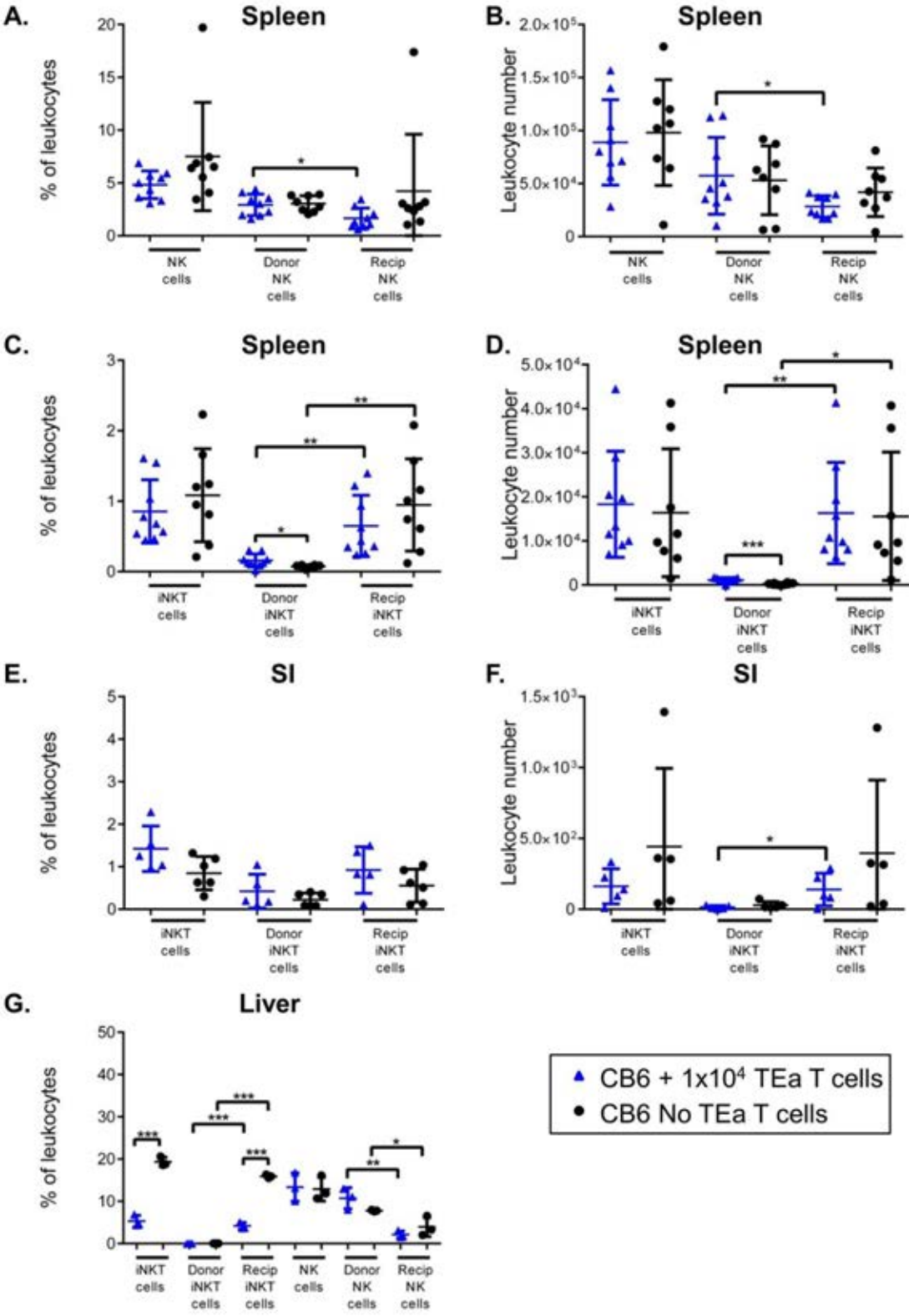
A.
Spleen
% of leukocytes
NK cells
Donor NK cells
Recip NK cells
B.
Spleen
Leukocyte number
C.
Spleen
% of leukocytes
iNKT cells
Donor iNKT cells
Recip iNKT cells
D.
Spleen
Leukocyte number
E.
SI
% of leukocytes
F.
SI
Leukocyte number
G.
Liver
% of leukocytes
iNKT cells
Donor iNKT cells
Recip iNKT cells
NK cells
Donor NK cells
Recip NK cells
CB6 + 1x10⁴ TEa T cells
CB6 No TEa T cells

Figure 3.12 NK cells are primarily of donor origin in mice that receive TEa T cells compared to BM controls while donor iNKT cells are increased in the spleen of mice that receive TEa T cells compared to BMT controls in terms of frequency and absolute number and are primarily recipient derived CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. Mice were harvested on Day 8, just before the onset of disease. Live cells were pre-gated on lymphocytes, live/dead and CD45 expression and further divided into iNKT cells (TCR-β^+PBS57 CD1d-tetramer$^+$) those which were either CD45.1$^+$(donor), CD45.2$^+$ (recipient) derived in the (A, B) spleen, (C, D) SI and (G) Liver. Results are analysed using unpaired t-tests. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, SI n=5-6 mice per group, Liver n=3 mice per group. See Appendix V for gating strategy.

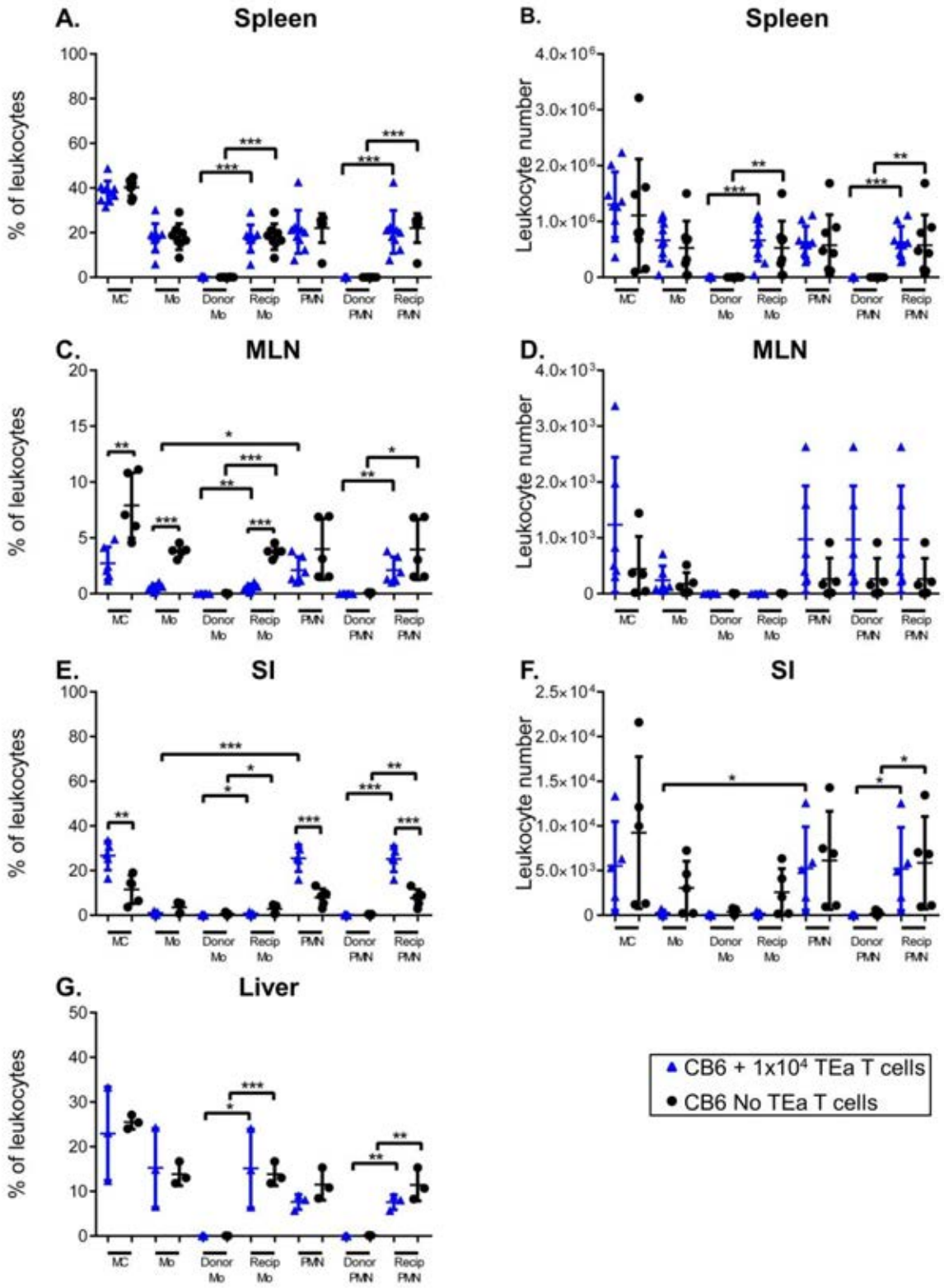
A.
Spleen
% of leukocytes
B.
Spleen
Leukocyte number
C.
MLN
% of leukocytes
D.
MLN
Leukocyte number
E.
SI
% of leukocytes
F.
SI
Leukocyte number
G.
Liver
% of leukocytes
MC
Mo
Donor Mo
Recip Mo
PMN
Donor PMN
Recip PMN
CB6 + 1x10⁴ TEa T cells
CB6 No TEa T cells

Figure 3.13 There was a decrease in the frequency of MC in the MLN in GVHD mice, while in the SI of GVHD mice MC were increased CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. Mice were harvested on Day 8, just before the onset of disease. Live cells were pre-gated on cells, live/dead and CD45 expression and further divided into $Gr1^+CD11b^+$(myeloid cells) which were either $Ly6C^+$ (monocytes) and $Ly6G^+$ (polymorphonuclear (PMN) leukocytes) and divided further into those which were either $CD45.1^+$(donor), $CD45.2^+$ (recipient) derived in the (A,B) spleen (C,D) MLN (E, F) SI and (G) Liver. Results are analysed using unpaired t-tests. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, MLN and SI n=5-6 mice per group, Liver n=3 mice per group. See Appendix V for gating strategy.

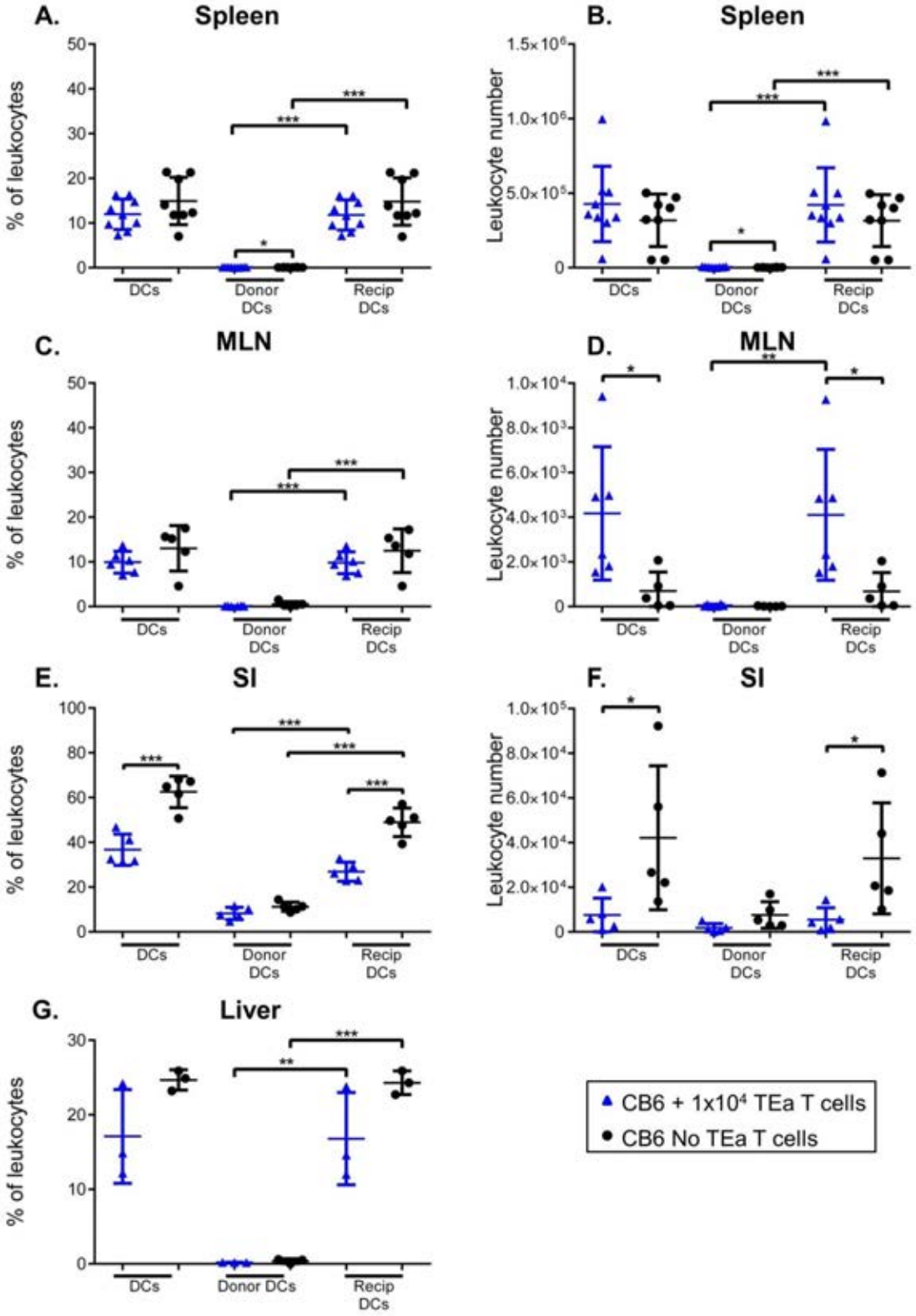
A.
Spleen
% of leukocytes
50
40
30
20
10
0
***
***
*
DCs
Donor DCs
Recip DCs
B.
Spleen
Leukocyte number
1.5x10⁶
1.0x10⁶
5.0x10⁵
0
***
***
*
DCs
Donor DCs
Recip DCs
C.
MLN
% of leukocytes
50
40
30
20
10
0
***
***
DCs
Donor DCs
Recip DCs
D.
MLN
Leukocyte number
1.0x10⁴
8.0x10³
6.0x10³
4.0x10³
2.0x10³
0
**
*
*
DCs
Donor DCs
Recip DCs
E.
SI
% of leukocytes
100
80
60
40
20
0
***
***
***
***
DCs
Donor DCs
Recip DCs
F.
SI
Leukocyte number
1.0x10⁵
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
*
*
DCs
Donor DCs
Recip DCs
G.
Liver
% of leukocytes
30
20
10
0
***
**
DCs
Donor DCs
Recip DCs
CB6 + 1x10⁴ TEa T cells
CB6 No TEa T cells

Figure 3.14 There was a perturbation in the frequency and numbers of DCs which varied by anatomical site CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ or no (control) TEa T cells via i.v. injection, on day 2. Mice were harvested on Day 8, just before the onset of disease. Live cells were pre-gated on cells, live/dead and CD45 expression and further divided into DCs ($CD11c^+$ MHC class II^+) , those which were either $CD45.1^+$ (donor) and $CD45.2^+$ (recipient) derived in the (A,B) spleen (C,D), MLN (E, F) SI and (G) Liver. Results are analysed using unpaired t-tests. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and include 3-4 independent experiments, spleen n=8-9 mice per group, MLN and SI n=5-6 mice per group, Liver n=3 mice per group. See Appendix V for gating strategy.

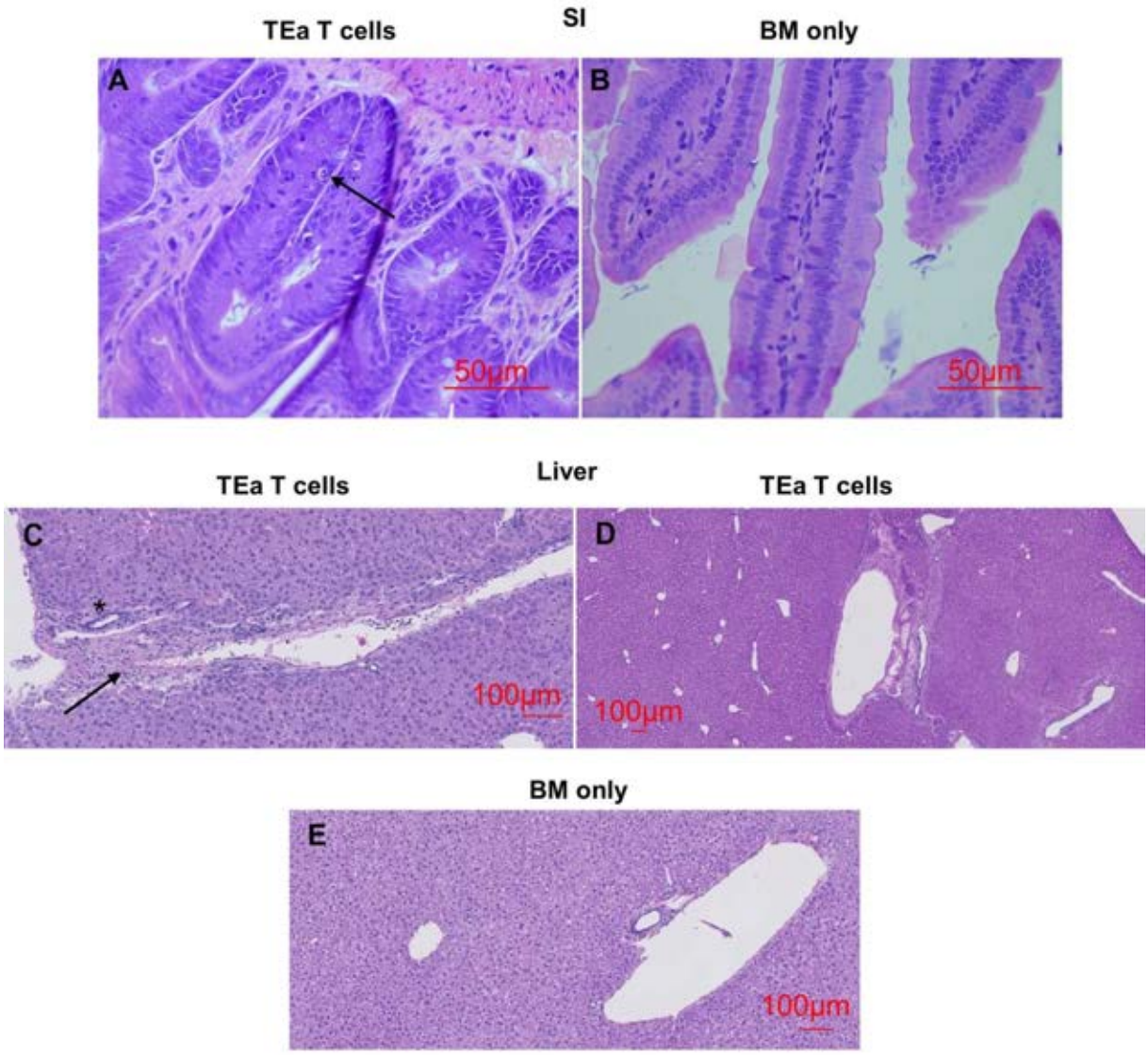

Figure 3.15 GVHD associated damage is observed in the Liver and SI which correlates with the presence of effector cells CB6F1 mice were lethally irradiated and given $5x10^6$ TCD CD45.1 B6 BM cells i.v. on day 0. Additionally, one group received the addition of $1x10^4$ TEa T cells. Mice were harvested on Day 8, just before the onset of disease, and lobes of liver and sections of SI were fixed and paraffin embedded and cut into 3 µm sections. Representative images show H&E stained SI at 40X magnification showing (A) apoptotic crypts in CB6F1 mice that received TEa T cells compared to (B) BM only control with no apoptotic crypts. Liver sections at 20X showing (C) bile duct damage (*) and (→) fibrotic tract and (B) perivascular infiltrates in mice that received TEa T cells, compared to (D) an hepatic portal triad from CB6F1 mice which received TCD B6 BM only. Representative images for n=6 mice for mice that received BM and TEa T cells, n=5 mice for mice that received BM only.

CHAPTER 4 EXPANDING iNKT CELLS *IN VITRO*

4.1. Introduction

iNKT cells have been shown to be able to suppress GVHD, while maintaining the anti-tumour effect and therefore present an attractive potential therapeutic strategy to treat GVHD (234, 238). The frequency of murine iNKT cells varies depending on location. They are rare population of cells in the mouse thymus, LNs and the blood where they make up 0.2-0.7% of lymphocytes (250-252). In contrast iNKT cells are enriched in the adipose tissue they make up 10-25% of T cells in the adipose tissue (or 2-8% of lymphocytes); while in the liver iNKT cells constitute 20-50% of T cells (246-254). We chose the spleen as the source of iNKT cells due to the prevalence of such cells (~1-2%) (250-253) and due to the fact we can easily isolate relatively high numbers of cells. Furthermore, spleen is part of the blood circulatory route and blood is the most easily acquired source of iNKT cells in humans.

The majority of iNKT cells in the spleen are described as Th1-like (or NKT1) cells, like Th1 cells they express T-bet and produce IFN-γ (320, 330, 331). NKT1 cells can

be either $CD4^+$ or $CD4^-$ and can be $NK1.1^+$, or $NK1.1^-$ (320). Although primarily thought of as proinflammatory, especially in the context of anti-tumour immunity (449), iNKT cell derived IFN-γ has also been shown to be involved in tolerance induction. In a fully mismatched model of heart transplant in which cardiac allograft rejection was blocked by means of co-stimulatory or integrin blockade, long term acceptance was observed in WT but not iNKT cell deficient mice (399). Furthermore, this study went on to show using IFN-$\gamma^{-/-}$ mice, that IFN-γ is critical for the iNKT cell mediated graft acceptance. This does not however, directly confirm that iNKT cells are the source of IFN-γ.

NKT2 cells express high levels of GATA3 and interferon regulatory factor 4 (IRF-4) and produce IL-4, IL-9 and IL-13 (310, 332, 333). NKT2 cells are the predominant subset in the spleens of BALB/c mice, however they are also found in B6 spleens (310, 330, 331). NKT2 cells have been described to exacerbate allergic inflammation in the lungs (310, 320, 333). However, they can also play a regulatory role. IL-13 has been shown to suppress anti-tumour immunity in a model of tumour growth-regression/recurrence and to be suppressive in a model of contact dermatitis, although it was not confirmed that the iNKT cells were the source of IL-13 in either case (450, 451). Similarly, IL-4 has been shown to be suppressive upon UV-light irradiation induced immune suppression, although again this IL-4 was not directly confirmed to be coming from the iNKT cells (452).

The spleen also plays host to a small population of IL-17 producing iNKT cells (NKT17) (320). IL-17 producing iNKT cells have been shown to be involved in airway inflammation (453, 454). Therefore, one might predict that they would not be beneficial in a disease such as GVHD. Finally, splenic iNKT cells produce very low

levels of IL-10 (331). NKT10 cells have been shown to be a distinct regulatory subset, with the capacity to impair anti-tumour immunity (320, 336). However, they can be induced in response to repeated activation with αGal and are enriched in adipose tissue (249, 336).

In humans, the expression of CD4 can be used to delineate functionally distinct iNKT cell subsets. $CD4^+$ iNKT cells make both NKT1 and NKT2 cytokines, while $CD4^-$ iNKT cells primarily make T-bet associated cytokines (330, 340, 341, 455). Although they are assumed to be proinflammatory this may not always be the case as increased numbers of $CD4^-$ iNKT cells in HSC transplants has been shown to reduce the risk of acute GVHD in humans (408). In mice $CD4^-NK1.1^-$ iNKT cells have been shown to be the main source of IL-17 in FI splenic iNKT cells (335). A study performed before the generation of CD1d-PBS57 tetramers showed that $NK1.1^-$ and $NK1.1^+$ iNKT cells reciprocally regulate GVHD in mice (456). In this study, $NK1.1^+$ T cells were found to suppress GVHD by an IL-4 dependent mechanism. Furthermore, $CD4^+$ iNKT cells have more recently been shown to protect from GVHD lethality in one model (126, 234).

iNKT cells make up between 0.001% and 1% of peripheral blood lymphocytes in humans (457, 458). Given the relative paucity of iNKT cells it is envisaged that they would need to be expanded in order to make any potential therapy translatable. Therefore, we set about expanding iNKT cells based on existing protocols. One protocol for expanding iNKT cells involves using BMDCs pulsed with αGal and a cocktail of cytokines (413), while another relied on αCD3 and αCD28 ligation with mAbs and various cytokines (273). The BMDC expansion protocol used bone-marrow leukocytes expanded/differentiated with GM-CSF and IL-4 to increase MHC

II and co-stimulatory molecules together with pulsing with αGal (459). In addition IL-2 and IL-7 were added to the culture as IL-7 is required for iNKT survival and homeostasis (460) and IL-2 is a general growth promoting cytokine for iNKT cells and can activate such cells independent of TCR-mediated recognition of glycolipids (231).

The second protocol relied on plate bound αCD3 and soluble αCD28 mAbs to trigger a TCR and co-stimulatory signal along with IL-2 and IL-7 (414). Unlike the first protocol where IL-12 would be produced by DCs (461), IL-12 was also added to the culture. IL-12 production has been shown to be TLR driven, especially in the context of microbial infection (461). Importantly, human iNKT cells can also be expanded, making them a viable cellular therapy. Indeed, Exp iNKT cells have been used in a phase 1 clinical trial for advanced melanoma and advanced and recurrent Non-Small Cell Lung carcinoma (396, 462). The Exp iNKT cells were characterised and were found to be functional and produce IFN-γ and IL-4. These trials showed that expanding and infusing *in vitro* generated Exp iNKT cells was both feasible as safe.

Although the phenotype of FI iNKT cells is well studied, the phenotype of Exp cells has not been extensively investigated. The secretion of cytokines by the Exp cells has been measured using enzyme-linked immunosorbent assays (ELISAs), but what cytokines the cells have the potential to make post expansion upon stimulation has not been rigorously examined at the single cell level (413, 414). Notably, the studies looking at secreted cytokines did not measure IL-10 production, a cytokine of interest to us due to its immunosuppressive properties. If a particular subset of iNKT cells was found to be suppressive, it would be preferable to isolate and expand such cells, or sort the cells post expansion.

4.2. Aims and hypothesis

As our ultimate aim was to investigate the ability of different subsets of Exp iNKT cells to influence GVHD following adoptive transfer we first phenotyped FI splenic iNKT cells. Due to the fact that the iNKT cells would require expansion in order to make any potential cellular therapy translational, we also aimed to optimise a protocol to expand these cells *in vitro* and fully characterise the Exp cells, so that ultimately we could investigate their suppressive effects. While the function of Exp iNKT cells may be different in humans, if suppressive activity was attributed to a specific subset of iNKT cells in mice, this information could be used to select human iNKT cells with analogous functions for expansion. We hypothesised that splenic iNKT cells could be expanded *in vitro*, that the cells would retain their original pattern of cytokine secretion and that such cells would be predominantly NKT1 cells.

4.3. Results

4.3.1. iNKT cells are a rare pleiotropic population of cells in the thymus, blood and peripheral LNs

Ultimately, we are interested in identifying the iNKT cells subset that may suppress GVHD. Therefore we initially set out to confirm that we could identify iNKT cells in B6 mice. Cells were isolated from the spleen, thymus, blood and several LNs and analysed by flow cytometry. We identified iNKT cells in the spleen where they made up ~1%, peripheral LNs and blood where they made up ~0.1% and the thymus where they made up ~0.2% of lymphocytes (Figure 4.1B). Given the relative frequency and the ease of isolating large number of cells from the spleen, we decided to proceed with characterising splenic iNKT cells with the ultimate aim of transferring them to bone-marrow transplant recipients to investigate their suppressive potential in GVHD.

We then set out to characterise subsets of iNKT cells is the spleen. FI splenocytes were stimulated with PMA and ionomycin in the presence of BFA and monensin and analysed by flow cytometry. The production of cytokines associated with the known subsets of iNKT cells namely IFN-γ, IL-4, IL-10, IL-13 and IL-17 were measured. Upon activation, the majority of iNKT cells produced IFN-γ (57%), while only a relatively minor population of iNKT cells were capable of producing IL-17 (~7%) (Figure 4.2A, B). In addition, iNKT cells produced IL-4 (~34%) and IL-13 (~25%) and only ~2% of cells were able to produce IL-10 (Figures 4.2A, B). Furthermore some iNKT cells were capable of producing more than one cytokine simultaneously for example approximately a third of iNKT cells could co-produce IL-4 and IL-13 (Figure 4.2C). However, IL-17 and IFN-γ tended to delineate separate subsets, as did IL-4 and IL-17A (Figure 4.2C).

Further to our investigation of cytokine production by splenic iNKT cells, the transcription factor expression in such cells was examined. Cells were stained for RORγT, T-bet and E4BP4 (NFIL3) and analysed by flow cytometry. Unsurprisingly, given the dominance of IFN-γ, T-bet was the dominant transcription factor, accounting for ~70% of iNKT cells (Figure 4.3A, B). In addition, RORγT was expressed in approximately 10% of iNKT cells while <3% of iNKT cells expressed E4BP4. Initial studies also involved staining for GATA3, however the antibody was deemed suboptimal. In addition GATA3 is not NKT2 specific (310). Therefore we decided to preferentially use IL-4 and IL-13 to delineate NKT2 cells.

In summary, we have shown that the majority of iNKT cells in the spleen are NKT1 iNKT cells, which express T-bet and IFN-γ. There is also a population of NKT2 cells and a minor population of NKT17 cells.

4.3.2. iNKT cells can be expanded *in vitro* using αGal pulsed BMDCs but fail to retain their original phenotype

Next, we set out to expand iNKT cells based on a previously published method (413). BM cells from B6 mice were cultured for 7 days in the presence of IL-4 and GM-CSF. On day 6, αGal was added to the culture (Figure 4.4A). The following day purified splenic iNKT cells (Figure 4.4B) were plated with irradiated BMDCs in the presence of IL-2 and IL-7 and expanded for 14 days (Figure 4.4C, D). Cells were monitored and split as required. Average fold expansion was 2.7.

On day 14 the Exp iNKT cells were briefly stimulated with PMA and ionomycin and analysed for expression of IFN-γ, IL-4, IL-10, IL-13 and IL-17. Surprisingly, few Exp

iNKT cells expressed IFN-γ (~9%). However, many more iNKT cells expressed IL-4 (~64%) and/or IL-17 (~28%) when compared to FI iNKT cells (Figure 4.5A, C). Furthermore, we saw the emergence of iNKT cells that were able to produce IL-10 (~31%), a property that was not present in FI cells (Figures 4.5A, C). We further investigated Exp iNKT cells in terms of transcription factor expression. Consistent with the presence of IL-10 producers we saw the emergence of E4BP4 expressing iNKT cells (~35%) (Figure 4.5B, D). RORγT^{+} iNKT cells were also found as were T-bet^{+} iNKT cells; however they were no longer the dominant population (Figures 4.5B, D).

In summary, the Exp cells showed a different phenotype to the FI cells and were no longer predominantly NKT1 cells. Of particular interest was the emergence of the IL-10 producing iNKT cells, given the propensity of IL-10 to act in an immunosuppressive manner.

4.3.3. iNKT cells expanded *in vitro* using anti-CD3 and anti-CD28 show an altered phenotype compared to FI iNKT cells

Given the time-consuming nature and inconsistency of producing BMDCs we sought to determine whether anti-CD3 and anti-CD28 mAbs could also expand iNKT cells *in vitro*. We set out to expand purified iNKT cells based on previously published methods (414). Purified iNKT cells were plated at a concentration of 5x10^{5}/mL with anti-CD3 and anti-CD28 in the presence of IL-2, 12 and later IL-7 (Figure 4.6). Cells were monitored daily, split as required and restimulated with anti-CD3 on day 8. We noted that there was a massive drop in cell numbers between days 0 and 2 (Average fold expansion was ~1.6 when calculated from day 0, but ~7 when calculated from

day 2). It is common when transferring cells *in vitro* that there is initially a large decrease in numbers to the stress of the artificial conditions. Therefore, we decided that going forward fold expansion would be calculated from day 2.

On day 14 the Exp iNKT cells were stimulated with PMA and ionomycin and analysed for cytokine production by flow cytometry. We observed no change in the ability of iNKT cells to produce IFN-γ or IL-4 however, it should be noted that IFN-γ expression was highly variable in the Exp cells (Figure 4.6B, C). Interestingly, we saw an increase in IL-10 production (from ~2% to 24%), in IL-13 (from ~25% to 84%) and in IL-17 (from ~8% to 33%) in the Exp cells compared to the FI cells (Figure 4.6B, C).

To confirm the observed increases in the production of certain cytokines, we further examined Exp iNKT cells for transcription factor expression. Consistent with the increase in IL-10 a marked increase in E4BP4 expression was observed (from ~3% amongst FI iNKT cells to 75% Exp iNKT cells). In addition, the increase in IL-17 production by Exp iNKT cells correlated with a significant increase in the proportion of cells expressing RORγT (from ~10% to 49% in FI compared to Exp iNKT cells, respectively; Figure 4.7A, B). Furthermore, there was no change in the expression of T-bet in the Exp cells but the expression was highly variable in the Exp iNKT cells (Figures 4.7A, B). Taken together these finding imply an altered phenotype in the Exp cells, wherein the Exp iNKT cells acquire the ability to produce IL-10 along with an increase in IL-17 and copious amounts of IL-13.

4.3.4. The cytokines produced by iNKT cells using PMA and Ionomycin are indicative of more physiological stimulation

PMA and ionomycin are chemicals used to bypass the need for surface receptor cross-linkage that is required under physiological conditions in order to initiate the signalling cascade that leads to T cell activation. This is an artificial means of activating cells and we wanted to investigate if the phenotype of the Exp cells was maintained under more physiological conditions. To this end, FI and Exp iNKT cells were stimulated with PMA/Ionomycin, anti-CD3/CD28 mAbs or αGal-pulsed BMDC.

Cells were stained for IFN-γ, IL-4, IL-10, IL-13 and IL-17. There was no significant difference in cytokine staining between PMA and ionomycin (Figures 4.8B, E), anti-CD3 and anti-CD28 (Figure 4.8C, E) and αGal pulsed BMDCs (Figure 4.8D, E) in terms of production of any of the aforementioned cytokines. iNKT cells can rapidly release cytokines in response to activation as they have pre-formed mRNA which allows the cells to respond quickly without the need for transcription (463, 464). We wondered whether different methods of stimulation might lead to an increase in transcription factor expression due to a rapid *de novo* transcription factor expression. We found that there was no difference in the expression of E4BP4, RORγT or T-bet between stimulation with PMA and ionomycin (Figure 3.10B, E), Anti-CD3 and anti-CD28 (Figure 3.10C, E) and αGal pulsed BMDCs (Figure 3.10D, E). Together, these data suggest that using PMA and ionomycin is representative of a genuine physiological response and not merely an artefact of chemical stimulation.

4.3.5. Exp iNKT cells take on complex effector phenotypes

As a result of the observation of altered phenotype in the Exp cells compared to the FI iNKT cells the differences in phenotype between such populations was further investigated. iNKT cells were expanded as described in section 4.3.3 and harvested on day 14. In this series of experiments we observed a decrease in IFN-γ production in the Exp cells (from ~80% in FI to 11% in Exp iNKT cells) and an increase in IL-13 expression (from 40% in FI to 83% in Exp iNKT cells; (Figure 4.10C). However, while IL-10 expression was higher in the Exp cells it was highly variable over these three experiments and therefore unlike in previous experiments no significant increase in the Exp cells was seen.

Interestingly, we saw the emergence of iNKT cells capable of producing between 2 and at least 4 of the cytokines examined (Figures 4.10 A, B, C). Whilst the only FI iNKT cells that could produce multiple cytokines were those that produced IL-4 and IL-13, within Exp iNKT cells there was the emergence of cells capable of producing IFN-γ and IL-10, IL-17 and IL-4, IL-17 and IL-10, IL-13 and IL-10 and IL-4 and IL-10 (Figures 4.10 A, B, C). However, IFN-γ and IL-17 remained largely mutually exclusive (Figures 4.10 A, B, C). Furthermore, within the Exp iNKT cells there were cells able to produce 3 cytokines (IL-13, IL-17 and IL-4 or IL-13 IL-10 and IL-4 or IL-4, IL-17 and IL-10; Figures 4.10 A, B, C) and even 4 cytokines (IL-4, IL-17, IL-10 and IL-13; Figure 4.10A, B, C). Clearly, these data suggested that expanding iNKT cells *in vitro* leads to emergence of a complex effector phenotype with Exp iNKT cells capable of producing multiple cytokines on reactivation.

4.3.6. The increased proportions of IL-17 producing iNKT cells following expansion is due to outgrowth of NKT17 cells rather than conversion of NKT1 to NKT17 cells

One robust observation when analysing the potential of iNKT cells to produce certain cytokines before and after expansion was that there was always a marked increase in cells able to produce IL-17 after expansion. The increase in NKT17s could be either due to the outgrowth of IL-17 producing iNKT cells, or due to conversion of IFN-γ producing iNKT cells into those that could produce IL-17. Given our observation that there was no significant increase in cells capable of producing both IL-17 and IFN-γ, (i.e. an intermediate population that one might expect if conversion was occurring; Figure 4.10C), we hypothesised that NKT17 cells were able to outgrow NKT1 under these conditions.

We planned to remove IL-17 producing cells, and expand the remaining iNKT cells to examine the possibility that NKT1 to NKT17 conversion. Originally we sought to delineate iNKT cell subsets based on surface markers expression which would enable us to sort specific subsets of iNKT cells prior to expansion. Indeed, $CD4^-$ $NK1.1^-$ cells have been reported to produce high levels of IL-17 (335). We observed that IFN-γ^+ iNKT cells were either $CD4^+NK1.1^+$, $CD4^+NK1.1^-$ or $CD4^-NK1.1^+$ but not $CD4^-NK1.1^-$ (Figure 4.11A). This suggests that removing the $CD4^-NK1.1^-$ population would be a useful means for removing IFN-γ^- cells. Furthermore, we found that IL-17^+ iNKT cells were mainly confined to $CD4^-NK1.1^-$ population (Figure 4.11B). This is in keeping with the observations by Coquet *et al.* who found that $CD4^-NK1.1^-$ cells produce IL-17 (335). However, given that divisions based on surface markers were not absolute and subject to variation, we decided that removing IL-17 producing cells would be better achieved using reporter mice.

In order to separate NKT17 from the pool of iNKT cells to be expanded, we sorted RORγT$^-$ iNKT cells from RORγT-GFP mice. Such cells devoid of NKT17 were then expanded *in vitro* using the anti-CD3 and anti-CD28 mAbs and cytokines as before. Expanding such cells, proved extremely difficult, which in itself is an argument that the cells capable of producing IL-17 proliferate more aggressively in these cultures. Indeed, the RORγT$^-$ iNKT cells expanded more poorly than poorly expanding FI iNKT cells (Figure 4.12A). Importantly, the Exp iNKT cells failed to make IL-17 upon stimulation with PMA and ionomycin (Figure 4.12B, C) but retained their ability to produce IFN-γ (Figure 4.12C). These data suggest that the increase in IL-17 producing cells is due to the outgrowth of NKT17 cells that out-compete poorly proliferative NKT1 cells.

4.4. Discussion

iNKT cells present an attractive potential cellular therapy due to the availability of ligands that specifically activate these cells. Although iNKT cells have been shown to exhibit potent immunomodulatory properties, they are not a homogenous population. Thus it is important to understand the complexity of their phenotype and functional heterogeneity in order take advantage of their potential as a cellular therapy. The phenotype of iNKT cells that we observed was consistent with what is reported in the literature. IFN-γ producing cells were the dominant subset observed in the spleen, with IL-4 and IL-13, IL-17 and very little IL-10 producing cells present (310, 320, 330, 331) (Figure 4.2).

One thought provoking observation was the emergence of IL-10 and E4BP4 expressing in splenic iNKT cells upon expansion (Figures 4.6-4.10).This was a surprising observation given that NKT10 cells are most commonly found in the adipose tissue in mice (249). In terms of IL-10 secretion by Th cells, *Wraith et al.* have shown that antigen strength controls the generation of IL-10 secreting cells (465). After repeated activation, these cells maintain the ability to produce IFN-γ but take on the ability to produce IL-10 and in this way act in a self-limiting manner (466). It is possible that a similar negative feedback mechanism is occurring when the cells are continually activated with a strong antigenic signal provided by either αGal or anti-CD3, throughout the expansion. Indeed, it has been demonstrated *in vivo* that repeated stimulation with αGal leads to induction of IL-10 production by iNKT cells (336). We observed a significant increase in iNKT cells that can produce IL-10 and IFN-γ in the Exp cells compared to the FI cells (Figure 4.6C).

NKT10 cells have been shown to be a distinct regulatory subset, with the ability to suppress anti-tumour immunity (336). Furthermore, iNKT cell derived IL-10 has been shown to be required for corneal graft survival (233). Therefore, we hypothesised that NKT10 cells may be suppressive in the context of GVHD.

IL-10 was not the only cytokine that was altered in the Exp cells, such cells also showed an increase in their ability to produce IL-13 and IL-17 (Figures 4.6). IL-13 is a NKT2 cytokine and has been shown to have an immunosuppressive role in a model of tumour growth-regression-recurrence (451); therefore NKT2 cells producing IL-13 might have a protective role against GVHD. However, it would be important to bear in mind that it might affect the GVT response.

IL-17 is generally regarded to be a proinflammatory cytokine; indeed IL-17 producing iNKT cells have been shown to be involved in inducing airway inflammation (453, 454). However, NKT17 cells have been shown to also produce IL-22 (467), and IL-22 produced by innate lymphoid cells (ILCs) has been shown to reduce susceptibility to GVHD (468, 469). The Exp iNKT cells were negative for IL-22 expression (data not shown). Therefore, NKT17 cells are unlikely to be protective. Furthermore, previous studies which have demonstrated the suppressive effects of FI iNKT cells have used $CD4^+$ iNKT cells to suppress GVHD (126, 234, 456) and $IL\text{-}17^+$ iNKT cells are mainly found in the $CD4^-NK1.1^-$ fraction (335). While we have seen that this segregation is not true of Exp splenic iNKT cells (data not shown), it does suggest that removing IL-17 producers might be useful.

In addition there were perturbations in IFN-γ expression. In one series of experiments there was a significant decrease in IFN-γ production in the Exp iNKT cells compared

to the FI cells (Figures 4.8, 9). However, this was not as robust at the alterations in other cytokines as this decrease was not shown to be consistent upon further repeating this experiment (Figure 4.6).

It was interesting to observe that there was no difference in the expression of cytokines between the different methods of stimulation namely PMA and ionomycin, anti-CD3 and anti-CD28 and αGal pulsed BMDCs (Figures 4.8, 9). PMA activates protein kinase C which leads to NFκB signalling. Ionomycin is a $Ca2^{+}$ ionophore, which triggers $Ca2^{+}$ release which is required for NFAT signalling. NFκB and NFAT are important transcription factors for T cell activation. As such, using PMA and Ionomycin is an artificial mode of stimulation and thus it was noteworthy that there was no significant difference between the alternative methods of staining. In the future, it would also be useful to include an unstimulated control to see the background level of cytokines production. It would also help to determine if residual signal remains from stimulation by anti-CD3 and anti-CD28 during the expansion.

A previous study by *Coquet et al.* saw a difference between anti-CD3 and anti-CD28 stimulation, which the authors suggested was a result of the requirement of spleen iNKT cells for DC derived factors such as IL-12 and IL-18 (335). However, in this experiment the authors were looking at secreted cytokines using ELISAs. This means that some of the cytokines could be coming from the BMDCs as they have the capacity to make many of the same cytokines. In addition in this study FI cells were used, while we were comparing Exp cells. As the phenotype of the Exp iNKT cells is different to that of FI they may not necessarily respond in the same way to stimulation. It is also important to note that this is a different time point as we looked at 4 h while the earliest time point investigated in this study was 24 h. It is possible

that at a later time point we would have observed a difference in cytokine production between anti-CD3 and anti-CD28 and BMDC stimulation.

One striking observation was that not only did the Exp cells have altered cytokine expression they also took on complex effector phenotypes not observed in the FI cells. The Exp cells were capable of making at least four and possibly more cytokines (Figure 4.12). Given the propensity of iNKT cells to be involved in a myriad of immune responses generally attributed to a specific subtype of iNKT cells, this raises the question as to what the functionality of such a complex phenotype would be in the context of GVHD.

Further to the functional properties of these polyfunctional cells, this also raises the question as to whether some of these phenotypes are intermediate between the iNKT cell subtypes, and whether peripheral iNKT cells are committed to a specific lineage or can behave in a more promiscuous manner. The functional plasticity of iNKT cells is not well understood. iNKT cells can express a range of cytokines, transcription factors and chemokines and can respond to their local microenvironment (309, 470-472). Furthermore, the phenotype of iNKT cells is tissue dependent, and stimulating iNKT cells with APCs from different tissues can lead to different cytokine response (289, 309, 334). This suggests that peripheral iNKT cells may possess at least some degree of functional plasticity. In addition, as previously mentioned IL-17 production can be induced in peripheral iNKT cells under the right conditions and this is also the case with FOXP3 expression in both mice and humans (454, 473-475). However, there is no direct evidence as to which if any peripheral iNKT cells possess functional plasticity and to definitively prove the existence of such a phenomenon cell tracing and single cell studies would be required.

It is also possible that some of the changes in frequency observed are due to outgrowth of subsets of iNKT cells due to their superior capacity to expand. However, this does not explain the ability of the Exp cells to produce multiple cytokines. It is possible that certain subsets of iNKT cell are taking on the ability to make multiple cytokines or, that there is peripheral conversion of iNKT cells occurring, or indeed a combination of both.

To address the possibility that outgrowth or peripheral conversion of particular subsets was occurring we made use of RORγT reporter mice, as a means to sort out the predominant population in the FI cells (namely IFN-γ^+). We found that RORγT$^-$ iNKT cells were extremely difficult to expand, supporting our hypothesis that IL-17 producing iNKT cells are more proliferative. Furthermore, the Exp cells were predominantly IFN-γ^+. It is likely that outgrowth of IL-17 producing cells is at least a contributing factor to their overrepresentation in the Exp cells compared to the FI cells.

We attempted to verify our hypothesis using IL-17$^+$ splenic iNKT cells. However the number of iNKT cells isolated was not sufficient to achieve a level of confluence compatible with expansion. It should also be noted that the reason for the increase in IL-10 and IL-13 production by the Exp cells was not examined directly, however as this population is not present in the FI cells it is likely to be occurring as a result of differentiation of the iNKT cells. However, the exact ontogeny of the various phenotypes has not been thoroughly investigated and it would be interesting to look at this in the future using cells from fate mapping mice. Furthermore, the stability of the Exp iNKT cells has not been investigated and it would be interesting to

investigate the stability of such cells perhaps to carry out epigenetic analysis of the relevant regions.

In conclusion, we have optimised a protocol for expanding iNKT cells *in vitro,* which leads to the alteration in the phenotypes of the Exp iNKT cells compared to those seen in their FI counterparts. Of note is the increase in IL-10 and IL-13 production, both of which have been shown to have suppressive effects in certain contexts. We also saw the emergence of iNKT cells capable of producing multiple cytokines which may have functional implications upon investigation of the propertied of such cells. This alteration in phenotypes is robust and occurs regardless of the method of stimulation employed. The expansion of these cells will enable us to thoroughly investigate their ability to influence alloreactive responses.

4.5. Figures

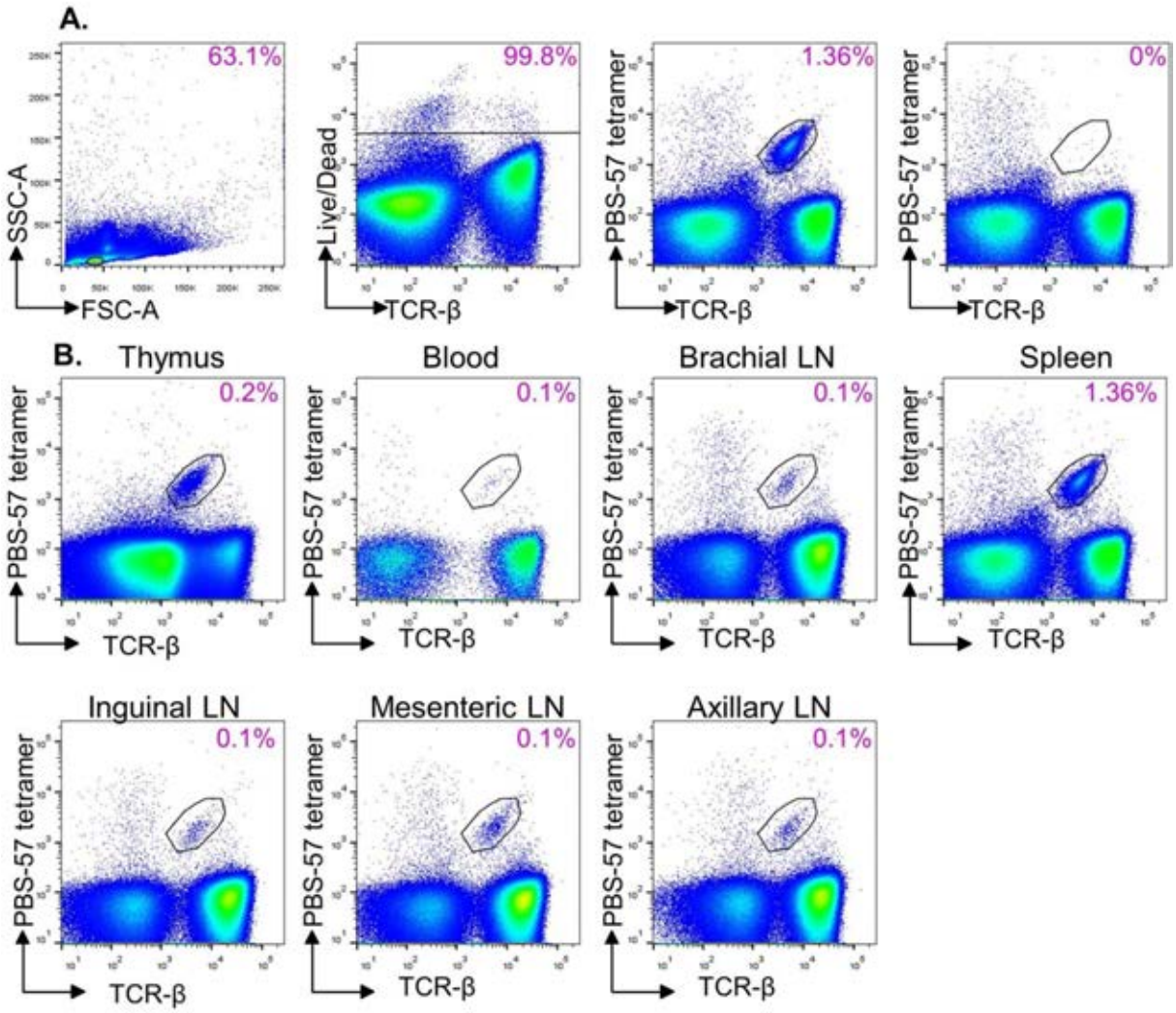

Figure 4.1 iNKT cells are a rare population of cells in the thymus, blood and peripheral LNs Cells were isolated from B6 mice, and were analysed by flow cytometry. (A) Leukocytes were gated on lymphocytes (FSC-A, SSC-A), live cells and TCR-β versus PBS-57 tetramer, tetramer positive cells were identified using an unloaded control. (B) iNKT cells were gated as the splenocytes in the thymus, blood, brachial LN, inguinal LN, MLN and axillary LN. n=3 mice.

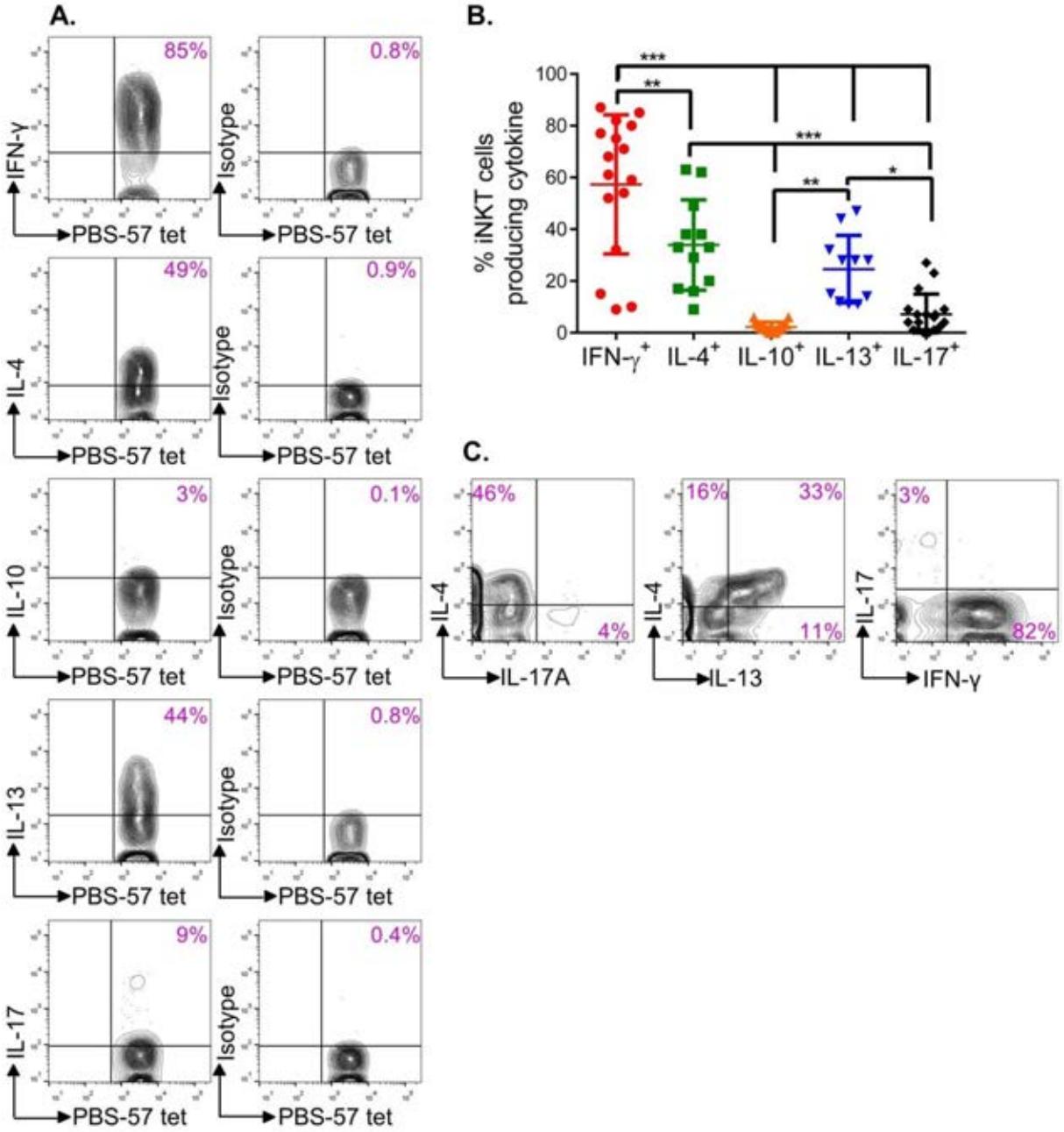

Figure 4.2 A high frequency of FI splenic iNKT cells secrete IFN-γ, but only a small frequency of such cells produce IL-17A Splenocytes were isolated from B6 mice and cells were stimulated with PMA and ionomycin and cultured in the presence of BFA and monensin. iNKT cells were gated as in Figure 4.1A. Cells were analysed by flow cytometry for intracellular production of IFN-γ, IL-4, IL-10, IL-13 and IL-17A (A) Representative dot plots showing cytokine production (B) Graph showing cytokine expression. (C). Representative dot plots of cytokine production. Data shown are mean ± SD (n=11-18 mice). Results are analysed using one-way ANOVA with multiple comparisons *p<0.05, **p<0.01, ***p<0.001.

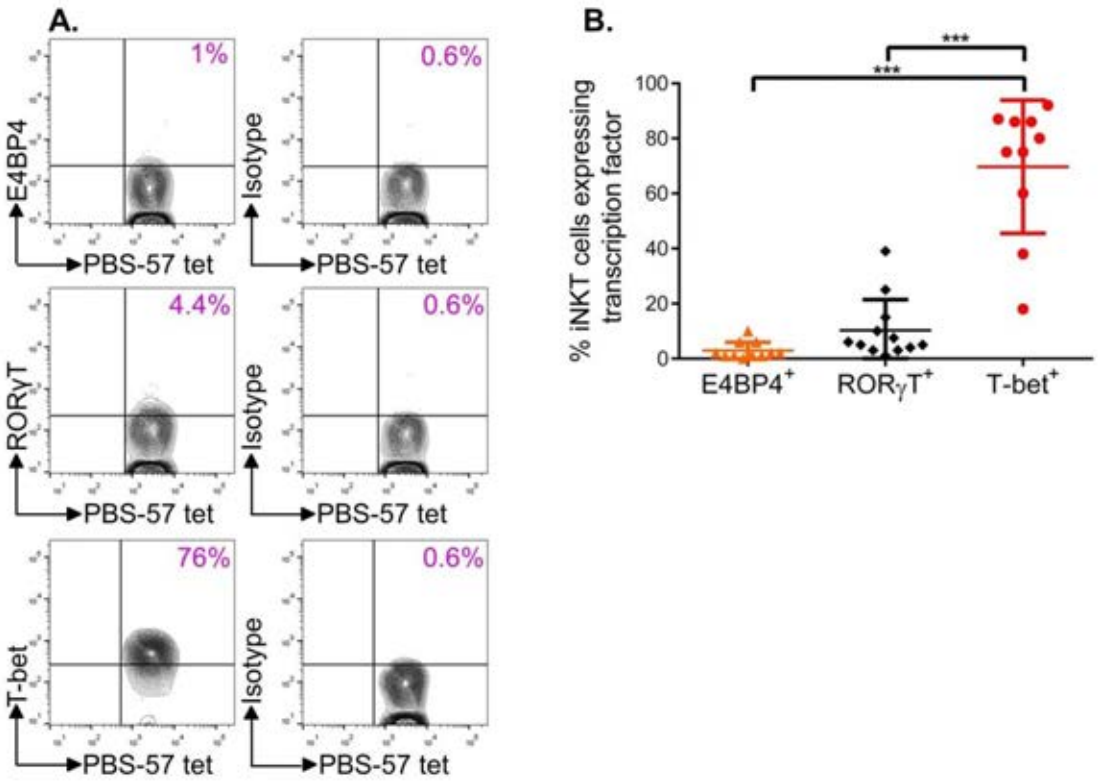

Figure 4.3 Fl splenic iNKT cells express high levels of T-bet and low levels of E4BP4 and RORγT Splenocytes were isolated from B6 mice and cells were stimulated with PMA and ionomycin in the presence of BFA and monensin. iNKT cells were gated as in Figure 4.1A. Cells were analysed by flow cytometry for expression of E4BP4, RORγT and T-bet. (A) Representative dot plots showing transcription factor expression (B) Average transcription factor expression. Data shown are mean ± SD (n=8-9 mice). Results are analysed using one-way ANOVA with multiple comparisons ***p<0.001.

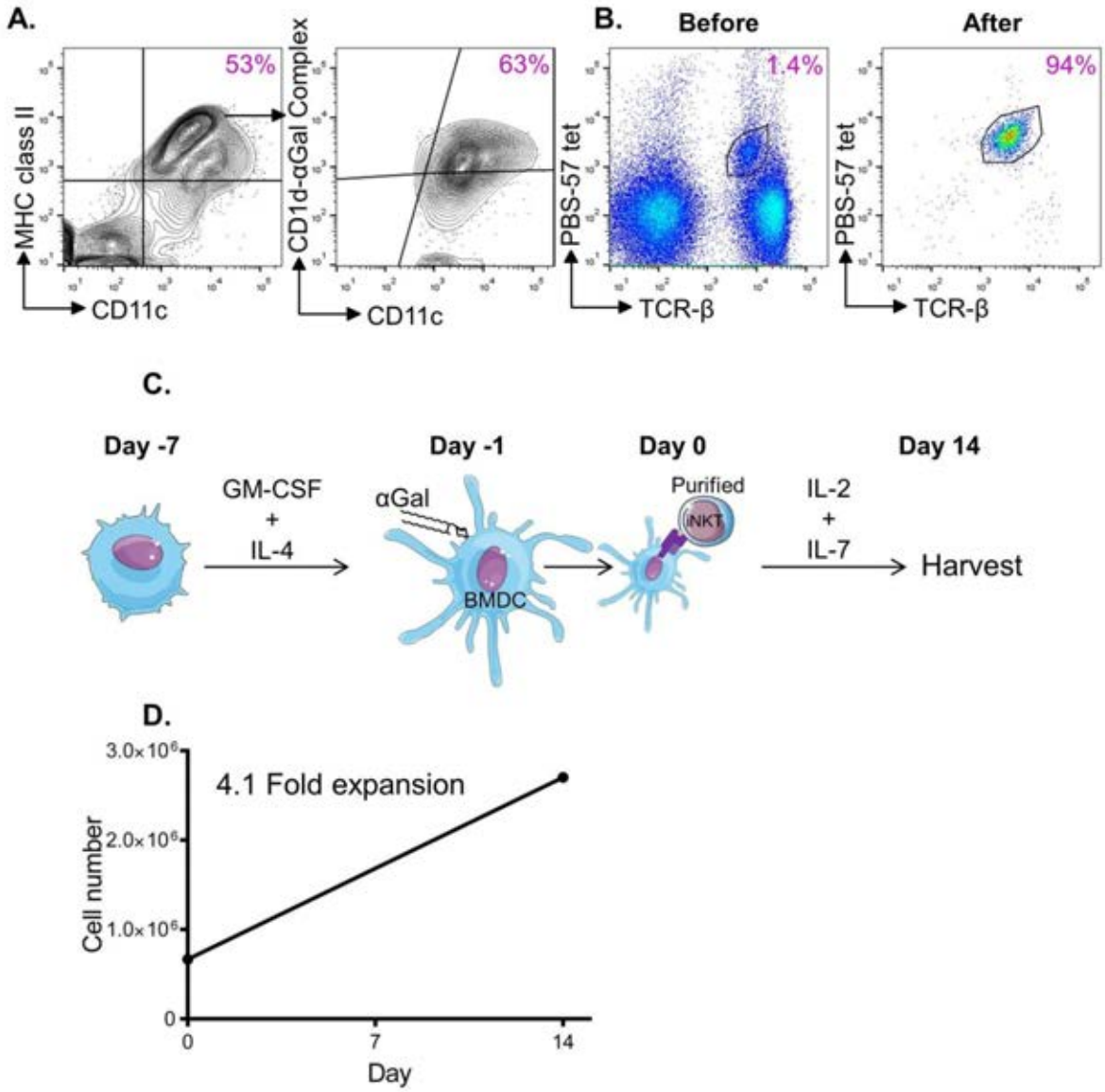

Figure 4.4 iNKT cells can be expanded using BMDCs pulsed with αGal (A) B6 BM cells were cultured for 7 days in the presence of GM-CSF and IL-4, αGal was added to the culture on day -1. Cells were irradiated on Day 0 and cultured with (B) purified iNKT cells from B6 spleens in the presence of IL-2 and IL-7. (C, D) The cells were monitored and split as required and harvested at Day 14. (D) Representative graph showing iNKT cell expansion, graph is representative of 4 independent experiments.

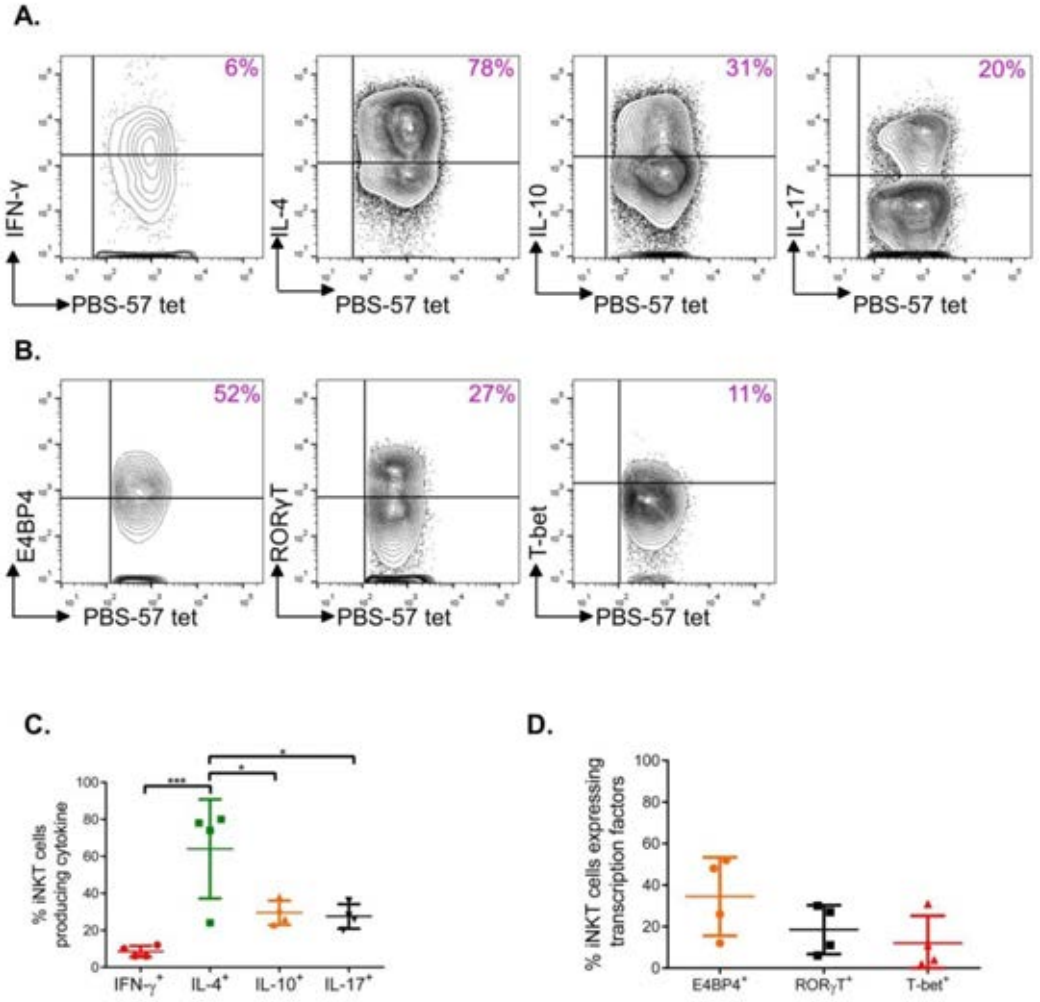

Figure 4.5 Exp iNKT cells predominantly produce IL-4, while a smaller frequency of such cells produce IFN-γ, IL-10 and IL-17 and additionally such cells differentially express transcription factors B6 BM cells were cultured for 7 days in the presence of GM-CSF and IL-4, αGal was added to the culture on day -1. Cells were irradiated on Day 0. Purified iNKT cells from B6 spleens were plated with BMDCs (at a ratio of 10:1) in the presence of IL-2 and IL-7, monitored and split as required and harvested at Day 14. On Day 14 Exp iNKT cells were stimulated with PMA and ionomycin and cultured in the presence of BFA and monensin and analysed by flow cytometry. Cells were gated on live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various cytokines (IFN-γ, IL-4, IL-10, and IL-17) or transcription factors (E4BP4, RORγT and T-bet). (A) Representative dot plots showing cytokine production. (B) Representative plots showing transcription factor expression. (C) Average cytokine production. (D) Average transcription factor expression. Data shown are mean ± SD from 4 independent experiments. Results are analysed using one-way ANOVA with multiple comparisons *p<0.05,***p<0.001.

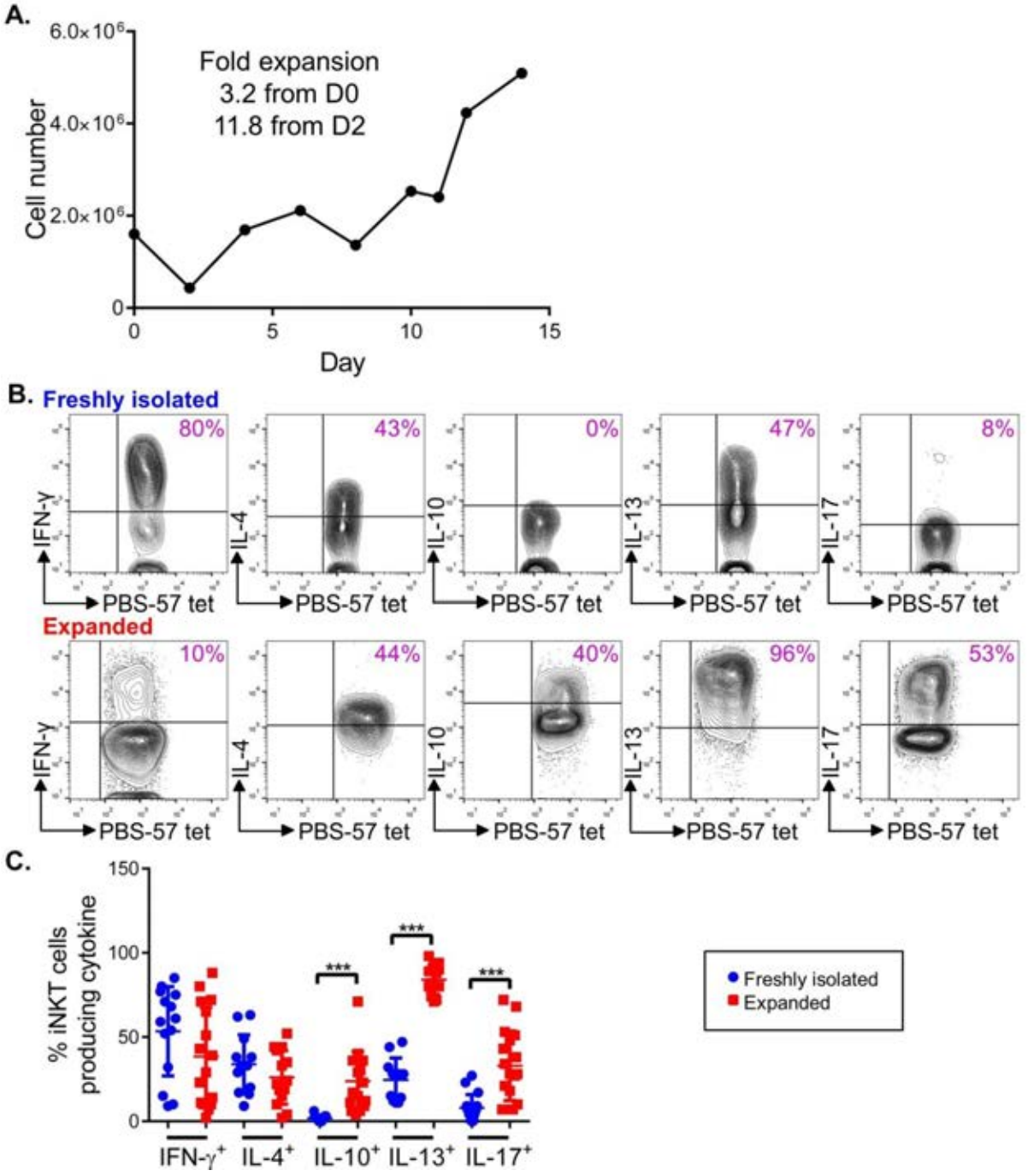

Figure 4.6 iNKT cells can be expanded using anti-CD3 and anti-CD28 and an increased frequency of Exp iNKT cells can produce IL-10, IL-13 and IL-17 compared to FI cells (A) Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 Exp iNKT cells were stimulated alongside FI iNKT cells with PMA and Ionomycin and cultured in the presence of BFA and monensin and analysed by flow cytometry. The production of IFN-γ, IL-4, IL-10, IL-13 and IL-17A was measured. Cells were gated on live lymphocytes or live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various cytokines. (B) Representative dot plots showing cytokines production, results are representative of 11-17 independent experiments (C) Graph showing cytokine production, data shown are mean ± SD. Results are analysed using t-tests. ***p<0.001.

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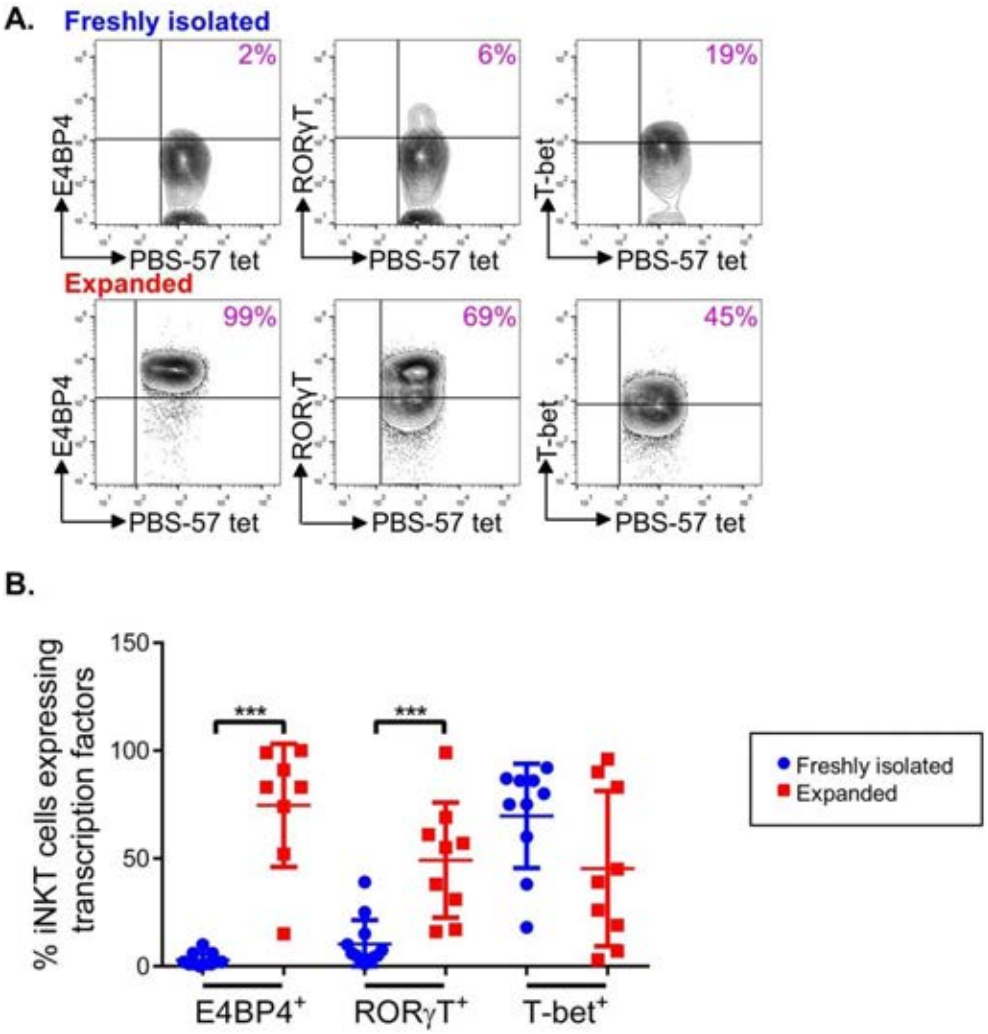


Figure 4.7 Exp iNKT cells exhibit increased expression of E4BP4 and RORγT compared to FI iNKT cells Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 the Exp iNKT cells were analysed by flow cytometry alongside FI iNKT cells for expression of E4BP4, RORγT and T-bet. Cells were gated on live lymphocytes or live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various transcription factors. (B) Representative dot plots showing transcription factor expression. Results are representative of 8-12 independent experiments (B) Graph showing transcription factor expression, data shown are mean ± SD. Results are analysed using t-tests***p<0.001.

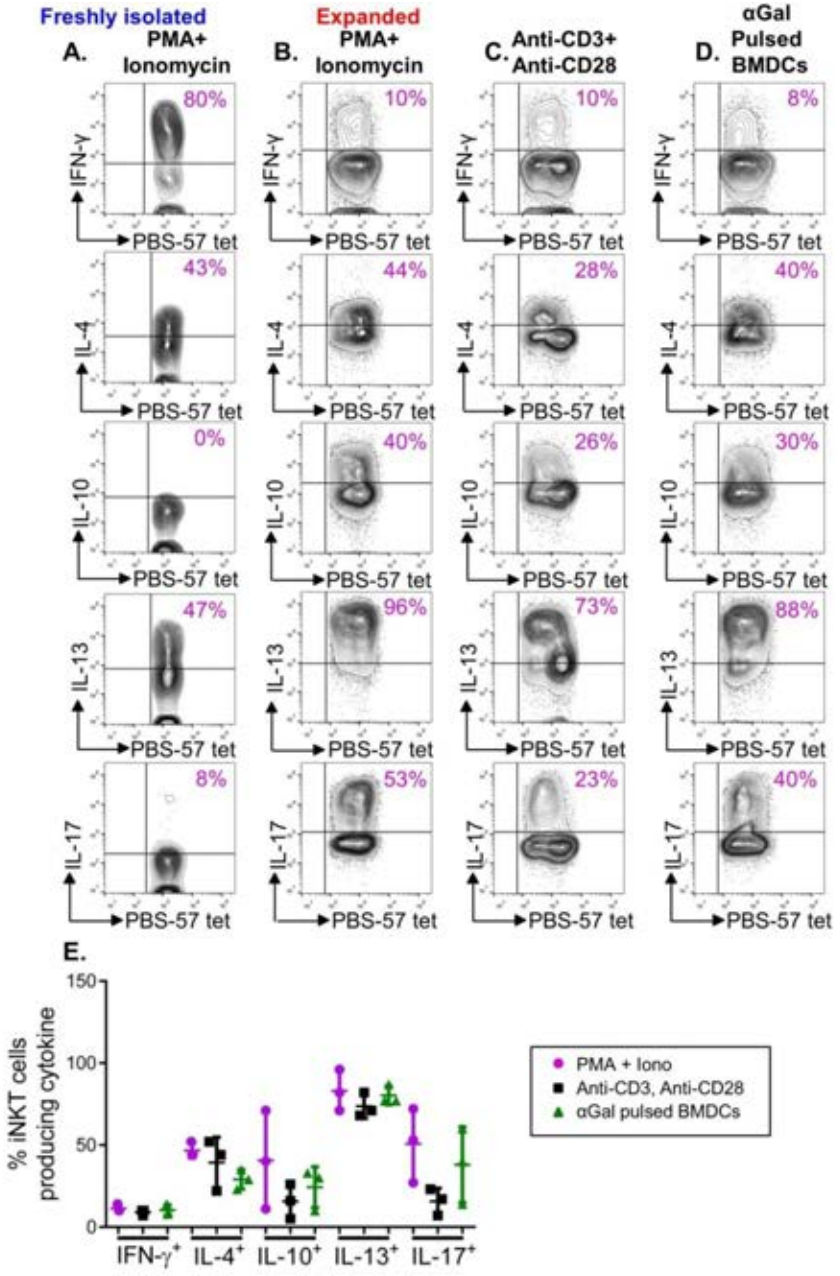


Figure 4.8 Chemical induced cytokine secretion by iNKT cells is representative of a physiological response Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 (A) FI iNKT cells were stimulated with PMA and Ionomycin in the presence of BFA and monensin and analysed by flow cytometry alongside Exp iNKT cells stimulated with (B) PMA and Ionomycin, (C) Anti-CD3 and Anti-CD28 or (D) BMDCs pulsed with αGal and the production of IFN-γ, IL-4, IL-10, IL-13 and IL-17A was measured. Cells were gated on live lymphocytes or expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various cytokines. Representative dot plots showing cytokines. (E) Graph showing cytokine production by expanded iNKT cells in 3 independent experiments, data shown are mean ± SD. Results are analysed using one-way ANOVAs with multiple comparisons. All n.s. non-significant.

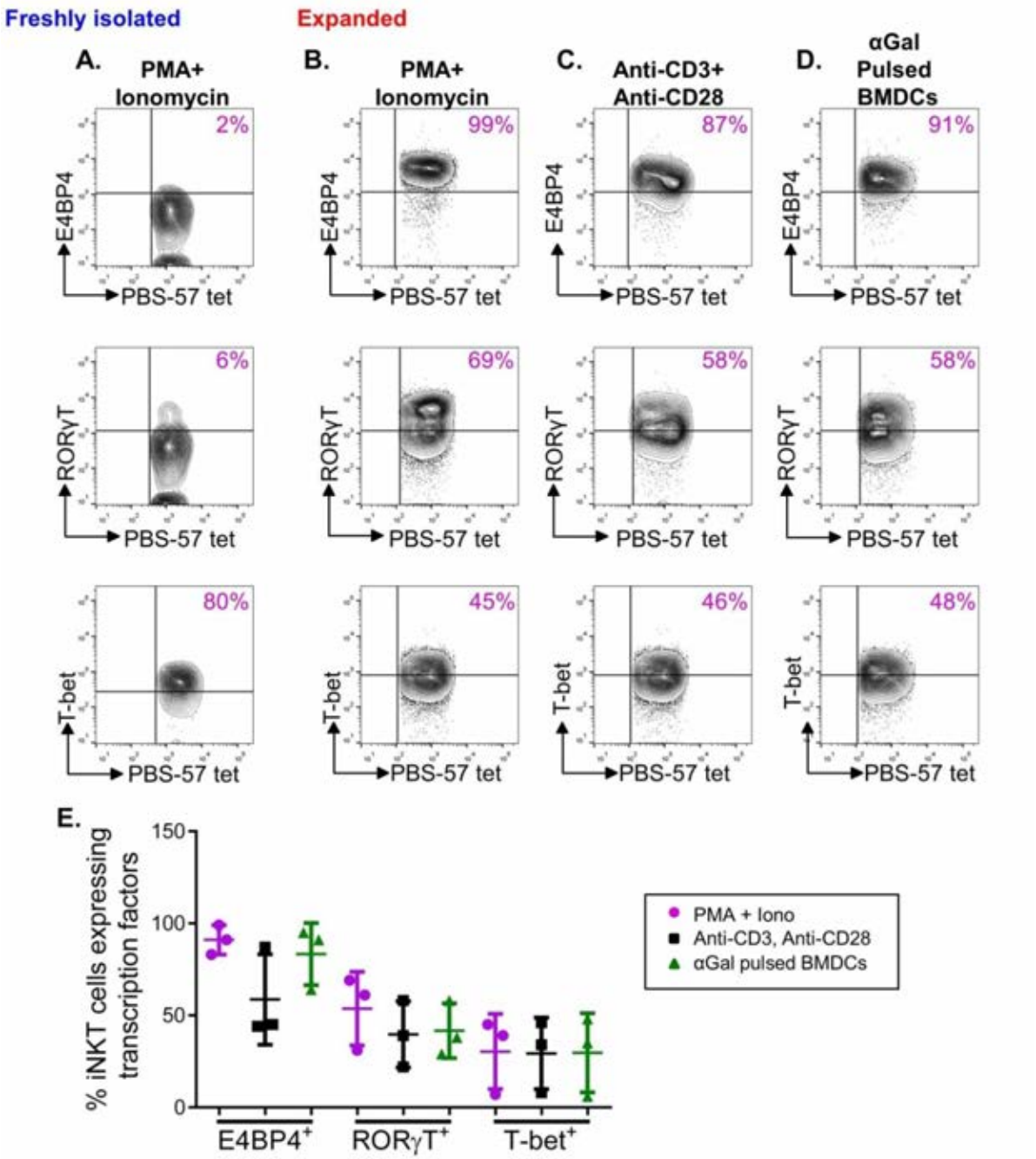


Figure 4.9 Confirmation that iNKT cells express the corresponding transcription factors associated with the cytokines secreted by such cells Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 (A) FI iNKT cells were stimulated with PMA and Ionomycin and in the presence of BFA and monensin and analysed by flow cytometry alongside Exp iNKT cells stimulated with (B) PMA and Ionomycin, (C) anti-CD3 and anti-CD28 or (D) BMDCs pulsed with αGal (all in the presence of BFA and monensin) and the expression of E4BP4, RORγT and T-bet was measured. Cells were gated on live lymphocytes or live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various transcription factors. Representative dot plots showing transcription factor (E) Graph showing transcription factor expression in 3 independent experiments. All n.s. non-significant.

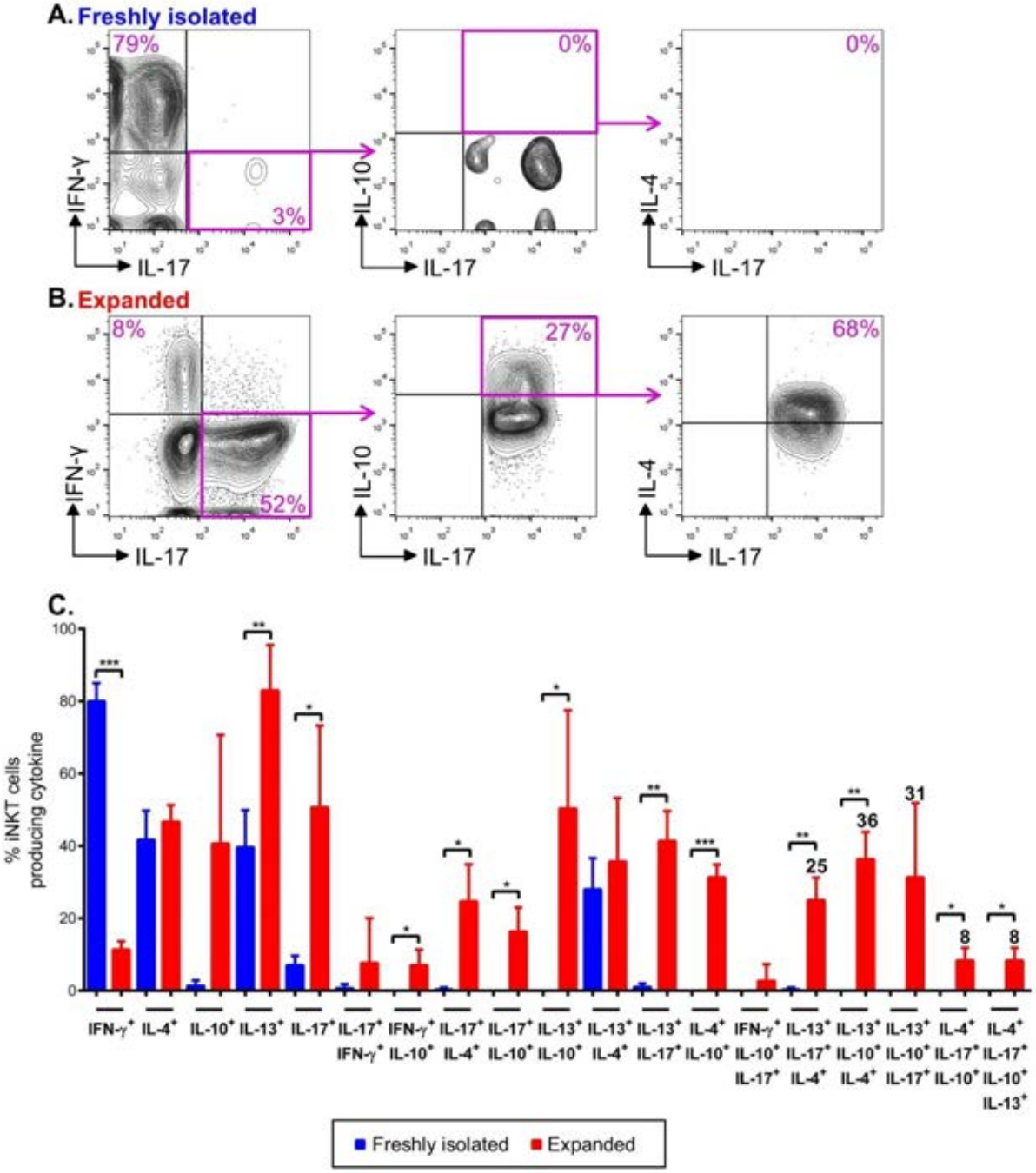


Figure 4.10 iNKT cells expanded using anti-CD3 and anti-CD28 exhibit complex effector phenotypes Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 the FI iNKT cells were stimulated alongside Exp iNKT cells and both FI and Exp iNKT cells were stimulated with PMA and Ionomycin, in the presence of BFA and monensin and analysed by flow cytometry, and the production of IFN-γ, IL-4, IL-10, IL-13 and IL-17A. Cells were gated on live lymphocytes or live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various cytokines (A) Representative dot plots showing cytokines production in FI iNKT cells (B) Representative dot plots showing cytokines production in Exp iNKT cells (C) Graph showing cytokine production in 3 independent experiments, data shown are mean ± SD. Results are analysed using t-tests *p<0.05, **p<0.01, ***p<0.001.

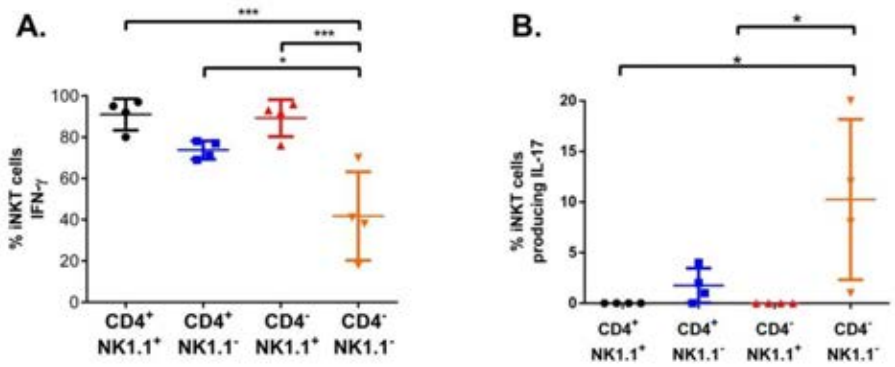


Figure 4.11 The distribution of IFN-γ and IL-17 producing iNKT cells cannot be confined to a particular subset based on CD4 and NK1.1 expression Cells were expanded using anti-CD3 and anti-CD28, in the presence of IL-2, IL-12 and IL-7 for 14 days. On Day 14 FI iNKT cells were stimulated with PMA and Ionomycin, in the presence of BFA and monensin and analysed by flow cytometry. Cells were gated on live lymphocytes or live expanded lymphocytes cells, followed by TCR-β versus PBS-57 tetramer and then divided into $CD4^+NK1.1^+$ $CD4^+NK1.1^-$ $CD4^-NK1.1^+$ and $CD4^-NK1.1^-$ and IFN-γ and IL-17 was investigated within the various subsets. (A). Comparison of IFN-γ production within the CD4 and NK1.1 based subsets (B) Comparison of IL-17 production within the CD4 and NK1.1 based subsets. Graphs showing cytokine expression in 4 independent experiments. Results were analysed using one-way ANOVAs *p<0.05, ***p<0.001.

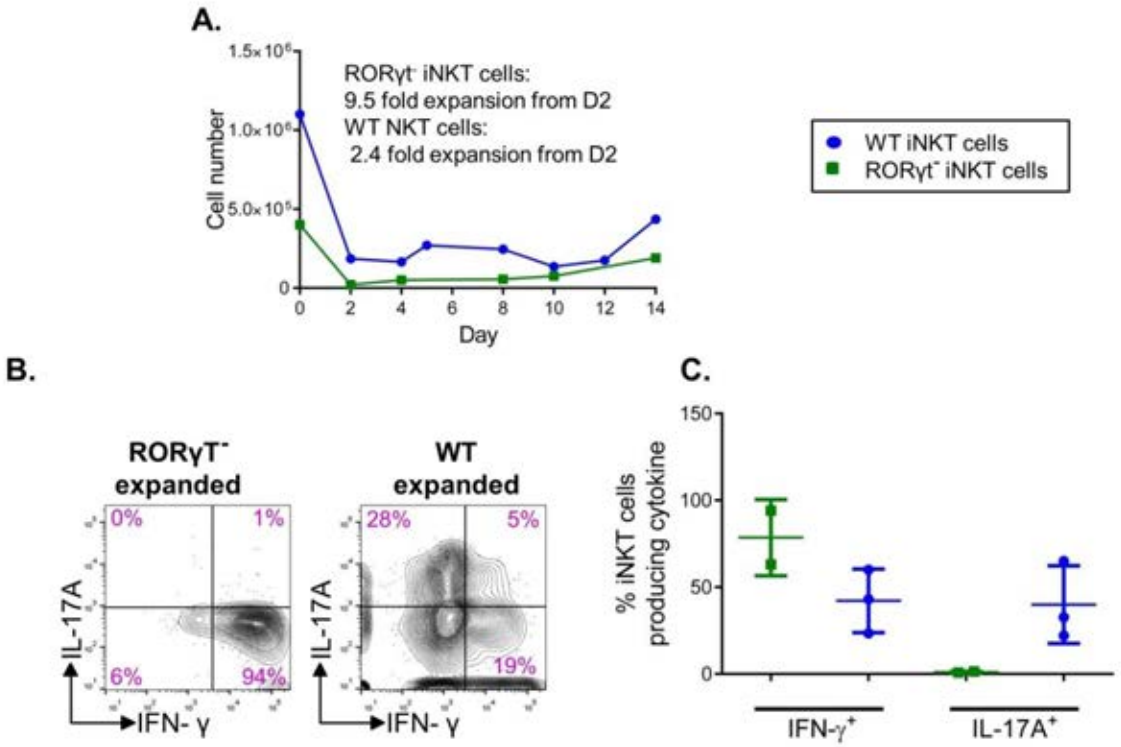


Figure 4.12 IFN-γ$^+$ and IL-17$^+$ iNKT cells primarily expand as separate lineages On day 0, GFP- iNKT cells from RORγT reporter mice and WT NKT cells from B6 mice were isolated and (A) all cells were expanded using anti-CD3 and anti-CD28. for 14 days in the presence of IL-2, IL-12 and IL-7. On Day 14 the expanded iNKT cells were stimulated with PMA and Ionomycin, in the presence of BFA and monensin and analysed by flow cytometry. Cells were gated on live lymphocytes or live expanded lymphocytes, followed by TCR-β versus PBS-57 tetramer, followed by the various IFN-γ and IL-17 (B) Representative examples of IL-17 versus IFN-γ production in RORγT– and WT Exp iNKT cells. (C) Cumulative data from 2-3 independent expression showing IFN-γ versus IL-17 production in RORγT– and WT Exp iNKT cells.

CHAPTER 5 INVESTIGATING THE SUPPRESSIVE CAPACITY OF INKT CELLS *IN VITRO*

5.1. Introduction

In Chapter 4 we found that Exp iNKT cells show an increased capacity to produce IL-10 and IL-13 upon activation. *In vivo* studies have reported that multiple injections with αGal leads to iNKT cells producing IL-10, which have been shown to have a protective effect when transferred into Jα18$^{-/-}$ mice with EAE (336). They were also shown to suppress anti-tumour immunity (336). iNKT derived IL-10 has also been demonstrated to be essential for corneal graft survival (233). Given the suppressive role of IL-10 secreted by iNKT cells after repeat αGal injections, we reasoned that Exp iNKT cells, which also have the capacity to produce IL-10, may also display an enhanced ability to suppress T cell responses.

In vitro suppression assays are a useful and efficient way to investigate the suppressive activity of a population of cells. They are commonly used in the Treg field to investigate the ability of Treg populations to suppress T cells and to identify

the molecules involved in Treg mediated suppression. The first Treg suppression assays were described 20 years ago (476, 477) and contributed to the paradigm that $CD25^+$ T cells are able to suppress Tcon. In addition, such assays require few reagents and facilitate the investigation of several factors in one experiment. T cells are typically activated with either APCs or anti-CD3 and anti-CD28 and proliferation measured by labelling the Tcon with fluorescent dyes or assessing uptake of tritiated thymidine. It is also possible to investigate antigen specific responses using transgenic mice (478).

Several studies have demonstrated that Exp human iNKT cells can suppress T cells *in vitro,* however to our knowledge, to date no such suppression assays have been published investigating the suppressive effects of FI or Exp murine iNKT cells. This may be due to the rarity of these cells making it difficult to carry out these experiments. There is also one example of a suppression assay preformed with $CD8^+$NKT-like cells, which were shown to be able to suppress alloreactive T cells by killing antigen bearing DCs (479). Given that we have expanded iNKT cells *in vitro* we felt there was a distinct possibility that testing the suppressive capacity of such cells *in vitro* would be a convenient and fruitful method to test the suppressive properties of Exp iNKT cells *in vitro*. Using such a system would allow the investigation of not only whether Exp iNKT cells are suppressive but also whether the suppression is mediated by immunosuppressive cytokines (given that they readily produce IL-10) or cell surface interactions.

IL-10 was originally discovered by Mosmann in 1989, as a cytokine that inhibits the effects IL-2 and IFN-γ (480). It was originally called ‘‘cytokine synthesis inhibitory factor’’ (CSIF)(480). IL-10 was originally thought to be solely produced by T cells, but

it is now known that NK cells, B cells, DCs, cytotoxic T cells, γδ T cells, mast cells and other granulocytes also produce IL-10 (481-489). IL-10 works through binding to the IL-10r (IL-10r), which is composed of two chains IL-10r1 and IL-10r2 and intracellular signalling mainly occurs via Janus kinase (JAK)/signal transducer of activation (STAT) pathway (490).

IL-10 has an inhibitory effect on immunity and can suppress proinflammatory cellular activity, and can simultaneously promote anti-inflammatory functions of such cells. For example, it can suppress the secretion of TNF-α, IL-1β, IL-6 IL-8, G-CSF and GM-CSF by macrophages, while enhancing anti-inflammatory molecules such as IL-1r and soluble TNFr (491-495). As well as acting on APCs, IL-10 can also directly suppress T cell activity by suppressing T cell mediated production of IL-2, IFN-γ and IL-4 production (496, 497). IL-10 has also been shown to be critical for Treg to mediate tolerance to alloantigen in the context of skin transplantation in mice (498).

In addition to immunosuppressive cytokines, co-inhibitory receptors are also employed by the immune system to maintain self-tolerance and as such prevent autoimmunity (499). Co-inhibitory molecule ligands can be expressed by tumours, immunoprivileged tissues or by APCs whereby they are able to directly interact with and suppress Tcon. Alternatively, co-inhibitory molecules can be expressed by immunomodulatory cells such as Treg and play a role in cell-mediated suppression.

An example of a co-inhibitory molecule is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which is constitutively expressed on Treg and can inhibit T cell responses by attenuating the ability of APCs to present antigen and hence reducing T cell priming, or by inducing the indoleamine 2,3-dioxygenase (IDO) production thereby

suppressing T cell proliferation (500, 501). IDO acts by inhibiting tryptophan catabolism which is required for T cell proliferation. (502). Other co-inhibitory molecules that are expressed on Treg and have been shown to mediate immunosuppression by either directly suppressing T cells or inhibiting antigen presentation include immunoreceptor tyrosine-based inhibition motif domain (TIGIT), Lymphocyte activation gene 3 (LAG-3), PD1 and T cell immunoglobulin and mucin domain 3 (TIM-3) (503-507). In conclusion, as we have seen Exp iNKT cells are functionally distinct from their freshly-isolated counterparts. However, whether Exp iNKT cells are immunosuppressive has yet to be determined. In this chapter we will test the suppressive ability of both Exp iNKT and FI iNKT cells *in vitro*.

5.2. Aims and hypothesis

Given our observation in chapter 4 that Exp iNKT cells have an altered cytokine secretion pattern compared to the FI iNKT cells we hypothesized that such cells may have additional suppressive properties.

Therefore, we aimed to test whether Exp and/or FI iNKT cells could suppress alloreactive T cell responses *in vitro*. Furthermore, we determined the cell-surface expression/function of co-inhibitory molecules by iNKT cells and tested whether selected molecules played a role in iNKT cell-mediated suppression.

5.3. Results

5.3.1. Alloreactive T cell proliferation is suppressed in the presence of αGal activated Exp iNKT cells

In chapter 4 we showed that Exp iNKT gained the ability to produce IL-10 following *in vitro* culture. Given the propensity of IL-10 to act in an immunosuppressive manner (508), we reasoned that the Exp cells might show enhanced immunosuppressive properties compared to FI cells. In order to investigate the immunomodulatory potential of Exp iNKT cells, we designed an *in vitro* suppression assay that was reflective of the GVHD model described in Chapter 3. To this end, purified CFSE labelled TEa T cells were cultured with irradiated BMDC from CB6F1 ($H2^{b/d}$) mice that had or had not been pulsed for 24 h with αGal. Such cells were cultured in the presence or absence of Exp iNKT cells. Cultures were left for 4 days before being analysed by flow cytometry.

Strikingly, there was a significant decrease in the frequency of divided TEa T cells in the presence activated iNKT cells (from 79±5% proliferation to 13 ±2% proliferation), which was evident at ratios as low as 1:0.1 (47±7% proliferation) (Figure 5.1A and B). Furthermore, there was a decrease in the number of proliferating cells; once again this was true of ratios as low as 1:0.1 (Figure 5.1C). Importantly, suppression was occasionally but inconsistently observed when cells were not activated with αGal. However the level of suppression was minor compared to that of Exp iNKT cells activated with αGal (Figure 5.1A-C).

Interestingly when we looked at the CFSE Median Fluorescence intensity (MFI) of the divided cells there was no difference in the level of CFSE of divided TEa T cells in the presence or absence of activated Exp iNKT cells suggesting that although there were less dividing TEa T cells in the presence of activated iNKT cells the cells that were dividing did so at the same rate as in control cultures (Figure 5.1D).

5.3.2. Exp iNKT cells are more potent at suppressing alloreactive T cell proliferation than their FI counterparts.

The difference in cytokine secretion (particularly with respect to IL-10) between FI and Exp iNKT cells led us to speculate that immunosuppression may be a feature acquired by Exp iNKT cells that was not shared by FI iNKT cells. In order to test this hypothesis we set-up *in vitro* assays (as described in section 5.2.1.) with either Exp iNKT cells or FI iNKT cells.

Activated Exp iNKT suppressed TEa T cell proliferation as previously shown (from 78±1% proliferation to 27±1% proliferation) (Figure 5.2A-C). However, the addition of FI iNKT cells to cultures resulted in no decrease in the percentage of dividing cells 67±10% proliferation) and only a small albeit significant decrease in TEa T cell number (Figure 5.2A-C). In terms of MFI, we observed that the addition of FI cells in the absence of αGal to the suppression assay lead to a decreased MFI, however this was not observed in repeat experiments. This suggests that, as was the case with the Exp iNKT cells, in the presence of FI iNKT cells there were less dividing TEa T cells however the cells that were dividing did so at the same rate as in control cultures (Figure 5.2D). In conclusion, we found that Exp iNKT cells potently suppressed the T cell response to alloantigen *in vitro*. This was dependent on the re-

activation of Exp iNKT cells at the point of T cell recognition of alloantigen and was confined to Exp iNKT cells. This further suggests that following *in vitro* expansion, iNKT cells acquire potent immunomodulatory properties that are not seen in FI cells.

5.3.3. IL-10 and IFN-γ are not required for the immunosuppressive properties of activated Exp iNKT cells

IL-10 and IFN-γ can both act in an immunosuppressive manner in the context of transplantation (233, 399, 498, 509-511). We therefore investigated whether either IL-10 or IFN-γ were required for Exp iNKT cell-mediated suppression. CFSE labelled TEa T cells were activated with irradiated CB6F1 BMDCs pulsed with αGal and were cultured with or without Exp iNKT cells (at a ratio of 1:0.25). In selected wells, anti-IL-10r or anti-IFN-γ mAbs were added to such cultures. As before, cells were cultured for 4 days and analysed by flow cytometry.

Exp iNKT cells suppressed TEa T cells in terms of the frequency and number of dividing cells (Figure 5.3A-C). The addition of either anti-IL-10r or anti-IFN-γ failed to significantly affect the suppression of alloreactive T cells in terms of frequency (Figure 5.3A, B). The number of proliferating cells was also reduced in the presence of anti-IL-10r or anti-IFN-γ mAbs (Figure 5.3C). Taken together these findings imply that the mechanism of suppression utilized by Exp iNKT cells is independent of IL-10 or IFN-γ.

5.3.4. Exp iNKT cell suppression of alloreactive T cells is not mediated by iNKT cell consumption of IL-2

Activated T cells and iNKT cells both express the high affinity IL-2r α chain, CD25, we wanted to test whether iNKT cells might be starving activated TEa T cells of IL-2 thus preventing their proliferation. First, given that Exp iNKT cells were better at suppressing than the FI cells, we investigated to see if the level of CD25 between such populations varied. We found that there was an increase in the frequency of Exp iNKT cells expressing CD25 compared to the FI cells, indeed almost all the Exp cells expressed CD25 (Figure 5.4A and B). It was therefore feasible that the Exp iNKT cells were limiting IL-2 availability for activated TEa T cells. To investigate this possibility *in vitro* suppression cultures were carried out in an excess of exogenous IL-2.

Interestingly, we found that Exp iNKT cells were still able to suppress TEa T cells in the presence of IL-2 (Figure 5.4-C-E). Together, these findings suggest that Exp iNKT cells do not suppress T cell responses by starving responding Tcon of IL-2.

5.3.5. Anti-CD3 and anti-CD28 stimulated iNKT cells do not suppress TEa T cells *in vitro*

Thus far we had used BMDC to drive both alloreactive T cells and iNKT cells raising the possibility that suppression was via signalling to the APC. Therefore, we asked whether Exp iNKT cells could suppress TEa T cells in an APC free system using anti-CD3 and anti-CD28 mAbs to stimulate both iNKT cells and conventional T cells.

Surprisingly, we found that not only did the Exp iNKT cells not suppress TEa T cells when stimulated with anti-CD3 and anti-CD28 but the addition of Exp iNKT cells

resulted in an increase in the percentage of TEa T cells that had divided as well as in absolute TEa T cell number (Figure 5.5A-C). These data therefore suggest that either there is a requirement for BMDCs to mediate the suppressive effect or that activation by anti-CD3 and anti-CD28 renders T cells resistant to suppression.

5.3.6. The expression of co-inhibitory receptors is increased in activated iNKT cells

Due to our observation that Exp iNKT cells could suppress TEa T cells stimulated with BMDCs but not anti-CD3 and anti-CD28, we next sought to investigate if BMDCs affected the phenotype of Exp iNKT cells in terms of co-inhibitory molecules expression. A previous study in our lab looked at the alteration in gene expression in iNKT cells, 48 hrs after *in vivo* activation with αGal. Since we were also looking at cells which have been activated (albeit during the course of the expansion), we decided to look to this microarray for cell surface molecules that are upregulated during activation which have known suppressive effects. The microarray showed the upregulation of several candidate molecules namely PD1, Lag-3, TIM-3 and TIGIT (Figure 5.6A-C).

As a result of these observations we decided to investigate if there were differences in the expression of some of these markers in Exp iNKT cells following culture with unpulsed BMDCs, anti-CD3 and anti-CD28 or αGal pulsed BMDCs. The expression of markers proved to be highly variable depending on the experiment, possibly due to the fact that the Exp iNKT cells have a more varied phenotype than the FI iNKT cells. However, in all three independent experiments, stimulation with αGal pulsed BMDCs resulted in the highest frequency of Exp iNKT cells expressing LAG-3 (Figure 7A, D,

E), PD1 (Figure 7B, D, E) and TIGIT (Figure 7C, D, E). Our observation that Exp iNKT cells express LAG-3, PD1 and TIGIT after activation combined with the microarray the data suggest that one or more co-inhibitory molecules such as PD1, TIGIT and LAG-3 could be responsible for mediating the suppressive effect of Exp iNKT cells *in vitro*.

Given the fact that only activated Exp iNKT cells potently suppressed T cell responses *in vitro* we next examined the expression of LAG-3, PD1 and TIGIT on activated FI iNKT cells. To this end, FI iNKT cells were stimulated with either anti-CD3/CD28 or αGal pulsed BMDCs and analysed after 6 h. The frequency of FI iNKT cells expressing either LAG-3, PD1 or TIGIT was low in the FI iNKT cells regardless of activation (Figure 5.8). Given the fact that these molecules are co-inhibitory receptors we sought to determine if one or more of them were responsible for the suppressive effect of the Exp iNKT cells.

Durgan *et al* showed that there was increased anti-tumour immunity in the absence of PDL1:PD1 interactions (512). Therefore we decided to test whether PDL:PD1 interactions were important for Exp iNKT cell suppression. To this end, a suppression assay was performed using a ratio of 1:1 TEa T cells to Exp iNKT cells which we have previously shown results in robust suppression of T cell responses *in vitro* (Figure 5.1). Blocking antibodies for PD1 (which is expressed on Exp iNKT cells) and PDL1 and PDL2, which are expressed on BMDCs (unpublished data by Jones *et al.)*, were added to test their involvement in the suppressive mechanism. Blocking PD1 or PDL1/2 did not impact the ability of Exp iNKT cells to suppress TEa T cells in terms of either frequency or number of proliferating TEa T cells (Figure 5.9A-C). The level

of CFSE was not changed in the presence of anti-PDL1 and anti-PDL2 blockade, (Figure 5.9D).

Taken together these findings show that Exp iNKT cells differentially express a panoply of co-inhibitory surface markers. Although we have shown a lack of involvement of the PD1/PDL1/2 pathway there are other candidate immunomodulatory molecules which may play a role in iNKT cells mediated suppression.

5.4. Discussion

The most significant finding of this chapter was that Exp iNKT cells but not FI iNKT cells potently suppress an alloreactive T cell response *in vitro* (Figure 5.1). This finding is validated by three human studies that Exp human iNKT cells were shown to suppress alloreactive T cells *in vitro* (408, 513, 514). However, as these studies used unseparated T cells as responder cells it is possible that Treg were also involved in the suppressive mechanism. Furthermore, these studies did not investigate if FI human iNKT cells can suppress alloreactive T cells *in vitro*. One of these studies details the mechanism of suppression, which in this case occurs via iNKT cell mediated lysis of the DCs (408). This would be an interesting experiment to carry out in the future using murine iNKT cells.

Phenotypic analysis of Exp iNKT cells revealed that they functionally distinct compared to FI iNKT (Figures 4.6 and 4.8). In particular, Exp iNKT cells produced high levels of IL-10, IL-13 and IL-17 whilst maintaining IFN-γ secretion (Figure 4.6). IL-10 and IFN-γ have both been found to be immunosuppressive in the context of transplantation (although IFN-γ is classically a proinflammatory cytokine) (233, 399, 498, 509-511, 515). However, blocking the IL-10r or neutralising IFN-γ failed to affect suppression (Figure 5.3). It is possible however that the suppressive effect could be mediated by other cytokines that are secreted by Exp iNKT cells such as IL-13. IL-13 has been shown to suppress anti-tumour immunity in a model of tumour growth-regression/recurrence and to be suppressive in a model of contact dermatitis, although it was not confirmed that the iNKT cells were the source of IL-13 in either case (450, 516).

One way to test if soluble factors, such as cytokines, are involved in suppression would be to activate T cells in the presence of supernatant harvested from re-activated Exp iNKT cells. Although soluble molecules were not exhaustively tested we might argue that as suppression is consistent but the cytokines that Exp iNKT cells produced at the end of an expansion varied, the modulation of cytokine production in Exp iNKT cells may not relate to the suppressive effect of these cells. However, although the signature cytokines may not be involved in suppression another cytokine could be involved. Such a cytokine could be common to all subsets of iNKT cell or unique to one or more subset. In addition we have not investigated the stability of Exp iNKT cells and thus we don't know if they still make these cytokines during the response. Indeed preliminary analyses of supernatants harvested from such suppression assays showed variable cytokine detection between experiments (data not shown).

Another possibility was that given that CD25 is highly expressed in Exp iNKT cells they could be out-competing the TEa T cells for IL-2 and therefore starving the TEa T cells of IL-2 which is required for survival and proliferation. There is precedence for this as this mechanism of suppression has been reported to be used by Treg (477, 517, 518). However, performing assays in the presence of excess IL-2 did not affect the suppression by Exp iNKT cells therefore; the mechanism of suppression is unlikely to involve IL-2 sequestration by iNKT cells.

In addition to secreted molecules such as cytokines, Exp iNKT cells may suppress via the expression of certain cell surface molecules with immunomodulatory potential. Ideally, we would have performed a microarray of the Exp cells compared to the FI cells, to see if there were significant changes in the expression of genes related to T

cell suppression. However, due to time and money constraints we looked to a microarray that was performed on *in vivo* activated iNKT cells. The array showed that there was upregulation of many co-inhibitory molecules on activated iNKT cells after 48h (Figure 5.6). We looked at the expression of such molecules on FI and Exp iNKT cells and found that consistent with the array data they were upregulated on the Exp cells and were further increased following re-activation with BMDCs presenting αGal the most efficient mode of activation (Figure 5.7). However, the upregulation of molecules such as LAG-3, PD1 and TIGIT was confined to Exp iNKT cells as most FI iNKT cells did not express these molecules even after stimulation (Figure 5.8).

There are many suppressive co-inhibitory molecules that could be responsible for suppression either directly or indirectly, and this would be an interesting line of investigation. Indeed similar mechanisms of suppression have been observed to be mediated by Treg for example TIGIT is expressed on a distinct subset of Treg and can suppress Th1 and Th17 cells by inducing the expression of fibrinogen-like protein 2 (Fgl2) a soluble suppressive molecule that promotes Treg mediated suppression of Tcon (503). In addition, TIGIT-Fc has been shown to alleviate acute GVHD by binding to CD155 on DCs (504). This promotes DCs to develop a regulatory phenotype with attenuated antigen presentation capacity and the ability to suppress $CD8^+$ T cells via IL-10 production (504). It is possible that TIGIT could be a suppressive mechanism employed by Exp iNKT cells to suppress alloreactive T cells. This could occur via either Fgl2, or through interactions with CD155 on DCs promoting to the generation of immunoregulatory DCs in a similar manner to Treg mediated suppression.

LAG-3 is another co-inhibitory molecule which confers a suppressive ability upon Treg, enabling them to suppress Tcon, while Treg from $LAG3^{-/-}$ mice exhibit diminished suppressive capacity (505). Upon engagement of MHC class II on $CX3CR1^{+}$ macrophages LAG-3^{+} Treg cells have been shown to inhibit IL-1β and IL-23 production by such cells, thereby promoting immune tolerance (519).

PD1 is another co-inhibitory molecule that has also been shown to play a role in Treg mediated suppression of Tcon, $PD1^{-/-}$ mice retain normal levels of FOXP3, but lack functionally suppressive Treg (506). TIM-3 may also play a suppressive role, it has been shown to be expressed on 40% of $FOXP3^{+}$ T cells which infiltrate in a model of skin-graft rejection and were shown to robustly suppress Tcon *in vitro* (507). Furthermore, $FOXP3^{+}$ TIM-3^{+} Treg have been shown to inhibit Tcon in RA and Head and Neck cancer although the exact mechanism by which this occurs is not understood (520, 521).

In many cases it is unclear how these molecules function to suppress Tcon responses. They may rely on cell-cell contact to transmit inhibitory signals to mediate their suppressive effects, or ligation to their ligands may help them to maintain a suppressive phenotype, or to induce other cell surface or secretory molecules or even to act through another cell. Whatever the case may be work by the Brenner Lab as part of the Immunological Genome Project revealed that co-inhibitory receptors are expressed on iNKT cells and thus may play a role in any potential iNKT mediated suppression (522). Furthermore, we examined the role of PD1/PDL1 in immunomodulation by Exp iNKT cells but found that disruption of this pathway failed to impact suppression. However, as we found that LAG-3 and TIGIT were expressed upon stimulation with αGal BMDCs (Figure 5.7), it would be interesting to investigate

the involvement of co-inhibitory molecules such as these in the mechanism of suppression. This could occur either through direct T cell suppression or indirectly though BMDCs. Indeed, the ligand for LAG-3 is MHC class II is expressed on BMDCs (Appendix V) and one of the ligands for TIGIT is CD155 is also expressed on BMDCs (505).

Another interesting possible mechanism of suppression could be that the Exp iNKT cells are killing the TEa T cells, either directly, or through killing the BMDCs and thereby preventing antigen presentation. Indeed, human $CD8^{+}$NKT-like cells have been reported to suppress T cells *in vitro* by killing antigen bearing DCs. (479). Similarly, human $CD4^{-}$ iNKT cells have been shown to suppress Tcon in a human *in vitro* suppression assay, by mediating the lysis of DCs and thereby inhibiting T cell proliferation (408). In addition, iNKT cells from the spleen, liver and thymus were shown to be able to kill A2 lymphoma cells with or without CD1d transfection (523). Furthermore, this study also showed that killing is CD1d and antigen dependent *in vivo* and that killing is more potent in the presence of a strong iNKT cells antigen like αGal than a weak stimulator like OCH. Interestingly, iNKT cell mediated killing was shown to be mediated through the CD95/CD178 (Fas-Fas-L) *in vivo* (523). It remains a possibility that Exp iNKT cells prevent T cell activation using a similar mechanism therefore it would be worthwhile blocking Fas-Fas-L interactions to see if this had any effect on the suppression. It would also be useful to look at apoptosis of both BMDCs and TEa T cells. Furthermore, it would be interesting to investigate if Exp iNKT cell mediated suppression is maintained when BMDCs are pulsed with C20.2 instead of αGal.

Finally, we noted that Exp iNKT cells were unable to suppress alloreactive T cells that were stimulated with anti-CD3 and anti-CD28. There could be several reasons for this. One possibility is that the activation of T cells with anti-CD3/CD28 results in a response that is resistant to suppression. Alternatively, it may be the anti-CD3/CD28 stimulation of Exp iNKT cells fails to elicit the mechanism of suppression. It is also possible that the mechanism of suppression involves the modification of allogeneic, glycolipid presenting APC.

It would be interesting to investigate if the αGal has to be presented by the BMDCs presenting alloantigen to the T cells or whether suppression occurs as long as both T cell and Exp iNKT cells are activated by BMDC. This could be tested by mixing αGal pulsed B6 BMDCs to provide iNKT activation and CB6F1 BMDCs to activate the TEa T cells. If suppression is not seen in this system then this would imply that Exp iNKT cells suppress TEa T cells by modification of the alloantigen-presenting APC. This could work by Exp iNKT cells inducing a phenotypic change in the BMDCs reducing their ability to stimulate TEa T cells (such as loss of co-stimulatory molecules) or killing of BMDC or inducing the BMDC to express molecules that suppress T cell responses themselves, one such molecule being IDO. Treg have been reported to use both mechanisms via the expression of co-inhibitory molecule CTLA-4 to suppress T cells (500, 501).

At this point we have shown that there are cells with different functional activities present within the Exp iNKT cells (Figure 4.12). It is possible that all of the cells share a common suppressive mechanism or that the ability to suppress T cell responses is restricted to Exp iNKT cells with a certain phenotype. It would be interesting to test the various subsets of iNKT cells to see if their ability to suppress differs. This could

be achieved by sorting functional subsets using cytokine or transcription factor reporter mice, although as we have seen some subsets do not expand well so this could be difficult in the mouse. If a specific subset of iNKT cells harbours the suppressive activity then performing RNA-Seq studies may reveal a cell surface molecular signature for the cells that could be used to sort the immunosuppressive Exp iNKT cells to maximise functional activity before transfer into HSC/solid organ transplant recipients.

In conclusion, we found that re-activated Exp iNKT cells but not FI iNKT cells potently suppress the response of alloreactive T cells *in vitro*. Although the mechanisms of suppression used by Exp iNKT cells remain to be elucidated this provides us with the exciting prospect that Exp iNKT cells have developed suppressive properties which may impact alloreactive T cell-driven pathology *in vivo* in conditions such as GVHD.

5.5. Figures

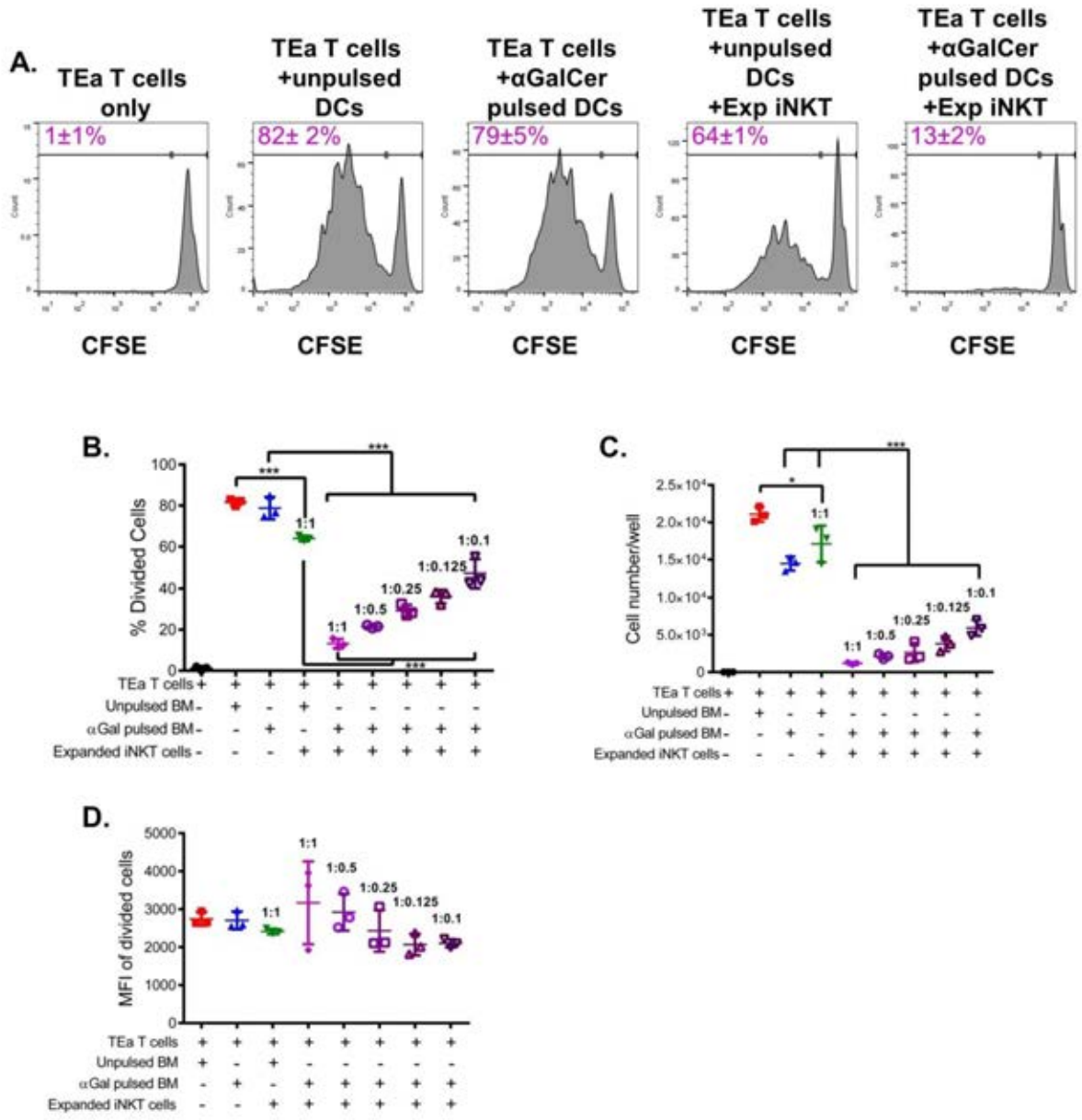


Figure 5.1 Activated Exp iNKT cells suppress alloreactive T cell responses in vitro TEa T cells were purified and labelled with CFSE and stimulated with BMDCs ± αGal ± Exp iNKT cells (at decreasing ratios from 1:1 to 0.1). Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated on live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (A) Representative plots showing CFSE profile of TEa T cells. (B) Proliferating TEa T cells percentage. (C) Proliferating TEa T cell number. (D) Proliferating TEa T cells MFI. Data is representative of 2 independent experiments, each experiment done in triplicate for the titration and 4 independent experiments, each in triplicate for all other conditions. Results are analysed using one-way ANOVA with multiple comparisons. *p<0.05 ,***p<0.001.See Appendix V for gating strategy.

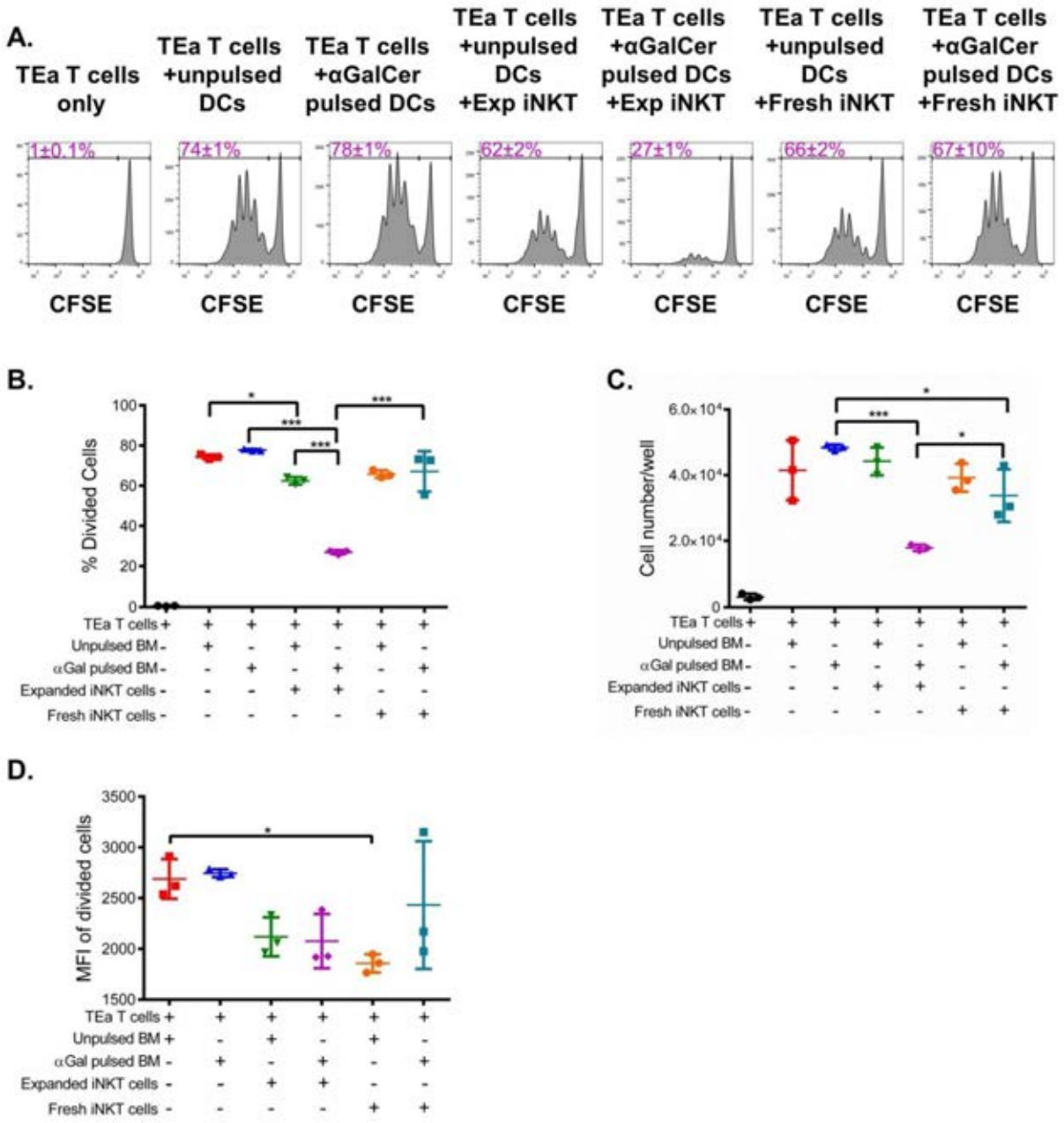


Figure 5.2 Activated Exp iNKT cells but not FI iNKT cells can suppress alloreactive T cells *in vitro* TEa T cells were purified and labelled with CFSE and stimulated with BMDCs ± αGal ± Exp or FI iNKT cells (at a 1:1 ratio). Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated on live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (A) Representative plots showing CFSE profile of TEa T cells (B) Proliferating TEa T cells percentage. (C) Proliferating TEa T cell number. (D) Proliferating TEa T cells MFI. Data is representative of 3 independent experiments, each done in triplicate. Results are analysed using one-way ANOVA with multiple comparisons. *p<0.05, ***p<0.001. See Appendix V for gating strategy.

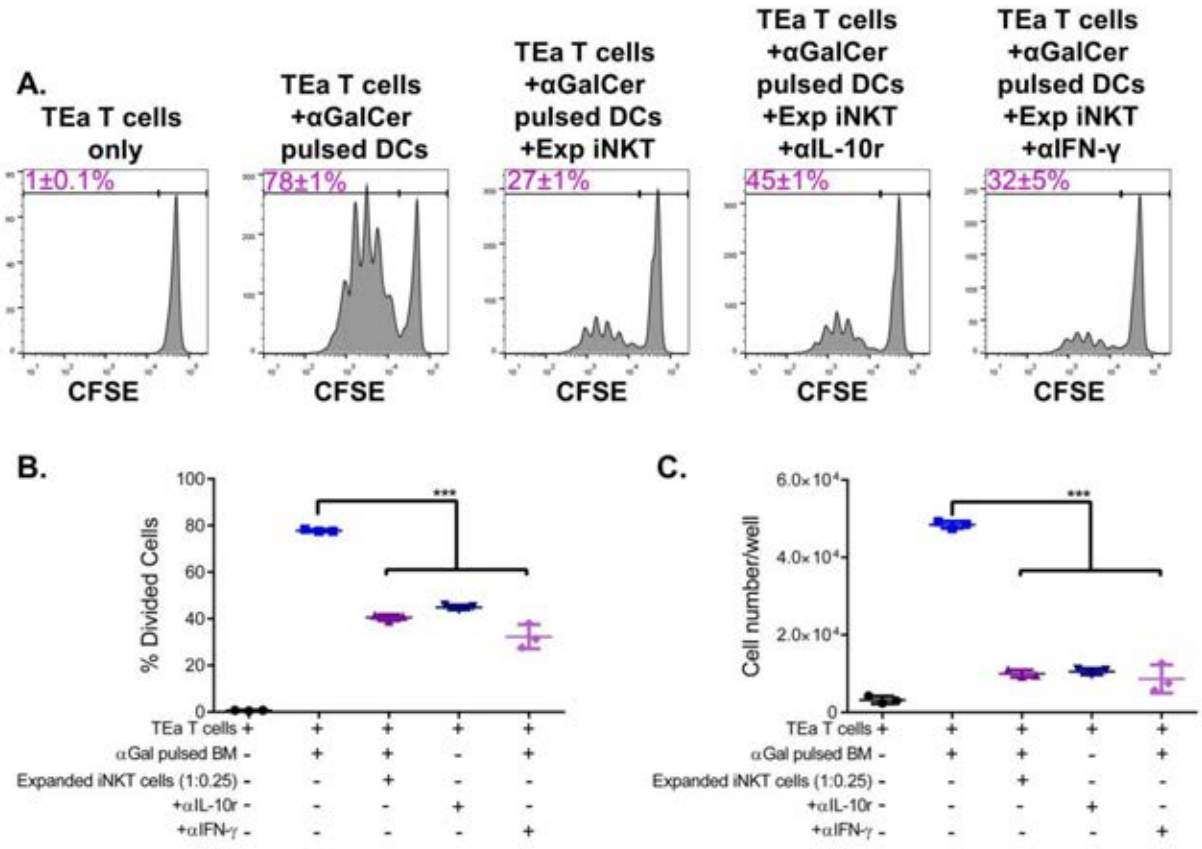


Figure 5.3 IL-10 and IFN-γ are not required for the suppression of alloreactive T cells by activated Exp iNKT cells TEa T cells were labelled with CFSE and stimulated with BMDCs ± αGal ± Exp iNKT cells (at a ratio of 1:0.25) ± αIL-10r or ±IFN-γ. Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated on live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (A) Representative plots showing CFSE profile of TEa T cells. (B) Proliferating TEa T cells percentage. (C) Proliferating TEa T cell number. Data is representative of 3 independent experiments, each done in triplicate. Results are analysed using one-way ANOVA with multiple comparisons. ***p<0.001. See Appendix V for gating strategy.

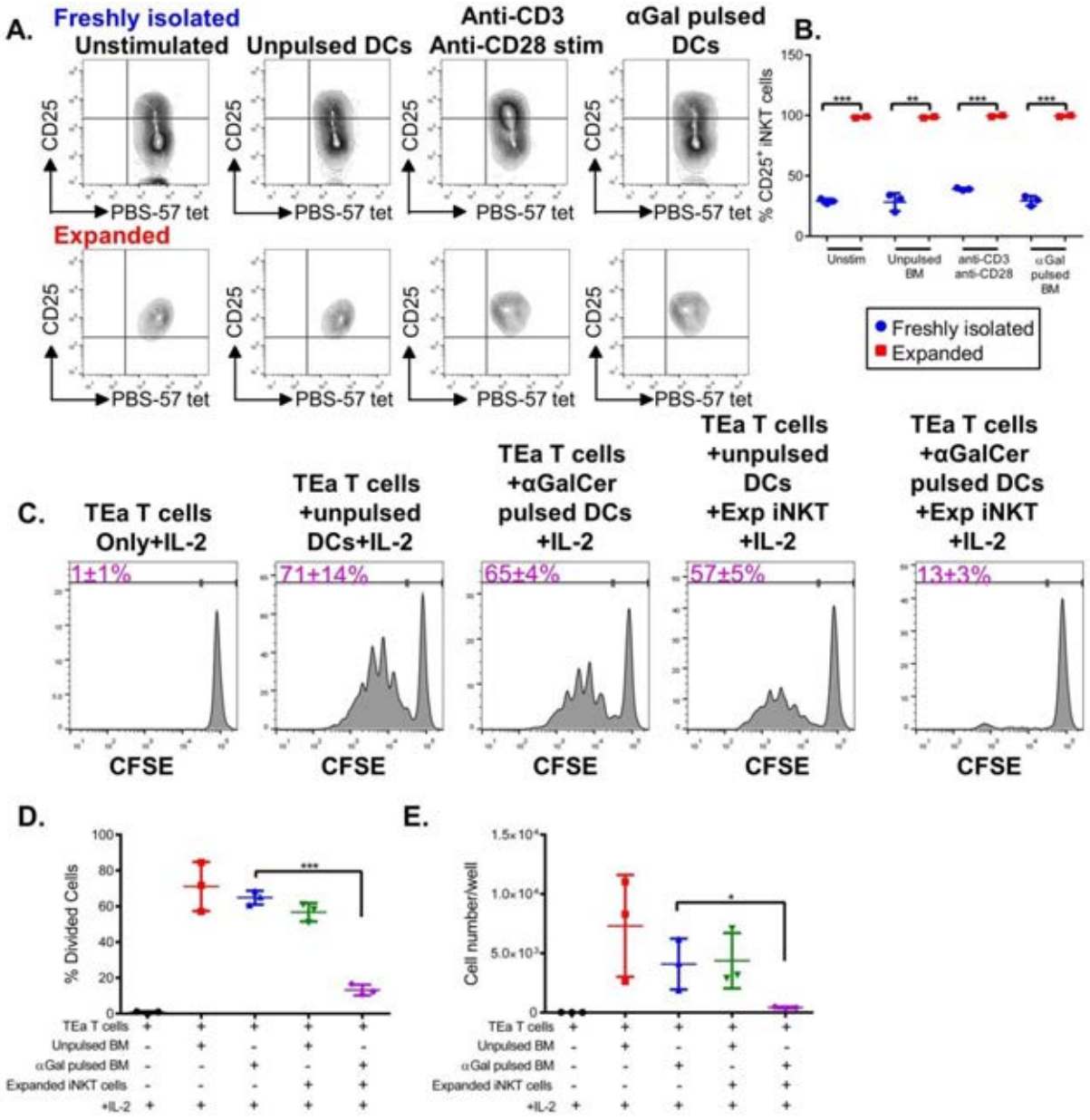


Figure 5.4 Activated Exp iNKT cells suppress alloreactive T cells *in vitro* through an IL-2 independent mechanism (A, B) Exp iNKT cells were stained for CD25, and gated on live lymphocytes, TCR-β and PBS-57 tetramer and CD25. TEa T cells were purified and labelled with CFSE and stimulated with BMDCs ± αGal ± Exp iNKT cells in the presence of excess IL-2. Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (C) Representative plots showing CFSE profile of TEa T cells (D) Proliferating TEa T cell percentage. (E) Proliferating TEa T cell number. Data is representative of 2 independent experiments, each done in triplicate. Results are analysed using t-tests.*p<0.05 **p<0.01 ***p<0.001. See Appendix V for gating strategy.

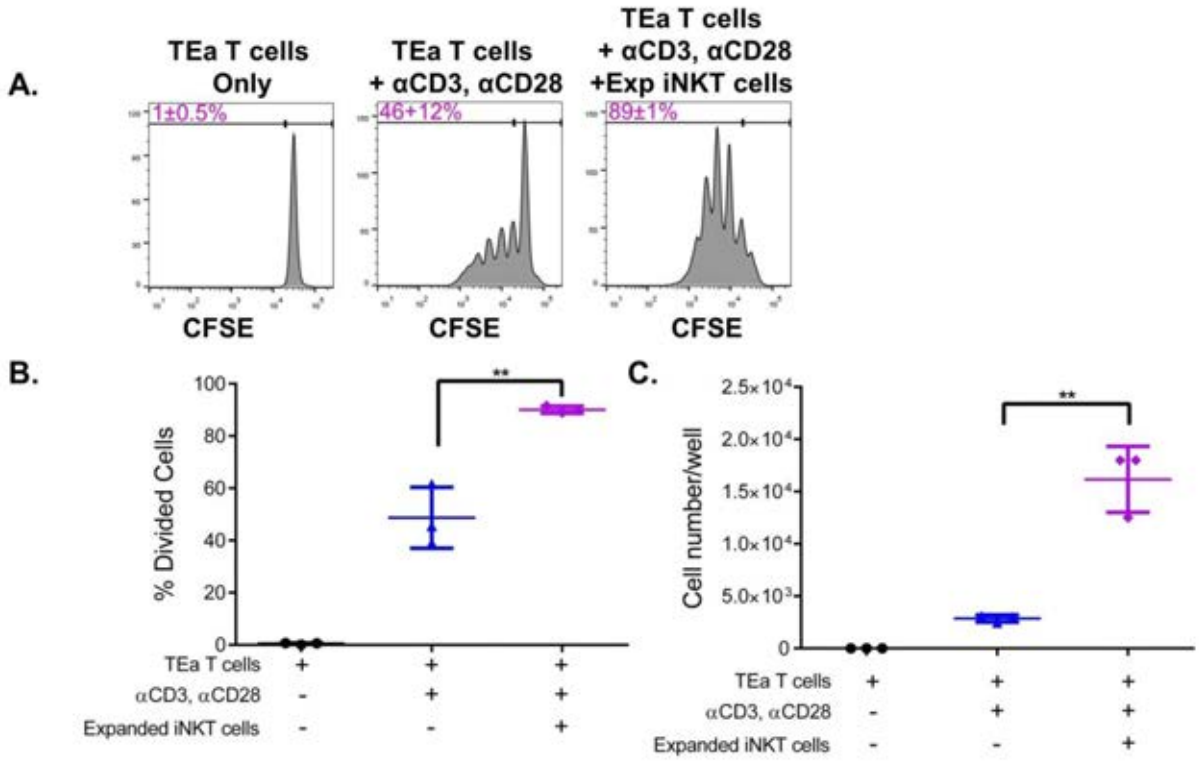


Figure 5.5 Activated Exp iNKT cells cannot suppress anti-CD3, anti-CD28 stimulated alloreactive T cells *in vitro* TEa T cells were purified and labelled with CFSE and stimulated with αCD3 and αCD28 ± Exp iNKT cells. Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated on live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (A) Representative plots showing CFSE profile of TEa T cells. Data is representative of 2 independent experiments. (B) Proliferating TEa T cell percentage. (C) Proliferating TEa T cell number. Data is representative of 2 independent experiments, each done in triplicate. Results are analysed using t-tests. **p<0.01. See Appendix V for gating strategy.

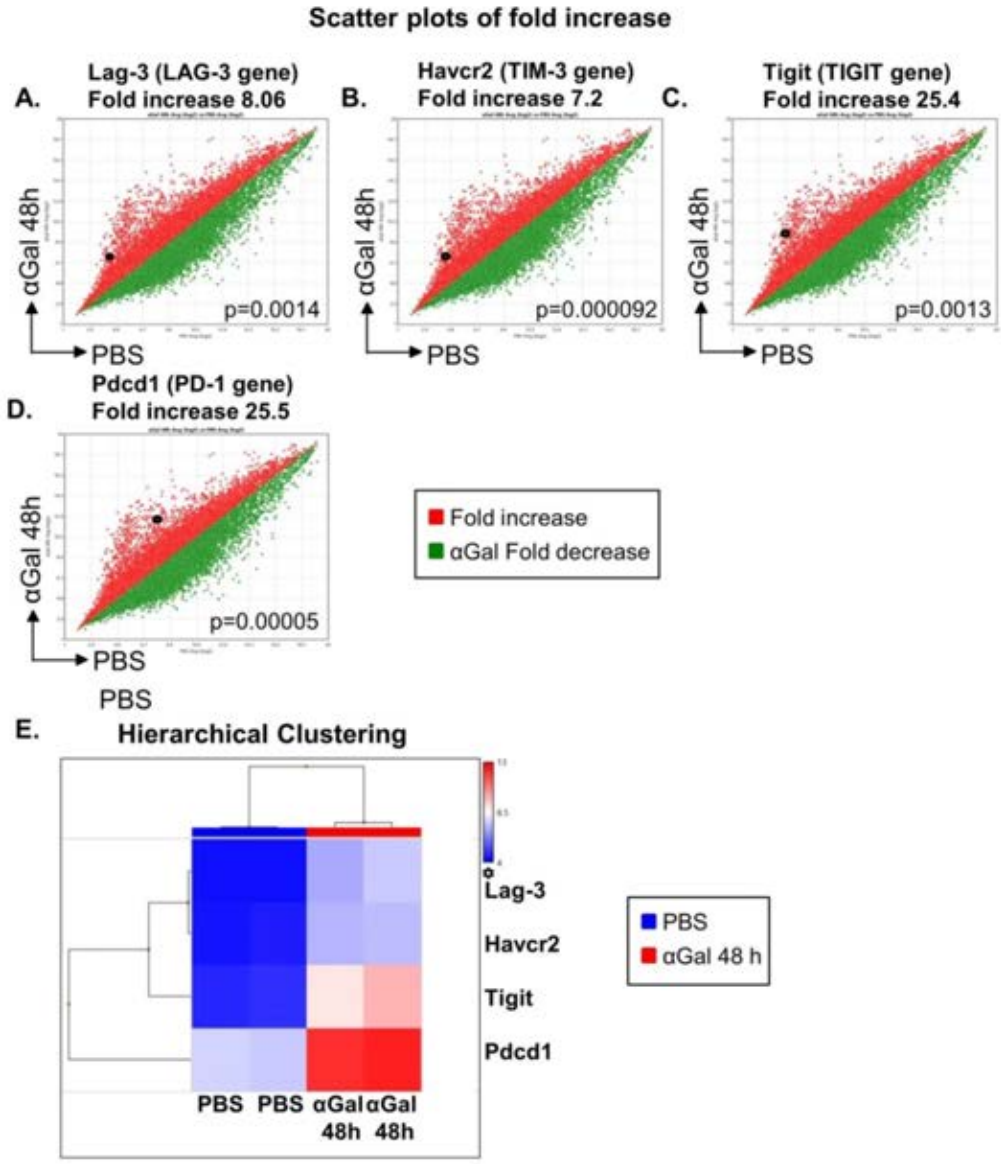


Figure 5.6 Activated iNKT cells show an increased expression of immune checkpoint molecules LAG-3, PD1 and TIGIT IFN-γ reporter mice were given i.p. 2μg of αGal or PBS. After 48h iNKT cells were isolated from αGal or PBS injected mice using beads following by FACS sorting and RNA was extracted and a microarray was performed. Relative expression of (A) Lag-3 (LAG-3 gene) (B) Havcr2 (TIM-3 gene) (C) Tigit (TIGIT gene) (D) Pdcd1 (PD-1 gene) (E) Hierarchical Clustering of Lag-3, Havcr2, Tigit and Pdcd1. Figures are from 2 pooled experiments. Results are analysed using ANOVAs with eBayes empirical parameter improvement.

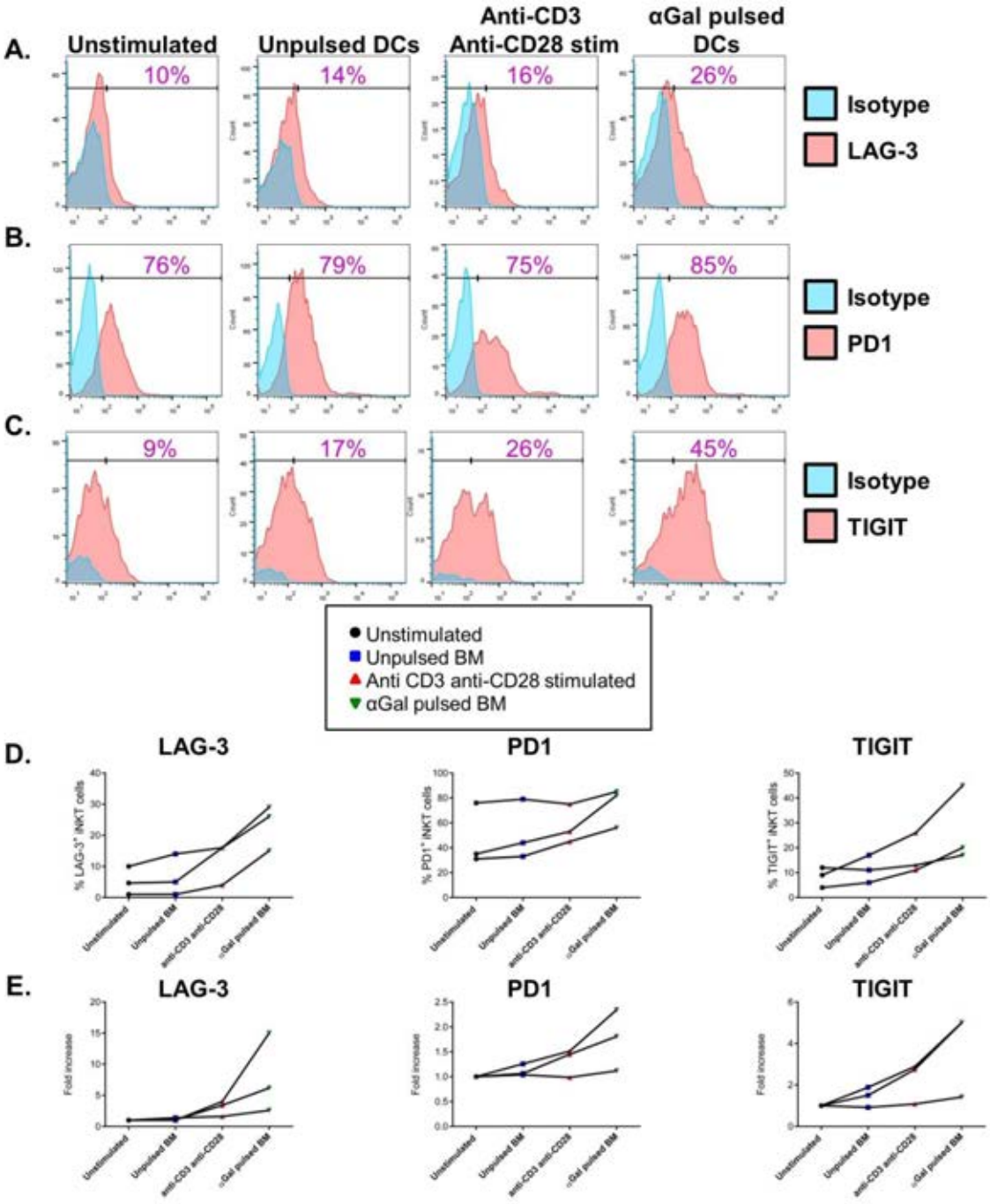


Figure 5.7 Activated Exp iNKT cells show an increased expression of immune checkpoint molecules LAG-3, PD1 and TIGIT Exp iNKT cells were either left in unstimulated or stimulated with unpulsed BMDCs, anti-CD3 and anti-CD28 or αGal pulsed BMDCs and incubated for 6 h. Cells were analysed by Flow Cytometry and gated on expanded lymphocytes, live cells and TCR-β versus PBS-57 tetramer followed by LAG-3, PD1 or TIGIT. Representative plots of Exp iNKT cells stained for **(**A) LAG-3, (B) PD1 and (C) TIGIT. (D) Percentage expression of LAG-3, PD1 and TIGIT in Exp iNKT cells. (E) Normalised expression of LAG-3, PD1 and TIGIT, such expression is measured relative to unstimulated expression which is set at 100%. Plots are representative of 3 independent experiments, and graphs are from 3 compiled experiments. Results are analysed using one-way ANOVA with multiple comparisons. All results were non-significant.

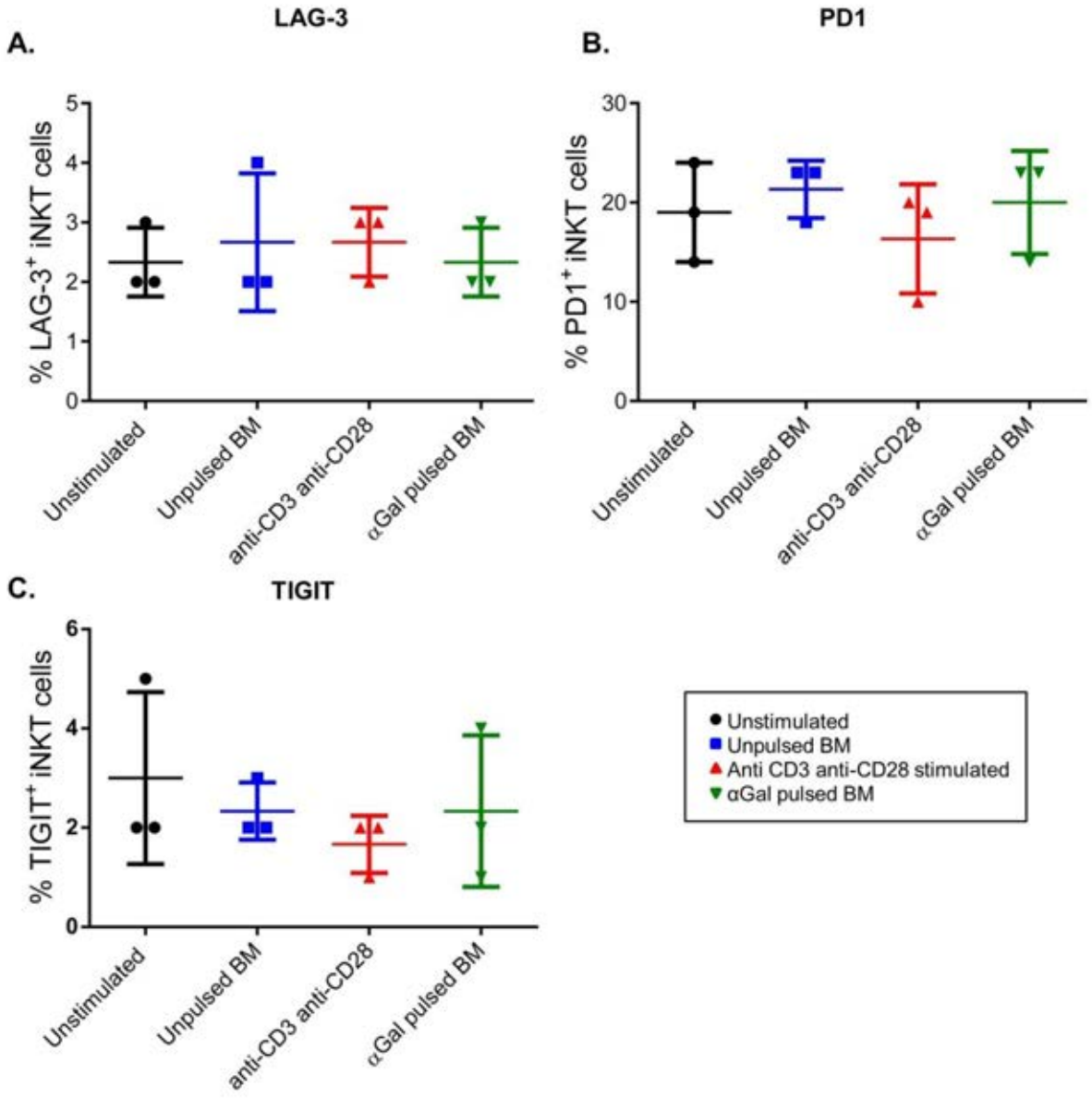


Figure 5.8 Activated FI iNKT cells show no difference in expression of LAG-3, PD1 and TIGIT regardless of the mode of activation FI iNKT cells were either left in unstimulated or stimulated with unpulsed BMDCs, anti-CD3 and anti-CD28 or αGal pulsed BMDCs and incubated for 6 h. Cells were analysed by Flow Cytometry and gated on lymphocytes, live and TCR-β versus PBS-57 tetramer followed by (A) LAG-3, (B) PD1 and (C) TIGIT. Graphs are from 3 compiled experiments. Results are analysed using one-way ANOVA with multiple comparisons. All results were non-significant.

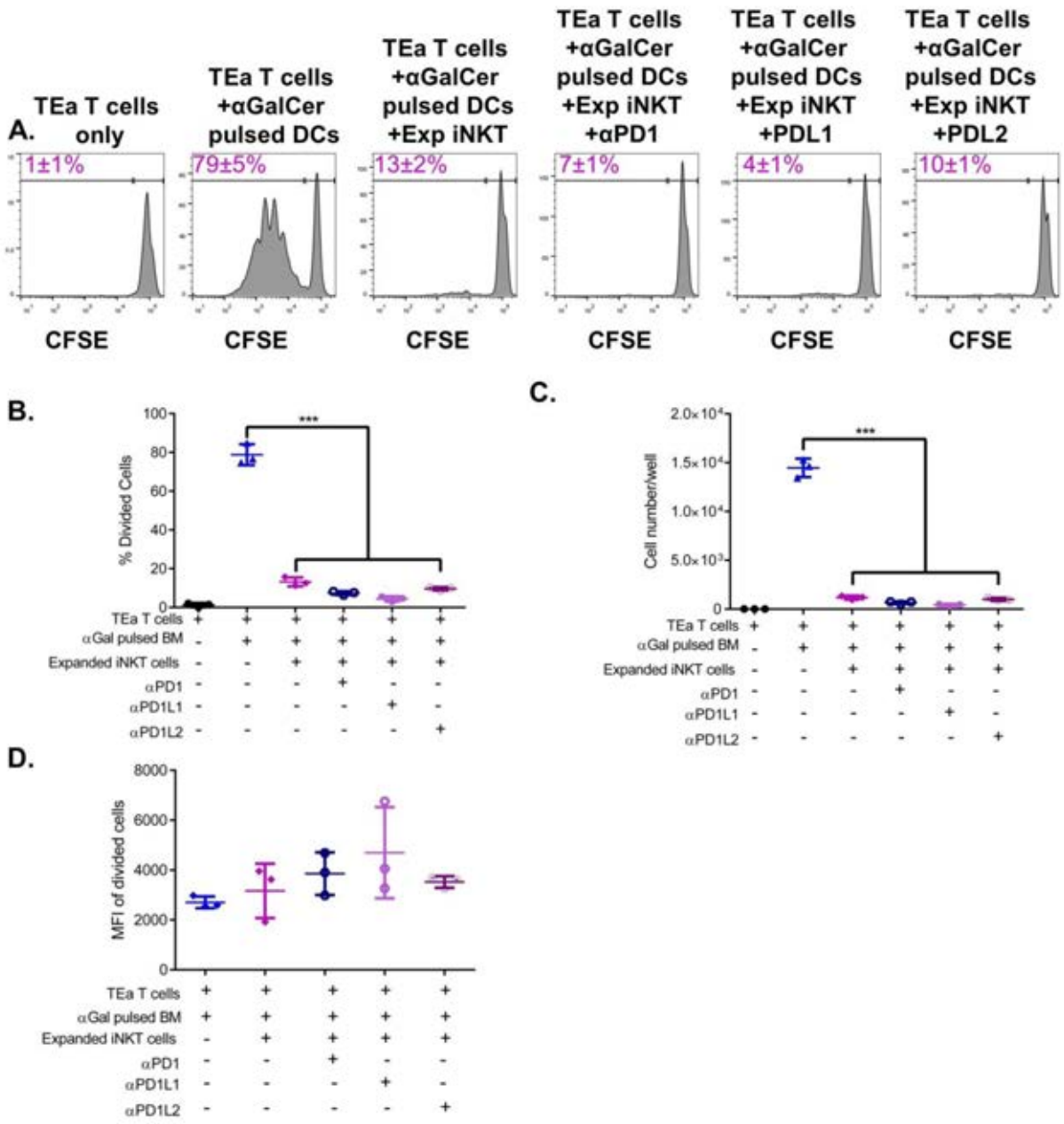


Figure 5.9 Activated Exp iNKT cells suppress alloreactive T cells *in vitro* through a PDL1/2 independent mechanism *in vitro* TEa T cells were purified and labelled with CFSE and stimulated with BMDCs ± αGal ± Exp iNKT cells at a 1:1 ratio) ±PD1 or PDL1 or PDL2. Cells were analysed by Flow Cytometry on Day 4 and proliferating TEa T cells were gated live cells, TCR-β and CD4, Vα2 and proliferating cells based on CFSE staining. (A) Representative plots showing CFSE profile of TEa T cells. (B) Proliferating TEa T cell percentage. (C) Proliferating TEa T cell number. (D) Proliferating TEa T cell MFI. Data is representative of 1 experiment, done in triplicate. Results are analysed using one-way ANOVA with multiple comparisons. **p<0.01 ***p<0.001. See Appendix V for gating strategy.

CHAPTER 6 INVESTIGATING THE ABILITY OF iNKT CELLS TO INFLUENCE GVHD

6.1. Introduction

iNKT cells were originally thought to contribute to GVHD, and it wasn't until 1999 that it was observed that depletion of $NK1.1^+$ cells from bone-marrow grafts led to an increased occurrence of GVHD in mice (456). In addition, observations in humans have found an association between the increased number of iNKT cells in HSC transplants and the incidence and severity of GVHD. One study found that there was a decreased incidence of grade II-IV acute GVHD in patients that received in excess of the median number of contaminating iNKT cells (408). Furthermore, greater numbers of iNKT cells in the HSC transplant was found to correlate with both improved GVHD and progression free survival (409). Another study showed that the recovery of iNKT cells post-transplant was associated with reduced non-relapse mortality without a risk of relapse and increased the overall survival rate (410).

As a result of these observations, attempts have been made to artificially manipulate the frequency of the remaining iNKT cells by using fractionated non-myeloablative irradiation of lymphoid tissues and anti-T cell antibodies. This was shown to lead to over representation of $NK1.1^+$ and $DX5^+$ T cells in B6 and BALB/c mice respectively and to prevent GVHD in BALB/c mice that had received fully mismatched B6 transplants (524). Using IL-$4^{-/-}$ mice this was shown to be mediated by either donor or host derived IL-4. The same group later showed that host iNKT cells could induce the expansion of Treg which was IL-4 dependent and was lost in J$\alpha18^{-/-}$ recipients (525).

There have also been several clinical trials which validate the use of reduced intensity conditioning regimes. Patients that received total lymphoid irradiation (where only lymphoid organs are exposed to irradiation) and T cell depletion and HCT transplants to treat haematopoietic malignancies had a very low risk of acute GVHD and other transplant related mortalities (447). Two more recent trials confirmed this and in addition patients showed that the anti-tumour effect was maintained, however none of these trials have examined the role that iNKT cells may play in this protection (447, 526).

As well as boosting the number of iNKT cells through conditioning regimes, the ability of adoptively transferred (AT) iNKT cells to suppress GVHD has also been explored. The first study transferred small numbers of $DX5^+$ cells (40% of which are iNKT cells) into a model of semi-allogeneic BMT and found that this led to decreased clinical symptoms of GVHD and increased survival (235). The Negrin lab later specifically isolated $CD4^+$ NKT cells and showed that they could suppress GVHD at a low dose, through an IL-4 dependent mechanism (234). They later went on to show that this effect was mediated by $CD4^+$ iNKT cell mediated expansion of $CD11b^+Gr\text{-}1^{int}$ MDSCs

which lead to the expansion of $CD4^{+}CD25^{+}FoxP3^{+}$Treg which are required for the protective effect to be mediated (126, 238). Furthermore, this effect could still be obtained using third party iNKT cells (126).

The ability of glycolipids to activate iNKT cells and hence manipulate GVHD has also been investigated. Hashimoto *et al.* have reported that a single injection with αGal significantly decreases morbidity and mortality in a B6 to BALB/c model of GVHD, and this protective effect did not occur in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipient mice (527). The opposite was seen by Kuns *et al.* who showed that αGal injection induced hyperacute GVHD and early mortality in B6 to $B6D2F_1$ and BALB/c to B6 models (528). The difference between the responses to αGal is likely due to the recipient mice used. This suggests a role for remaining recipient iNKT cells. BALB/c mice are known to be Th2 biased (529-531), and therefore it is likely that the IL-4 rich environment contributed to the protective effect. In support of this hypothesis, C20.2 an αGal analogue that is biased towards Th2 cytokines (278), failed to exacerbate GVHD in B6 to $B6D2F_1$ and BALB/c to B6 GVHD models and in most settings inhibited it (528).

iNKT cells have been reported be able to prevent GVHD while maintaining the anti-tumour effect. Existing therapies that involve depleting T cells in the graft can reduce GVHD, however this comes at a cost as it results in impaired engraftment and reduces the anti-tumour effect (148, 532). The Holy Grail in the treatment of GVHD is the dampening down of the GVHD response while still maintaining the anti-tumour effect. Higher numbers and ratios of iNKT to Tcon have been shown to be associated with decreased GVHD occurrence and increased tumour progression free survival in humans (409, 410). In addition, in a mouse model of total lymphoid irradiation, host iNKT cells were shown to be protective, by preventing the tissue damage induced by

donor T cells. However despite this the donor T cells maintained their cytotoxic function and could still kill the B cell lymphoma cells (533). CD1d$^{-/-}$ or Jα18$^{-/-}$ recipient mice were able to clear the tumour cells but died from GVHD (533). Furthermore, adoptively transferring iNKT cells did not affect the eradication of a B cell lymphoma (202, 234). In terms of the clinical relevance of these findings, reduced conditioning regimes in humans have also been shown to preserve the GVL effect and patients showed a significant increase in the percentage of iNKT cells in the blood (534).

In order to be clinically translatable it is likely that iNKT cells would need to be expanded *in vitro*. Indeed two studies have shown that Exp iNKT cells can provide some protection from GVHD (237, 513). Yang *et al.* observed an increased survival with Exp iNKT cells compared to Tcon only along with higher IL-4 in the serum of Exp iNKT cell treated mice. The mice treated with Exp iNKT cells exhibited 80% survival at day 60, but all mice died within 38 days of the control group (237). This is in contrast to studies where for example 80% of mice adoptively transferred with 2.5x10^4 FI iNKT cells survived past day 100 (234). It is worth noting that this study used very high numbers of Exp iNKT cells (1x10^7). A recent study transferred Exp human iNKT cells into a xenograft model of GVHD and found that mice treated with Exp human iNKT cells showed approximately 63% survival on day 56, which was a significant increase compared to control xenograft GVHD mice which all died by approximately day 50 (513).

Interestingly, another study injected BMT recipient mice with 1x10^6 Exp iNKT and showed that survival was prolonged to between 60 days with syngeneic Exp iNKT cells and 50 days with allogeneic Exp iNKT cells. However, importantly Jα18$^{-/-}$ BALB/c recipient mice did not survive beyond ~25 days, implying that the Exp cells

may have had an effect early but it was the residual host iNKT cells which lead to prolonged survival at least in this model (535). This study calls into question the contribution of the AT iNKT cells in prolonging the survival and suggests that AT cells play a role earlier in the course of disease that is then taken over by residual recipient cells.

Much study is required to understand the relative contribution of residual versus donor iNKT cells. In addition, while it is likely that IL-4 is involved in the protection mediated at least in part by donor Exp iNKT cells, the exact mechanism has not been fully elucidated and it has not been determined if Treg are involved. Furthermore, to date donor FI cells have been more potent and effective than Exp cells in prolonging survival and the phenotype of the Exp iNKT cells might be one explanation for this. In addition the role of specific subsets of iNKT cells in protection from GVHD has not been investigated. While clearly, there is a lot that remains unknown about the protective effects of Exp iNKT cells in GVHD, they are a potentially viable and safe therapy for GVHD, as they have been shown to be feasible and safe in a stage 1 clinical trial for advanced melanoma and advanced and non-small cell lung carcinoma (396, 462).

6.2. Aims and hypothesis

As iNKT have been observed to suppress GVHD in a B6 to BALB/c model of GVHD (126, 234), we aimed to investigate the ability of such cells to suppress alloreactive transgenic T cells in the model we established in chapter 3. We aimed to investigate the ability of both FI and Exp iNKT cells to suppress GVHD. Furthermore, we planned to look at the suppressive ability of different subsets of iNKT cells as well as the propensity of different glycolipids to influence the alloreactive response.

6.3. Results

6.3.1. αGal activated FI iNKT cells exacerbate initial weight loss after GVHD

FI iNKT cells have been shown to be suppressive in mouse models of GVHD in which B6 cells were transferred to BALB/c recipients (126, 234, 238). Furthermore, activating iNKT cells with αGal in a B6 to BALB/c model of GVHD was shown to be protective as they were required to polarize donor T cells towards a Th2 response (527). Therefore, we set out to investigate if the adoptive transfer of FI iNKT cells could ameliorate TCR-transgenic T cell induced GVHD (set up in chapter 3).

To this end, mice received TBI and TCD BM on day 0, and on day 2 $1x10^5$ FI B6 iNKT cells (the highest dose used in previous studies) were i.v. injected concomitantly with disease causing TEa T cells i.v., with or without αGal i.p. (Figure 6.1). Such mice were compared to mice that received TBI and TCD BM on day 0 and TEa only, FI iNKT only or FI iNKT and αGal on day 2. Mice that received FI iNKT cells and αGal lost significantly more weight compared to mice that received FI iNKT only, however all mice gradually increased weight with time and survived past day 50 without registering clinical scores (Figure 6.1A, D, E). These data imply that activating iNKT cells can lead to increased weight loss even in the absence of alloreactive T cells via the activation of residual recipient iNKT cells or BM derived iNKT cells as well as the AT FI iNKT cells.

Following from this, mice that received TEa T cells + FI iNKT cells + αGal lost more weight between days 4-6, than mice that received TEa T cells alone (Figure 6.1B). There was no difference in the survival of such mice, however they did exhibit significantly higher clinical scores on day 9 (Figure 6.1B, D, E). This suggests that the adoptive transfer and activation of FI iNKT cells in the context of disease-causing

TEa T cells exacerbates GVHD. Mice that received TEa T cells + FI iNKT + αGal showed increased weight loss and clinical scores and decreased survival compared to mice that received FI iNKT cells and αGal alone (Figure 6.1C) confirming that the TEa T cells caused GVHD. Mice that received TEa T cells + FI iNKT with or without αGal cells exhibited increased clinical scores on day 9 compared to mice that received TEa T cells only (Figure 6.1E). Together these data suggest that activating iNKT cells with αGal exacerbates GVHD. However, it does not tell us the relative contribution of recipient or donor BM derived cells compared to AT FI iNKT cells.

6.3.2. Activated iNKT cells increase the frequencies of TEa T cells in the spleen

Next, we sought to investigate if activating iNKT cells during the GVHD-causing TEa T cell response impacted the frequency or number of other immune cells. To this end mice that received TEa T cells + iNKT +αGal were compared to mice that received TEa T cells + iNKT cells alone. Mice were sacrificed when they reached 20% weight loss or the maximum clinical severity allowed and the spleen and MLN analysed. Mice were sacrificed between days 10 and 14.

Notably there was an increase in the frequency of TEa T cells in the spleens of mice that received TEa T cells + iNKT +αGal compared to TEa T cells + iNKT alone (Figure 6.2A). However, this was not reflected in absolute numbers (Figure 6.2B). In addition, no alteration in the frequency or number of donor or recipient-derived T cells were seen in the MLN (Figure 6.2C, D). In terms of other lymphocytes populations there was no change in the frequency or number of B cells (Figure 6.3A-D) or NK cells (Figure 6.3E-H) in either the spleen or MLN.

Interestingly, there was a significant increase in the overall frequency of iNKT cells and the frequency of AT iNKT cells in the spleens of mice that received TEa T cells + iNKT cells +αGal compared to TEa T cells + iNKT cells alone, as well as an increase in the number of AT iNKT cells (Figure 6.3E, F). In the MLN the only difference in iNKT cells was that there was an increase in the frequency of AT iNKT cells in the mice that received iNKT cells +αGal compared to TEa T cells + iNKT cells alone (Figure 6.3G). In terms of innate populations there was no difference in the frequency or number of myeloid cells or DCs in the spleen or MLN (Figure 6.4A-H).

Collectively, these data suggest that activating iNKT cells leads to an increase in the frequency of AT iNKT cells in the spleen and MLN, and that this increase results in an increase in the frequency of TEa T cells. There were no other perturbations observed in other immune populations that were investigated, when iNKT cells were activated with αGal. This suggests that decreased survival was due to the activation of the AT iNKT cells.

6.3.3. Transferred iNKT cells that have been activated by αGal *in vivo* persist and retain their ability to secrete IFN-γ

In order to investigate if the AT iNKT cells survive long term as well as the nature of the phenotype of such cells in terms of cytokines production, mice that received either iNKT cells alone or iNKT cells +αGal were analysed on day 50. We observed an increase in iNKT cell frequency in the spleen of mice that received iNKT cells + αGal compared to iNKT cells alone (Figure 6.5A), however similar numbers of iNKT cells were present in both sets of mice (Figure 6.5B). There was also no difference in the frequency or absolute numbers of iNKT cells in the MLN. (Figure 6.5 C, D).

Interestingly, spleen iNKT cells that had been previously activated demonstrated an enhanced frequency of IFN-γ producing iNKT cells (Figure 6.5E). However, we did not identify the provenance of these iNKT cells so they could be AT iNKT cells or of donor or host origin, or likely a combination of all three. Together these data suggest that there is an increase in the frequency of iNKT cells in mice that received iNKT + αGal compared to iNKT cells alone, even 50 days post-transplant. However as the numbers of iNKT cells are very low, it seems unlikely that such cells could have a biological effect at this late time point.

6.3.4. Activating iNKT cells with C20.2 in GVHD exacerbates initial weight loss and does not lead to a significant increase in survival

Due to our observation that αGal administration leads to exacerbation of GVHD (Figure 6.1), we decided to explore the possibility of using an alternative glycolipid. C20.2 is an αGal analogue, which has been shown in most settings to inhibit GVHD in B6 to B6D2F$_1$ and BALB/c to B6 GVHD models (528). Therefore, we set out to investigate the effects of C20.2 in our model.

Mice were given TBI and TCD BM on day 0, followed by TEa T cells + iNKT cells + i.p. C20.2 or TEa T cells only or C20.2 only on day 2. Mice that received C20.2 lost significantly less weight compared to mice that received TEa T cells + iNKT +C20.2 or TEa T cells alone, and ultimately went on to gain weight (Figure 6.6A). Such mice showed 100% survival (Figure 6.6B) and did not register clinical scores (Figure 6.6C).

On day 5 and 6, mice that received TEa T cells + iNKT cells + i.p. C20.2 lost significantly more weight and had enhanced clinical scores compared to mice that received TEa T cells only (Figure 6.6A and C). This led to the death of 80% of mice by day 5 (Figure 6.6B), suggesting that activating the AT iNKT cells with C20.2 exacerbates the initial decrease in weight loss that occurs after irradiation (Figure 6.6A). Interestingly, the remaining surviving mice went on to show a 2 day delay in death (Figure 6.6B), onset of weight loss (Figure 6.6A) and clinical scores (Figure 6.6C) compared to those that received TEa T cells only.

Together these data suggest that although there is no significant increase in survival in mice that received TEa T cells + iNKT +C20.2, compared to those that receive TEa T cells alone, if the exacerbation of the initial weight loss after irradiation was prevented then iNKT cells may diminish GVHD.

6.3.5. iNKT cell numbers increase in the spleens of mice that received C20.2 without affecting the number or frequency of TEa T cells

In order to investigate if C20.2 has an effect on iNKT cells frequency and number, cells were isolated from the spleens of mice that received TEa T cells only or TEa T cells + iNKT cells +C20.2 at the time of disease onset which was between days 10 and 12. There was no change in the frequency or number of TEa T cells between the 2 groups (Figure 6.7A, B) despite an increase in the number of AT FI iNKT cells in mice that received TEa T cells + iNKT +C20.2 (Figure 6.7D). However, there was no increase observed in the overall number of iNKT cells or in the number of recipient or donor derived iNKT cells (Figure 6.7D). In addition the frequency of iNKT cells was also unaffected (Figure 6.7C). Together these data suggest that while C20.2 can impact the number of AT iNKT cells, unlike αGal, C20.2 does not affect the frequency

or number of TEa T cells. This is in keeping with the mice all exhibiting weight loss and clinical signs of GVHD as all mice had succumbed to GVHD by day 13 (Figure 6.6).

6.3.6. Residual or BM derived iNKT cells and not the transferred iNKT cells are responsible for modest increase in survival

As a result of the observation that mice that received TEa T cells + iNKT + C20.2 seemed to have a modest increase in survival, we sought to determine whether the remaining recipient or donor derived iNKT cells or the AT FI iNKT cells were mediating this effect. Equally important, we also investigated whether the exacerbated weight loss observed after irradiation was attributable to the activation of recipient or donor derived iNKT cells or the AT FI iNKT cells. To this end, mice received TBI and TCD BM and either TEa T cells alone or together with C20.2. Importantly, no additional iNKT cells were transferred to these mice.

In keeping with previous experiment mice that received TEa T cells only exhibited weight loss from day 8 onwards (Figure 6.8A). However there was no significant difference between mice that received a single or multiple injections of C20.2 (Figure 6.8B). Mice that received a single shot of C20.2, showed 100% survival (Figure 6.8E) and did not register clinical scores (Figure 6.8F). Receiving multiple shots of C20.2 did not significantly affect weight loss, however one mouse had to be sacrificed on day 5 (Figure 6.8B, E).

Interestingly, on day 8 and 9 mice that received TEa T cells only, lost significantly more weight than those that received TEa T cells and C20.2 (Figure 6.8C). This occurred concomitantly with an increase in survival in mice that received TEa T cells and C20.2 compared to TEa T cells alone (Figure 6.8E) and a decrease in clinical scores in the presence of C20.2 (Figure 6.8F). Furthermore, the exacerbated weight loss seen following adoptive transfer of iNKT cells and C20.2 (Figure 6.6A) was not observed on administration of C20.2 in the absence of adoptive transfer of iNKT cells (Figure 6.8C).

Together, these data suggest that the modest increase in survival observed when C20.2 is administered is a result of activating either residual iNKT cells or contaminating iNKT cells in the BMT. It also suggests while multiple shots of C20.2 are tolerable it does cause initial weight loss, so such use in future experiments would need to be approached with caution.

6.3.7. T-bet$^{-/-}$ iNKT cells activated with C20.2 exacerbate GVHD

In Figure 6.6 we observed that weight loss was exacerbated when FI iNKT cells were injected concomitantly with C20.2 but that mice that survived beyond this point went on to do marginally better. Subsequently, we showed that the exacerbated initial weight loss was caused by the AT iNKT cells. However, as iNKT cells exist as multiple subsets with distinct effector functions it was possible that this unwanted side-effect was attributable to a particular subset. Given that ~57% of FI iNKT cells produce IFN-γ (Figure 4.2), and that αGal a glycolipid that is known to leads to production of Th1 cytokines exacerbates GVHD (Figure 6.1), we reasoned that

removing Th1 type cytokines might eradicate the pronounced weight loss following irradiation. Therefore, iNKT cells were purified from T-bet$^{-/-}$ mice and were transferred to mice that also received irradiation, TCD BM and TEa T cells (Figure 6.9).

Surprisingly, we observed that mice that received TEa T cells + T-bet$^{-/-}$ iNKT cells + C20.2 lost significantly more weight between days 4-6 than mice that received TEa T cells alone which resulted in one mouse having to be sacrificed (Figure 6.9A, B, C). In addition, such mice also lost significantly more weight, presented with decreased survival and increased clinical scores compared to mice that received TEa T cells +C20.2 on days 8 and 9 (Figure 6.9A, C, D).

Despite the fact that TEa T cells + WT iNKT cells + C20.2 did not show exacerbated weight loss after irradiation as in Figure 6.6, they still showed a modest decrease in survival compared to mice that received TEa T cells + C20.2 (Figure 6.9A, C).

Together these data suggest that the NKT1 subset of iNKT cells is not responsible for increased weight loss after irradiation and indeed these cells may temper the impact of other subsets on weight.

6.3.8. RORγt^{-} iNKT cells activated with C20.2 exacerbate GVHD

Given that exacerbated weight loss after irradiation was not attributable to NKT1 cells we decided to determine whether it was IL-17 producing NKT17 cells that were mediating this effect. Although the spleen only hosts a small proportion of IL-17 producing iNKT cells (8) IL-17 producing iNKT cells have been shown to be involved in proinflammatory responses in airway inflammation and as such one might predict

that they would not be beneficial in the setting of bone-marrow transplantation (453, 454).

To this end, RORγT$^-$ iNKT cells were purified from RORγT reporter mice and were transferred to mice that also receive TBI, TCD BM and TEa T cells (Figure 6.10). We observed that in this experiment, there was no increased weight loss after irradiation observed from day 4-6, however as this occurred in the presence of both WT and RORγT$^-$ iNKT cells, we cannot conclude that there was any benefit to transferring RORγT$^-$ iNKT cells (Figure 6.10A).

We observed that mice that received TEa T cells + WT or RORγT$^-$ iNKT +C20.2 mice lost significantly more weight than mice that received BMT only (Figure 6.10A). This corresponded with decreased survival and an increase in clinical scores (Figure 6.10B, C). Mice that received TEa T cells + RORγT$^-$ iNKT cells +C20.2 also did worse in terms of survival than mice that received TEa T cells + WT iNKT cells +C20.2 or TEa T cells alone, which was also reflected in increased clinical scores (Figure 6.10B, C).

Even in the absence of the decreased weight loss after irradiation, mice did not show increased survival (Figure 6.10B). Importantly, while none of the differences in survival were substantial, the fact that RORγT$^-$ iNKT cells +C20.2 lead to a decrease in survival implies that removing such cells from FI iNKT cells was futile.

6.3.9. Exp iNKT cells can survive and can be identified *in vivo* following BMT

Given the lack of success at alleviating GVHD with FI iNKT cells we sought to test whether iNKT cells that had been expanded *in vitro* would have a different impact in

this setting. Indeed, we found that Exp iNKT cells were better at suppressing alloreactive T cell responses *in vitro* in Chapter 5.

First we conducted a pilot experiment to test whether Exp iNKT cells could survive following transfer *in vivo* (Figure 6.11). B6 iNKT cells were expanded for 14 days with anti-CD3 and anti-CD28 with IL-2, IL-12 and IL-7 before being transferred to mice that had received TBI and TCD BM on day 0. One mouse also received C20.2 to activate the transferred cells. The transferred Exp iNKT cells were 75% pure and produced IFN-γ (39%), IL-4 (50%), IL-10 (35%), IL-13 (88%) and IL-17 (38%) on re-stimulation (Figure 6.11A, B). The transferred Exp iNKT cells proved to be easily detectable in the spleen (Figure 6.11C), MLN (Figure 6.11D) and SI (Figure 6.11E).

6.3.10. GVHD mice that receive Exp iNKT cells activated with C20.2, show increased survival compared to mice that receive Exp iNKT cells alone

As we could successfully detect the Exp iNKT cells *in vivo*, we proceeded to investigate their ability to suppress GVHD. Mice were given TBI and TCD BM on day 0, followed on day 2 by TEa T cells + Exp iNKT cells with and without C20.2 or TEa T cell + C20.2 alone. The injected cells were 72% pure and produced IFN-γ (88%), IL-4 (16%), IL-10 (20%), IL-13 (98%) and IL-17 (28%) upon *in vitro* re-stimulation (Figure 6.12A, B).

Mice that received TEa T cells +Exp iNKT with C20.2 showed no difference in weight loss however they did show significantly increased survival and lower clinical scores from day 9-11 compared to mice that received TEa T cells +Exp iNKT without C20.2 (Figure 6.12C, D, E). However, neither group showed increased survival even

compared to the group that received TEa T cells +C20.2 alone (Figure 6.12D). Collectively these data show that while activating iNKT cells with C20.2 incurs a marginal survival benefit, and delayed onset of clinical scores compared to mice that do not receive C20.2, these benefits are not significantly increased with AT Exp iNKT cells, and therefore the modest protective effect is likely predominantly mediated by host residual iNKT cells.

6.4. Discussion

This chapter set out to investigate the ability of both FI and Exp iNKT cells to influence GVHD in a model where GVHD is caused by TCR-transgenic T cells (TEa T cells) that recognise a BALB/c-derived H2I-Eα peptide presented by H2I-A^b in CB6F1 recipients. In addition we sought to investigate the impact of specific subsets of iNKT cells on GVHD. We observed that residual iNKT cells can incur a slight survival benefit when activated with C20.2, however transferring FI or Exp iNKT cells did not lead to attenuation of GVHD or an increase in survival. Both recipient-derived and AT iNKT cells have previously been shown to attenuate GVHD in other models (126, 234, 236-238, 524).

The use of reduced intensity irradiation has been shown to lead to over representation of $DX5^+$ T cells in BALB/c mice and to prevent GVHD in BALB/c mice that had received fully mismatched B6 transplants. This was shown to be dependent on recipient derived IL-4 (524, 525). Furthermore, the majority of studies that have shown the beneficial effect of AT FI iNKT cells in models of GVHD have also been carried out using BALB/c recipient mice (126, 234, 238). BALB/c mice are known to be Th2 biased (529), and IL-4 production has been shown to be important in the mechanism observed in these models (234). A single injection with αGal significantly decreases morbidity and mortality in a B6 to BALB/c model of GVHD but neither B6 to $B6D2F_1$ nor BALB/c to B6 GVHD models (527, 528).The protective effect observed in BALB/c recipients does not occur in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipient mice (527). In contrast, C20.2 an αGal analogue that is biased towards Th2 cytokines (278), failed to exacerbate GVHD in B6 to $B6D2F_1$ and BALB/c to B6 GVHD models and in most settings inhibited it (528).

This requirement for IL-4 at least for this mechanism, may be the reason that much of the success suppressing GVHD with iNKT cells has been achieved in BALB/c recipients or using glycolipids to promote Th2 type responses. We observed only a modest increase in survival as a result of activating recipient iNKT cells (Figure 6.8). The CB6F1 recipient mice are a cross between B6 and BALB/c mice. A study of *leismania major* showed that while B6 and BALB/c mice show Th1 and Th2 responses respectively in the first 5 months of infection, CB6F1 show a response in which both type 1 and type 2 cytokines are elevated (536). Therefore it is possible that they do not confer protection to the same extent as BALB/c recipients due to their intermediate phenotype between BALB/c and B6. Furthermore, although we adoptively transferred B6 iNKT cells as was used in previous studies, as the recipients are CB6F1 mice they may differentially impact the phenotype of the transferred cells compared to BALB/c recipients.

Treg have also been repeatedly shown to be required by iNKT cells to suppress GVHD in BALB/c mice. The same group that used reduced intensity conditioning regime to prevent GVHD via iNKT cells, later went on to show that host iNKT cells induced the expansion of Treg which was IL-4 dependent and was lost in Jα18$^{-/-}$ recipients (525). Furthermore, the Negrin group showed that the protective effect observed when iNKT cells are transferred into a B6 to BALB/c model of GVHD also requires Treg (238). In our studies we did not transfer Treg as TEa T cells have a re-arranged TCR genes which precludes selection of Treg raising the possibility that suppression would have been observed with the co-transfer of Treg.

However, we do not know if mechanisms exist in which FI iNKT cells or subsets of such cells can suppress independently of Treg. In Chapter 5, we observed that both FI and Exp iNKT cells could suppress TEa T cells *in vitro* without the requirement for Treg. Similar results have been reported with Exp human iNKT cells where the iNKT cells suppressed T cell activation by the cytolysis of DCs (408). These results suggest a mechanism of suppression by which Exp iNKT cells can suppress alloreactive T cells in a cytokine and Treg independent pathway. Whether suppression can occur independently of Treg and IL-4 *in vivo* is currently unknown. However, it is unlikely that cytolysis of DCs occurs *in vivo,* due to the sheer number of iNKT cells that would be required to kill all alloantigen bearing APCs, and to simultaneously maintain APCs to present glycolipids to the iNKT cells.

While we did not observe suppression of GVHD with Exp iNKT cells, there could be several reasons for this. It is possible that Exp iNKT cells also require Treg in order to suppress GVHD (although they could suppress alloimmune responses *in vitro* in their absence; Chapter 5, (Figure 5.1). It should be noted that many of the reports of iNKT cells suppressing GVHD involve the transfer of splenocytes which would include Treg, so we cannot rule out the requirement for Treg in Exp iNKT cell mediated suppression (237, 513). Another possibility is that the Exp cells have a complex phenotype as seen in Chapter 4, and that any suppressive effect of a particular subset is masked by other proinflammatory subsets. To test this hypothesis it would be necessary to sort the cells, preferable post expansion, as preferentially expanding some subsets is difficult as seen in the data presented in chapter 4.

A further possibility is that suppression might have been seen on transfer of increased numbers of Exp iNKT cells. We transferred $1x10^5$ Exp iNKT cells, which is in line with the numbers used in other studies that used FI cells and 10 fold more cells than the disease-causing TEa T cells (234). One previous study used $1x10^7$ Exp iNKT cells to suppress GVHD whilst in a xenograft model of GVHD $5x10^5$ iNKT cells were transferred to prevent GVHD (237, 513). However, as we had observed in Chapter 5 (Figure 5.2), Exp iNKT cells are more potent at suppressing alloreactive T cells *in vitro* we decided to start with $1x10^5$ iNKT cells. Furthermore, in the study that utilized $1x10^7$Exp iNKT cells mice had lost up to 35% of their initial weight before recovering, and we were mindful of breaching our project license which only allowed up to 20% weight loss.

Another study used BM and $1x10^7$ splenocytes to induce GVHD along with $1x10^6$ Exp iNKT cells (535). This study showed that it was host-residual iNKT cells that prolong survival in this context. $J\alpha18^{-/-}$ BALB/c recipient mice did not survive beyond ~25 days. This suggests that Exp iNKT cells may have an effect early on but it is the residual host iNKT cells which lead to prolonged survival in this model. The previously described studies did not address the relative contribution of the AT Exp iNKT cells versus recipient iNKT cells, and therefore it is possible that the suppression in these models is at least partly mediated by host cells. Therefore, it is unclear if increasing the number of Exp iNKT cells would lead to suppression of GVHD in our model.

The relative contribution of donor versus recipient iNKT cells in the suppression of GVHD is not well understood. iNKT cells are radioresistant and express high-levels of anti-apoptotic protein Bcl-2 (443). In the studies carried out by the Negrin Lab, they do not definitively show whether recipient iNKT cells are involved at least in initiating the suppressive effects of FI iNKT cells. Mice that receive conventional T cells without iNKT cells are compared to those in which FI iNKT cells are also added. However, $CD1d^{-/-}$ or $J\alpha18^{-/-}$ recipients were not tested for their ability to prevent GVHD (126, 234). We observed that the marginal protective effect observed with C20.2 was seen regardless of the presence of AT iNKT cells, which supports the idea that host iNKT cells are important in early protection from GVHD.

To definitively confirm this we would have to use $CD1d^{-/-}$ or $J\alpha18^{-/-}$ donor B6 BM, however as we depleted to T cells including the iNKT cells from the graft donor BM derived iNKT cells are unlikely to contribute. We also looked at iNKT cells 2 days post BM transplant in the spleen and MLN to determine the extent of iNKT cell survival post TBI. However, although detectable, numbers of both recipient and donor iNKT cells were vanishingly small (data not shown). Therefore, it is possible that donor or recipient iNKT cells may mediate the minimal attenuation of GVHD that was seen it is unlikely. However, we cannot rule out potential local effects so it may have been more revealing to assess iNKT cell number in GVHD target organs.

In addition to recipient iNKT cells modestly extending survival in the GVHD model, somewhat surprisingly, we found that transfer of either FI or Exp iNKT cells followed by activation with either αGal or C20.2 led to increased weight loss after irradiation. This may be due to the cytokines being produced by the AT iNKT cells leading to increased inflammation. Indeed in the case of αGal there was an increase in the

frequency of disease causing TEa T cells upon administration to mice that received TEa T cells + iNKT cells (Figure 6.2).

Due to this exacerbated weight loss we hypothesized that removing potentially pathogenic subsets of iNKT cells such as NKT1 cells or NKT17 cells might alleviate this exacerbated weight loss after irradiation and perhaps lead to an increase in overall survival. The dominant subset of iNKT cells found in the spleen is IFN-γ secreting NKT1s however transferring T-bet$^{-/-}$ iNKT cells did not prevent the increased weight loss and exacerbated death through GVHD (Figure 6.9). This was surprising as T-bet$^{-/-}$ mice have been shown to produce increased levels of IL-4, which has been shown to be protective in models of GVHD in which BALB/c recipients are used (234, 238, 310, 525). These data would suggest that IFN-γ or perhaps something else under the transcriptional control of T-bet in fact has beneficial effect in GVHD in this model, which would point to another population being the pathogenic subset. However, transferring iNKT cells that were devoid of NKT17 cells also promoted weight loss and GVHD (Figure 6.10).

These seemingly multifarious observations, could be explained by the fact that we are transferring the FI iNKT cells into an inflammatory environment in which irradiation has led to tissue damage and there has been an expansion of inflammatory cells and alloreactive T cells. Indeed bystander activation of iNKT cells can occur by means of IL-2 produced by alloreactive T cells (231). Furthermore, the phenotype of the iNKT cells may be influenced by the microenvironment into which they are transferred or the activation with glycolipids. This may lead to the AT iNKT cells exhibiting an inflammatory phenotype regardless of the cells transferred and

perhaps in the absence of one proinflammatory cytokine the cells compensate by making increased quantities of other pathogenic subsets.

Although we did not thoroughly investigate the stability of the phenotype of either the AT FI or Exp iNKT, there is evidence that the outcome of iNKT cell activation is strongly influenced by the context in which the iNKT cells are activated. For example, the co-culture of thymic iNKT cells with APCs from the spleen in the presence of αGal, leads to a significant increase in IFN-γ expression, which was reduced when thymic iNKT cells were co-cultured with thymic APCs (334). This is thought to be as a result of the fact that co-stimulatory molecules are expressed at different levels depending on their tissue of origin.

As was the case with the remaining recipient iNKT cells, the genetic background of the recipient mouse may also play a role. In previous studies that AT FI iNKT cells B6 BM and cells were transferred into BALB/c recipients (126, 234, 238). As previously mentioned the CB6F1 mice show a response in which both type 1 and type 2 cytokines are elevated (536). Therefore it is possible that their ability to skew the immune response towards a type 2 response may not be sufficient to incur iNKT cells with sufficient ability to produce IL-4. Studies have shown that B6 lineage diversity is intrinsically determined and the phenotype of these cells was not perturbed by exposure to BALB/c cells during development or in BM chimeras (310, 537).

However, as we have discussed in the context of GVHD the iNKT cells are being transferred into an inflammatory environment. The influence of GVHD on the phenotype of AT B6 iNKT cells has not been studied, although serum from such mice was Th2 biased and suppression did not occur if IL-$4^{-/-}$ iNKT cells were transferred

(126, 238). Transferred iNKT cells being either NKT1 or NKT17 biased by the microenvironment could be an explanation for the detrimental effect of T-bet$^{-/-}$ or RORγT^{-} iNKT cells in our model of GVHD. Although it is important to remember, that if Treg are required in our model, even sufficient IL-4 production by iNKT cells would not induce protection.

In conclusion, although we did not observe any increase in survival during GVHD with either FI or Exp iNKT cells this could be as a result of disparities between GVHD models, or a requirement for Treg. Both of these possibilities can be addressed in future studies. In addition it will be important to understand the relative contribution of donor versus recipient iNKT cells and the stability of AT cells in order to understand how adoptively transferring iNKT cells in humans can be both safe and effective in the context of GVHD.

6.5. Figures

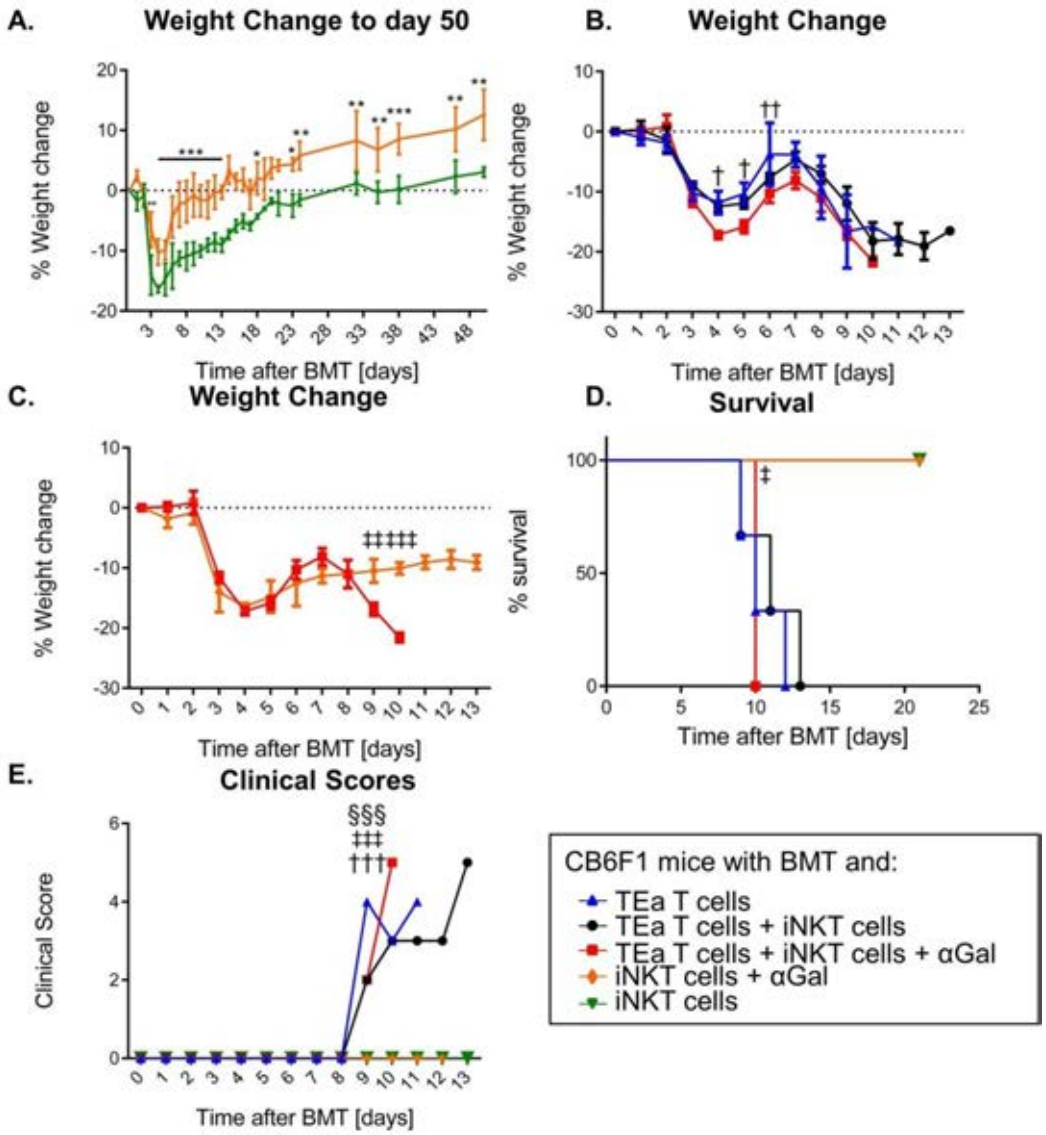


Figure 6.1 Activated iNKT cells exacerbate initial weight loss and GVHD CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received either 1x10^4 TEa T cells, TEa T cells + 1x10^5 FI iNKT cells, TEa T cells + iNKT cells + αGal, iNKT cells + αGal or iNKT cells only, on day 2. Mice were monitored and (A) iNKT vs iNKT + αGal weight loss to day 50 (B) Weight loss TEa only versus TEa T cells + iNKT versus TEa T cells + iNKT + αGal. (C) Weight loss TEa T cells + iNKT + αGal versus iNKT + αGal. (D) Survival of all groups (E) Clinical scores of all groups. Results were analysed using repeated measure 2-way ANOVA with post hoc Tukey's comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n=3 mice per group.

*= iNKT vs iNKT +αGal†= TEa T cells vs. TEa T cells + iNKT + αGal

‡= TEa T cells + iNKT + αGal vs. iNKT + αGal

§= TEa T cells vs. TEa T cells +iNKT

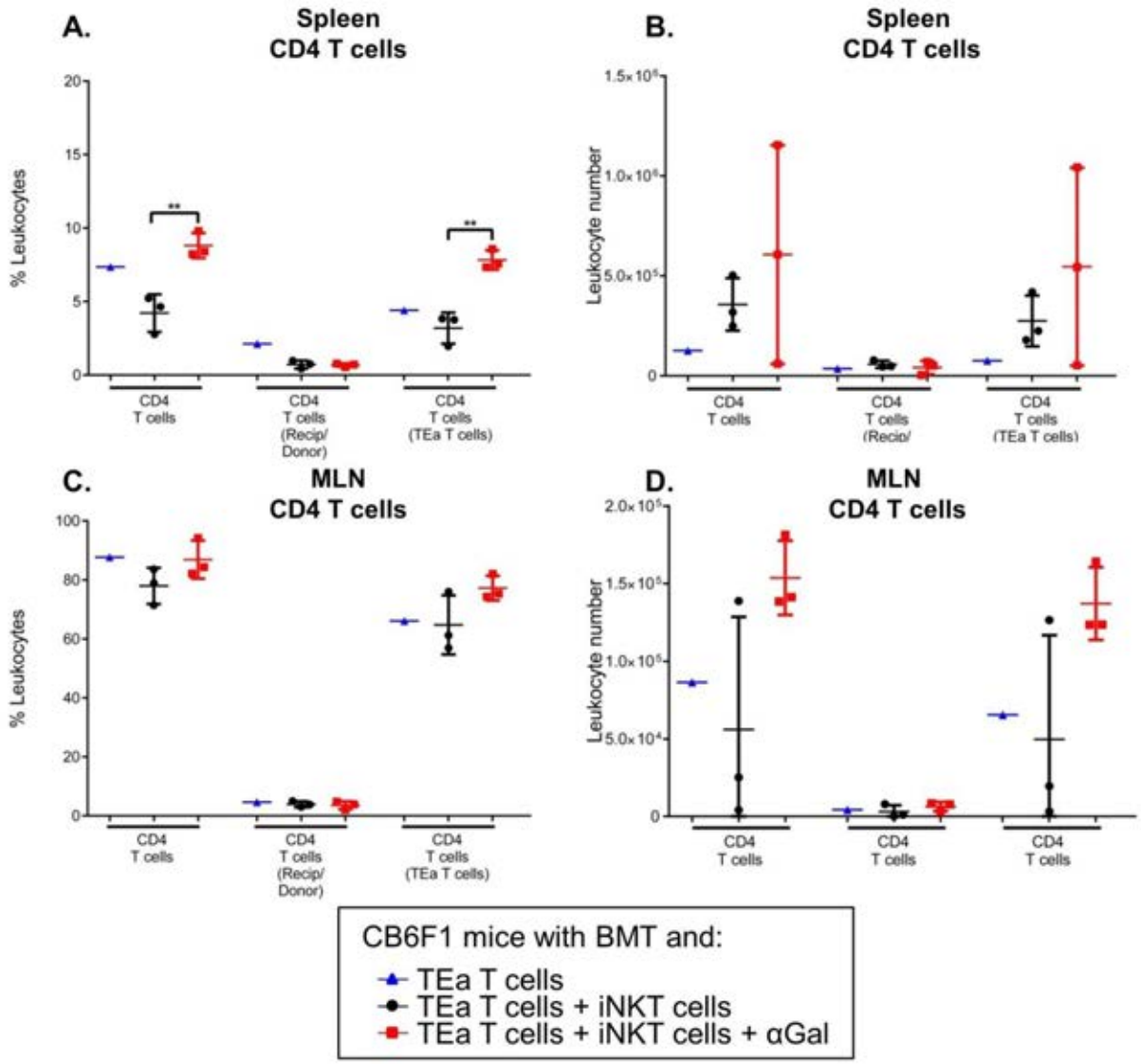
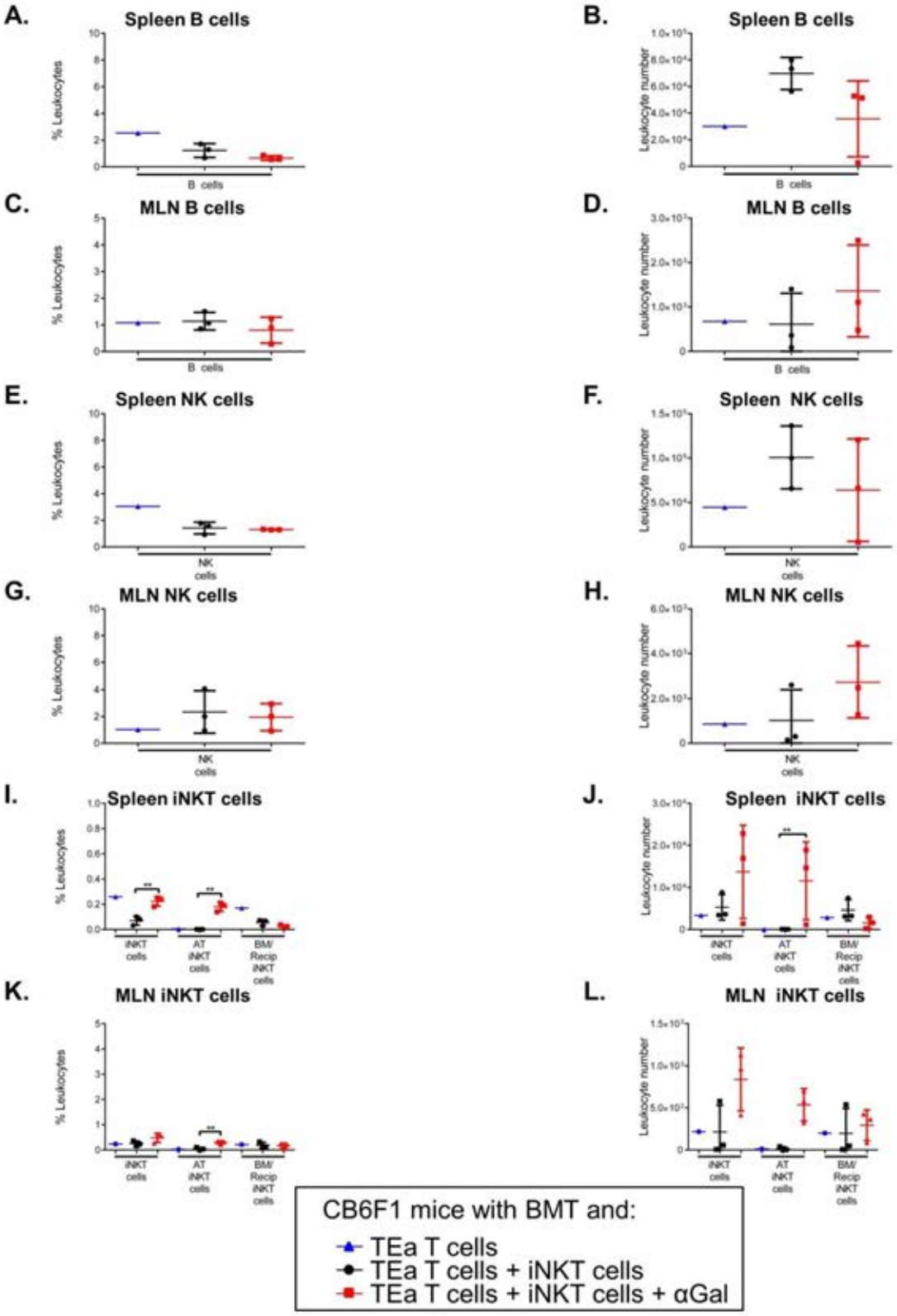


Figure 6.2 There is an increase in the frequency of total CD4$^+$ T cells and TEa T cells in the spleens of mice that received TEa T cells + iNKT + αGal compared to those that received TEa T cells + iNKT cells only CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received either 1x10^4 TEa T cells, TEa T cells + 1x10^5 iNKT cells or TEa T cells + iNKT cells + αGalon day 2. Mice were sacrificed when they reached 20% weight loss, or were deemed to be at the maximum clinical symptoms allowed by our license. Spleen and MLN derived leukocytes were analysed by flow cytometry. Results are expressed as CD4 T cells were gated live lymphocytes and TCR-β and CD4 and CD45.1 (donor iNKT cells) or CD45.2 (Donor or recipient) or CD45.1 and Vα2 (TEa T cells) in (A, B) the spleen and (C, D) the MLN. Results are analysed using t-tests to compare TEa T cells + iNKT versus TEa T cells + iNKT + αGal. Mouse that received TEa T cells only mouse is included as a reference point. **p<0.01. Results are presented as mean ± SD, and n=3 mice per group. See Appendix V for gating strategy.


A.
Spleen B cells
% Leukocytes
B cells
B.
Spleen B cells
Leukocyte number
B cells
C.
MLN B cells
% Leukocytes
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D.
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B cells
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MLN NK cells
Leukocyte number
NK cells
I.
Spleen iNKT cells
% Leukocytes
iNKT cells
AT iNKT cells
BM/ Recip iNKT cells
J.
Spleen iNKT cells
Leukocyte number
iNKT cells
AT iNKT cells
BM/ Recip iNKT cells
K.
MLN iNKT cells
% Leukocytes
iNKT cells
AT iNKT cells
BM/ Recip iNKT cells
L.
MLN iNKT cells
Leukocyte number
iNKT cells
AT iNKT cells
BM/ Recip iNKT cells
CB6F1 mice with BMT and:
TEa T cells
TEa T cells + iNKT cells
TEa T cells + iNKT cells + αGal

Figure 6.3 There is an increase in the number and frequency of AT iNKT cells in the spleens of mice that received TEa T cells + iNKT + αGal compared to those that received TEa T cells + iNKT cells only CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ TEa T cells, TEa T cells + $1x10^5$ FI iNKT cells or TEa T cells + FI iNKT cells + αGalon day 2. Mice were sacrificed when they reached 20% weight loss, or were deemed to be at the maximum clinical symptoms allowed by our license. Spleen and MLN derived leukocytes were analysed by flow cytometry. Results are expressed as (A, C, E, G, I, K) percentage live leukocytes and (B, D, F, H, J, L) absolute numbers of live leukocytes. B cells were on live lymphocytes and B220 in the spleen (A, B) and MLN (C, D). NK cells were gated on live lymphocytes and $NK1.1^+$ $TRC\text{-}\beta^-$ in the (E, F) spleen and (G, H) MLN. iNKT cells were gated on live lymphocytes and TCR-β and PBS-57 tetramer in the (I, J) spleen and (K, L) MLN. Results are analysed using t-tests to compare TEa T cells + iNKT versus TEa T cells + iNKT + αGal. Mouse that received TEa T cells only mouse is included as a reference point. **p<0.01. Results are presented as mean ± SD, and n=3 mice per group. See Appendix V for gating strategy.

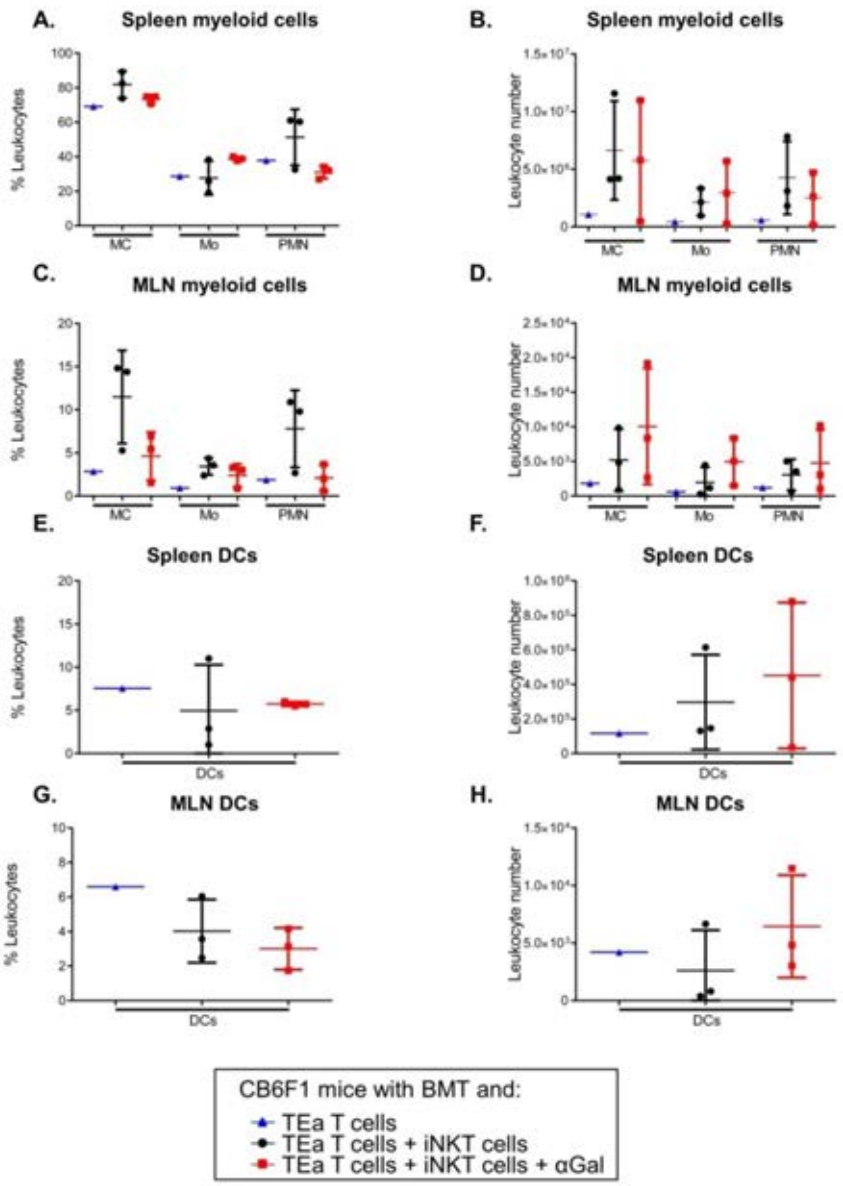


Figure 6.4 There is no difference in the number or frequency of myeloid cells in the spleens or MLN of mice that received TEa T cells + iNKT + αGal compared to those that received TEa T cells + iNKT cells only CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ TEa T cells, TEa T cells + $1x10^5$ FI iNKT cells, orTEa T cells + FI iNKT cells + αGalon day 2. Mice were sacrificed when they reached 20% weight loss, or were deemed to be at the maximum clinical symptoms allowed by our license. Spleen and MLN derived leukocytes were analysed by flow cytometry. Results are expressed as (A, C, E, G) percentage live leukocytes and (B, D, F, H) absolute numbers of live leukocytes. Cells were gated on live leukocytes and $Gr1^+CD11b^+$(myeloid cells (MC)) which were either $Ly6C^+$ (monocytes (Mo)) and $Ly6G^+$ (polymorphonuclear (PMN) leukocytes) in (A, B) the spleen and (C, D) the MLN. DCs were gated on live leukocytes and CD11c and MHC class II in the (E, F) spleen and the (G, H) MLN. Results are analysed using t-tests to compare TEa T cells + iNKT versus TEa T cells + iNKT + αGal. Mouse that received TEa T cells only mouse is included as a reference point. Results are presented as mean ± SD, and n=3 mice per group. See Appendix V for gating strategy.

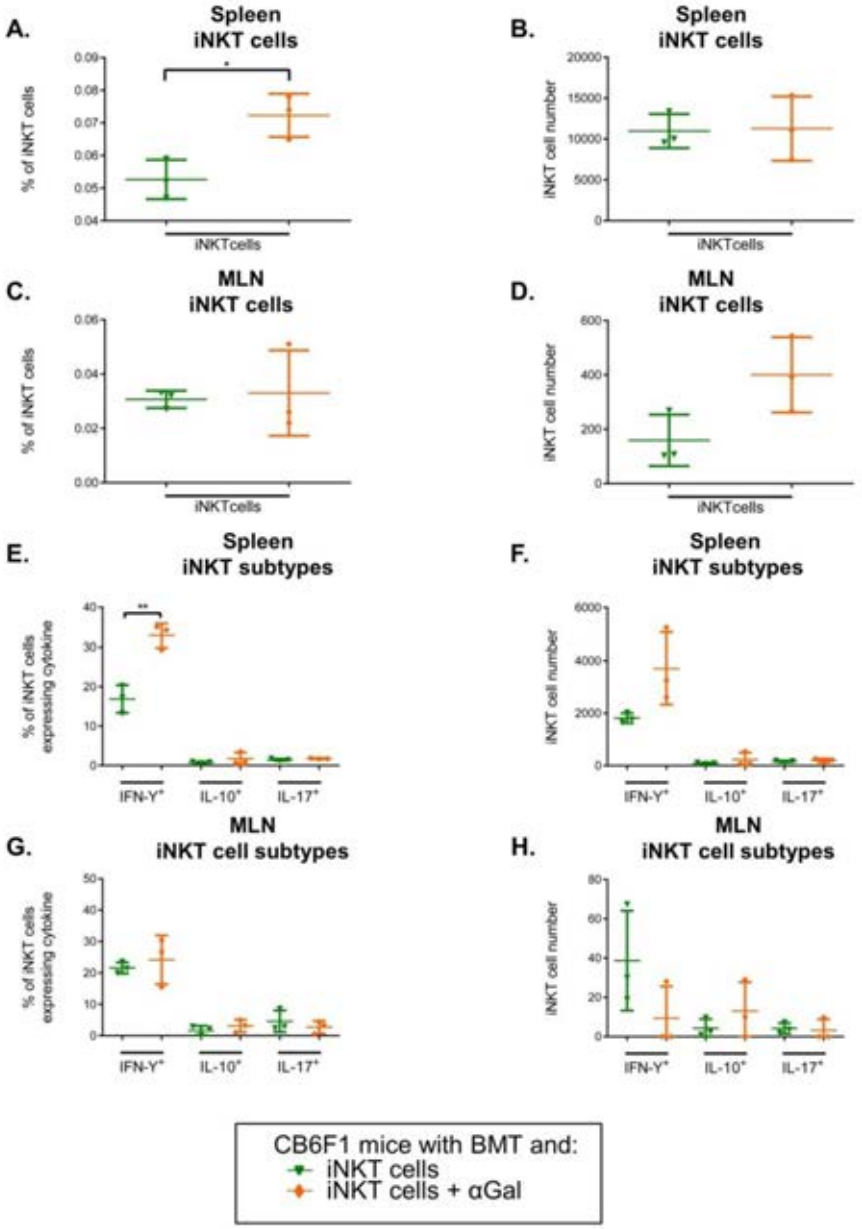


Figure 6.5 There is an increase in the absolute number of IFN-γ$^+$ iNKT cells in the spleen and a decrease in IL-17$^+$ iNKT cells in the MLN of mice with activated iNKT cells CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received FI iNKT cells + αGal or FI iNKT cells only, on day 2. Such mice were let go and harvested on day 50. iNKT cells were gated on live lymphocytes and TCR-β and PBS-57 tetramer. Results are expressed as percentage of live leukocytes in the (A) the spleen and (C) the MLN and absolute numbers of live leukocytes in (C) the spleen and (D) the MLN and frequency and number of iNKT cells expressing IFN-γ, IL-10 and IL-17 in the (E, F) spleen and (G, H) MLN. Results are analysed by unpaired t-tests. *p<0.05, **p< 0.01. Results are presented as mean ± SD, and n=3 mice per group.

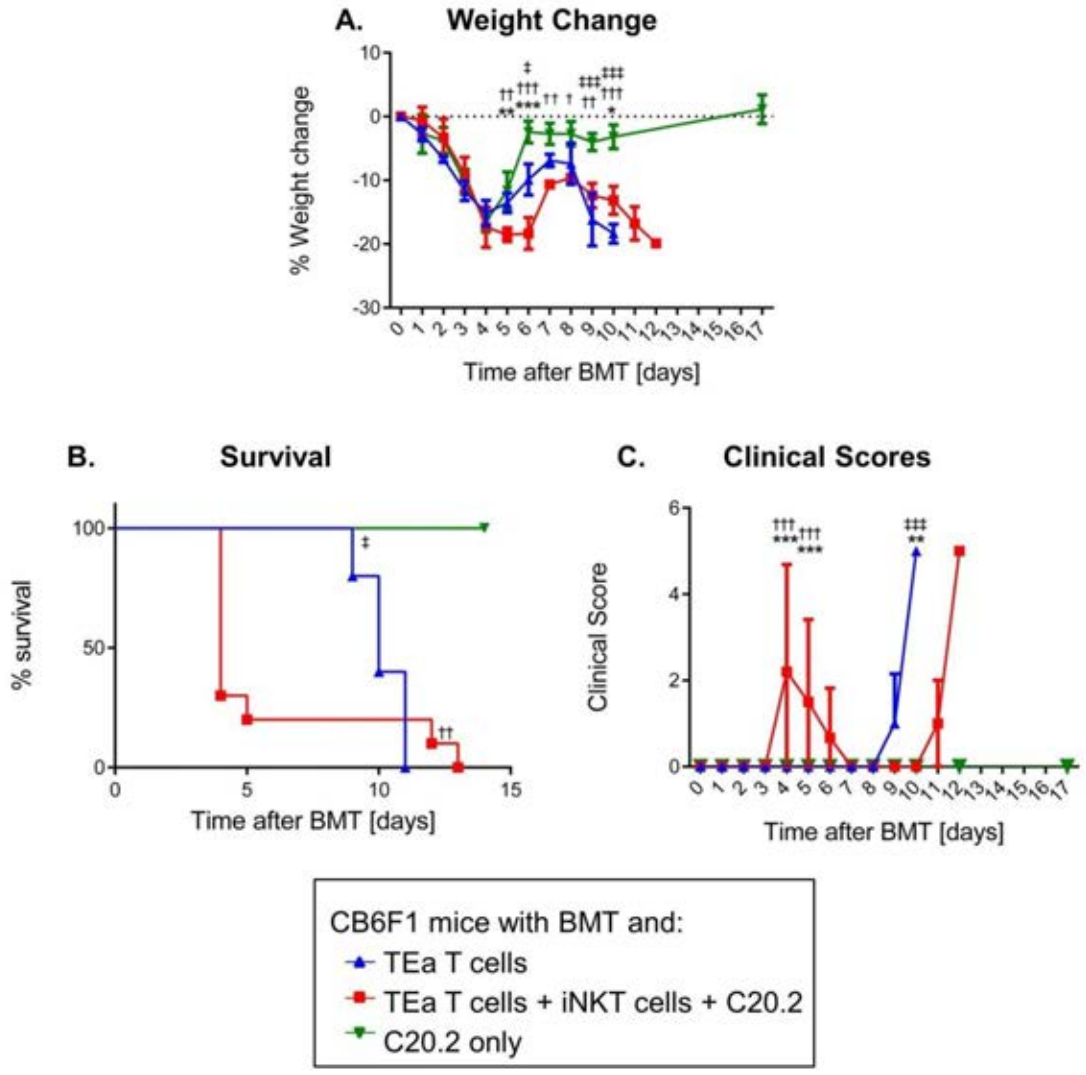


Figure 6.6 Activating iNKT cells with C20.2 in GVHD exacerbates initial weight loss and does not lead to a significant increase in survival CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ TEa T cells, TEa T cells + $1x10^5$ FI iNKT cells + C20.2 or C20.2 only, on day 2. Mice were monitored and (A) Weight loss (B) Survival and (D) Clinical scores were recorded. Results are analysed using repeated measure 2-way ANOVA with post hoc Bonferroni comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n=5 mice per group for TEa T cell only group and n=10 mice for TEa T cells + iNKT +C20.2, n=3 for C20.2 alone. Data is pooled from 2 independent experiments.

*= TEa T cells vs. TEa T cells + iNKT + C20.2

†= TEa T cells vs. C20.2

‡= TEa T cells + iNKT + C20.2 vs. C20.2

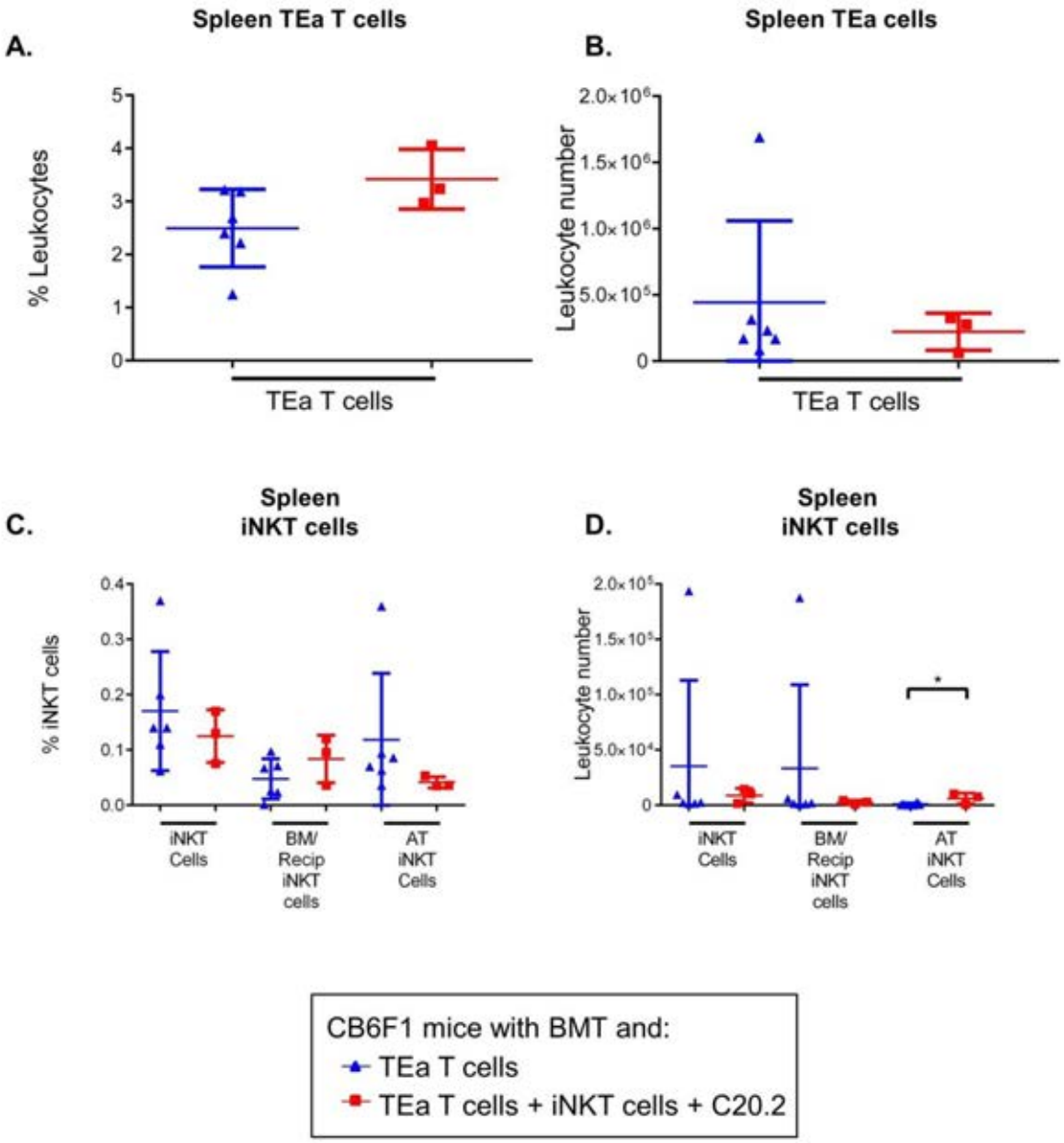


Figure 6.7 There is an increase in the number of iNKT cells in the spleens of mice that received TEa T cells + iNKT cells + C20.2 compared to TEa T cells alone CB6F1 mice were lethally irradiated and given 5x10^6 TCD depleted B6 BM cells i.v. on day 0. Such mice received either 1x10^4 TEa T cells or TEa T cells + 1x10^5 FI iNKT cells + C20.2on day 2. Mice were sacrificed when they reached 20% weight loss, or were deemed to be at the maximum clinical symptoms allowed by our license. Spleen derived leukocytes were analysed by flow cytometry. Results are expressed as (A, C) percentage live leukocytes and (B, D) absolute numbers of live leukocytes in the spleen. (A, B) TEa T cells were gated on live lymphocytes, TCR-β and CD4 and CD45.1 and Vα2$^+$in the spleen. (C,D) iNKT cells were gated on live lymphocytes and TCR-β and PBS-57 tetramer of total, recipient (CD45.2$^+$) and donor (CD45.2$^+$CD45.2$^+$) origin. Results are analysed by unpaired t-tests. *p<0.05. Results are presented as mean ± SD, and n=6 mice per group for TEa T cell only group and TEa T cells + iNKT +C20.2, n=3.

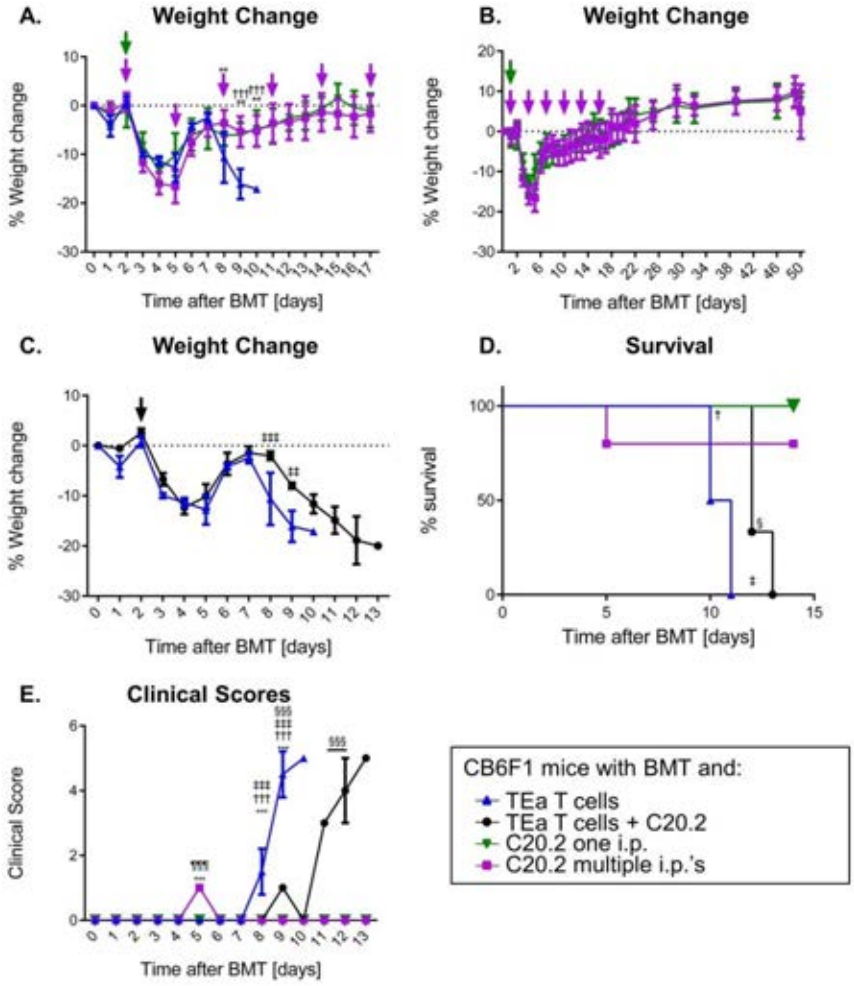


Figure 6.8 Modest increase in survival in mice that receive TEa T cells +C20.2 compared to those that received TEa T cells alone is caused by residual iNKT cells CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ TEa T cells, TEa T cells + C20.2 or C20.2 only, on day 2. An additional group of mice received further i.p.'s of C20.2 on days 5, 8, 11, 14 and 17. Mice were monitored and weight loss was monitored in mice that received (A) C20.2 single shot, versus C20.2 multiple shots versus TEa T cells only (B) FI iNKT only versus iNKT +C20.2 (C) TEa T cells versus TEa T cells + C20.2 and (D) survival and (E) clinical scores were recorded in all groups. Results are analysed using repeated measure 2-way ANOVA with post hoc Bonferroni comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n=2 mice for TEa T cells, n=5 mice for C20.2 multiple i.p.'s C20.2 n=3 for C20.2 one i.p. and n=3 for TEa T cells + one i.p. C20.2. *= TEa T cells vs. C20.2 multiple i.p.'s

†=. TEa T cells vs. C20.2 one shot

‡= TEa T cells vs. TEa T cells + C20.2 one i.p.

§= C20.2 one i.p. vs. TEa T cells + C20.2 one i.p.

¶= C20.2 one i.p. vs. C20.2 multiple i.p.'s.

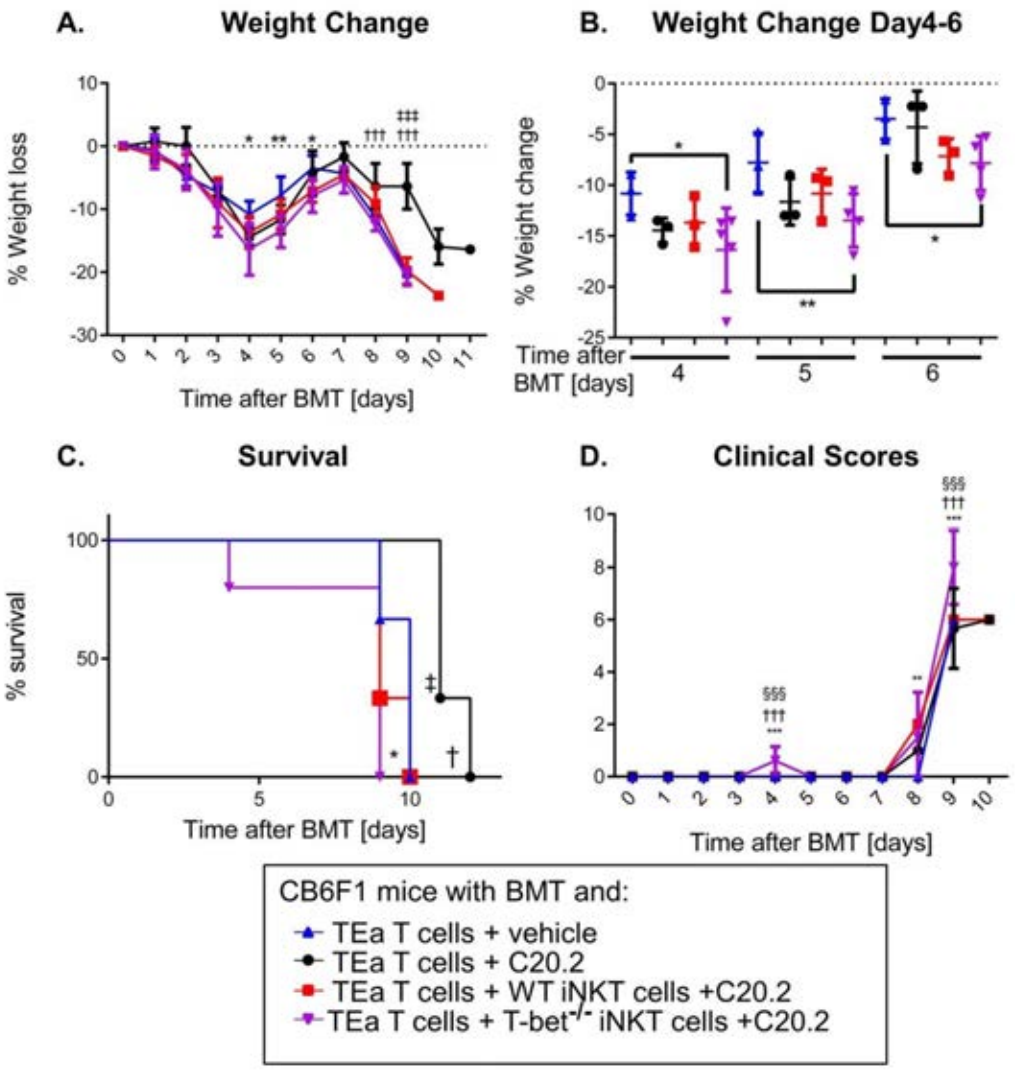


Figure 6.9 iNKT cells from T-bet$^{-/-}$ mice exacerbate GVHD CB6F1 mice were lethally irradiated and given 5x10^{6} TCD B6 BM cells i.v. on day 0. Such mice received either 1x10^{4} TEa T cells, TEa T cells + C20.2 or TEa T cells + 1x10^{5} WT or T-bet$^{-/-}$ FI iNKT cells + C20.2 on day 2. Mice were monitored and (A, B) Weight loss and (C) Survival and (D) Clinical scores were recorded. Results are analysed using repeated measure 2-way ANOVA with post hoc Bonferroni comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n=5 mice for TEa T cells + T-bet$^{-/-}$ iNKT cells + C20.2, n=3 mice for all other groups. See Appendix V for phenotype of T-bet$^{-/-}$ mice.

*= TEa T cells + T-bet$^{-/-}$ iNKT +C20.2 vs. TEa T cells + vehicle

†= TEa T cells + T-bet$^{-/-}$ iNKT +C20.2 vs. TEa T cells + C20.2

‡= TEa T cells + WT iNKT +C20.2 vs. TEa T cells + C20.2

§= TEa T cells + T-bet$^{-/-}$ iNKT +C20.2 vs. TEa T cells + WT iNKT cells + C20.2

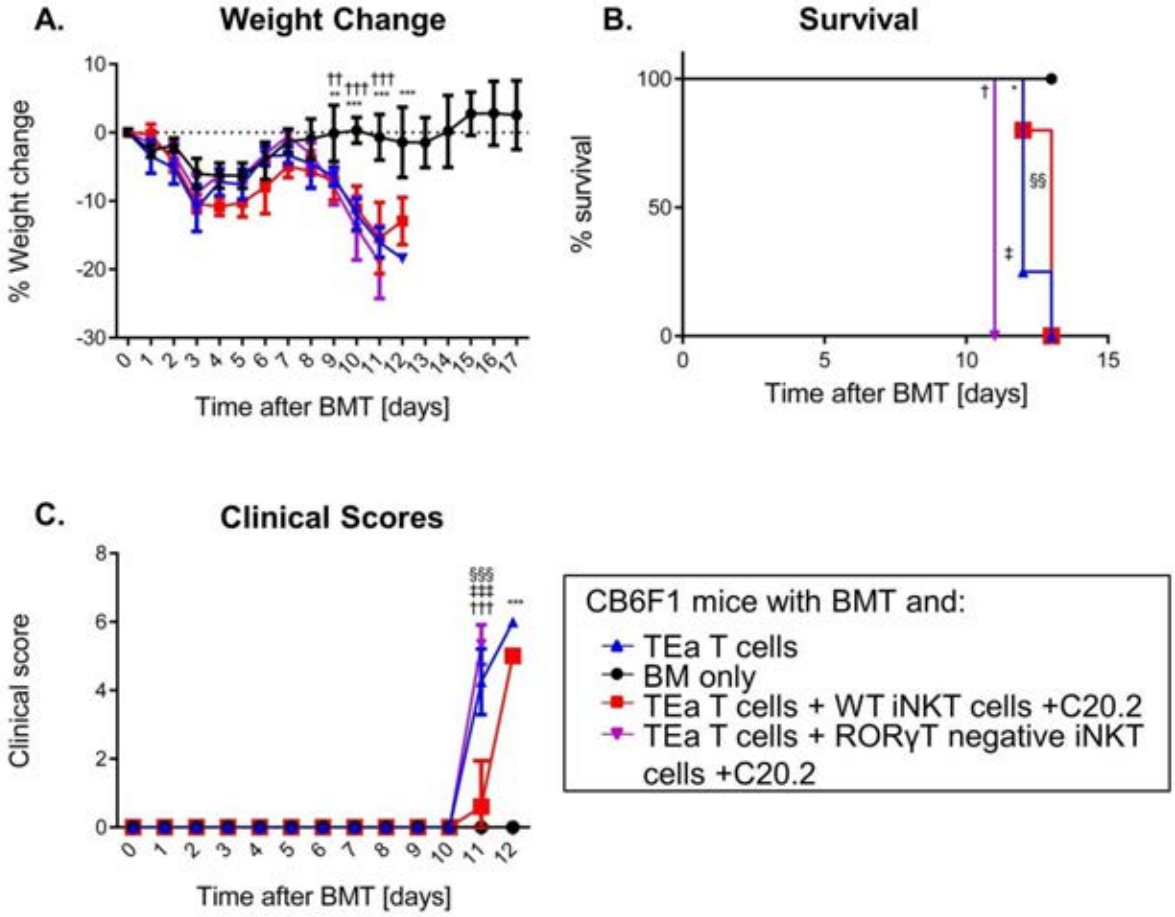


Figure 6.10 RORγT negative iNKT cells do not increase survival in GVHD CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received either 1x10^4 TEa T cells, TEa T cells + 1x10^5 WT or RORγT negative FI iNKT cells + C20.2 or no injection on day 2. Mice were monitored and (A) Weight loss and (B) Survival and (C) Clinical scores were recorded. Results are analysed using repeated measure 2-way ANOVA with post hoc Bonferroni comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n= 4 mice for TEa T cells only, n= 5 mice for TEa T cells + WT iNKT + C20.2, n=3 mice for TEa T cells + RORγT negative iNKT cells + C20.2 and n=2 mice for BM only.

*= BM versus vs. TEa T cells + WT iNKT +C20.2

†= BM versus vs. TEa T cells + RORγT negative iNKT +C20.2

‡= TEa T cells vs. TEa T cells + RORγT negative iNKT +C20.2

§= TEa T cells + WT iNKT +C20.2 vs. TEa T cells + RORγT negative iNKT +C20.2

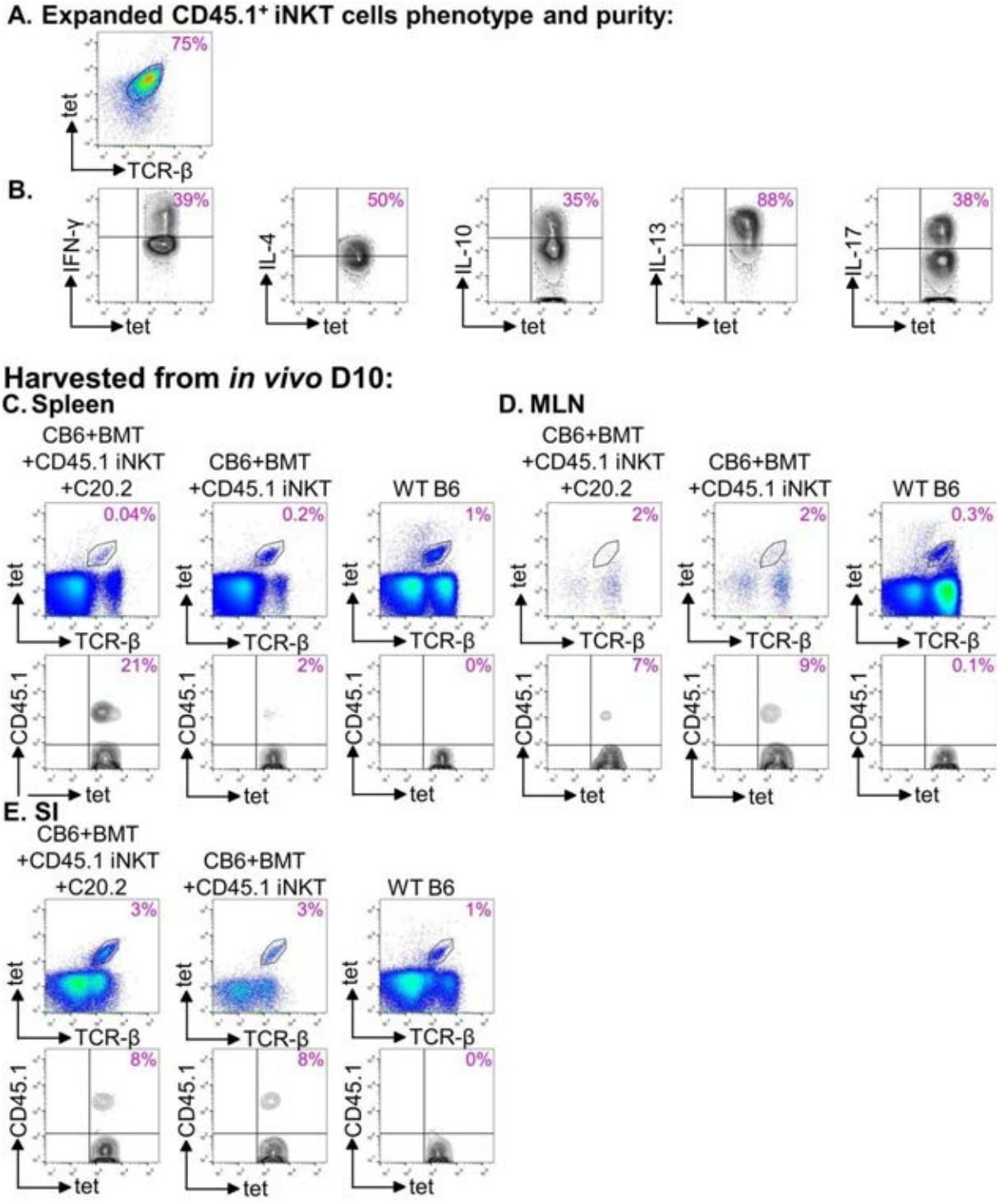


Figure 6.11 AT Exp iNKT cells can be identified in the spleen, MLN and SI of mice with BMT CB6F1 mice were lethally irradiated and given 5x10^6 TCD B6 BM cells i.v. on day 0. Such mice received 1x10^5 Exp iNKT cells ± C20.2 on day 2. (A) iNKT cells were gated on live and TCR-β and PBS-57 tetramer. Exp iNKT cells were phenotyped for (B) Cytokines before injection. Mice were sacrificed on day 10 and donor CD45.1$^+$ iNKT cells were identified in the (C) spleen, (D) MLN and (E) SI. n=1 mouse per group (as this was a pilot experiment).

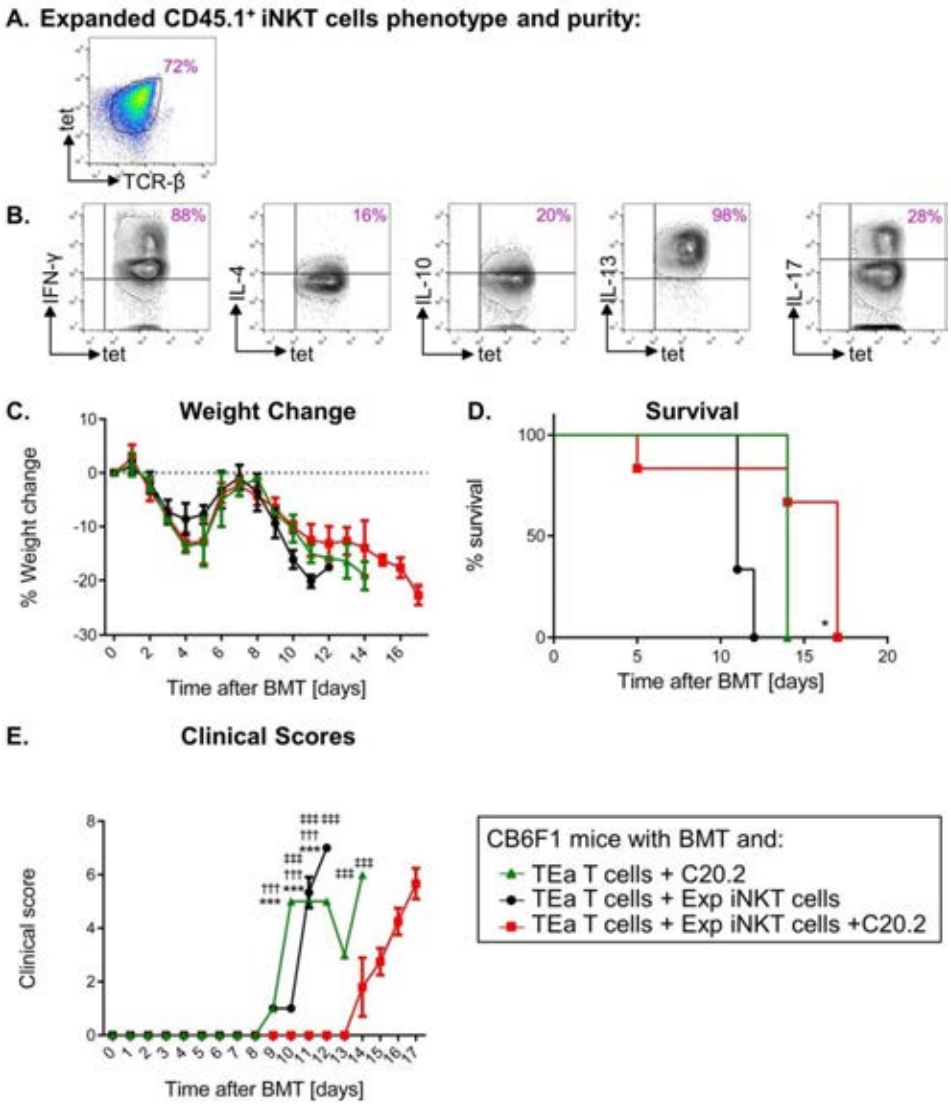


Figure 6.12 Mice that receive TEa T cells + Exp iNKT cells activated with C20.2, show increased survival compared to mice that receive TEa T cells + Exp iNKT cells CB6F1 mice were lethally irradiated and given $5x10^6$ TCD B6 BM cells i.v. on day 0. Such mice received either $1x10^4$ TEa T cells, TEa T cells + $1x10^5$ Exp iNKT cells or TEa T cells + Exp iNKT cells + C20.2 on day 2. (A) Prior to injection, Exp iNKT cells were phenotyped for (B) Cytokines. Mice were monitored and (C) Weight loss and (D) Survival and (E) Clinical scores were recorded. Results are analysed using repeated measure 2-way ANOVA with post hoc Bonferroni comparison for weight loss and clinical scores and Log-rank tests were used to analyse survival. *p<0.05, **p<0.01 ***p<0.001. Results are presented as mean ± SD, and n= 2 mice for TEa T cells + C20.2, n= 6 mice for TEa T cells + Exp iNKT cells + C20.2, n=3 mice for TEa T cells + Exp iNKT cells.

*= TEa T cells + expanded iNKT cells vs. TEa T cells + expanded iNKT cells +C20

†= TEa T cells +C20.2 vs. TEa T cells + expanded iNKT cells +C20

‡= TEa T cells + C20.2 vs. TEa T cells + expanded iNKT cells

CHAPTER 7 DISCUSSION

7.1. Summary of data

7.1.1. Summary of Chapter 3 data

In chapter 3 we aimed to set up a model of GVHD in which we could track the response of disease-causing alloreactive T cells. To this end, we established a model based on a previously report in which CB6F1 mice endogenously produce and present an H2IEα peptide in the context of $H2IA^b$ enabling recognition by TEa T cells (424, 432, 433). Initial experiments showed that TEa T cells could induce weight loss, increased clinical scores and death upon transfer to irradiated CB6F1 mice (Figures 3.2, 3.3). This occurred on a timescale similar to previous studies (424, 433). This was true of numbers of TEa T cells ranging from $5x10^4$ down to $1x10^3$ (Figures 3.2-3.4). However, at lower numbers incomplete penetrance was observed and the optimal induction of GVHD that still resulted in complete penetrance was obtained using $1x10^4$ TEa T cells (Figure 3.3). Therefore, we decided to proceed with using this dose for subsequent experiments.

Concomitant with the onset of GVHD we detected TEa T cells in the spleens and MLN of diseased mice (Figures 3.2, 3.5). Furthermore, there was an increase in the frequency of myeloid cells in the spleens of GVHD mice, which is consistent with the onset of GVHD (Figure 3.6). In addition, we could detect TEa T cells and $Gr1^+$ cells in the spleens of mice with GVHD using confocal (Figure 3.7).

Further to this we examined the spleens, MLN and two target organs namely the liver and SI just prior to disease onset (day 8). We observed an expansion of TEa T cells in all these anatomical locations in an allogeneic setting but not following transfer to syngeneic mice, confirming that the expansion of TEa T cells was allospecific and not due to homeostatic proliferation (Figure 3.9). Interestingly, we observed that recipient iNKT cells were increased in number and frequency compared to donor iNKT cells, suggesting that a radioresistant population of iNKT cells remained which could impact GVHD (Figure 3.12). Furthermore, we detected signs of damage on day 8 in the SI and the liver, two target organs of GVHD (Figure 3.15).

Our reason for choosing this particular model was due to the high precursor frequency of alloantigen specific cells, which would not be present in models which use polyclonal T cells to induce GVHD. This would this enable us to stringently test the capacity of iNKT cells to suppress GVHD, and assign those properties to iNKT cells specifically, and not any contaminating Treg. However, one caveat of the model is that the high precursor frequency and lack of Treg resulted in the model being extremely acute which may be difficult to control with immunoregulatory cells.

Another caveat is that this model is driven solely by $CD4^+$ T cells, as are many mouse models of GVHD in which successful suppression of GVHD has been seen

(418, 538). This ignores the contribution of $CD8^+$ T cells to GVHD, which is known to occur in humans in both MHC matched and MHC mismatched settings (539-542). Another issue that the disease is caused essentially by a monoclonal population of T cells recognising a single MHC-peptide which may not exactly mirror what occurs when polyclonal T cells recognise multiple alloantigens. Despite these caveats, this model was chosen due to its potential to enable detailed mechanistic dissection and elucidation of the role of specific cell populations such as iNKT cells in suppressing GVHD. Collectively, the observations in chapter 3 demonstrate that we have established a robust model of GVHD in which we can follow the response of alloreactive T cells in lymphoid and non-lymphoid tissues.

7.1.2. Summary of Chapter 4 data

Ultimately we sought to investigate the capacity of different subsets of iNKT cells to impact GVHD, therefore we first phenotyped iNKT cells. Furthermore, as iNKT cells would likely have to be expanded *in vitro* in order to make their use as a cellular therapy clinically viable, we set about expanding and characterising the resultant iNKT cells *in vitro*.

In line with previous reports we found that iNKT cells made up approximately 1% of splenic iNKT cells (Figure 4.1) (250-253). Upon stimulation with PMA and Ionomycin, IFN-γ (57%) was dominant cytokine produced by FI iNKT cells while only ~7% of such cells produced IL-17 (Figures 4.2). In addition, FI iNKT cells produced IL-4 (~34%) and IL-13 (~25%), while only ~2% of cells were able to produce IL-10 (Figures 4.2). In line with these observations T-bet was the dominant transcription

factor with ~70% of the cells expressing T-bet, while ~10% expressed RORγT (Figure 4.3).

iNKT cells could be expanded with BMDCs pulsed with αGal or ant-CD3 and anti-CD28 (Figures 4.4, 4.6). This was a difficult process and expansion did not always generate sufficient cells to be used in functional assays, however as human cells are easier to expand this would not be an issue in terms of translation (543). Indeed human iNKT cells have been sufficiently expanded to use in two phase 1 clinical trials for advanced melanoma and advanced and recurrent Non-Small Cell Lung carcinoma (396, 462).

When stimulated with PMA and Ionomycin, the iNKT cells expanded using αGal pulsed BMDCs produced less IFN-γ (~9%) than FI iNKT cells, IL-4 (~28%) was found to be similar but and interestingly we observed the emergence of iNKT cells able to express IL-10 (~31%) which were not present in their FI counterparts (Figure 4.5). Expansion with anti-CD3 and anti-CD28 also led to an increase in IL-10 (from ~2% to 24%), in IL-13 (from ~25% to 84%) and in IL-17 (from ~8% to 33%) with no changes in IFN-γ and IL-4 production compared to FI iNKT cells (Figure 4.6).

The changes in the cytokine producing ability of Exp iNKT cells was also reflected in changes in transcription factor expression. For example, an increase in E4BP4 expression was observed (from ~3% amongst FI iNKT cells to 75% Exp iNKT cells) (Figure 4.7). Similarly, the proportion of cells expressing RORγT was markedly increased (from ~10% to 49% in FI compared to E iNKT cells, respectively; (Figure 4.7). The Exp iNKT cells produced similar cytokines upon stimulation with anti-CD3 and anti-CD28 or αGal pulsed BMDCs compared to PMA and Ionomycin (Figure 4.8).

The Exp iNKT cells also took on complex effector phenotypes with some Exp iNKT cells capable of making between two and at least four cytokines on reactivation, which was not observed in the FI iNKT cells, suggesting that some the Exp iNKT cells took on the ability to produce cytokines not seen in the FI cells (Figure 4.10). Although this is clearly interesting and potentially a confounding factor with using these cells to manipulate disease we did not investigate the stability of these phenotypes *in vivo*. Furthermore, Exp human iNKT cells have a different phenotype (396, 462, 513, 544). However our observations do emphasises the importance of investigating the phenotype of human Exp iNKT cells extensively when these cells are used in clinical trials as this may affect the function, effectiveness and safety of such cells.

Further, to this we investigated the lineage diversification of Exp iNKT cells. Exp sorted RORγT$^-$ iNKT cells proved to be very difficult to expand perhaps suggesting that the increase in IL-17 producing iNKT cells following expansion was the result of outgrowth of the NKT17 population present at the beginning of cultures (Figure 4.12).

Together, these observations suggest that Exp iNKT cells take on the ability to produce multiple additional cytokines upon *in vitro* expansion and constitute a potential population of immunomodulatory cells to be investigated *in vitro* and *in vivo* for their ability to influence alloresponses.

7.1.3. Summary of Chapter 5 data

As we observed in Chapter 4, Exp iNKT cells have complex effector phenotypes compared to FI iNKT cells, in terms of their pattern of cytokine secretion. In this

chapter we aimed to investigate the suppressive properties of such cells *in vitro*. In order to investigate their suppressive capacity *in vitro* we designed a suppression assay in which CFSE labelled TEa T cells were stimulated with α-Gal-pulsed allogeneic (CB6F1) BMDCs. The assay was then used to test the impact of FI and Exp iNKT cells on a T cell alloresponse.

Interestingly, we found that activated Exp iNKT cells suppressed TEa T cell proliferation (from 79%±5% proliferating cells to 13±2% at a ratio of 1:1 TEa T cells to Exp iNKT cells; Figure 5.1). Furthermore, the Exp iNKT cells were found to suppress proliferation at ratios as low as 1:0.1 (Figure 5.1). This was also true in terms of numbers. Furthermore, we found that activated Exp iNKT cells were significantly more potent at suppressing TEa T cells than FI iNKT cells (Figure 5.2).

We investigated the mechanism used by iNKT cells to suppress T cell responses *in vitro* and found that that neither IL-10 nor IFN-γ was required (Figure 5.3). In addition, iNKT cells did not simply out-compete the TEa T cells for IL-2 (Figure 5.4). Interestingly we found that Exp iNKT cells could not suppress TEa T cells when they were stimulated with anti-CD3 and anti-CD28 (Figure 5.5). This suggests a possible involvement of the allogeneic DCs in the suppressive mechanism, or that the anti-CD3 anti-CD28 stimulated T cells were too potent to suppress. A caveat with this experiment was that anti-CD3 and anti-CD28 would also activate the iNKT cells and therefore could also affect their suppressive function. Moreover, iNKT cells have previously been reported to become anergic in the presence of a strong TCR signal with co-stimulation (545). While one recent *in vitro* human study showed that Exp human iNKT cells can suppress T cells stimulated with anti-CD3 and anti-CD28 the

source of conventional T cells also contained Treg which may have contributed to suppression (513).

In order to explore candidate molecules that could be involved in immunosuppression we analysed microarray results for the modulation of cell surface molecules on iNKT cells which had been activated with αGal for 48h compared to PBS controls (Figure 5.6). We found that LAG-3, PD-1 and TIGIT were all upregulated in the activated cells (Figure 5.6). To confirm these molecules were expressed on Exp iNKT cells we stimulated the Exp cells with either αGal pulsed BMDCs or anti-CD3 and anti-CD28 (Figure 5.7). The expression of these molecules proved to be highly variable depending on the experiment, possibly due to the fact that the Exp iNKT cells have a more varied phenotype than the FI iNKT cells. However, in all three independent experiments, stimulation with αGal pulsed BMDCs resulted in the highest frequency of Exp iNKT cells expression LAG-3, PD1 and TIGIT (Figure 5.7). Combined with the microarray these data suggest that one or more co-inhibitory molecules such as PD1, TIGIT and LAG-3 could be responsible for mediating the suppressive effect mediated by Exp iNKT cells *in vitro*.

We also analysed the expression of LAG-3, PD1 and TIGIT in FI iNKT cells and found that the expression of all three molecules was low regardless of whether they were activated with anti-CD3 and anti-CD28 or αGal pulsed BMDCs (Figure 5.8). However, we showed that the PD1 pathway was not involved in Exp iNKT cell mediated suppression (Figure 5.9). However, we did not examine if LAG-3 or TIGIT was utilized by Exp iNKT cells. Unfortunately, we were unable to perform RNA-Seq of FI and Exp iNKT cells which may have revealed additional molecules that may contribute to the suppression seen by Exp iNKT cells.

Together, these findings suggest that Exp iNKT cells alone can suppress TEa T cells, and their suppressive activity is more potent than FI iNKT cells. This suppression is not mediated by IFN-γ, IL-10, IL-2 sequestration by iNKT cells, or the PD1 pathway. It would be interesting to investigate in further experiments if suppression was mediated by a soluble factor. One way to do this would be to investigate the suppressive properties of supernatant derived from activated Exp iNKT cells or perhaps to conduct experiments where the iNKT cells and conventional T cells were separated by a transwell. It would also be worthwhile to repeat some of the experiments using C20.2 instead of αGal to activate the Exp iNKT cells as this is reported to promote a Th2-type response (278). Finally it would be interesting to see if the glycolipid and alloantigen needed to be on the same APC. To do this αGal pulsed B6 BMDCs (to provide iNKT cell activation) and unpulsed CB6F1 BMDCs (to provide TEa stimulation) could have been used. If suppression still occurred it would suggest that it is not important that iNKT cells and TEa T cells are stimulated by the same BMDC.

These *in vitro* studies have been validated by three studies that showed that Exp human iNKT cells are able to suppress alloreactive T cells *in vitro* (408, 513, 514). However, these studies used unseparated T cells as responder cells and as such Treg may also have contributed the mechanism of suppression. One of these studies showed that the iNKT mediated suppression occurred through iNKT mediated lysis of the DCs (408). This would be an important experiment to carry out in future work. Interestingly, it was recently shown that in addition to suppressing alloreactive T cell responses Exp iNKT cells mounted a robust GVL response against HLA matched allogeneic leukaemia cells *in vitro* (514). It would be interesting to investigate whether

Exp iNKT cells could also mediated an anti-tumour effect. Nonetheless, as far as we are aware our study represents the first demonstration that murine iNKT cells can suppress alloreactive T cells *in vitro,* and that Exp iNKT cells are more potent suppressors than FI cells.

7.1.4. Summary of Chapter 6 data

In this chapter we aimed to use the model we established in Chapter 3 to investigate the ability of both FI and Exp iNKT cells to suppress GVHD. Our initial experiment set out to investigate the ability of FI iNKT cells with and without αGal to suppress GVHD. We found that even in the absence of alloreactive T cells iNKT cells activated with αGal exacerbated initial radiation-induced weight loss (Figure 6.1). In addition, TEa T cells administered with iNKT cells and αGal exacerbated GVHD in terms of clinical scores, weight loss and survival (Figure 6.1).

As a result of these findings we next sought to investigate if activating iNKT cells with C20.2 which has been shown to lead to the inhibition of GVHD in a B6 to B6D2F$_1$ model would be a beneficial alternative to activation with αGal (528). However, we again found that the administration of C20.2 exacerbated the initial weight loss, which in some cases led to death. Furthermore, no significant increase in survival was observed (Figure 6.6).

Intriguingly, the mice that survived the initial radiation induced weight loss went on to show a 2 day delay in GVHD related death (Figure 6.6). We went on to show that mice did not present with exacerbated weight loss following the administration of C20.2 in the absence of AT iNKT cells but still survived longer than controls (Figure

6.8). This suggests that the AT iNKT cells were mediating the initial weight loss and the host residual iNKT cells were responsible for the modest increase in survival.

The observation that the AT iNKT cells exacerbated the initial weight loss after irradiation, led us to postulate that removing potentially pathogenic subsets of iNKT cells might prevent this exacerbated weight loss. However, transfer of either T-bet$^{-/-}$ or RORγT^{-} iNKT cells exacerbated GVHD (Figure 6.9, 6.10). One potential explanation for this is as cells are being transferred into an inflammatory environment upon adoptive transfer, and it is possible that the absence of one pathogenic phenotype leads to the cells compensating by increasing the production of other proinflammatory cytokines. This could be investigated using fate-mapping mice. Another possibility is that the cells having a pathogenic phenotype that is not under the control of RORγT or T-bet, for example IL-2 production, to test this it would be necessary to block IL-2 using mAb.

In Chapter 5 we observed that Exp iNKT cells were more potent suppressors of alloreactive T cell responses than FI iNKT cells *in vitro*. As a result of this observation and due to our lack of success suppressing GVHD using FI iNKT cells, we sought to test whether Exp iNKT cells would have a better outcome in GVHD. We found that anti-CD3 anti-CD28 Exp iNKT cells could survive following *in vivo* transfer (Figure 6.11). A caveat is that it would have been useful to investigate the stability of their phenotype upon transfer. Mice that received TEa T cells + Exp iNKT cells +C20.2, showed extended survival compared to mice that received TEa T cells + Exp iNKT cells (Figure 6.12). However, neither group showed increased survival compared to mice that received TEa T cells + C20.2 alone, suggesting that any increase in

survival was primarily mediated by recipient iNKT cells and not the AT Exp iNKT cells (Figure 6.12).

There could be several reasons why we did not observe GVHD suppression, despite the ability of these cells to suppress alloreactive T cells *in vitro*. As discussed at length in Chapter 6, despite our observation that Exp iNKT cells alone can suppress alloreactive T cells *in vitro* they may require other cells such as Treg to mediate suppression in an *in vivo* setting (238, 525). Another possibility is that due to the complex effector phenotype of Exp iNKT cells observed in chapter 4, any suppressive effect of a particular subset is masked by other proinflammatory properties. It is also possible that increasing the number of iNKT cells transferred might enable suppression to occur. However, in a previous study that saw increased survival in a B6 to DBA/2 model of GVHD due to transfer of Exp iNKT cells, mice lost 30-35% of their original body weight (237). This would exceed the moderate severity limit in our licence. We also encountered this problem when testing the suppressive ability of the FI iNKT cells. In several previous studies in which transferring FI iNKT cells lead to a protective effect in GVHD, mice exceeded 20% weight loss but went on to recover from GVHD. It is likely that amending our licence to allow for more severe weight loss would provide us with a better opportunity to investigate the suppressive ability of iNKT cells in this model of GVHD.

7.2. Potential factors in the therapeutic potential of iNKT cells

7.2.1. The timing of infusion may influence the therapeutic potential of iNKT cells

In all studies to date that have shown suppression of GVHD with the iNKT cells (FI or Exp) have either been co-infused with BM transplant and disease causing cells, or administered one day later (126, 234-238, 513). In the model of GVHD we employed we infused iNKT cells with the Tcon and an effect on GVHD was not observed. Our reasoning for the timing of infusion was that iNKT cells are rapidly activated and therefore pre-infusion might not be necessary and in fact it might be too early for them to have an effect on the Tcon.

Many studies have examined the ability of AT Treg to suppress GVHD, and it has been reported that the timing of the Treg infusion has a profound influence on GVHD in that it can affect both GVHD and the GVT effect (546). Although the exact timing of Treg infusion is not likely to be directly translatable due to the differences in iNKT and Treg activation and response rates, it does emphasise that the timing of the delivery of immunomodulatory cells can impact their ability to control disease (546). nTreg infusion 2 days prior to the infusion of Tcon has been shown to lead to nTreg expansion and a ten-fold decrease in the number of Treg needed to suppress GVHD and infusing Treg prior to Tcon also lead to increase iTreg potency (547, 548). It would be interesting to investigate if injecting iNKT cells before, during and after Tcon injection impacted their ability to influence GVHD.

7.2.2. The disease setting and local microenvironment can influence the iNKT response

In the model of GVHD that was used in the presented studies the adoptive transfer of iNKT cells caused exacerbated weight loss after irradiation. While there are many explanations as to why this may have occurred, including the absence of Treg transfer, iNKT cells can play a protective or pathogenic role in different models of the same disease. This has been shown to occur in Inflammatory Bowel disease (IBD) a disease in which iNKT cells have been shown to have both protective and pathogenic roles. For example, activating iNKT cells using either αGal or OCH has been shown to suppress Dextran sulfate sodium induced IBD (549, 550). While in contrast in the Oxazolone colitis model of IBD αGal activated iNKT cells have been shown to be detrimental, an effect mediated by IL-13 production (551). This may be due to differences in the disease microenvironment of the target organs such as the intestine in different models having an effect on the phenotype or function of the transferred iNKT cells.

A hallmark of iNKT cells is their ability to rapidly secrete cytokines upon activation and this property is conferred by epigenetic regulation during development (326, 335, 463, 552). However, iNKT cell phenotype is not fixed and can be altered peripherally as the phenotype of iNKT cells can be influenced by the microenvironment in which they are situated (334).

Another study showed that the nature of the APC presenting glycolipid to the iNKT cell determines the polarity of the immune response (289). It goes on to suggest that the reason that iNKT cells respond differently to different glycolipids is because αGal requires presentation by DCs, while presentation of Th2-promoting glycolipid variants

is promiscuous and is not effected by the ablation of CD1d on DCs (289). Co-stimulation has also been suggested to influence the polarity of the iNKT cell response with anti-CD28 and anti-CD40 promoting Th1 responses whereas Th2 responses are only driven by anti-CD28 stimulation (553).

There is precedence for iNKT cells behaving in a plastic manner in response to cytokines. For example iNKT cells capable of producing IL-17 are thought to originate in the thymus; however IL-17 production can be induced under inflammatory conditions such as in the presence of IL-1β and IL-23 with TGF-β (454, 475).

Epigenetic changes are involved in human disease in addition to development; however the epigenetic regulation of peripheral iNKT cells has not been extensively studied. iNKT cells in the intestine are activated by lipid antigens from either pathogenic bacteria or indeed commensals presented by APCs. Whether iNKT cells are pathogenic or protective can be influenced by the nature of the lipid antigens, the cytokine milieu and the type of APC presenting glycolipid (554). In the steady state CD1d mediated lipid presentation by $CD11c^{+}$ cells and intestinal epithelial cells have been shown to be import in the maintenance of intestinal homeostasis (555, 556).

The intestinal microbiome has been shown to affect the phenotype and function of iNKT cells (557). Exposing germ-free mice to bacteria which carry iNKT cell specific antigens led to the iNKT cells maturing from less mature to fully mature iNKT cells (557). In this study we have observed an inflammatory infiltrate and signs of intestinal damage in GVHD (Figure 3.15). Given the propensity of the phenotype of iNKT cells to be altered in response to the intestinal microbiome, theoretically it would be possible for iNKT cells in a disease such as GVHD which causes epithelial damage

and hence weakens the integrity of the intestine to alter the phenotype of iNKT cells through releasing previously sequestered lipid antigens.

iNKT cells have also been shown to be amenable to *ex vivo* reprogramming (558). Splenic iNKT cells were harvested from mice with 2,4,6-trinitrobenzenesulfonic acid colitis (oxazolone colitis) and were exposed to colitis extracted proteins which resulted in a shift away from NKT1 cytokines and towards NKT2 cytokines (558). Adoptive transfer of such cells alleviated colitis. Although the mechanism by which this occurred was not investigated it is likely that it occurred by means of epigenetic regulation.

More research is required into the plasticity of peripheral iNKT cells and if indeed this plasticity is mediated by epigenetic regulation. Furthermore, it will be important in the context of therapeutically transferring iNKT cells to understand how the local and systemic disease microenvironment can potentially influence the phenotype of transferred iNKT cells.

7.2.3. The site of infection or injury can influence the iNKT cell response

iNKT cells can play paradoxical roles in organ transplantation depending on the anatomical location of the transplanted organ (Jones, unpublished observation). iNKT cells have been shown to be required for graft acceptance in vascularised cardiac transplant models in which tolerance was induced either by blocking co-stimulatory molecules, integrins or co-receptors (232, 399, 404). In contrast, iNKT cells produce IL-17 post skin transplantation and promote graft rejection (231).

In these models of heart transplantation the recipients were B6 and the hearts were vascularised and hence drained to the spleen (559-562). The majority of iNKT cells in the spleen of B6 mice are IFN-γ producing (310, 320, 330, 331). On the other hand skin transplants drain to the LNs and in B6 mice IL-17 is the predominant cytokine produced by iNKT cells in the skin and draining LNs (407). Therefore, it is likely that as a result of the dominance of different subtypes of iNKT cells at different locations, namely the draining secondary lymphoid organs, iNKT cells have the ability to both facilitate and impede allograft acceptance. iNKT cells also play diametrically opposing roles in various viral infections, which has been suggested to be influenced at least in part by the primary site of infection (563).

In terms of GVHD, the skin, lungs, liver and intestine are affected, in addition to the iNKT cells potentially having an anti-tumour response against blood borne cancer cells. With multiple organs being affected by GVHD, iNKT cells may have to behave in the same suppressive manner against alloreactive T cells in multiple anatomical locations. However, the GI tract is thought to be particularly important in the initiation of GVHD and the perpetuation of the cytokine storm (172). Furthermore, intestinal damage has been shown to be important in the pathophysiology of GVHD (154). Therefore, as iNKT cells respond rapidly on activation, iNKT cells may mediate their effects early in disease before GVHD has progressed to multiple organs.

In the model of GVHD characterised in Chapter 3, we observed the infiltration of immune cells, and damage to the liver and SI on day 8 which is prior to the onset of disease (Figure 3.15). Although we did not observe any clinical signs of skin GVHD, the damage that was present in the SI and Liver was sufficient for the animals to reach their severity limit in terms of either weight loss or clinical score. Given these

observations and the significant weight loss observed in disease mice, we hypothesise that in order to see suppression mediated by iNKT cells in this model it will be important for them to have a suppressive effect in the GI tract.

7.2.4. The stage of disease can influence the iNKT cell response

In the model of GVHD we employed, we have observed that activation of AT iNKT cells exacerbates initial weight loss after irradiation (Figure 6.6, 6.8). However, although the majority of mice that received AT iNKT cells and C20.2 had to be culled as a result of this exacerbated weight loss, those that survived did so for longer than control mice (Figure 6.6). While this effect was most likely mediated by recipient iNKT cells (Figure 6.8), it suggests that iNKT cells albeit from different origins had different roles depending on the stage of the disease. Due to project licence regulations we had to cull mice that reach 20% weight due to exacerbated weight loss post-irradiation, however these mice appeared to be otherwise healthy and did not register a clinical score. Therefore, it is impossible to say if such mice would have also gone on to show improved survival.

iNKT cells have previously been shown to have pathogenic and immunoregulatory roles even within the same disease depending on the disease stage. An example of this is in relapsing remitting MS, in which decreased peripheral blood iNKT cells correlated with onset of relapse (370). In contrast, remission periods have been associated with Th2 biased NKT cells (371).

NOD mice, which are frequently used to study diabetes, have significant defects in the frequency of iNKT cells and cytokines they produced (564). Adoptive transfer of WT iNKT cells, or restoration of iNKT cell function using αGal, or overexpression of CD1d or TCR can prevent the development of diabetes in NOD mice (564-566). This suggests that iNKT cells have a protective role during the development of diabetes. These observations led to investigation into the mechanism by which iNKT cells mediate this protective effect. As a result of this iNKT cells have been found to impair the development of autoreactive T cells into effector Th1 cells, by directly suppressing autoreactive $CD4^+$ T cells via IFN-γ and by inducing the development of non-inflammatory DCs (567-569).

In contrast, iNKT cells have also been shown to have a pathogenic role in diabetes through enhancing the activation, expansion and IFN-γ production of diabetogenic $CD8^+$ T cells (570). This suggests that iNKT cells may have a different effect on $CD4^+$ and $CD8^+$ T cells and while they may act to prevent diabetes early in disease once autoreactive $CD8^+$ T cells become activated they may exacerbate disease pathogenesis.

7.2.5. The type of glycolipid and route of administration can impact the response of iNKT cells

As previously mentioned the nature of the glycolipids used to activate iNKT cells influences the polarity of the immune response. αGal leads to the production of predominantly Th1 type cytokines such as IFN-γ, while OCH and C20.2 lead to preferential production of IL-4 and other Th2 type cytokines (271, 277, 278).

In Chapter 6 we observed no improved survival of mice which received AT iNKT cells and αGal i.p. Kuns *et al.* showed that αGal injection induced hyperacute disease induction and early mortality in B6 to $B6D2F_1$ and BALB/c to B6 GVHD models while C20.2 failed to exacerbate GVHD in B6 to $B6D2F_1$ and BALB/c to B6 GVHD models and in most settings inhibited it (528). Therefore we decided to use C20.2 to activate the AT iNKT cells. However, we found that this led to only a very modest increase in survival, which was most likely mediated by radio-resistant recipient iNKT cells (Figure 6.8). This observation is in agreement with a previous study which investigated the role of host residual cells upon adoptive transfer of Exp iNKT cells into a B6 to BALB/c mice model of GVHD (535). Using $J\alpha18^{-/-}$ recipient mice the authors demonstrated that host-residual iNKT cells play an important role in reducing the severity of GVHD, and are required for the prolongation of survival by AT Exp iNKT cells (535).

The route by which the glycolipid is administered can also influence the nature of the immune response. For example type 3 innate lymphoid cells (ILC3s) express CD1d and can present glycolipids to iNKT cells (571). An increase in IL-22 production by ILC3s is observed when αGal is administered by oral gavage rather that i.v. injection (571).

In the model of GVHD we employed we administered αGal by i.p. injection. This was due to it being the mode of delivery employed in previous GVHD experiments, and the proximity of the intestine which is affected by GVHD (527, 528). However, given that one study has found that ILC3 derived IL-22 is critical for prevention of intestinal GVHD (468); it might be beneficial to investigate the influence of αGal delivery via oral gavage.

7.2.6. The local animal facility or supplier and microbiota may influence the iNKT cell response

The local animal facility and animal supplier has an impact on the microbiome of mice (572, 573). As previously discussed the intestinal microbiome has been shown to affect the phenotype and function of iNKT cells (557). iNKT cells can recognise intestinal glycolipids presented on CD1d expressed by $CD11c^+$ cells and intestinal epithelial cells which has been shown to be importance in the maintenance of intestinal homeostasis (555, 556).

In the model of GVHD we employed, Baytril (enrofloxacin) was given in drinking water for one week prior to and two weeks post BMT. Antibiotics are commonly administered prior to BMT as the immune system has been ablated by the irradiation which leaves the mice vulnerable to infection. However, interestingly in a model of collagen induced arthritis Baytril has been shown to aggravate the disease (574). It does this through partially depleting the intestinal microbiota when administered orally compared to positive control mice. *In vitro*, auxiliary LN and MLN cells from collagen induced arthritis mice show increased production of IFN-γ and lL-17 and a decrease in IL-4 production compared to controls (574).

Given this observation and the propensity of iNKT cells to respond to microflora lipid antigens in the intestine and hence maintain homeostasis it is conceivable that alterations in the microflora results in the AT iNKT cells having a detrimental impact in our experiments, in that they cause exacerbated weight loss after irradiation. This could theoretically be influenced by the microbiome present in the local animal facility or animal supplier (572, 573). Indeed in mice and humans, broad spectrum antibiotics have been shown to give rise to the dominance of families of bacteria which are usually subdominant and in some cases these bacteria are multi-drug resistant (575-580). Furthermore, decreased diversity is related to poor clinical outcomes in clinical GVHD calling into question which antibiotics should be used and the timing of antibiotic administration (575-580). One study showed that a decrease in the *Lachnospiraceae* and *Ruminococcaceae* families and increase in the *Enterobacteriaceae* family correlated with an imbalance in the ratio of Treg/Th17 observed in GVHD patients compared to non-GVHD patients (579).

Together these studies suggest that broad spectrum antibiotics can alter the microbiome and decrease diversity and this is associated with onset of GVHD and poor outcomes. In mice studies, the remaining microbiome post antibiotic treatment could be influenced by the type of antibiotic used as well as the timing of administration. It is also likely that the original microbiome present in a particular animal facility or supplier prior to antibiotic treatment could influence what bacteria are present after treatment which one might predict could impact the effect of the microbiome of GVHD, depending on the dominant family of bacteria present. It is possible that in our animal house treatment with broad-spectrum antibiotic Baytril leads to the dominance of bacteria that promote GVHD and the death of bacteria that

promote regulatory cells, similar to what has been shown to occur in collagen induced arthritis (574). Given the propensity of iNKT cells to recognise lipids, iNKT cells could have exacerbated this. Indeed in another disease setting (NOD mice) an increase in NKT17 cells has been observed in the intestine which was associated high *Bacterioidales* and low *Clostridiales* abundance (581). Of course only a detailed phenotyping of the microbiome of our recipient mice would enable us to examine this hypothesis.

7.2.7. Summary of known and potential effects on the therapeutic potential of iNKT cells

Multiple factors are known to influence the iNKT cells response, which may have a bearing on their ability to be used therapeutically to suppress GVHD. These include the timing of infusion, the microenvironment in which they are situated, the site of injury, the stage of disease and the type of glycolipid antigen and mode of delivery employed. Furthermore, other factors such as the influence of the local animal facility and supplier and antibiotic treatment and the timing of iNKT infusion for adoptive transfer based therapies have not been investigated in the context of iNKT cells but may impact their response none the less. Greater understanding of these factors in the context of GVHD would result in improved study design for investigating the ability of iNKT cells to suppress GVHD. Furthermore, this information would be useful in unravelling the ability of iNKT cells to maintain anti-tumour immunity following HSC transplantation.

7.3. Graft-versus-tumour effect.

FI isolated $CD4^+$ iNKT cells or $DX5^+$ $CD4^+$ NKT cells have been reported to be capable of suppressing GVHD while maintaining the anti-tumour effect (234, 238). Current therapies for GVHD involve blocking or depleting T cells in the graft and while this can reduce GVHD, it results in impaired engraftment and reduces the GVT effect (148, 532). The ultimate goal in the treatment of HSC transplantation is to prevent the GVHD response while still maintaining the anti-tumour effect. Exp iNKT cells have been shown to suppress GVHD, however these studies have not thoroughly investigated the impact of Exp iNKT cells on the tumour (236, 237, 513).

Decreased GVHD and increased progression free survival has been observed in humans who received higher numbers and ratios of iNKT to Tcon (409, 410). Reduced conditioning regimes in patients have also been shown to preserve the GVT effect and patients showed a significant increase in the frequency of iNKT cells in the blood (534). Furthermore, iNKT cells were shown to mediate protection by preventing the tissue damage induced by donor T cells in a mouse model of GVHD, while simultaneously maintaining the GVT response of donor T cells against B cell lymphoma cells (533). In addition, although $CD1d^{-/-}$ or $J\alpha18^{-/-}$ recipient mice clear tumour cells they ultimately succumb to GVHD (533).

The mechanism by which iNKT cells prevent GVHD while maintaining the anti-tumour response has not been demonstrated. As we observed in Chapter 4 iNKT cells are a heterogeneous population of cells (Figures 4.2, 4.3). It is possible that in studies where iNKT cells were observed to suppress GVHD while maintaining the GVT effect, different subsets were responsible for these two roles.

As discussed at length in the introduction, iNKT cells can have a different phenotype depending on their location and the type of disease. As GVHD occurs as a result of HSC transplantation to treat leukaemia or lymphoma, there are effectively two diseases being treated which may require dichotomous immune responses in order to restore immune homeostasis. The anatomical locations affected by GVHD and the cancer are different as leukaemia, lymphoma and myeloma mainly affect the blood, BM, LNs and lymphoid tissue and GVHD affects the skin, lung, liver and GI tract. Therefore, it is possible that the iNKT cell subsets are present in the different locations and are having different effects.

iNKT cell derived IFN-γ produced as a result of stimulation with αGal, stimulates NK cells and $CD8^+$ T cells which go on to lyse tumour cells and iNKT cell derived IL-12 primes DCs and enhancing their ability to stimulated tumour specific $CD8^+$ T cells (582). In contrast NKT cell derived IL-4 has been shown to be required for NKT cell mediated suppression of GVHD (234). Therefore, it is possible that different subsets of iNKT cells are responsible for suppressing GVHD and maintaining the anti-tumour response.

While, is has been shown that iNKT cells can maintain the GVT response, whether or not iNKT cells can contribute directly to tumour clearance, or merely do not affect the anti-tumour effect of the T cells has not been definitely demonstrated. In humans and mouse models iNKT cells can mediate their effects directly by killing CD1d expressing tumour cells, as can occur in many forms of cancer including AML and acute lymphoblastic leukaemia, myelomas and myeloid leukaemias (390-392). Furthermore, it has been reported that iNKT cells play a role in anti-tumour immunity in leukaemia and lymphoma (386) and a decrease in the number of iNKT cells or

defects in their function has been observed in leukaemia, lymphoma and myeloma (583).

There is some evidence that iNKT cell can mediate anti-tumour immunity in the context of HSC transplantation as they have been shown, using bioluminescence imaging, to have modest anti-tumour activity against A20 but not BCL-1 lymphoma cell lines, and such anti-tumour immunity lead to a significant survival benefit (238). However, B cell lymphoma lines are known to grow variably *in vivo* and to be particularly sensitive to anti-tumour immunity (538). Furthermore, the mechanism of killing used is dependent on the cell line (584) which is also likely to be the case in human leukaemia. Therefore, inducing leukaemia using retroviral insertion of oncogenes may be more clinically relevant (146, 585). Furthermore, this study does not make evident that iNKT cells are mediating this effect directly (238). Further evidence that iNKT cells can mediate anti-tumour immunity comes from a recent *in vitro* study that demonstrated that Exp human iNKT cells can suppress alloreactive T cells and the same cells Exp iNKT cells show a robust anti-tumour response against HLA matched allogeneic patient leukaemia cells *in vitro* (514).

iNKT cells have also been shown to mediate anti-tumour immunity indirectly. Such cells have been shown to be required for $CD8^+$ T cell mediated cytotoxicity upon administration of potent G-CSF analogues (586). iNKT cells may enhance tumour clearance indirectly via recruitment and activation of other cells or by regulating immunosuppressive cells in the tumour microenvironment or directly by tumour cell lysis (587). An example iNKT cells indirectly effecting tumours is via regulating the tumour microenvironment, for example by killing tumour-associated macrophages which suppress anti-tumour immunity (394, 588).

Similar to iNKT cells, Treg have been shown to suppress GVHD while allowing the anti-tumour effect to still occur in some mouse models (200-202). This is thought to be due to the fact that there is relatively high precursor frequency of allospecific T cells in many models of GVHD. Under such conditions, major expansion of CTLs may not be required for the anti-tumour effect. Effectively this means that is some models of GVHD, the maintenance of the anti-tumour effect is thought to be due to the fact that Treg inhibit proliferation, but not activation (589). It is possible that iNKT mediated suppression of GVHD and maintenance of the GVT response is occurring in a similar manner either directly by the iNKT cells, or indirectly via the Treg. Indeed, Treg have been shown to be required for iNKT cell mediated suppression in some models of GVHD (238, 525).

7.4. Challenges in translation of cellular therapies to a clinical setting

Cellular therapy has the potential to transform the treatments of many diseases; however several challenges exist in order for a therapy to be successfully translated into the clinic. The most important requirements are that the therapy is safe, consistent and cost effective and achieving these requirements is not trivial. Furthermore, as most preclinical studies are conducted in mice cell therapy faces the same challenges as any translational therapy in that results do not always translate to humans in terms of relative dose or efficacy. This is further complicated by the fact that the mechanism of action of cellular therapies often rely on the intrinsic properties of the cells, and therefore the success of the therapy in the clinic is dependent on whether human immune cells have the same phenotype and activity.

As many cells require expansion is order to obtain numbers capable of mediating their effect *in vivo* a manufacturing process that produces consistent numbers of cells of a consistent phenotype at a relatively low cost is required in order for the therapy to be financially viable. Cells are known to lose their therapeutic potential with increased time in culture, however sufficient numbers of cells have to be produced which can take time (590). Furthermore, even slight alterations in conditions can drastically alter the phenotype of the cells, for example changes in oxygen concentration in the culture media of embryonic stem cells can lead to changes in the phenotype of the cells (591, 592).

Maintenance of the phenotype and function of the cells is of quintessential importance for the efficacy of the therapy (593). Maintaining such processing under Good Manufacturing practice (GMP) presents a unique challenge (594). Traditional production of biotherapeutics involves isolating products from cells but does not require recovery of the cells, whereas cell therapies require the cells to be re-isolated.

Pathogen transfer is also a concern especially as patients receiving the cell therapy are often immunocompromised. Potency assays have been suggested to be a useful means of determining the quality, stability and consistency of the cells and it has been suggested that they should be in place at an early stages of development and be validated prior to commencement of clinical trials (595).

Variation in the expanded products can occur either as a result of variation in the cells being input (which may be genetic) or as a result of the conditions during the expansion (596). In some cases cells can come from a third party i.e. can be

allogeneic, which makes the process more straightforward as the cells for expansion can be carefully chosen and a single batch administered to multiple patients. Automating the expansion process can help decrease variability in the conditions (597-600). Manufacturing the cells in a closed process would also be preferable as it would reduce the risk of pathogen transfer. It would also allow for facilities in which multiple batches of cells could be produced in the same room, whereas a semi-closed system needs to be separated or there would be a risk of cross-contamination (601).

Existing expansion protocols require hands on labour intensive processes which may be difficult to automate and scale-up. For example mesenchymal stem cells (MSCs) which are currently in ongoing stage 3 clinical trials for steroid refractory GVHD. The maximum number of cells that can be generated per lot is currently ~21 billion cells (601). The steroid refractory GVHD therapy requires 100 million cells per dose, which would mean that 213 doses could be produced per lot and multiple infusions may be required per patient to treat GVHD (602). There are approximately 30,000 HSC transplants performed annually world-wide, where ~10 to 80% get GVHD depeding on the risk factors (603). Of those that develop GVHD, 50% develop steroid refractory GVHD (208). This means that even a single infusion per patient would present a significant challenge with current technologies.

Given the difficulties which lead to expense in establishing closed and automated manufacturing processes, it has been proposed that for Phase 1 trials a semi-closed, manual process should be used which could be up-scaled to an automatic closed process if the therapy was successful in early stages.

Cell therapies that cannot utilize third party cells present additional challenges. One issue is the inability to choose the donor can lead to a variable starting population of cells to be expanded. Furthermore, this kind of therapy is a highly personalised medicine which is extremely expensive. For example Sipuleucel-T (Provenge), an individualised DC vaccine for prostate cancer, currently costs $93,000 for a course of three treatments (604). In addition, the shelf life of these therapies can be limited for Provenge it is only 18 h at 2-8°C (605). Furthermore, some potential therapies are ineffective after freezing and therefore need to be manufactured on site.

7.5. Where do iNKT cells fit into potential cellular therapies?

Existing cellular therapies in trials for GVHD consist of MSCs which are in ongoing stage 3 clinical trials and Treg which are in stage 1 clinical trials (206, 207, 602). Similarly to iNKT cells, Treg have been shown to be able to suppress GVHD while allowing the GVT effect to occur (206).

In addition to being able to maintain the anti-tumour effect while suppressing GVHD iNKT cells have the advantage of being specifically activated by glycolipids, and studies in mice have shown that specific glycolipids can influence the polarity of the immune response (271, 277, 278). Furthermore, this means that the iNKT cells are effectively antigen specific as they respond to the same glycolipids, whereas Treg are polyclonal and the precursor frequency of antigen specific cells would not be as high. Depending the on the relative potency of iNKT cells and Treg this may mean that less iNKT cells are required in order to mediate an effect.

Several protocols exist for the *in vitro* expansion of human iNKT cells (462, 513, 543, 544, 606-611). These protocols rely on combinations of αGal pulsed APCs, IL-2, IL-12 and anti-CD3 and some of these protocols vary only slightly. Furthermore, these cells can be cryopreserved and have been deemed safe in two stage 1 clinical trials (396, 462). Exp iNKT cells have been successfully expanded for a stage 1 clinical trial for advanced melanoma based on scaled up versions of two of the aforementioned methods (396, 606, 607). Another clinical trial used Exp iNKT cells for advanced and recurrent Non-Small Cell Lung carcinoma (462).

In Chapter 4, we saw Exp murine iNKT cells have a different phenotype than their FI counterparts. In addition, we observed the immergence of complex phenotype, with some Exp cells capable of making multiple cytokines, although we do not know the stability of this phenotype upon *in vivo* transfer. Several studies have investigated the phenotype of Exp human iNKT cells.

Two studies investigated the capacity of Exp iNKT cells to produce cytokines using IC staining and found that following stimulation with PMA and Ionomycin that the Exp iNKT cells were capable of producing IFN-γ and IL-4, with IFN-γ production predominating (396, 513). One of the studies also reported that IL-10 was produced by iNKT cells in only one patient out of eight after 4 h stimulation. However, a recent study found that Exp human $CD4^+$ iNKT cells produced predominantly IL-4 with lower frequencies in IFN-γ and IL-17 producing cells, these difference may be due to variations in the expansion protocols (514). Several other studies have stimulated Exp iNKT cells with αGal and measured cytokine levels being produced by enzyme-linked immunosorbent assay (ELISA) after 24h or 48h incubation and generally found intermediate levels of IFN-γ, low levels of IL-4 and high levels of IL-13. (396, 462,

513, 544). Additionally, one study measured IL-10 production which was produced at a low level in the supernatants after 24 h (544). The stability of the phenotype of Exp cells upon administration has not been investigated although cytokine production by PBMCs has been shown to be IFN-γ dominant (396).

We observed that Exp murine iNKT cells produce IL-10 (Figure 4.5, 4.6), while production of IL-10 in Exp human iNKT cells appears to be variable. While IL-10 might be useful in the suppression of GVHD, it would not be preferable in terms of the anti-tumour response. However, if IL-10 or indeed another cytokine was found to be detrimental in the case of either GVHD or the GVT effect, gene editing techniques such as Clustered Regularly Interspaced Short Palindromic Repeats (Crispr)/CRISPR associated protein 9 (Cas9) or Transcription activator-like effector nucleases (TALENs) could be employed in order to knockout the expression of the implicated gene. Indeed these techniques have been shown to be feasible in primary isolated immune cells (612).

It should be noted, that in the clinical trial for advanced melanoma the Exp iNKT cells were autologous and hence coming from patients with advanced melanoma. iNKT cells isolated from prostate cancer patients have previously been observed to have diminished capacity to proliferate as well as to have a decreased ability to produce IFN-γ compared to healthy controls (396, 613). However, cytokines levels were comparable to those from healthy donors in the case of advanced melanoma (126).

Murine studies have indicated that third party iNKT cells can be used to suppress GVHD (126). Individuals have differences in the phenotype of peripheral blood iNKT cells even before expansion, for example there are differences in the cytokine

profiles of iNKT cells in both men and women as well as the profile of cytokines produced by bystander cells (610). Therefore, it is not surprising that the phenotype of human Exp iNKT cells seems to be variable in terms of cytokine production (396, 462, 513, 544). If third party iNKT cells could be utilized in humans as has been shown in mice this would be useful as it would allow some control of the starting material. Additionally, as discussed in the previous section, non-autologous cellular therapies are easier to scale up and are therefore likely to be more cost effective.

In addition to the safety and consistent phenotype of Exp cells, large number of iNKT cells would needed to be generated in order to make a potential therapy financially viable. In the clinical trial for advanced melanoma, iNKT cells were successfully expanded between to $1.1x10^7$-$12.26x10^9$ from a starting number of ~$1x10^6$ cells per patient (396). Therefore, human iNKT cells expand well and if it were possible to use third party iNKT cells it may be possible to scale up production further and constantly manufacture these cells.

Chimeric antigen receptor (CAR) or recombinant (r)iNKT cells would be an interesting area to explore for the treatment of GVHD. CAR and riNKT cells have been investigated in the context of cancers such as lymphoma, but to date their ability to suppress GVHD has not been investigated. As iNKT cells possess an endogenous TCR, they have been suggested to contribute to anti-tumour immunity directly as described previously, as well as through their CAR or rTCR. CAR-iNKT cells against B cell lymphomas have been shown to stably express CD19 CARs and to kill APCs expressing the relevant tumour antigen *in vitro* as well as exhibiting enhanced anti-tumour activity *in vivo* (614). Interestingly, CD19 CAR-iNKT cells have been shown to

be more effective at killing tumour cells than CD19 CAR-T cells *in vitro* and *in vivo* (615). Given this enhanced anti-tumour effect and the propensity of iNKT cells to suppress GVHD while maintaining the anti-tumour effect, investigating the propensity of CAR-iNKT cells to suppress GVHD while maintaining the GVT effect would be an interesting area of future research.

In conclusion, though a lot of additional research needs to be carried out before clinical translation, Exp iNKT cells present an attractive potential therapeutic for the treatment for GVHD as they have been shown to prevent GVHD while maintaining the GVT response in mice, they can be specifically activated and they are amenable to expansion.

7.6. Conclusions/Future work

The Holy Grail in the treatment of GVHD has long since been to supress GVHD without interfering with the GVT effect. Cellular therapy using Exp iNKT cells provides an attractive therapeutic prospect due to their ability to be activated with specific glycolipids and the fact that they are effectively antigen specific.

In this study we have set up a model of GVHD using transgenic T cells, in which the mechanism by which iNKT cells suppress GVHD could be investigated. We extensively phenotyped Exp iNKT cells and found the emergence of complex effector phenotypes and such cells were potent suppressors of alloreactive T cell responses *in vitro*. Experiments thus far have not found these cells capable of suppressing GVHD. However, further investigation is warranted in order to determine if these Exp

iNKT cells can suppress GVHD in this model and if so to investigate the mechanism by which this occurs.

Appendix I Mice

Breed	**Origin**	**Age at initiation of experiment**	**Allotype**	**Haplotype**
B6.SJL-Ptprca Pepcb/BoyJ (BoyJ)	Bred in house	8-12 weeks of age	CD45.1	b
CB6F1	Charles River	8-12 weeks of age	CD45.2	b/d
C57BL/6	Charles River	8-12 weeks of age	CD45.2	b
IFN-γ reporter (C57BL/6)	Bred in house	8-12 weeks of age	CD45.2	b
RORγT reporter mice (C57BL/6)	Bred in house	8-12 weeks of age	CD45.2	b
TEa (C57BL/6)	Bred in house (Kind gift from Dr. W. Gao and Prof. T. Strom (Harvard Medical School, Boston, MA)	8-12 weeks of age	CD45.1 or $CD45.1^{+}CD45.2^{+}$	b
T-bet knockout (C57BL/6)	Bred in house	8-12 weeks of age	CD45.2	b

Clinical Scoring

Criteria	Grade 0	Grade 1	Grade 2
Posture	Normal	Slight hunching noted only at rest	Hunching only at rest
Activity	Normal	Slightly decreased	Mild to moderate decrease in activity
Fur texture	Normal	Slight Ruffling	Mild to moderate ruffling
Hair loss	None	Slight loss	Severe loss
Eye Integrity	Normal	Obvious pain	Eyes closed
Skin Integrity	Normal	Erythema	Erythema + Scaling of paws/tail
Diarrhoea	None	None	Anal staining

Appendix II Solutions

cRPMI

Penicillin-streptomycin 100 U ml-1 (Sigma Aldrich)

L-glutamine 2mM (Sigma Aldrich)

2-mercaptoethanol 5.5 µM

FCS 10% (vol/vol)

1X RPMI 1640 Sodium Bicarbonate without L-Glutamine (Sigma)

FACS buffer

Azide 0.02% (w/v) (Sigma Aldrich)

1X PBS (See PBS)

HBSS w/EDTA

HBSS 500 ml

EDTA 2mM

HBSS w/FCS

HBSS 500 ml

FCS 2% v/v

iNKT cell medium

non-essential amino acids 0.1 mM (Sigma Aldrich)

Penicillin-streptomycin 100 U ml-1

L-glutamine 2mM

Hepes 10 nM (Sigma Aldrich)

Sodium Pyruvate 1 nM (Sigma Aldrich)

2-mercaptoethanol 5.5 µM (Sigma Aldrich)

FCS 10% (vol/vol)

1X RPMI-1640 Medium M/L-Glutamine (Sigma Aldrich)

MACS buffer

1X PBS (See PBS)

EDTA 2 mM

FCS 2 %(v/v)

PBS (without calcium and magnesium)

1 PBS tablet (Life technologies/Fisher)

1L of ddH2O

Appendix III Cytometry antibodies/viability dyes

Surface marker	Fluorochrome	Clone	Antibody Subclass	Company	Stock Concentration	Working Concentration	Dilution
7-aminoactinomycin D (7-AAD)	PECy5	n/a	n/a	eBioscience	0.5 mg/ml	10 µg/ml	1/50
CD11b	APCy7	M1/70	Rat IgG2b, κ	BD Biosciences	0.2 mg/ml	0.5 µg/ml	1/400
CD11c	PECy7	HL3	Armenian Hamster IgG1, λ2	BD Biosciences	0.2 mg/ml	1 µg/ml	1/200
CD19	PE	6D5	Rat IgG2a, κ	Biolegend	0.2 mg/ml	1 µg/ml	1/200
CD1d tetramer (PBS57)	APC			NIH Tetramer Core Facility	1.5 mg/ml	3.75 µg/ml	1/400
CD1d tetramer (unloaded)	APC			NIH Tetramer Core Facility	1.5 mg/ml	3.75 µg/ml	1/400
CD1d-αGal complex	PE	L363	Mouse IgG2a,k	eBiosciences	0.2 mg/ml	2 µg/ml	1/100
CD3ε	PE/Cy7	145-2C11	Armenian Hamster IgG	eBioscience	0.2 mg/ml	1 µg/ml	1/200
CD4	PerCP5.5	RM4-5	Rat IgG2aκ	BD	0.2 mg/ml	0.5 µg/ml	1/400
CD4	FITC						
CD5	PECy7	53-7.3	Rat-IgG2a,k	Biolegend	0.5 mg/ml	2.5 µg/ml	1/200
CD8α	BV786	53-6.7	Rat-IgG2a,κ	BD	0.2 mg/ml	0.25 µg/ml	1/800
CD45.1	BV510	A20	IgG2a, κ	Biolegend	0.2 mg/ml	2 µg/ml	1/100
CD45.1	FITC	A20	Mouse (A.SW) IgG2a, k	Biolegend	0.5 mg/ml	1.25 µg/ml	1/400

CD45.2	APCy7	104	Mouse IgG2a, k	eBiosciences	0.5 mg/mL	2.5 µg/ml	1/200
CD45-bio	n/a	30-F11	Rat LOU IgG2b, k	BD Biosciences	0.5 mg/ml	2.5 µg/ml	1/200
CD25	BV605	PC61	Rat IgGa, ramda	Biolegend	0.2 mg/ml	1 µg/ml	1/200
DAPI	BV421	n/a	n/a	Sigma Aldrich	0.1 mg/ml	1 µg/ml	1/100
Gr1	APC	RB6-8C5	Rat IgG2b, κ	eBioscience	0.2 mg/ml	0.5 µg/ml	1/400
H2 (IA-IE)	V500	M5/114 15.2	Rat IgG2b, κ	BD Biosciences	0.2 mg/ml	1 µg/ml	1/200
LAG-3 (CD223)	BV785	C9B7W	Rat IgG1, κ	Biolegend	0.2 mg/ml	1 µg/ml	1/200
Ly6C	BV421	AL-21	Rat IgM, κ	BD Bioscience	0.2 mg/ml	1 µg/ml	1/200
Ly6G	PerCp5.5	1A8	Rat IgG2a, κ	Biolegend	0.2 mg/ml	0.5 µg/ml	1/400
MHC class II	V500						1/200
NK1.1	BV605	PK136	Mouse IgG2a,κ	BD	0.1 mg/ml	1 µg/ml	1/100
PD-1 (CD279)	BV421	29F.1A12	Rat IgG2a, k	Biolegend	0.2 mg/ml	1 µg/ml	1/200
Streptavidin	APC-eFluor780	n/a	n/a	eBioscience	0.2 mg/mL	1 µg/ml	1/200
Streptavidin	BV605	n/a	n/a	Biolegend	0.1 mg/ml	.25 µg/ml	1/400
TIGIT	PE	1G9	Mouse IgG1, κ	Biolegend	0.2 mg/mL	1 µg/ml	1/200
TRC-β	BV510	H57-597	Armenian Hamster IgG	Biolegend	0.1 mg/ml	0.5 µg/ml	1/200
TCR-β	PE						
Vα2-biotin	n/a	B20.1	Rat IgG2a	eBioscience	0.5 mg/ml	1.25 µg/ml	1/400
TCR-Vα2	Pacific blue	B20.1	Rat IgG2a, ramda	Biolegend	0.5 mg/ml	5 µg/ml	1/100
TCR-Vα2	PE-Cy7	B20.1	Rat-IgG2a	eBioscience	0.2 mg/ml	0.25 µg/ml	1/800
Zombie-dye	PE-texas red	n/a	n/a	Biolegend	n/a	n/a	1/1000

Intracellular antibodies

Intracellular marker	Fluorochrome	Clone	Antibody subclass	Company	Stock Concentration	Working concentration	Dilution
E4BP4	PE	S2M-E19	Rat IgG2a, κ	eBioscience	0.2 mg/ml	4 µg/ml	1/50
IFN-γ	PE	XMG1.2	Rat IgG1, κ	eBioscience	0.2 mg/ml	2 µg/ml	1/100
IL-4	Alexa488	11B11	Rat-IgG1k	eBioscience	0.5 mg/ml	4 µg/ml	1/50
IL-10	PE/Cy7	JES5-16E3	Rat-IgG2b,κ	Biolegend	0.2 mg/ml	4 µg/ml	1/50
IL-13	PE	eBio13A	Rat IgG1, k	eBioscience	0.2 mg/ml	4 µg/ml	1/50
IL-17	FITC	eBio17B7	Rat-IgG2aκ	eBioscience	0.5 mg/ml	10 µg/ml	1/50
IL-17	BV711	TC11-18H10.1	Rat IgG1, k	Biolegend	0.2 mg/ml	4 µg/ml	1/50
FOXP3	APC	FJK-16s	Rat-IgG2a,k	eBioscience	0.2 mg/ml	4 µg/ml	1/50
RORγT	PE	B2D	Rat IgG1k	eBioscience	0.2 mg/ml	4 µg/ml	1/50
T-bet	PerCP5.5	4B10	Mouse IgG1,k	Biolegend	0.2 mg/ml	4 µg/ml	1/50

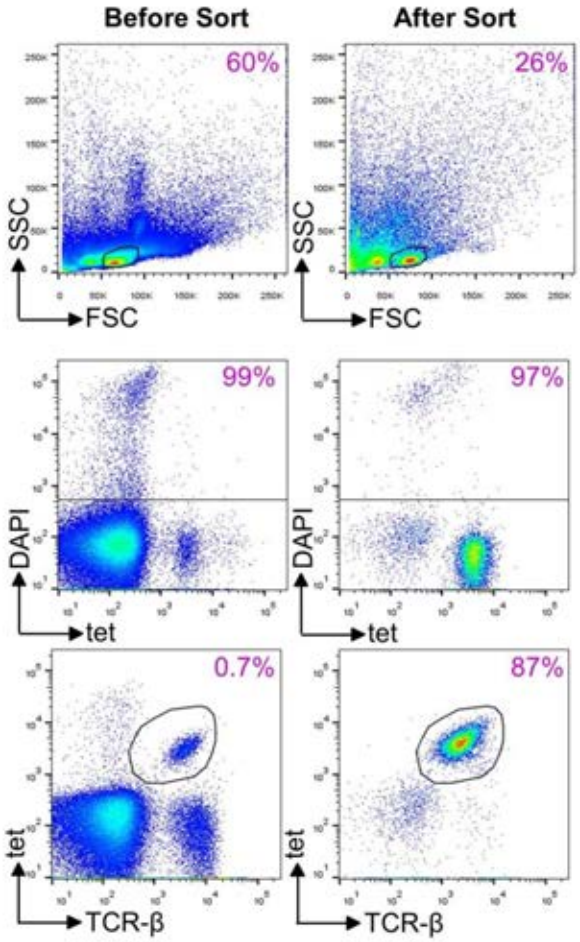
Isotype controls

Isotype	Fluorochrome	Clone	Antibody Subclass	Company	Stock Concentration	Working Concentration	Dilution
Golden Syrian hamster IgG	PerCP	n/a	Golden Syrian hamster IgG	eBioscience	0.2 mg/mL	10 µg/ml	1/50
Rat IgG 2b	PE/Cy7	RTK2071	Rat IgG1, κ	Biolegend	0.2 mg/ml	10 µg/ml	1/50
RatIgG1	PE	eBRG1	Rat IgG1, κ	eBioscience	0.2 mg/ml	2 µg/ml (IFN-γ), 4 µg/ml (RORγT)	1/100 (IFN-γ) 1/50 (RORγT)
RatIgG1, k	BV711	RTK2071	Isotype Control RatIgG1, k	Biolegend	0.2 mg/ml	10 µg/ml	1/50
RatIgG1κ488	FITC	eBRG1	Rat IgG1, κ	Biolegend	0.5 mg/ml	10 µg/ml	1/50

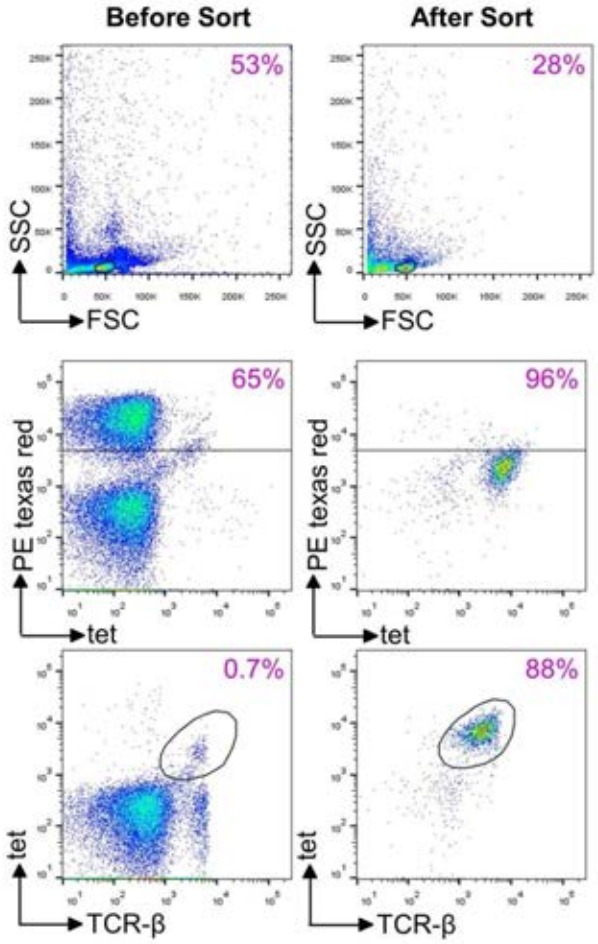
Appendix IV Confocal microscopy

Surface marker	Fluorochrome	Clone	Antibody Subclass	Company	Stock Concentration	Working Concentration	Dilution
CD45.1	FITC	A20	Mouse (A.SW) IgG2a, k	Biolegend	0.5 mg/ml	5 µg/ml	1/100
Gr-1	eFluor660	RB6-8C5	Rat IgG2b, κ	eBioscience	0.2 mg/mL	0.4 µg/ml	1/500

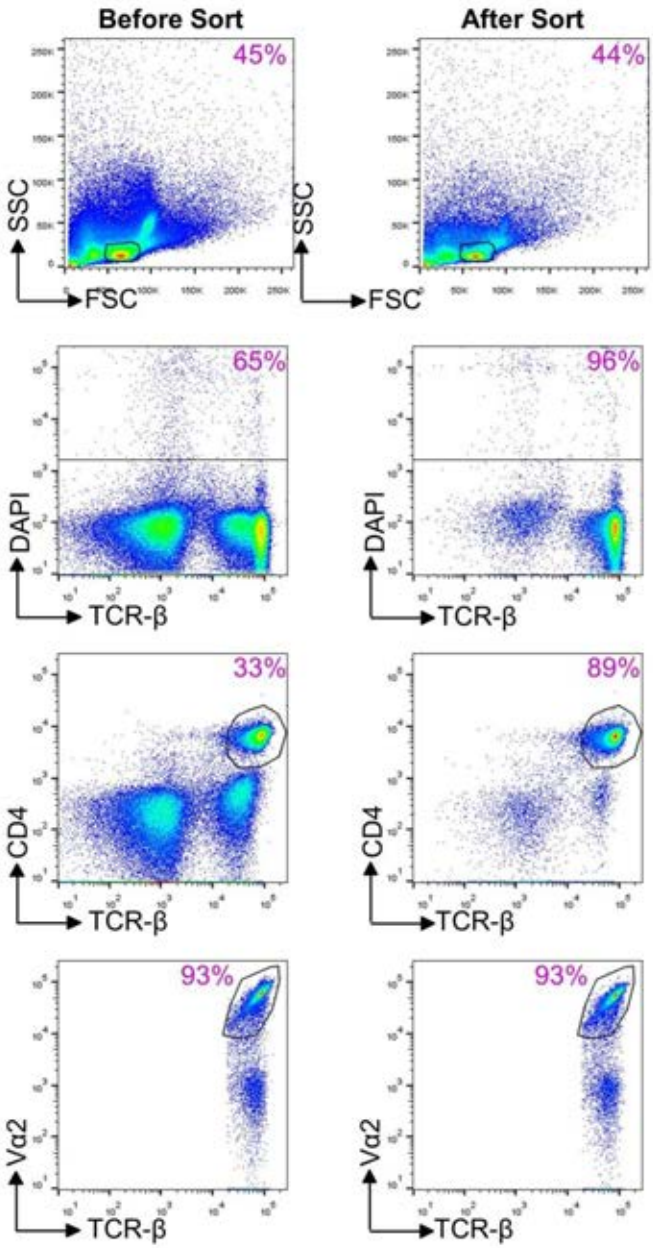
Appendix V Purity checks and gating strategies



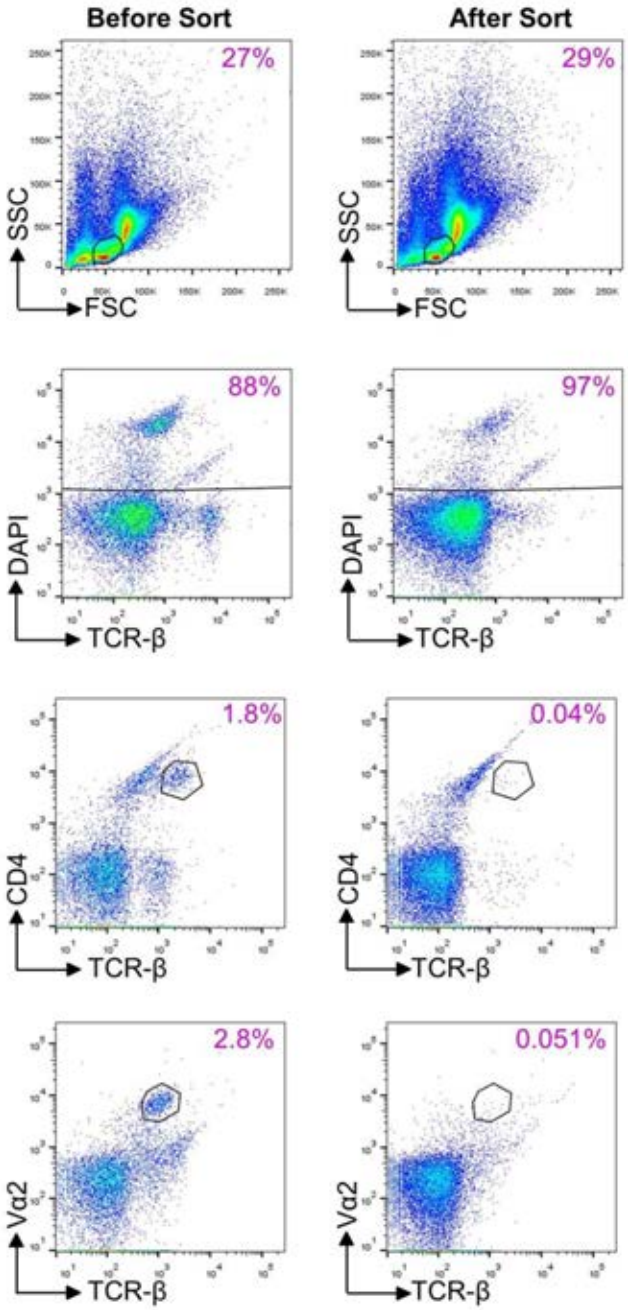
Purity check iNKT cells post bead sort. Mean frequency post sort 85%±11%. n=43.



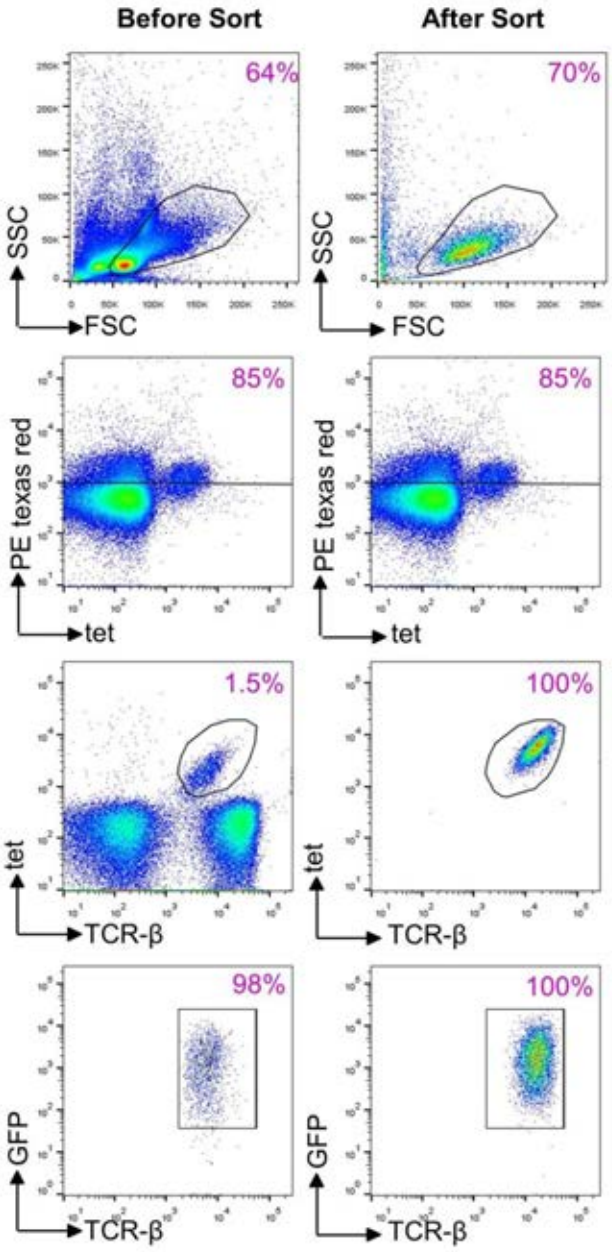
Purity check iNKT cells post bead and sorter sort. Mean frequency post sort 85%±0%. n=5.



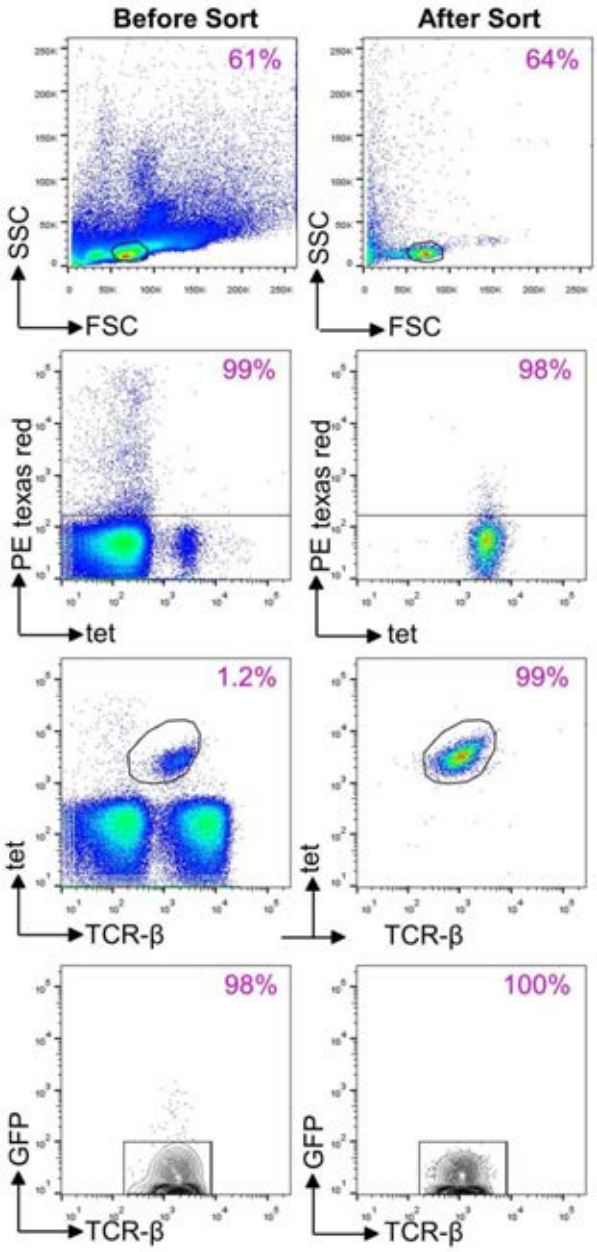
Purity check TEa T cells. Mean frequency post sort 87%±0.08%. n=20.



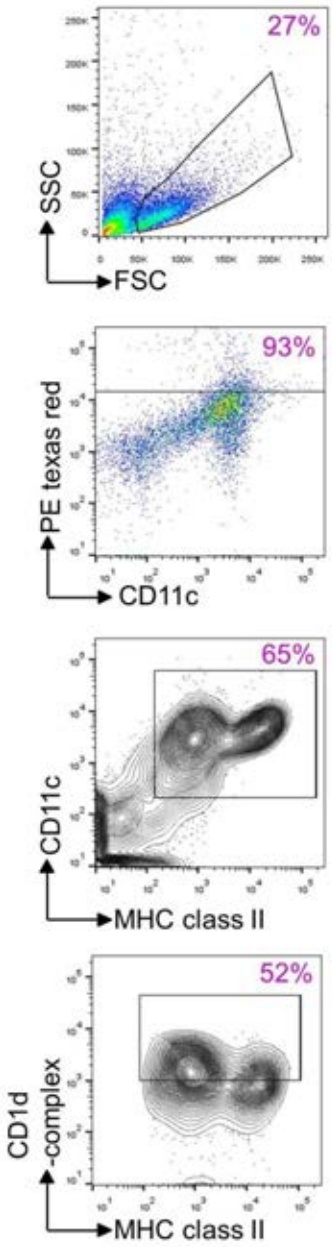
Purity check T cell depletion of BM. Mean frequency post sort of contaminating $CD4^+$ T cells 0.4±0.7%. $CD8^+$ T cells 0.2±0.1%. n=15.



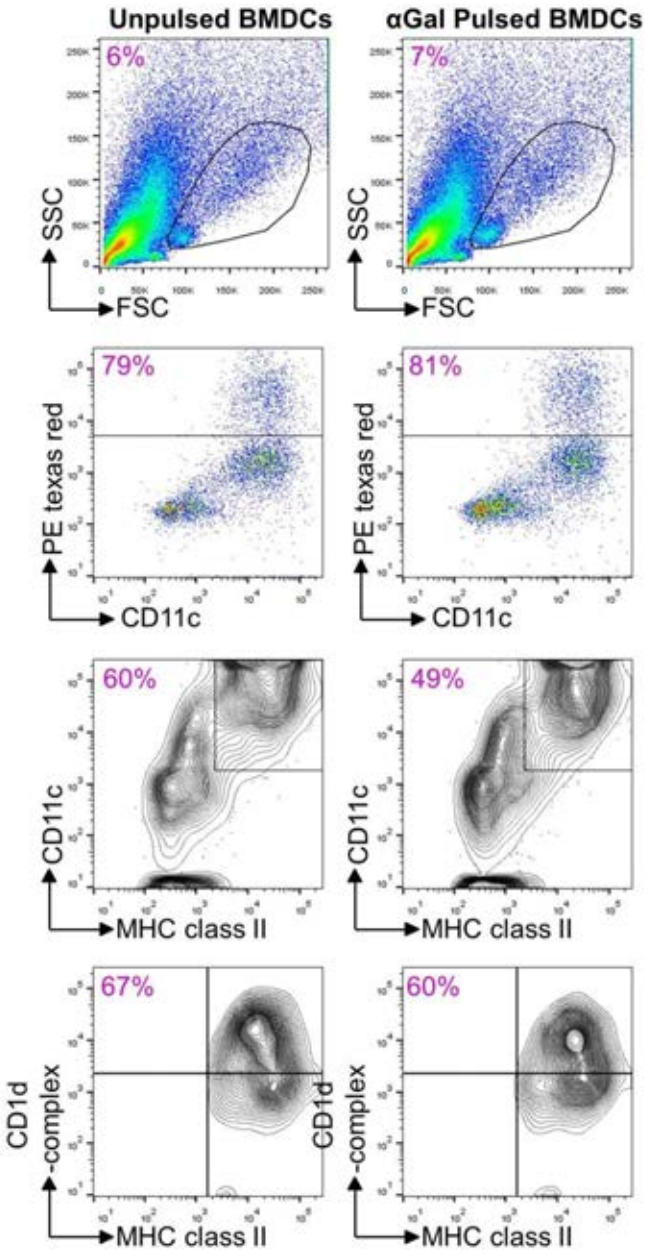
Purity check IFN-γ^{+} iNKT cells post bead and sorter sort. Mean frequency post sort 94%±6%. n=5.



Purity check RORγT^{-} iNKT cells post bead and sorter sort. Mean frequency post sort 87%±12%. n=3.

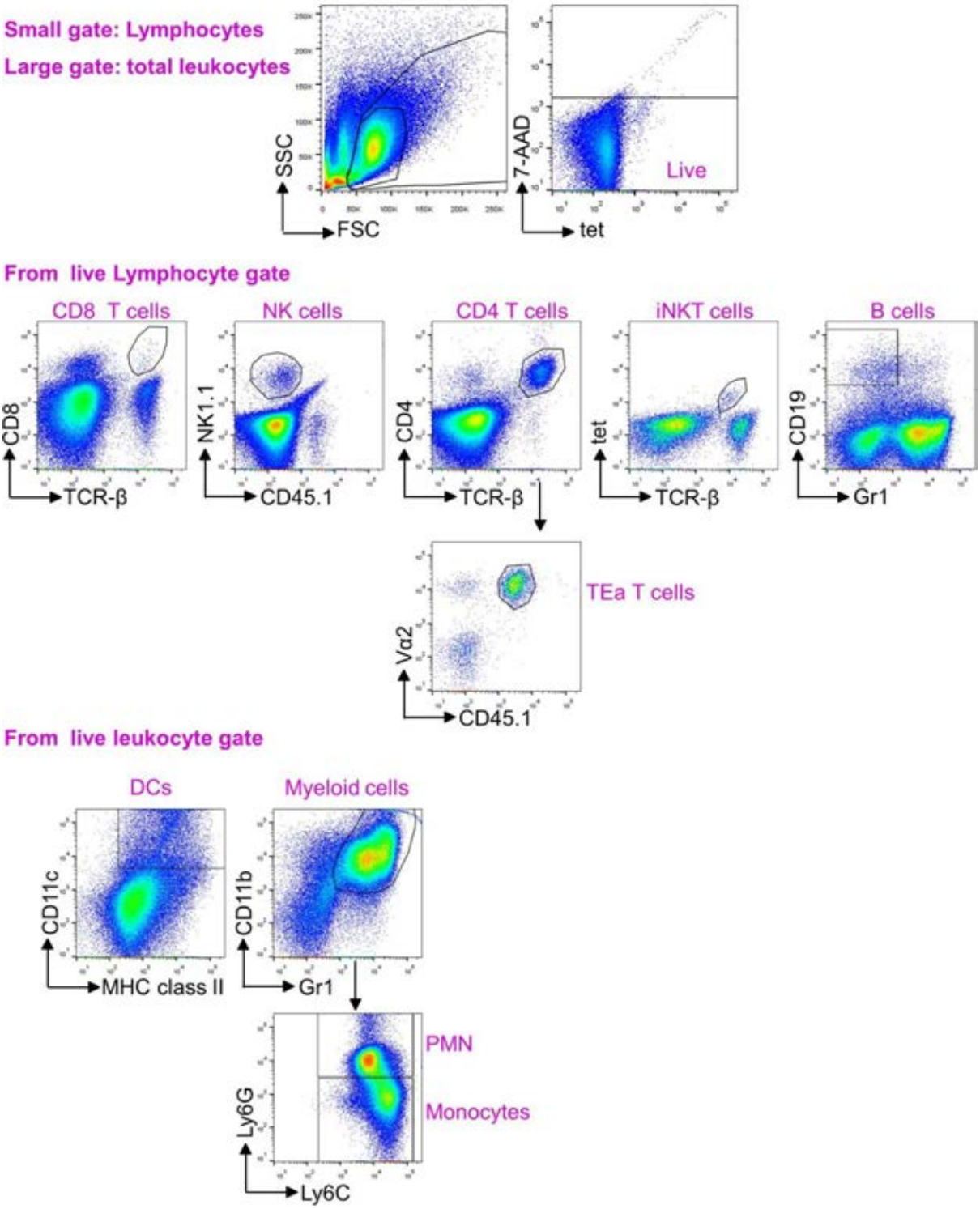


Purity check B6 BMDCs 53%±10%. n=5.

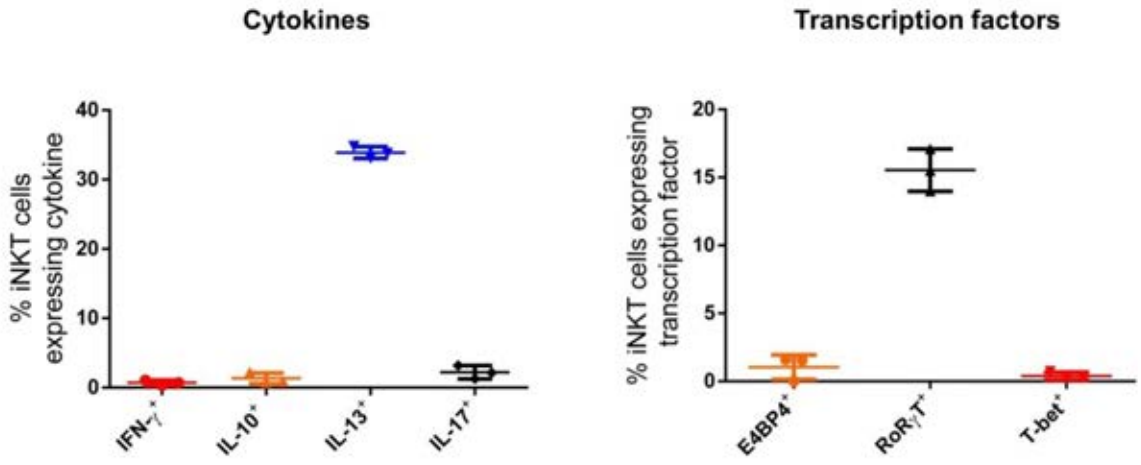


Purity check CB6F1 BMDCs 38%±16%. n=5.

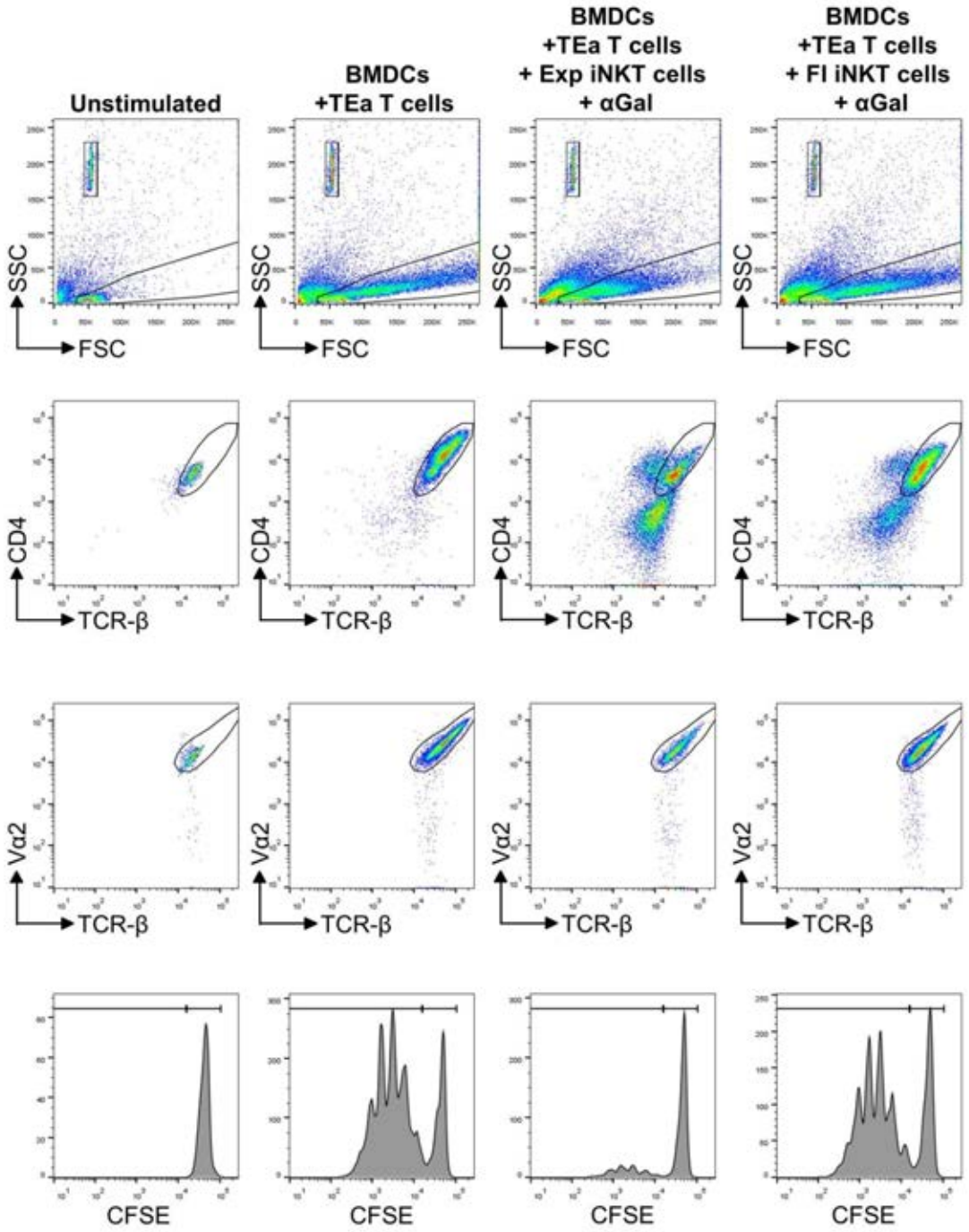
Bone marrow transplant and GVHD mice basic gating strategy



T-bet$^{-/-}$ mice phenotype of iNKT cells



Suppression assay sample gating strategy



References

1. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annual review of immunology. 2002;20:197-216.
2. Walsh KP, Mills KH. Dendritic cells and other innate determinants of T helper cell polarisation. Trends Immunol. 2013;34:521-30.
3. Creagh EM, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol. 2006;27:352-7.
4. Murphy K, Travers P, Walport M, Janeway C. Janeway's immunobiology. 8th ed. New York: Garland Science; 2012.
5. Forthal DN. Functions of Antibodies. Microbiology spectrum. 2014;2(4):1-17.
6. Andersen MH, Schrama D, thor Straten P, Becker JC. Cytotoxic T Cells. Journal of Investigative Dermatology. 2006;126(1):32-41.
7. Zhu J, Paul WE. Heterogeneity and plasticity of T helper cells. Cell research. 2010;20(1):4-12.
8. Radosavljevic M, Cuillerier B, Wilson MJ, Clement O, Wicker S, Gilfillan S, et al. A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. Genomics. 2002;79:114-23.
9. Complete sequence and gene map of a human major histocompatibility complex. The MHC sequencing consortium. Nature. 1999;401(6756):921-3.
10. Marsh SG. Nomenclature for factors of the HLA system, update December 2010. Tissue antigens. 2011;77(4):362-3.
11. Howard CA, Fernandez-Vina MA, Appelbaum FR, Confer DL, Devine SM, Horowitz MM, et al. Recommendations for donor human leukocyte antigen assessment and matching for allogeneic stem cell transplantation: consensus opinion of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN). Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2015;21(1):4-7.
12. Dzierzak-Mietla M, Markiewicz M, Siekiera U, Mizia S, Koclega A, Zielinska P, et al. Occurrence and Impact of Minor Histocompatibility Antigens' Disparities on Outcomes of Hematopoietic Stem Cell Transplantation from HLA-Matched Sibling Donors. Bone marrow research. 2012;2012:257086.
13. Major Histocompatibility Complex (MHC): Mouse. eLS.
14. IMGT®, the international ImMunoGeneTics information system® http://www.imgt.org [Internet].
15. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nature Reviews Immunology. 2012;12:557.
16. King DP, Jones PP. Induction of Ia and H-2 antigens on a macrophage cell line by immune interferon. Journal of immunology (Baltimore, Md : 1950). 1983;131(1):315-8.
17. Gonalons E, Barrachina M, Garcia-Sanz JA, Celada A. Translational control of MHC class II I-A molecules by IFN-gamma. Journal of immunology (Baltimore, Md : 1950). 1998;161(4):1837-43.
18. Fowlkes BJ, Edison L, Mathieson BJ, Chused TM. Early T lymphocytes. Differentiation in vivo of adult intrathymic precursor cells. The Journal of experimental medicine. 1985;162(3):802-22.
19. Naquet P, MacDonald HR, Brekelmans P, Barbet J, Marchetto S, Van Ewijk W, et al. A novel T cell-activating molecule (THAM) highly expressed on CD4-CD8- murine thymocytes. Journal of immunology (Baltimore, Md : 1950). 1988;141(12):4101-9.
20. Wilson A, Day LM, Scollay R, Shortman K. Subpopulations of mature murine thymocytes: properties of CD4-CD8+ and CD4+CD8- thymocytes lacking the heat-stable antigen. Cellular immunology. 1988;117(2):312-26.
21. Godfrey DI, Kennedy J, Suda T, Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. Journal of immunology (Baltimore, Md : 1950). 1993;150(10):4244-52.

22. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science (New York, NY). 1990;248(4962):1517-23.
23. Ma Y, Pannicke U, Schwarz K, Lieber MR. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell. 2002;108(6):781-94.
24. Dudley EC, Petrie HT, Shah LM, Owen MJ, Hayday AC. T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. Immunity. 1994;1(2):83-93.
25. Capone M, Hockett RD, Zlotnik A. Kinetics of T Cell Receptor β , γ , and δ Rearrangements during Adult Thymic Development: T Cell Receptor Rearrangements are Present in CD44⁺CD25⁺ Pro-T Thymocytes. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(21):12522-7.
26. Born W, Yagüe J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor beta-chain genes during T-cell development. Proceedings of the National Academy of Sciences. 1985;82(9):2925-9.
27. Okada CY, Weissman IL. Relative V beta transcript levels in thymus and peripheral lymphoid tissues from various mouse strains. Inverse correlation of I-E and Mls expression with relative abundance of several V beta transcripts in peripheral lymphoid tissues. The Journal of experimental medicine. 1989;169(5):1703-19.
28. Groettrup M, Baron A, Griffiths G, Palacios R, von Boehmer H. T cell receptor (TCR) beta chain homodimers on the surface of immature but not mature alpha, gamma, delta chain deficient T cell lines. The EMBO journal. 1992;11(7):2735-45.
29. Groettrup M, Ungewiss K, Azogui O, Palacios R, Owen MJ, Hayday AC, et al. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. Cell. 1993;75(2):283-94.
30. Aifantis I, Buer J, von Boehmer H, Azogui O. Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. Immunity. 1997;7(5):601-7.
31. Casanova JL, Romero P, Widmann C, Kourilsky P, Maryanski JL. T cell receptor genes in a series of class I major histocompatibility complex-restricted cytotoxic T lymphocyte clones specific for a Plasmodium berghei nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. The Journal of experimental medicine. 1991;174(6):1371-83.
32. Thompson SD, Pelkonen J, Rytkonen M, Samaridis J, Hurwitz JL. Nonrandom rearrangement of T cell receptor J alpha genes in bone marrow T cell differentiation cultures. Journal of immunology (Baltimore, Md : 1950). 1990;144(7):2829-34.
33. Thompson SD, Pelkonen J, Hurwitz JL. First T cell receptor alpha gene rearrangements during T cell ontogeny skew to the 5' region of the J alpha locus. The Journal of Immunology. 1990;145(7):2347-52.
34. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature. 1988;334(6181):395-402.
35. Nikolich-Žugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nature Reviews Immunology. 2004;4:123.
36. von Boehmer H, Teh HS, Kisielow P. The thymus selects the useful, neglects the useless and destroys the harmful. Immunology Today. 1989;10(2):57-61.
37. Robey E, Fowlkes BJ. Selective events in T cell development. Annual review of immunology. 1994;12:675-705.
38. Starr TK, Jameson SC, Hogquist KA. Positive and Negative Selection of T Cells. Annual review of immunology. 2003;21(1):139-76.
39. Perry JS, Hsieh CS. Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen. Immunol Rev. 2016;271(1):141-55.

40. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science (New York, NY). 2002;298(5597):1395-401.
41. Hinterberger M, Aichinger M, Prazeres da Costa O, Voehringer D, Hoffmann R, Klein L. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. Nature immunology. 2010;11(6):512-9.
42. Aschenbrenner K, D'Cruz LM, Vollmann EH, Hinterberger M, Emmerich J, Swee LK, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nature immunology. 2007;8(4):351-8.
43. Hogquist KA, Xing Y, Hsu FC, Shapiro VS. T Cell Adolescence: Maturation Events Beyond Positive Selection. Journal of immunology (Baltimore, Md : 1950). 2015;195(4):1351-7.
44. Parrish HL, Deshpande NR, Vasic J, Kuhns MS. Functional evidence for TCR-intrinsic specificity for MHCII. Proc Natl Acad Sci U S A. 2016;113(11):3000-5.
45. Krovi SH, Gapin L. Revealing the TCR bias for MHC molecules. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(11):2809-11.
46. Fekety FR. The Clonal Selection Theory of Acquired Immunity. The Yale Journal of Biology and Medicine. 1960;32(6):480-.
47. Zinkernagel RM. Restriction by H-2 gene complex of transfer of cell-mediated immunity to Listeria monocytogenes. Nature. 1974;251(5472):230-3.
48. Madrenas J, Chau LA, Smith J, Bluestone JA, Germain RN. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. The Journal of experimental medicine. 1997;185(2):219-29.
49. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature reviews Immunology. 2013;13(4):227-42.
50. Cunningham AJ, Lafferty KJ. A simple conservative explanation of the H-2 restriction of interactions between lymphocytes. Scandinavian journal of immunology. 1977;6(1-2):1-6.
51. Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc Natl Acad Sci U S A. 1990;87(13):5031-5.
52. June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Molecular and cellular biology. 1987;7(12):4472-81.
53. Scott P. IL-12: initiation cytokine for cell-mediated immunity. Science (New York, NY). 1993;260(5107):496-7.
54. Cantrell D. T cell antigen receptor signal transduction pathways. Annual review of immunology. 1996;14:259-74.
55. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. The Journal of Immunology. 1982;129(5):2293-300.
56. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, et al. Identification of a putative second T-cell receptor. Nature. 1986;322(6075):145-9.
57. Rudd CE, Trevillyan JM, Dasgupta JD, Wong LL, Schlossman SF. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc Natl Acad Sci U S A. 1988;85(14):5190-4.
58. Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell. 1988;55(2):301-8.
59. Weiss A. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. Cell. 1993;73(2):209-12.
60. Birnbaum ME, Berry R, Hsiao Y-S, Chen Z, Shingu-Vazquez MA, Yu X, et al. Molecular architecture of the αβ T cell receptor–CD3 complex. Proceedings of the National Academy of Sciences. 2014;111(49):17576-81.

61. Sun ZJ, Kim KS, Wagner G, Reinherz EL. Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. Cell. 2001;105(7):913-23.
62. Arnett KL, Harrison SC, Wiley DC. Crystal structure of a human CD3-epsilon/delta dimer in complex with a UCHT1 single-chain antibody fragment. Proc Natl Acad Sci U S A. 2004;101(46):16268-73.
63. Kjer-Nielsen L, Dunstone MA, Kostenko L, Ely LK, Beddoe T, Mifsud NA, et al. Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. Proc Natl Acad Sci U S A. 2004;101(20):7675-80.
64. Call ME, Schnell JR, Xu C, Lutz RA, Chou JJ, Wucherpfennig KW. The Structure of the ζζ Transmembrane Dimer Reveals Features Essential for Its Assembly with the T Cell Receptor. Cell. 2006;127(2):355-68.
65. Berry R, Headey SJ, Call MJ, McCluskey J, Tregaskes CA, Kaufman J, et al. Structure of the Chicken CD3εδ/γ Heterodimer and Its Assembly with the αβT Cell Receptor. The Journal of Biological Chemistry. 2014;289(12):8240-51.
66. Altin JG, Sloan EK. The role of CD45 and CD45-associated molecules in T cell activation. Immunology and cell biology. 1997;75(5):430-45.
67. Gauen LK, Zhu Y, Letourneur F, Hu Q, Bolen JB, Matis LA, et al. Interactions of p59fyn and ZAP-70 with T-cell receptor activation motifs: defining the nature of a signalling motif. Molecular and cellular biology. 1994;14(6):3729-41.
68. Thome M, Duplay P, Guttinger M, Acuto O. Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/zeta complex. The Journal of experimental medicine. 1995;181(6):1997-2006.
69. Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: The ZAP-70 Tyrosine Kinase Substrate that Links T Cell Receptor to Cellular Activation. Cell. 1998;92(1):83-92.
70. Bubeck Wardenburg J, Fu C, Jackman JK, Flotow H, Wilkinson SE, Williams DH, et al. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J Biol Chem. 1996;271(33):19641-4.
71. Braiman A, Barda-Saad M, Sommers CL, Samelson LE. Recruitment and activation of PLCgamma1 in T cells: a new insight into old domains. The EMBO journal. 2006;25(4):774-84.
72. Stoica B, DeBell KE, Graham L, Rellahan BL, Alava MA, Laborda J, et al. The Amino-Terminal Src Homology 2 Domain of Phospholipase Cγ1 Is Essential for TCR-Induced Tyrosine Phosphorylation of Phospholipase Cγ1. The Journal of Immunology. 1998;160(3):1059-66.
73. Yablonski D, Kadlecek T, Weiss A. Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. Molecular and cellular biology. 2001;21(13):4208-18.
74. Clements JL, Boerth NJ, Lee JR, Koretzky GA. Integration of T cell receptor-dependent signaling pathways by adapter proteins. Annual review of immunology. 1999;17:89-108.
75. Huse M. The T-cell-receptor signaling network. Journal of cell science. 2009;122(Pt 9):1269-73.
76. Scollay R, Smith J, Stauffer V. Dynamics of Early T Cells: Prothymocyte Migration and Proliferation in the Adult Mouse Thymus. Immunological Reviews. 1986;91(1):129-58.
77. Abraham RT, Weiss A. Jurkat T cells and development of the T-cell receptor signalling paradigm. Nature Reviews Immunology. 2004;4:301.
78. Janeway CA, Jr. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. Annual review of immunology. 1992;10:645-74.
79. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986. Journal of immunology (Baltimore, Md : 1950). 2005;175(1):5-14.
80. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature. 1996;383(6603):787-93.

81. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nature reviews Immunology. 2002;2(12):933-44.
82. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology. 1989;7:145-73.
83. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. The Journal of experimental medicine. 2005;201(2):233-40.
84. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem. 2003;278(3):1910-4.
85. Hirota K, Duarte JH, Veldhoen M, Hornsby E, Li Y, Cua DJ, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. Nature immunology. 2011;12(3):255-63.
86. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, et al. Late developmental plasticity in the T helper 17 lineage. Immunity. 2009;30(1):92-107.
87. Cheng LE, Öhlén C, Nelson BH, Greenberg PD. Enhanced signaling through the IL-2 receptor in CD8(+) T cells regulated by antigen recognition results in preferential proliferation and expansion of responding CD8(+) T cells rather than promotion of cell death. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(5):3001-6.
88. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. Annual review of immunology. 2002;20:323-70.
89. Antigen presentation by chemically modified splenocytes induces antigen- specific T cell unresponsiveness in vitro and in vivo. The Journal of experimental medicine. 1987;165(2):302-19.
90. Deeths MJ, Kedl RM, Mescher MF. CD8+ T cells become nonresponsive (anergic) following activation in the presence of costimulation. Journal of immunology (Baltimore, Md : 1950). 1999;163(1):102-10.
91. Jameson SC, Bevan MJ. T cell receptor antagonists and partial agonists. Immunity. 1995;2(1):1-11.
92. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. Nature. 1993;363(6425):156-9.
93. Ryan KR, Evavold BD. Persistence of peptide-induced CD4+ T cell anergy in vitro. The Journal of experimental medicine. 1998;187(1):89-96.
94. Jeon M-S, Atfield A, Venuprasad K, Krawczyk C, Sarao R, Elly C, et al. Essential Role of the E3 Ubiquitin Ligase Cbl-b in T Cell Anergy Induction. Immunity. 2004;21(2):167-77.
95. Schartner JM, Simonson WT, Wernimont SA, Nettenstrom LM, Huttenlocher A, Seroogy CM. Gene related to anergy in lymphocytes (GRAIL) expression in CD4+ T cells impairs actin cytoskeletal organization during T cell/antigen-presenting cell interactions. J Biol Chem. 2009;284(50):34674-81.
96. Critchfield J, Racke M, Zuniga-Pflucker J, Cannella B, Raine C, Goverman J, et al. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. Science (New York, NY). 1994;263(5150):1139-43.
97. Chen Y, Inobe J, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature. 1995;376(6536):177-80.
98. Liblau RS, Tisch R, Shokat K, Yang X, Dumont N, Goodnow CC, et al. Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. Proc Natl Acad Sci U S A. 1996;93(7):3031-6.
99. Lenardo MJ. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature. 1991;353(6347):858-61.
100. Van Parijs L, Ibraghimov A, Abbas AK. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. Immunity. 1996;4(3):321-8.
101. Takeuchi M, Kosiewicz MM, Alard P, Streilein JW. On the mechanisms by which transforming growth factor-β2 alters antigen-presenting abilities of macrophages on T cell activation. European journal of immunology. 1997;27(7):1648-56.

102. Gershon RK, Cohen P, Hencin R, Liebhaber SA. Suppressor T cells. Journal of immunology (Baltimore, Md : 1950). 1972;108(3):586-90.
103. Janeway CA, Jr. Do suppressor T cells exist? A reply. Scandinavian journal of immunology. 1988;27(6):621-3.
104. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. The Journal of Immunology. 1995;155(3):1151-64.
105. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. The Journal of experimental medicine. 1996;184(2):387-96.
106. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nature immunology. 2003;4(4):330-6.
107. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science (New York, NY). 2003;299(5609):1057-61.
108. Godfrey VL, Wilkinson JE, Rinchik EM, Russell LB. Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. Proc Natl Acad Sci U S A. 1991;88(13):5528-32.
109. Godfrey VL, Wilkinson JE, Russell LB. X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. The American journal of pathology. 1991;138(6):1379-87.
110. van der Vliet HJJ, Nieuwenhuis EE. IPEX as a Result of Mutations in FOXP3. Clinical and Developmental Immunology. 2007;2007:89017.
111. Moraes-Vasconcelos D, Costa-Carvalho BT, Torgerson TR, Ochs HD. Primary immune deficiency disorders presenting as autoimmune diseases: IPEX and APECED. Journal of clinical immunology. 2008;28 Suppl 1:S11-9.
112. Lin X, Chen M, Liu Y, Guo Z, He X, Brand D, et al. Advances in distinguishing natural from induced Foxp3(+) regulatory T cells. International Journal of Clinical and Experimental Pathology. 2013;6(2):116-23.
113. Shevach EM, Thornton AM. tTregs, pTregs, and iTregs: Similarities and Differences. Immunological reviews. 2014;259(1):88-102.
114. Cowan JE, Parnell SM, Nakamura K, Caamano JH, Lane PJL, Jenkinson EJ, et al. The thymic medulla is required for Foxp3(+) regulatory but not conventional CD4(+) thymocyte development. The Journal of experimental medicine. 2013;210(4):675-81.
115. Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. Journal of immunology (Baltimore, Md : 1950). 2004;172(9):5213-21.
116. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. The Journal of experimental medicine. 2003;198(12):1875-86.
117. Fu S, Zhang N, Yopp AC, Chen D, Mao M, Chen D, et al. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2004;4(10):1614-27.
118. Mills KH. Regulatory T cells: friend or foe in immunity to infection? Nature reviews Immunology. 2004;4(11):841-55.
119. Garba ML, Pilcher CD, Bingham AL, Eron J, Frelinger JA. HIV Antigens Can Induce TGF-β₁-Producing Immunoregulatory CD8⁺ T Cells. The Journal of Immunology. 2002;168(5):2247-54.
120. Haynes LM, Vanderlugt CL, Dal Canto MC, Melvold RW, Miller SD. CD8⁺ T cells from Theiler’s virus-resistant BALB/cByJ mice downregulate pathogenic virus-specific CD4⁺ T cells. Journal of Neuroimmunology. 2000;106(1):43-52.

121. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nature reviews Immunology. 2009;9(3):162-74.
122. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest. 2007;117(5):1155-66.
123. Fujimura T, Mahnke K, Enk AH. Myeloid derived suppressor cells and their role in tolerance induction in cancer. Journal of dermatological science. 2010;59(1):1-6.
124. Wu T, Sun C, Chen Z, Zhen Y, Peng J, Qi Z, et al. Smad3-deficient CD11b(+)Gr1(+) myeloid-derived suppressor cells prevent allograft rejection via the nitric oxide pathway. Journal of immunology (Baltimore, Md : 1950). 2012;189(10):4989-5000.
125. Nakamura T, Nakao T, Ashihara E, Yoshimura N. Myeloid-derived Suppressor Cells Recruit CD4(+)/Foxp3(+) Regulatory T Cells in a Murine Cardiac Allograft. Transplantation proceedings. 2016;48(4):1275-8.
126. Schneidawind D, Baker J, Pierini A, Buechele C, Luong RH, Meyer EH, et al. Third-party CD4+ invariant natural killer T cells protect from murine GVHD lethality2015 2015-05-28 00:00:00. 3491-500 p.
127. Arora M, Weisdorf DJ, Spellman SR, Haagenson MD, Klein JP, Hurley CK, et al. HLA-identical sibling compared with 8/8 matched and mismatched unrelated donor bone marrow transplant for chronic phase chronic myeloid leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27(10):1644-52.
128. Rogers NJ, Lechler RI. Allorecognition. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2001;1(2):97-102.
129. Chakraverty R, Sykes M. The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia. Blood. 2007;110(1):9-17.
130. Wang X, Li H, Matte-Martone C, Cui W, Li N, Tan HS, et al. Mechanisms of antigen presentation to T cells in murine graft-versus-host disease: cross-presentation and the appearance of cross-presentation. Blood. 2011;118(24):6426-37.
131. Markey KA, Koyama M, Gartlan KH, Leveque L, Kuns RD, Lineburg KE, et al. Cross-dressing by donor dendritic cells after allogeneic bone marrow transplantation contributes to formation of the immunological synapse and maximizes responses to indirectly presented antigen. Journal of immunology (Baltimore, Md : 1950). 2014;192(11):5426-33.
132. Game DS, Lechler RI. Pathways of allorecognition: implications for transplantation tolerance. Transplant immunology. 2002;10(2-3):101-8.
133. Lombardi G, Sidhu S, Daly M, Batchelor JR, Makgoba W, Lechler RI. Are primary alloresponses truly primary? International immunology. 1990;2(1):9-13.
134. Merkenschlager M, Ikeda H, Wilkinson D, Beverly PC, Trowsdale J, Fisher AG, et al. Allorecognition of HLA-DR and -DQ transfectants by human CD45RA and CD45R0 CD4 T cells: repertoire analysis and activation requirements. European journal of immunology. 1991;21(1):79-88.
135. Veerapathran A, Pidala J, Beato F, Yu XZ, Anasetti C. Ex vivo expansion of human Tregs specific for alloantigens presented directly or indirectly. Blood. 2011;118(20):5671-80.
136. Ashwell JD, Chen C, Schwartz RH. High frequency and nonrandom distribution of alloreactivity in T cell clones selected for recognition of foreign antigen in association with self class II molecules. Journal of immunology (Baltimore, Md : 1950). 1986;136(2):389-95.
137. Suchin EJ, Langmuir PB, Palmer E, Sayegh MH, Wells AD, Turka LA. Quantifying the frequency of alloreactive T cells in vivo: new answers to an old question. Journal of immunology (Baltimore, Md : 1950). 2001;166(2):973-81.
138. Benichou G, Valujskikh A, Heeger PS. Contributions of direct and indirect T cell alloreactivity during allograft rejection in mice. Journal of immunology (Baltimore, Md : 1950). 1999;162(1):352-8.
139. Boardman DA, Jacob J, Smyth LA, Lombardi G, Lechler RI. What Is Direct Allorecognition? Current Transplantation Reports. 2016;3(4):275-83.

140. Colf LA, Bankovich AJ, Hanick NA, Bowerman NA, Jones LL, Kranz DM, et al. How a single T cell receptor recognizes both self and foreign MHC. Cell. 2007;129(1):135-46.
141. Thomas ED, Lochte HL, Jr., Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. J Clin Invest. 1959;38:1709-16.
142. Felfly H, Haddad GG. Hematopoietic stem cells: potential new applications for translational medicine. Journal of stem cells. 2014;9(3):163-97.
143. Park B, Yoo KH, Kim C. Hematopoietic stem cell expansion and generation: the ways to make a breakthrough. Blood research. 2015;50(4):194-203.
144. Mahla RS. Stem Cells Applications in Regenerative Medicine and Disease Therapeutics. International journal of cell biology. 2016;2016:6940283.
145. Marmont A, Horowitz M, Gale R, Sobocinski K, Ash R, van Bekkum D, et al. T-cell depletion of HLA-identical transplants in leukemia. Blood. 1991;78(8):2120-30.
146. Matte-Martone C, Liu J, Jain D, McNiff J, Shlomchik WD. CD8+ but not CD4+ T cells require cognate interactions with target tissues to mediate GVHD across only minor H antigens, whereas both CD4+ and CD8+ T cells require direct leukemic contact to mediate GVL. Blood. 2008;111(7):3884-92.
147. Bleakley M, Riddell SR. Molecules and mechanisms of the graft-versus-leukaemia effect. Nature Reviews Cancer. 2004;4:371.
148. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990;75(3):555-62.
149. Xun CQ, Thompson JS, Jennings CD, Brown SA, Widmer MB. Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice. Blood. 1994;83(8):2360-7.
150. Chang RJ, Lee SH. Effects of interferon-gamma and tumor necrosis factor-alpha on the expression of an Ia antigen on a murine macrophage cell line. Journal of immunology (Baltimore, Md : 1950). 1986;137(9):2853-6.
151. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science (New York, NY). 2001;293(5528):293-7.
152. Chen Y, Zhao Y, Cheng Q, Wu D, Liu H. The Role of Intestinal Microbiota in Acute Graft-versus-Host Disease. Journal of Immunology Research. 2015;2015:145859.
153. Cooke KR, Hill GR, Crawford JM, Bungard D, Brinson YS, Delmonte J, Jr., et al. Tumor necrosis factor- alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. J Clin Invest. 1998;102(10):1882-91.
154. Hill GR, Crawford JM, Cooke KR, Brinson YS, Pan L, Ferrara JL. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood. 1997;90(8):3204-13.
155. Clift RA, Buckner CD, Appelbaum FR, Bearman SI, Petersen FB, Fisher LD, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. Blood. 1990;76(9):1867-71.
156. Nash RA, Pepe MS, Storb R, Longton G, Pettinger M, Anasetti C, et al. Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. Blood. 1992;80(7):1838-45.
157. Teshima T, Ordemann R, Reddy P, Gagin S, Liu C, Cooke KR, et al. Acute graft-versus-host disease does not require alloantigen expression on host epithelium. Nature medicine. 2002;8(6):575-81.
158. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, et al. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science (New York, NY). 1999;285(5426):412-5.

159. Tivol E, Komorowski R, Drobyski WR. Emergent autoimmunity in graft-versus-host disease. Blood. 2005;105(12):4885-91.
160. Matzinger P. The danger model: a renewed sense of self. Science (New York, NY). 2002;296(5566):301-5.
161. Sallusto F, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. Arthritis research. 2002;4 Suppl 3:S127-32.
162. Murphy WJ, Blazar BR. New strategies for preventing graft-versus-host disease. Curr Opin Immunol. 1999;11(5):509-15.
163. Schroeder MA, DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. Dis Model Mech. 2011;4(3):318-33.
164. Goker H, Haznedaroglu IC, Chao NJ. Acute graft-vs-host disease: pathobiology and management. Experimental hematology. 2001;29(3):259-77.
165. Mowat AM. Antibodies to IFN-gamma prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. Immunology. 1989;68(1):18-23.
166. Dickinson AM, Sviland L, Dunn J, Carey P, Proctor SJ. Demonstration of direct involvement of cytokines in graft-versus-host reactions using an in vitro human skin explant model. Bone Marrow Transplant. 1991;7(3):209-16.
167. Nestel FP, Price KS, Seemayer TA, Lapp WS. Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. The Journal of experimental medicine. 1992;175(2):405-13.
168. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, Anasetti C, et al. Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORgammat in mice. Blood. 2011;118(18):5011-20.
169. Serody JS, Burkett SE, Panoskaltsis-Mortari A, Ng-Cashin J, McMahon E, Matsushima GK, et al. T-lymphocyte production of macrophage inflammatory protein-1alpha is critical to the recruitment of CD8(+) T cells to the liver, lung, and spleen during graft-versus-host disease. Blood. 2000;96(9):2973-80.
170. Murai M, Yoneyama H, Harada A, Yi Z, Vestergaard C, Guo B, et al. Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. J Clin Invest. 1999;104(1):49-57.
171. Serody JS, Cook DN, Kirby SL, Reap E, Shea TC, Frelinger JA. Murine T lymphocytes incapable of producing macrophage inhibitory protein-1 are impaired in causing graft-versus-host disease across a class I but not class II major histocompatibility complex barrier. Blood. 1999;93(1):43-50.
172. Hill GR, Ferrara JL. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. Blood. 2000;95(9):2754-9.
173. Graubert TA, DiPersio JF, Russell JH, Ley TJ. Perforin/granzyme-dependent and independent mechanisms are both important for the development of graft-versus-host disease after murine bone marrow transplantation. Journal of Clinical Investigation. 1997;100(4):904-11.
174. Graubert T, Russell J, Ley T. The role of granzyme B in murine models of acute graft-versus-host disease and graft rejection. Blood. 1996;87(4):1232-7.
175. van den Brink MR, Burakoff SJ. Cytolytic pathways in haematopoietic stem-cell transplantation. Nature reviews Immunology. 2002;2(4):273-81.
176. Hattori K, Hirano T, Miyajima H, Yamakawa N, Tateno M, Oshimi K, et al. Differential effects of anti-Fas ligand and anti-tumor necrosis factor alpha antibodies on acute graft-versus-host disease pathologies. Blood. 1998;91(11):4051-5.
177. Herve P, Flesch M, Tiberghien P, Wijdenes J, Racadot E, Bordigoni P, et al. Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acute graft-versus-host disease. Blood. 1992;79(12):3362-8.

178. Gilliam AC, Whitaker-Menezes D, Korngold R, Murphy GF. Apoptosis Is the Predominant Form of Epithelial Target Cell Injury in Acute Experimental Graft-Versus-Host Disease. Journal of Investigative Dermatology. 1996;107(3):377-83.
179. R. Cooke K, R. Hill G, Gerbitz A, Kobzik L, Martin T, Crawford J, et al. Tumor necrosis factor-alpha neutralization reduces lung injury after experimental allogeneic bone marrow transplantation2000. 272-9 p.
180. Abhyankar S, G Gilliland D, Ferrara J. Interleukin-1 is a critical effector molecule during cytokine dysregulation in graft versus host disease to minor histocompatibility antigens1994. 1518-23 p.
181. Hill GR, Teshima T, Gerbitz A, Pan L, Cooke KR, Brinson YS, et al. Differential roles of IL-1 and TNF-alpha on graft-versus-host disease and graft versus leukemia. J Clin Invest. 1999;104(4):459-67.
182. McCarthy PL, Jr., Abhyankar S, Neben S, Newman G, Sieff C, Thompson RC, et al. Inhibition of interleukin-1 by an interleukin-1 receptor antagonist prevents graft-versus-host disease. Blood. 1991;78(8):1915-8.
183. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. Blood. 2002;100(10):3479-82.
184. Jacobsohn DA, Vogelsang GB. Acute graft versus host disease. Orphanet J Rare Dis. 2007;2:35.
185. Martin PJ, McDonald GB, Sanders JE, Anasetti C, Appelbaum FR, Deeg HJ, et al. Increasingly frequent diagnosis of acute gastrointestinal graft-versus-host disease after allogeneic hematopoietic cell transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2004;10(5):320-7.
186. Akpek G, Boitnott JK, Lee LA, Hallick JP, Torbenson M, Jacobsohn DA, et al. Hepatitic variant of graft-versus-host disease after donor lymphocyte infusion. Blood. 2002;100(12):3903-7.
187. Team SC. Clinical Commissioning Policy: Treatments for Graft versus Host Disease (GvHD) following Haematopoietic Stem Cell Transplantation Reference: NHS England: 16069/P. In: Service NH, editor. Electronic format only.: NHS England; 31 March 2017.
188. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974;18(4):295-304.
189. Thomas E, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, et al. Bone-marrow transplantation (first of two parts). The New England journal of medicine. 1975;292(16):832-43.
190. Thomas ED, Storb R, Clift RA, Fefer A, Johnson L, Neiman PE, et al. Bone-marrow transplantation (second of two parts). The New England journal of medicine. 1975;292(17):895-902.
191. Hahn T, McCarthy PL, Jr., Zhang MJ, Wang D, Arora M, Frangoul H, et al. Risk factors for acute graft-versus-host disease after human leukocyte antigen-identical sibling transplants for adults with leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26(35):5728-34.
192. Gratwohl A, Hermans J, Apperley J, Arcese W, Bacigalupo A, Bandini G, et al. Acute graft-versus-host disease: grade and outcome in patients with chronic myelogenous leukemia. Working Party Chronic Leukemia of the European Group for Blood and Marrow Transplantation. Blood. 1995;86(2):813-8.
193. Cahn JY, Klein JP, Lee SJ, Milpied N, Blaise D, Antin JH, et al. Prospective evaluation of 2 acute graft-versus-host (GVHD) grading systems: a joint Societe Francaise de Greffe de Moelle et Therapie Cellulaire (SFGM-TC), Dana Farber Cancer Institute (DFCI), and International Bone Marrow Transplant Registry (IBMTR) prospective study. Blood. 2005;106(4):1495-500.
194. Stuehler C, Mielke S, Chatterjee M, Duell J, Lurati S, Rueckert F, et al. Selective depletion of alloreactive T cells by targeted therapy of heat shock protein 90: a novel strategy for control of graft-versus-host disease. Blood. 2009;114(13):2829-36.

195. Watkins BK, Tkachev V, Furlan SN, Hunt DJ, Betz K, Yu A, et al. CD28 blockade controls T cell activation to prevent graft-versus-host disease in primates. J Clin Invest. 2018;128(9):3991-4007.
196. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. The Journal of experimental medicine. 2002;196(3):389-99.
197. Johnson BD, Konkol MC, Truitt RL. CD25+ immunoregulatory T-cells of donor origin suppress alloreactivity after BMT. Biology of Blood and Marrow Transplantation. 2002;8(10):525-35.
198. Cohen JL, Trenado A, Vasey D, Klatzmann D, Salomon BL. CD4(+)CD25(+) immunoregulatory T Cells: new therapeutics for graft-versus-host disease. The Journal of experimental medicine. 2002;196(3):401-6.
199. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood. 2002;99(10):3493-9.
200. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, et al. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J Clin Invest. 2003;112(11):1688-96.
201. Jones SC, Murphy GF, Korngold R. Post-hematopoietic cell transplantation control of graft-versus-host disease by donor CD425 T cells to allow an effective graft-versus-leukemia response. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2003;9(4):243-56.
202. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nature medicine. 2003;9(9):1144-50.
203. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, et al. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. Blood. 2005;105(5):2220-6.
204. Casares N, Rudilla F, Arribillaga L, Llopiz D, Riezu-Boj JI, Lozano T, et al. A peptide inhibitor of FOXP3 impairs regulatory T cell activity and improves vaccine efficacy in mice. Journal of immunology (Baltimore, Md : 1950). 2010;185(9):5150-9.
205. Klages K, Mayer CT, Lahl K, Loddenkemper C, Teng MW, Ngiow SF, et al. Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer research. 2010;70(20):7788-99.
206. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011;117(14):3921-8.
207. Center HLMC, Research I. Ex-vivo Expanded Donor Regulatory T Cells for Prevention of Acute Graft-Versus-Host Disease 2019 [updated December. Available from: https://ClinicalTrials.gov/show/NCT01795573.
208. Martin PJ, Schoch G, Fisher L, Byers V, Anasetti C, Appelbaum FR, et al. A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. Blood. 1990;76(8):1464-72.
209. Arai S, Margolis J, Zahurak M, Anders V, Vogelsang GB. Poor outcome in steroid-refractory graft-versus-host disease with antithymocyte globulin treatment. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2002;8(3):155-60.
210. Hill L, Alousi A, Kebriaei P, Mehta R, Rezvani K, Shpall E. New and emerging therapies for acute and chronic graft versus host disease. Ther Adv Hematol. 2018;9(1):21-46.
211. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. British Journal of Pharmacology. 2006;148(3):245-54.
212. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. Immunopharmacology. 2000;47(2-3):119-25.
213. Thomson AW, Bonham CA, Zeevi A. Mode of action of tacrolimus (FK506): molecular and cellular mechanisms. Therapeutic drug monitoring. 1995;17(6):584-91.

214. Hart JW, Shiue LH, Shpall EJ, Alousi AM. Extracorporeal photopheresis in the treatment of graft-versus-host disease: evidence and opinion. Therapeutic Advances in Hematology. 2013;4(5):320-34.
215. Poiré X, van Besien K. Alemtuzumab in allogeneic hematopoetic stem cell transplantation. Expert opinion on biological therapy. 2011;11(8):1099-111.
216. Levin AD, Wildenberg ME, van den Brink GR. Mechanism of Action of Anti-TNF Therapy in Inflammatory Bowel Disease. Journal of Crohn's & colitis. 2016;10(8):989-97.
217. Malard F, Labopin M, Yakoub-Agha I, Chantepie S, Guillaume T, Blaise D, et al. Rituximab-based first-line treatment of cGVHD after allogeneic SCT: results of a phase 2 study. Blood. 2017;130(20):2186-95.
218. Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clinical biochemistry. 1998;31(5):335-40.
219. Houghton PJ. Everolimus. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16(5):1368-72.
220. Perner F, Schnoder TM, Ranjan S, Wolleschak D, Ebert C, Pils MC, et al. Specificity of JAK-kinase inhibition determines impact on human and murine T-cell function. Leukemia. 2016;30(4):991-5.
221. Vogelsang GB, Hess AD, Santos GW. Thalidomide for treatment of graft-versus-host disease. Bone Marrow Transplant. 1988;3(5):393-8.
222. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 2000;47(2-3):85-118.
223. Robak T, Lech-Maranda E, Korycka A, Robak E. Purine nucleoside analogs as immunosuppressive and antineoplastic agents: mechanism of action and clinical activity. Current medicinal chemistry. 2006;13(26):3165-89.
224. Sathe A, Ortega SB, Mundy DI, Collins RH, Karandikar NJ. In Vitro Methotrexate as a Practical Approach to Selective Allodepletion. Biology of Blood and Marrow Transplantation. 2007;13(6):644-54.
225. Cornelissen JJ, Breems D, van Putten WL, Gratwohl AA, Passweg JR, Pabst T, et al. Comparative analysis of the value of allogeneic hematopoietic stem-cell transplantation in acute myeloid leukemia with monosomal karyotype versus other cytogenetic risk categories. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(17):2140-6.
226. Sprent J, Schaefer M, Gao EK, Korngold R. Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences. I. L3T4+ cells can either augment or retard GVHD elicited by Lyt-2+ cells in class I different hosts. The Journal of experimental medicine. 1988;167(2):556-69.
227. Korngold R. Lethal graft-versus-host disease in mice directed to multiple minor histocompatibility antigens: features of CD8+ and CD4+ T cell responses. Bone Marrow Transplant. 1992;9(5):355-64.
228. Martin PJ. Increased disparity for minor histocompatibility antigens as a potential cause of increased GVHD risk in marrow transplantation from unrelated donors compared with related donors. Bone Marrow Transplant. 1991;8(3):217-23.
229. Gonzalez M, Quezada SA, Blazar BR, Panoskaltsis-Mortari A, Rudensky AY, Noelle RJ. The balance between donor T cell anergy and suppression versus lethal graft-versus-host disease is determined by host conditioning. Journal of immunology (Baltimore, Md : 1950). 2002;169(10):5581-9.
230. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nature reviews Immunology. 2013;13(2):101-17.
231. Jukes JP, Jones ND. Immunology in the Clinic Review Series; focus on host responses: invariant natural killer T cell activation following transplantation. Clinical & Experimental Immunology. 2012;167(1):32-9.

232. Jiang X, Kojo S, Harada M, Ohkohchi N, Taniguchi M, Seino Ki. Mechanism of NKT Cell-Mediated Transplant Tolerance. American Journal of Transplantation. 2007;7(6):1482-90.
233. Watte CM, Nakamura T, Lau CH, Ortaldo JR, Stein-Streilein J. Ly49 C/I-dependent NKT cell-derived IL-10 is required for corneal graft survival and peripheral tolerance. Journal of leukocyte biology. 2008;83(4):928-35.
234. Leveson-Gower DB, Olson JA, Sega EI, Luong RH, Baker J, Zeiser R, et al. Low doses of natural killer T cells provide protection from acute graft-versus-host disease via an IL-4-dependent mechanism. Blood. 2011;117(11):3220-9.
235. Margalit M, Ilan Y, Ohana M, Safadi R, Alper R, Sherman Y, et al. Adoptive transfer of small numbers of DX5+ cells alleviates graft-versus-host disease in a murine model of semiallogeneic bone marrow transplantation: a potential role for NKT lymphocytes. Bone Marrow Transplantation. 2004;35:191.
236. Kuwatani M, Ikarashi Y, Iizuka A, Kawakami C, Quinn G, Heike Y, et al. Modulation of acute graft-versus-host disease and chimerism after adoptive transfer of in vitro-expanded invariant Vα14 natural killer T cells. Immunology Letters. 2006;106(1):82-90.
237. Yang J, Gao L, Liu Y, Ren Y, Xie R, Fan H, et al. Adoptive therapy by transfusing expanded donor murine natural killer T cells can suppress acute graft-versus-host disease in allogeneic bone marrow transplantation. Transfusion. 2010;50(2):407-17.
238. Schneidawind D, Pierini A, Alvarez M, Pan Y, Baker J, Buechele C, et al. CD4+ invariant natural killer T cells protect from murine GVHD lethality through expansion of donor CD4+CD25+FoxP3+ regulatory T cells. Blood. 2014;124(22):3320-8.
239. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. The Journal of experimental medicine. 1994;180:1097-106.
240. Koseki H, Asano H, Inaba T, Miyashita N, Moriwaki K, Lindahl KF, et al. Dominant expression of a distinctive V14+ T-cell antigen receptor alpha chain in mice. Proc Natl Acad Sci U S A. 1991;88(17):7518-22.
241. Arase H, Arase N, Ogasawara K, Good RA, Onoe K. An NK1.1+ CD4+8- single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor V beta family. Proc Natl Acad Sci U S A. 1992;89(14):6506-10.
242. Hayakawa K, Lin BT, Hardy RR. Murine thymic CD4+ T cell subsets: a subset (Thy0) that secretes diverse cytokines and overexpresses the V beta 8 T cell receptor gene family. The Journal of experimental medicine. 1992;176(1):269-74.
243. Dellabona P, Casorati G, Friedli B, Angman L, Sallusto F, Tunnacliffe A, et al. In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor alpha/beta CD4-8- subset. The Journal of experimental medicine. 1993;177(6):1763-71.
244. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. The Journal of experimental medicine. 1994;180(3):1171-6.
245. Porcelli S, Gerdes D, Fertig AM, Balk SP. Human T cells expressing an invariant V alpha 24-J alpha Q TCR alpha are CD4- and heterogeneous with respect to TCR beta expression. Human immunology. 1996;48(1-2):63-7.
246. Schipper HS, Rakhshandehroo M, van de Graaf SF, Venken K, Koppen A, Stienstra R, et al. Natural killer T cells in adipose tissue prevent insulin resistance. J Clin Invest. 2012;122(9):3343-54.
247. Huh JY, Kim JI, Park YJ, Hwang IJ, Lee YS, Sohn JH, et al. A novel function of adipocytes in lipid antigen presentation to iNKT cells. Molecular and cellular biology. 2013;33(2):328-39.
248. Ji Y, Sun S, Xu A, Bhargava P, Yang L, Lam KS, et al. Activation of natural killer T cells promotes M2 Macrophage polarization in adipose tissue and improves systemic glucose tolerance via interleukin-4 (IL-4)/STAT6 protein signaling axis in obesity. J Biol Chem. 2012;287(17):13561-71.

249. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. Immunity. 2012;37(3):574-87.
250. Berzins SP, Kyparissoudis K, Pellicci DG, Hammond KJ, Sidobre S, Baxter A, et al. Systemic NKT cell deficiency in NOD mice is not detected in peripheral blood: implications for human studies. Immunology and cell biology. 2004;82(3):247-52.
251. Hammond KJ, Pellicci DG, Poulton LD, Naidenko OV, Scalzo AA, Baxter AG, et al. CD1d-restricted NKT cells: an interstrain comparison. Journal of immunology (Baltimore, Md : 1950). 2001;167(3):1164-73.
252. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. The Journal of experimental medicine. 2000;192(5):741-54.
253. Benlagha K, Weiss A, Beavis A, Teyton L, Bendelac A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. The Journal of experimental medicine. 2000;191(11):1895-903.
254. Thomas SY, Scanlon ST, Griewank KG, Constantinides MG, Savage AK, Barr KA, et al. PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. The Journal of experimental medicine. 2011;208(6):1179-88.
255. Budd RC, Miescher GC, Howe RC, Lees RK, Bron C, MacDonald HR. Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes. The Journal of experimental medicine. 1987;166(2):577-82.
256. Fowlkes BJ, Kruisbeek AM, Ton-That H, Weston MA, Coligan JE, Schwartz RH, et al. A novel population of T-cell receptor alpha beta-bearing thymocytes which predominantly expresses a single V beta gene family. Nature. 1987;329(6136):251-4.
257. Imai K, Kanno M, Kimoto H, Shigemoto K, Yamamoto S, Taniguchi M. Sequence and expression of transcripts of the T-cell antigen receptor alpha-chain gene in a functional, antigen-specific suppressor-T-cell hybridoma. Proc Natl Acad Sci U S A. 1986;83(22):8708-12.
258. Koseki H, Imai K, Ichikawa T, Hayata I, Taniguchi M. Predominant use of a particular alpha-chain in suppressor T cell hybridomas specific for keyhole limpet hemocyanin. International immunology. 1989;1(6):557-64.
259. Ceredig R, Lynch F, Newman P. Phenotypic properties, interleukin 2 production, and developmental origin of a "mature" subpopulation of Lyt-2- L3T4- mouse thymocytes. Proc Natl Acad Sci U S A. 1987;84(23):8578-82.
260. Ballas ZK, Rasmussen W. NK1.1+ thymocytes. Adult murine CD4-, CD8- thymocytes contain an NK1.1+, CD3+, CD5hi, CD44hi, TCR-V beta 8+ subset. Journal of immunology (Baltimore, Md : 1950). 1990;145(4):1039-45.
261. Ohteki T, MacDonald HR. Major histocompatibility complex class I related molecules control the development of CD4+8- and CD4-8- subsets of natural killer 1.1+ T cell receptor-alpha/beta+ cells in the liver of mice. The Journal of experimental medicine. 1994;180(2):699-704.
262. Yoshimoto T, Paul WE. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. The Journal of experimental medicine. 1994;179(4):1285-95.
263. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. The Journal of experimental medicine. 1993;178(1):1-16.
264. Coles MC, Raulet DH. Class I dependence of the development of CD4+ CD8- NK1.1+ thymocytes. The Journal of experimental medicine. 1994;180(1):395-9.
265. Bendelac A, Killeen N, Littman D, Schwartz R. A subset of CD4+ thymocytes selected by MHC class I molecules. Science (New York, NY). 1994;263(5154):1774-8.

266. Bix M, Coles M, Raulet D. Positive selection of V beta 8+ CD4-8- thymocytes by class I molecules expressed by hematopoietic cells. The Journal of experimental medicine. 1993;178(3):901-8.
267. Adachi Y, Koseki H, Zijlstra M, Taniguchi M. Positive selection of invariant V alpha 14+ T cells by non-major histocompatibility complex-encoded class I-like molecules expressed on bone marrow-derived cells. Proc Natl Acad Sci U S A. 1995;92(4):1200-4.
268. Bendelac A, Lantz O, Quimby M, Yewdell J, Bennink, Brutkiewicz R. CD1 recognition by mouse NK1+ T lymphocytes. Science (New York, NY). 1995;268(5212):863-5.
269. Smiley ST, Kaplan MH, Grusby MJ. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. Science (New York, NY). 1997;275(5302):977-9.
270. Dascher CC, Brenner MB. Evolutionary constraints on CD1 structure: insights from comparative genomic analysis. Trends Immunol. 2003;24(8):412-8.
271. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science (New York, NY). 1997;278:1626-9.
272. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. Nature immunology. 2006;7(9):978-86.
273. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, 3rd, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature. 2005;434(7032):525-9.
274. Zhou D, Mattner J, Cantu C, 3rd, Schrantz N, Yin N, Gao Y, et al. Lysosomal glycosphingolipid recognition by NKT cells. Science (New York, NY). 2004;306(5702):1786-9.
275. Brennan PJ, Tatituri RV, Brigl M, Kim EY, Tuli A, Sanderson JP, et al. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. Nature immunology. 2011;12(12):1202-11.
276. Kain L, Webb B, Anderson BL, Deng S, Holt M, Costanzo A, et al. The identification of the endogenous ligands of natural killer T cells reveals the presence of mammalian alpha-linked glycosylceramides. Immunity. 2014;41(4):543-54.
277. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature. 2001;413(6855):531-4.
278. Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, et al. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. Proc Natl Acad Sci U S A. 2005;102(9):3383-8.
279. Albertson DG, Fishpool R, Sherrington P, Nacheva E, Milstein C. Sensitive and high resolution in situ hybridization to human chromosomes using biotin labelled probes: assignment of the human thymocyte CD1 antigen genes to chromosome 1. The EMBO journal. 1988;7(9):2801-5.
280. Calabi F, Milstein C. A novel family of human major histocompatibility complex-related genes not mapping to chromosome 6. Nature. 1986;323(6088):540-3.
281. Moseley WS, Seldin MF. Definition of mouse chromosome 1 and 3 gene linkage groups that are conserved on human chromosome 1: evidence that a conserved linkage group spans the centromere of human chromosome 1. Genomics. 1989;5(4):899-905.
282. Moseley WS, Watson ML, Kingsmore SF, Seldin MF. CD1 defines conserved linkage group border between human chromosomes 1 and mouse chromosomes 1 and 3. Immunogenetics. 1989;30(5):378-82.
283. Prigozy TI, Naidenko O, Qasba P, Elewaut D, Brossay L, Khurana A, et al. Glycolipid Antigen Processing for Presentation by CD1d Molecules. Science (New York, NY). 2001;291(5504):664-7.
284. Vartabedian VF, Savage PB, Teyton L. The processing and presentation of lipids and glycolipids to the immune system. Immunol Rev. 2016;272(1):109-19.
285. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, et al. The crystal structure of human CD1d with and without alpha-galactosylceramide. Nature immunology. 2005;6(8):819-26.

286. Zajonc DM, Cantu C, 3rd, Mattner J, Zhou D, Savage PB, Bendelac A, et al. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. Nature immunology. 2005;6(8):810-8.
287. McCarthy C, Shepherd D, Fleire S, Stronge VS, Koch M, Illarionov PA, et al. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. The Journal of experimental medicine. 2007;204(5):1131-44.
288. Oki S, Chiba A, Yamamura T, Miyake S. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. J Clin Invest. 2004;113(11):1631-40.
289. Bai L, Constantinides MG, Thomas SY, Reboulet R, Meng F, Koentgen F, et al. Distinct APCs explain the cytokine bias of alpha-galactosylceramide variants in vivo. Journal of immunology (Baltimore, Md : 1950). 2012;188(7):3053-61.
290. Im JS, Arora P, Bricard G, Molano A, Venkataswamy MM, Baine I, et al. Kinetics and cellular site of glycolipid loading control the outcome of natural killer T cell activation. Immunity. 2009;30(6):888-98.
291. Bai L, Sagiv Y, Liu Y, Freigang S, Yu KO, Teyton L, et al. Lysosomal recycling terminates CD1d-mediated presentation of short and polyunsaturated variants of the NKT cell lipid antigen alphaGalCer. Proc Natl Acad Sci U S A. 2009;106(25):10254-9.
292. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic ontogeny. Proc Natl Acad Sci U S A. 2005;102(14):5114-9.
293. Guo J, Hawwari A, Li H, Sun Z, Mahanta SK, Littman DR, et al. Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes. Nature immunology. 2002;3(5):469-76.
294. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, et al. Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity. 2005;22(6):705-16.
295. Villey I, de Chasseval R, de Villartay JP. RORgammaT, a thymus-specific isoform of the orphan nuclear receptor RORgamma / TOR, is up-regulated by signaling through the pre-T cell receptor and binds to the TEA promoter. European journal of immunology. 1999;29(12):4072-80.
296. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, Tcra rearrangement and the development of natural killer T cells. Nature immunology. 2010;11(3):240-9.
297. Dose M, Sleckman BP, Han J, Bredemeyer AL, Bendelac A, Gounari F. Intrathymic proliferation wave essential for Valpha14+ natural killer T cell development depends on c-Myc. Proc Natl Acad Sci U S A. 2009;106(21):8641-6.
298. Mycko MP, Ferrero I, Wilson A, Jiang W, Bianchi T, Trumpp A, et al. Selective Requirement for c-Myc at an Early Stage of Vα14i NKT Cell Development. The Journal of Immunology. 2009;182(8):4641-8.
299. Hu T, Simmons A, Yuan J, Bender TP, Alberola-Ila J. The transcription factor c-Myb primes CD4+CD8+ immature thymocytes for selection into the iNKT lineage. Nature immunology. 2010;11(5):435-41.
300. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in T_{reg} and iNKT cell development demonstrated by a novel fluorescent reporter mouse. The Journal of experimental medicine. 2011;208(6):1279-89.
301. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, et al. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity. 2007;27(5):751-62.
302. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annual review of immunology. 2007;25:297-336.

303. Benlagha K, Wei DG, Veiga J, Teyton L, Bendelac A. Characterization of the early stages of thymic NKT cell development. The Journal of experimental medicine. 2005;202(4):485-92.
304. Wang H, Hogquist KA. How Lipid-Specific T Cells Become Effectors: The Differentiation of iNKT Subsets. Frontiers in immunology. 2018;9:1450.
305. Kurobe H, Liu C, Ueno T, Saito F, Ohigashi I, Seach N, et al. CCR7-Dependent Cortex-to-Medulla Migration of Positively Selected Thymocytes Is Essential for Establishing Central Tolerance. Immunity. 2006;24(2):165-77.
306. Ueno T, Saito F, Gray DH, Kuse S, Hieshima K, Nakano H, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. The Journal of experimental medicine. 2004;200(4):493-505.
307. Witt CM, Raychaudhuri S, Schaefer B, Chakraborty AK, Robey EA. Directed migration of positively selected thymocytes visualized in real time. PLoS biology. 2005;3(6):e160.
308. Ehrlich LI, Oh DY, Weissman IL, Lewis RS. Differential contribution of chemotaxis and substrate restriction to segregation of immature and mature thymocytes. Immunity. 2009;31(6):986-98.
309. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. Immunity. 2015;43(3):566-78.
310. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. Nature immunology. 2013;14(11):1146-54.
311. Tuttle KD, Krovi SH, Zhang J, Bedel R, Harmacek L, Peterson LK, et al. TCR signal strength controls thymic differentiation of iNKT cell subsets. Nature communications. 2018;9(1):2650.
312. Seiler MP, Mathew R, Liszewski MK, Spooner CJ, Barr K, Meng F, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. Nature immunology. 2012;13(3):264-71.
313. Weinreich MA, Odumade OA, Jameson SC, Hogquist KA. T cells expressing the transcription factor PLZF regulate the development of memory-like CD8+ T cells. Nature immunology. 2010;11(8):709-16.
314. Lai D, Zhu J, Wang T, Hu-Li J, Terabe M, Berzofsky JA, et al. KLF13 sustains thymic memory-like CD8(+) T cells in BALB/c mice by regulating IL-4-generating invariant natural killer T cells. The Journal of experimental medicine. 2011;208(5):1093-103.
315. van Gisbergen KP, Kragten NA, Hertoghs KM, Wensveen FM, Jonjic S, Hamann J, et al. Mouse Hobit is a homolog of the transcriptional repressor Blimp-1 that regulates NKT cell effector differentiation. Nature immunology. 2012;13(9):864-71.
316. Carr T, Krishnamoorthy V, Yu S, Xue HH, Kee BL, Verykokakis M. The transcription factor lymphoid enhancer factor 1 controls invariant natural killer T cell expansion and Th2-type effector differentiation. The Journal of experimental medicine. 2015;212(5):793-807.
317. Berga-Bolanos R, Zhu WS, Steinke FC, Xue HH, Sen JM. Cell-autonomous requirement for TCF1 and LEF1 in the development of Natural Killer T cells. Molecular immunology. 2015;68(2 Pt B):484-9.
318. Gordy LE, Bezbradica JS, Flyak AI, Spencer CT, Dunkle A, Sun J, et al. IL-15 regulates homeostasis and terminal maturation of NKT cells. Journal of immunology (Baltimore, Md : 1950). 2011;187(12):6335-45.
319. Havenar-Daughton C, Li S, Benlagha K, Marie JC. Development and function of murine RORgammat+ iNKT cells are under TGF-beta signaling control. Blood. 2012;119(15):3486-94.
320. Watarai H, Sekine-Kondo E, Shigeura T, Motomura Y, Yasuda T, Satoh R, et al. Development and Function of Invariant Natural Killer T Cells Producing TH2- and TH17-Cytokines. PLoS biology. 2012;10(2):e1001255.
321. Holzapfel KL, Tyznik AJ, Kronenberg M, Hogquist KA. Antigen-dependent versus -independent activation of invariant NKT cells during infection. Journal of immunology (Baltimore, Md : 1950). 2014;192(12):5490-8.

322. Kaneko Y, Harada M, Kawano T, Yamashita M, Shibata Y, Gejyo F, et al. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. The Journal of experimental medicine. 2000;191(1):105-14.
323. Kawakami K, Kinjo Y, Yara S, Koguchi Y, Uezu K, Nakayama T, et al. Activation of Valpha14(+) natural killer T cells by alpha-galactosylceramide results in development of Th1 response and local host resistance in mice infected with Cryptococcus neoformans. Infection and immunity. 2001;69(1):213-20.
324. Duthie MS, Wleklinski-Lee M, Smith S, Nakayama T, Taniguchi M, Kahn SJ. During Trypanosoma cruzi infection CD1d-restricted NK T cells limit parasitemia and augment the antibody response to a glycophosphoinositol-modified surface protein. Infection and immunity. 2002;70(1):36-48.
325. Mieza MA, Itoh T, Cui JQ, Makino Y, Kawano T, Tsuchida K, et al. Selective reduction of V alpha 14+ NK T cells associated with disease development in autoimmune-prone mice. Journal of immunology (Baltimore, Md : 1950). 1996;156(10):4035-40.
326. Wesley JD, Tessmer MS, Chaukos D, Brossay L. NK cell-like behavior of Valpha14i NK T cells during MCMV infection. PLoS pathogens. 2008;4(7):e1000106.
327. Arase H, Arase N, Saito T. Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. The Journal of experimental medicine. 1996;183(5):2391-6.
328. Kuylenstierna C, Bjorkstrom NK, Andersson SK, Sahlstrom P, Bosnjak L, Paquin-Proulx D, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. European journal of immunology. 2011;41(7):1913-23.
329. Maeda M, Lohwasser S, Yamamura T, Takei F. Regulation of NKT cells by Ly49: analysis of primary NKT cells and generation of NKT cell line. Journal of immunology (Baltimore, Md : 1950). 2001;167(8):4180-6.
330. Hammond KJ, Pelikan SB, Crowe NY, Randle-Barrett E, Nakayama T, Taniguchi M, et al. NKT cells are phenotypically and functionally diverse. European journal of immunology. 1999;29(11):3768-81.
331. Stenstrom M, Skold M, Andersson A, Cardell SL. Natural killer T-cell populations in C57BL/6 and NK1.1 congenic BALB.NK mice-a novel thymic subset defined in BALB.NK mice. Immunology. 2005;114(3):336-45.
332. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nature medicine. 2003;9(5):582-8.
333. Monteiro M, Agua-Doce A, Almeida CF, Fonseca-Pereira D, Veiga-Fernandes H, Graca L. IL-9 Expression by Invariant NKT Cells Is Not Imprinted during Thymic Development. Journal of immunology (Baltimore, Md : 1950). 2015;195(7):3463-71.
334. Yang Y, Ueno A, Bao M, Wang Z, Im JS, Porcelli S, et al. Control of NKT Cell Differentiation by Tissue-Specific Microenvironments. The Journal of Immunology. 2003;171(11):5913-20.
335. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A. 2008;105(32):11287-92.
336. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. J Clin Invest. 2014;124(9):3725-40.
337. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue. Nature immunology. 2015;16(1):85-95.

338. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. Nature immunology. 2011;13(1):35-43.
339. King IL, Fortier A, Tighe M, Dibble J, Watts GF, Veerapen N, et al. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. Nature immunology. 2011;13(1):44-50.
340. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. The Journal of experimental medicine. 2002;195(5):625-36.
341. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. The Journal of experimental medicine. 2002;195(5):637-41.
342. Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. Journal of immunology (Baltimore, Md : 1950). 2003;170(3):1430-4.
343. Grubor-Bauk B, Arthur JL, Mayrhofer G. Importance of NKT cells in resistance to herpes simplex virus, fate of virus-infected neurons, and level of latency in mice. Journal of virology. 2008;82(22):11073-83.
344. Ashkar AA, Rosenthal KL. Interleukin-15 and natural killer and NKT cells play a critical role in innate protection against genital herpes simplex virus type 2 infection. Journal of virology. 2003;77(18):10168-71.
345. Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. Nature medicine. 2005;11(3):340-5.
346. Chung B, Aoukaty A, Dutz J, Terhorst C, Tan R. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. Journal of immunology (Baltimore, Md : 1950). 2005;174(6):3153-7.
347. Pasquier B, Yin L, Fondaneche MC, Relouzat F, Bloch-Queyrat C, Lambert N, et al. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. The Journal of experimental medicine. 2005;201(5):695-701.
348. Courtney AN, Nehete PN, Nehete BP, Thapa P, Zhou D, Sastry KJ. Alpha-galactosylceramide is an effective mucosal adjuvant for repeated intranasal or oral delivery of HIV peptide antigens. Vaccine. 2009;27(25-26):3335-41.
349. Kakimi K, Guidotti LG, Koezuka Y, Chisari FV. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. The Journal of experimental medicine. 2000;192(7):921-30.
350. van Dommelen SL, Tabarias HA, Smyth MJ, Degli-Esposti MA. Activation of natural killer (NK) T cells during murine cytomegalovirus infection enhances the antiviral response mediated by NK cells. Journal of virology. 2003;77(3):1877-84.
351. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. Nature medicine. 2008;14(6):633-40.
352. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. Nature immunology. 2006;7(8):835-42.
353. Gaya M, Barral P, Burbage M, Aggarwal S, Montaner B, Warren Navia A, et al. Initiation of Antiviral B Cell Immunity Relies on Innate Signals from Spatially Positioned NKT Cells. Cell. 2018;172(3):517-33.e20.
354. Sada-Ovalle I, Chiba A, Gonzales A, Brenner MB, Behar SM. Innate invariant NKT cells recognize Mycobacterium tuberculosis-infected macrophages, produce interferon-gamma, and kill intracellular bacteria. PLoS pathogens. 2008;4(12):e1000239.
355. Ishikawa H, Hisaeda H, Taniguchi M, Nakayama T, Sakai T, Maekawa Y, et al. CD4+ Vα14 NKT cells play a crucial role in an early stage of protective immunity against infection with Leishmania major. International immunology. 2000;12(9):1267-74.

356. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. European journal of immunology. 2003;33(12):3322-30.
357. Kawakami K, Kinjo Y, Uezu K, Yara S, Miyagi K, Koguchi Y, et al. Monocyte Chemoattractant Protein-1-Dependent Increase of Vα14 NKT Cells in Lungs and Their Roles in Th1 Response and Host Defense in Cryptococcal Infection. The Journal of Immunology. 2001;167(11):6525-32.
358. Cohen Nadia R, Tatituri Raju VV, Rivera A, Watts Gerald FM, Kim Edy Y, Chiba A, et al. Innate Recognition of Cell Wall β-Glucans Drives Invariant Natural Killer T Cell Responses against Fungi. Cell Host & Microbe. 2011;10(5):437-50.
359. Shiratsuchi T, Schneck J, Kawamura A, Tsuji M. Human CD1 dimeric proteins as indispensable tools for research on CD1-binding lipids and CD1-restricted T cells. Journal of immunological methods. 2009;345(1-2):49-59.
360. Urban BC, Ing R, Stevenson MM. Early interactions between blood-stage plasmodium parasites and the immune system. Current topics in microbiology and immunology. 2005;297:25-70.
361. Stanley AC, Zhou Y, Amante FH, Randall LM, Haque A, Pellicci DG, et al. Activation of Invariant NKT Cells Exacerbates Experimental Visceral Leishmaniasis. PLoS pathogens. 2008;4(2):e1000028.
362. Kaushal H, Bras-Gonçalves R, Avishek K, Kumar Deep D, Petitdidier E, Lemesre JL, et al. Evaluation of cellular immunological responses in mono-and polymorphic clinical forms of post-kala-azar dermal leishmaniasis in India. Clinical & Experimental Immunology. 2016;185(1):50-60.
363. Nakano Y, Hisaeda H, Sakai T, Ishikawa H, Zhang M, Maekawa Y, et al. Roles of NKT cells in resistance against infection with Toxoplasma gondii and in expression of heat shock protein 65 in the host macrophages. Microbes and infection. 2002;4(1):1-11.
364. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. The Journal of experimental medicine. 2001;194(12):1801-11.
365. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, et al. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. The Journal of experimental medicine. 2001;194(12):1789-99.
366. Furlan R, Bergami A, Cantarella D, Brambilla E, Taniguchi M, Dellabona P, et al. Activation of invariant NKT cells by alphaGalCer administration protects mice from MOG35-55-induced EAE: critical roles for administration route and IFN-gamma. European journal of immunology. 2003;33(7):1830-8.
367. Yanagihara Y, Shiozawa K, Takai M, Kyogoku M, Shiozawa S. Natural killer (NK) T cells are significantly decreased in the peripheral blood of patients with rheumatoid arthritis (RA). Clinical and experimental immunology. 1999;118(1):131-6.
368. Watanabe M, Nakamura Y, Matsuzuka F, Takamura Y, Miyauchi A, Iwatani Y. Decrease of intrathyroidal CD161+Valpha24+Vbeta11+ NKT cells in Graves' disease. Endocrine journal. 2008;55(1):199-203.
369. Beristain-Covarrubias N, Canche-Pool E, Gomez-Diaz R, Sanchez-Torres LE, Ortiz-Navarrete V. Reduced iNKT cells numbers in type 1 diabetes patients and their first-degree relatives. Immunity, inflammation and disease. 2015;3(4):411-9.
370. Demoulins T, Gachelin G, Bequet D, Dormont D. A biased Valpha24+ T-cell repertoire leads to circulating NKT-cell defects in a multiple sclerosis patient at the onset of his disease. Immunol Lett. 2003;90(2-3):223-8.
371. Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T. Th2 bias of CD4+ NKT cells derived from multiple sclerosis in remission. International immunology. 2003;15(2):279-88.
372. Fritz RB, Zhao ML. Regulation of experimental autoimmune encephalomyelitis in the C57BL/6J mouse by NK1.1+, DX5+, alpha beta+ T cells. Journal of immunology (Baltimore, Md : 1950). 2001;166(6):4209-15.

373. Mars LT, Laloux V, Goude K, Desbois S, Saoudi A, Van Kaer L, et al. Cutting edge: V alpha 14-J alpha 281 NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. Journal of immunology (Baltimore, Md : 1950). 2002;168(12):6007-11.
374. Parekh VV, Singh AK, Wilson MT, Olivares-Villagomez D, Bezbradica JS, Inazawa H, et al. Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids. Journal of immunology (Baltimore, Md : 1950). 2004;173(6):3693-706.
375. Iwamura C, Nakayama T. Role of NKT cells in allergic asthma. Curr Opin Immunol. 2010;22(6):807-13.
376. Akbari O, Faul JL, Hoyte EG, Berry GJ, Wahlstrom J, Kronenberg M, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. The New England journal of medicine. 2006;354(11):1117-29.
377. Matangkasombut P, Marigowda G, Ervine A, Idris L, Pichavant M, Kim HY, et al. Natural killer T cells in the lungs of patients with asthma. The Journal of allergy and clinical immunology. 2009;123(5):1181-5.
378. Reynolds C, Barkans J, Clark P, Kariyawasam H, Altmann D, Kay B, et al. Natural killer T cells in bronchial biopsies from human allergen challenge model of allergic asthma. The Journal of allergy and clinical immunology. 2009;124(4):860-2; author reply 2.
379. Hamzaoui A, Cheik Rouhou S, Grairi H, Abid H, Ammar J, Chelbi H, et al. NKT cells in the induced sputum of severe asthmatics. Mediators of inflammation. 2006;2006(2):71214.
380. Thomas SY, Chyung YH, Luster AD. Natural killer T cells are not the predominant T cell in asthma and likely modulate, not cause, asthma. The Journal of allergy and clinical immunology. 2010;125(5):980-4.
381. Vijayanand P, Seumois G, Pickard C, Powell RM, Angco G, Sammut D, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. The New England journal of medicine. 2007;356(14):1410-22.
382. Cui J, Watanabe N, Kawano T, Yamashita M, Kamata T, Shimizu C, et al. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Valpha14 natural killer T cells. The Journal of experimental medicine. 1999;190(6):783-92.
383. Matsuda H, Takeda K, Koya T, Okamoto M, Shiraishi Y, Miyahara N, et al. Plasticity of invariant NKT cell regulation of allergic airway disease is dependent on IFN-gamma production. Journal of immunology (Baltimore, Md : 1950). 2010;185(1):253-62.
384. Taniguchi M, Tashiro T, Dashtsoodol N, Hongo N, Watarai H. The specialized iNKT cell system recognizes glycolipid antigens and bridges the innate and acquired immune systems with potential applications for cancer therapy. International immunology. 2010;22(1):1-6.
385. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, et al. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science (New York, NY). 1997;278(5343):1623-6.
386. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: natural killer T cell defects and human disease. Nature reviews Immunology. 2011;11(2):131-42.
387. Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, et al. Differential antitumor immunity mediated by NKT cell subsets in vivo. The Journal of experimental medicine. 2005;202(9):1279-88.
388. Berzofsky JA, Terabe M. The contrasting roles of NKT cells in tumor immunity. Current molecular medicine. 2009;9(6):667-72.
389. Bellone M, Ceccon M, Grioni M, Jachetti E, Calcinotto A, Napolitano A, et al. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. PloS one. 2010;5(1):e8646.
390. Guo W, Dong A, Xing C, Lin X, Pan X, Lin Y, et al. CD1d levels in peripheral blood of patients with acute myeloid leukemia and acute lymphoblastic leukemia. Oncology Letters. 2014;8(2):825-30.
391. Dhodapkar MV, Geller MD, Chang DH, Shimizu K, Fujii S, Dhodapkar KM, et al. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. The Journal of experimental medicine. 2003;197(12):1667-76.

392. Dhodapkar MV, Richter J. Harnessing natural killer T (NKT) cells in human myeloma: progress and challenges. Clinical immunology (Orlando, Fla). 2011;140(2):160-6.
393. Nowak M, Arredouani MS, Tun-Kyi A, Schmidt-Wolf I, Sanda MG, Balk SP, et al. Defective NKT cell activation by CD1d+ TRAMP prostate tumor cells is corrected by interleukin-12 with alpha-galactosylceramide. PloS one. 2010;5(6):e11311.
394. Song L, Asgharzadeh S, Salo J, Engell K, Wu HW, Sposto R, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. J Clin Invest. 2009;119(6):1524-36.
395. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, et al. Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. International journal of cancer. 2001;91(4):523-8.
396. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive Transfer of Invariant NKT Cells as Immunotherapy for Advanced Melanoma: A Phase I Clinical Trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017;23(14):3510-9.
397. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8(12):3702-9.
398. Lynch L, O'Shea D, Winter DC, Geoghegan J, Doherty DG, O'Farrelly C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. European journal of immunology. 2009;39(7):1893-901.
399. Seino KI, Fukao K, Muramoto K, Yanagisawa K, Takada Y, Kakuta S, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. Proc Natl Acad Sci U S A. 2001;98(5):2577-81.
400. Toyofuku A, Yasunami Y, Nabeyama K, Nakano M, Satoh M, Matsuoka N, et al. Natural killer T-cells participate in rejection of islet allografts in the liver of mice. Diabetes. 2006;55(1):34-9.
401. Beilke JN, Kuhl NR, Van Kaer L, Gill RG. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. Nature medicine. 2005;11(10):1059-65.
402. Maier S, Tertilt C, Chambron N, Gerauer K, Huser N, Heidecke CD, et al. Inhibition of natural killer cells results in acceptance of cardiac allografts in CD28-/- mice. Nature medicine. 2001;7(5):557-62.
403. Oh K, Kim S, Park SH, Gu H, Roopenian D, Chung DH, et al. Direct regulatory role of NKT cells in allogeneic graft survival is dependent on the quantitative strength of antigenicity. Journal of immunology (Baltimore, Md : 1950). 2005;174(4):2030-6.
404. Jiang X, Shimaoka T, Kojo S, Harada M, Watarai H, Wakao H, et al. Cutting edge: critical role of CXCL16/CXCR6 in NKT cell trafficking in allograft tolerance. Journal of immunology (Baltimore, Md : 1950). 2005;175(4):2051-5.
405. Sonoda KH, Taniguchi M, Stein-Streilein J. Long-term survival of corneal allografts is dependent on intact CD1d-reactive NKT cells. Journal of immunology (Baltimore, Md : 1950). 2002;168(4):2028-34.
406. Yasunami Y, Kojo S, Kitamura H, Toyofuku A, Satoh M, Nakano M, et al. Valpha14 NK T cell-triggered IFN-gamma production by Gr-1+CD11b+ cells mediates early graft loss of syngeneic transplanted islets. The Journal of experimental medicine. 2005;202(7):913-8.
407. Doisne JM, Becourt C, Amniai L, Duarte N, Le Luduec JB, Eberl G, et al. Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. Journal of immunology (Baltimore, Md : 1950). 2009;183(3):2142-9.
408. Chaidos A, Patterson S, Szydlo R, Chaudhry MS, Dazzi F, Kanfer E, et al. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. Blood. 2012;119(21):5030-6.

409. Malard F, Labopin M, Chevallier P, Guillaume T, Duquesne A, Rialland F, et al. Larger number of invariant natural killer T cells in PBSC allografts correlates with improved GVHD-free and progression-free survival. Blood. 2016;127(14):1828-35.
410. Rubio MT, Moreira-Teixeira L, Bachy E, Bouillie M, Milpied P, Coman T, et al. Early posttransplantation donor-derived invariant natural killer T-cell recovery predicts the occurrence of acute graft-versus-host disease and overall survival. Blood. 2012;120(10):2144-54.
411. Boyson JE, Rybalov B, Koopman LA, Exley M, Balk SP, Racke FK, et al. CD1d and invariant NKT cells at the human maternal-fetal interface. Proc Natl Acad Sci U S A. 2002;99(21):13741-6.
412. Lappas CM, Day YJ, Marshall MA, Engelhard VH, Linden J. Adenosine A2A receptor activation reduces hepatic ischemia reperfusion injury by inhibiting CD1d-dependent NKT cell activation. The Journal of experimental medicine. 2006;203(12):2639-48.
413. Chiba A, Cohen N, Brigl M, Brennan PJ, Besra GS, Brenner MB. Rapid and reliable generation of invariant natural killer T-cell lines in vitro. Immunology. 2009;128(3):324-33.
414. Govindarajan S, Elewaut D, Drennan M. An Optimized Method for Isolating and Expanding Invariant Natural Killer T Cells from Mouse Spleen. J Vis Exp. 2015(105):e53256.
415. Korngold R, Sprent J. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. The Journal of experimental medicine. 1978;148(6):1687-98.
416. Sprent J, Boehmer HV, Nabholz M. Association of immunity and tolerance to host H-2 determinants in irradiated F1 hybrid mice reconstituted with bone marrow cells from one parental strain. The Journal of experimental medicine. 1975;142(2):321-31.
417. Ferrara JLM, Cooke KR, Deeg HJ. Graft-vs.-host disease. 3rd Edition ed. Marcel D, editor2005 p645.
418. Reddy P, Negrin R, Hill GR. Mouse Models of Bone Marrow Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2008;14(1 Suppl 1):129-35.
419. Ferrara JL, Abhyankar S, Gilliland DG. Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1. Transplantation proceedings. 1993;25(1 Pt 2):1216-7.
420. Blazar BR, Murphy WJ, Abedi M. Advances in graft-versus-host disease biology and therapy. Nature reviews Immunology. 2012;12(6):443-58.
421. Murai M, Yoneyama H, Ezaki T, Suematsu M, Terashima Y, Harada A, et al. Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. Nature immunology. 2003;4(2):154-60.
422. Panoskaltsis-Mortari A, Price A, Hermanson JR, Taras E, Lees C, Serody JS, et al. In vivo imaging of graft-versus-host-disease in mice. Blood. 2004;103(9):3590-8.
423. Beilhack A, Schulz S, Baker J, Beilhack GF, Wieland CB, Herman EI, et al. In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood. 2005;106(3):1113-22.
424. Koyama M, Kuns RD, Olver SD, Raffelt NC, Wilson YA, Don AL, et al. Recipient nonhematopoietic antigen-presenting cells are sufficient to induce lethal acute graft-versus-host disease. Nature medicine. 2012;18(1):135-42.
425. Baker MB, Altman NH, Podack ER, Levy RB. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. The Journal of experimental medicine. 1996;183(6):2645-56.
426. Braun MY, Lowin B, French L, Acha-Orbea H, Tschopp J. Cytotoxic T cells deficient in both functional fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-versus-host disease. The Journal of experimental medicine. 1996;183(2):657-61.
427. Jiang Z, Podack E, Levy RB. Major histocompatibility complex-mismatched allogeneic bone marrow transplantation using perforin and/or Fas ligand double-defective CD4(+) donor T cells: involvement of cytotoxic function by donor lymphocytes prior to graft-versus-host disease pathogenesis. Blood. 2001;98(2):390-7.

428. Maeda Y, Levy RB, Reddy P, Liu C, Clouthier SG, Teshima T, et al. Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. Blood. 2005;105(5):2023-7.
429. Iclozan C, Yu Y, Liu C, Liang Y, Yi T, Anasetti C, et al. T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2010;16(2):170-8.
430. Choi J, Ritchey J, Prior JL, Holt M, Shannon WD, Deych E, et al. In vivo administration of hypomethylating agents mitigate graft-versus-host disease without sacrificing graft-versus-leukemia. Blood. 2010;116(1):129-39.
431. Hoffmann P, Boeld TJ, Eder R, Albrecht J, Doser K, Piseshka B, et al. Isolation of CD4+CD25+ regulatory T cells for clinical trials. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2006;12(3):267-74.
432. Firpo EJ, Kong RK, Zhou Q, Rudensky AY, Roberts JM, Franza BR. Antigen-specific dose-dependent system for the study of an inheritable and reversible phenotype in mouse CD4+ T cells. Immunology. 2002;107(4):480-8.
433. Zhang P, Wu J, Deoliveira D, Chao NJ, Chen BJ. Allospecific CD4(+) effector memory T cells do not induce graft-versus-host disease in mice. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2012;18(10):1488-99.
434. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, et al. Host Dendritic Cells Alone Are Sufficient to Initiate Acute Graft-versus-Host Disease. The Journal of Immunology. 2004;172(12):7393-8.
435. Chewning JH, Zhang W, Randolph DA, Swindle CS, Schoeb TR, Weaver CT. Allogeneic Th1 Cells Home to Host Bone Marrow and Spleen and Mediate IFNγ-Dependent Aplasia. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2013;19(6):876-87.
436. Benard A, Ceredig R, Rolink AG. Regulatory T cells control autoimmunity following syngeneic bone marrow transplantation. European journal of immunology. 2006;36(9):2324-35.
437. Anderson BE, McNiff JM, Matte C, Athanasiadis I, Shlomchik WD, Shlomchik MJ. Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease. Blood. 2004;104(5):1565-73.
438. Komatsu N, Hori S. Full restoration of peripheral Foxp3+ regulatory T cell pool by radioresistant host cells in scurfy bone marrow chimeras. Proc Natl Acad Sci U S A. 2007;104(21):8959-64.
439. Bosco N, Swee LK, Benard A, Ceredig R, Rolink A. Auto-reconstitution of the T-cell compartment by radioresistant hematopoietic cells following lethal irradiation and bone marrow transplantation. Experimental hematology. 2010;38(3):222-32.e2.
440. Sanchez-Garcia J, Serrano J, Gomez P, Martinez F, Martin C, Roman-Gomez J, et al. The impact of acute and chronic graft-versus-host disease on normal and malignant B-lymphoid precursors after allogeneic stem cell transplantation for B-lineage acute lymphoblastic leukemia. Haematologica. 2006;91(3):340-7.
441. Garvy BA, Elia JM, Hamilton BL, Riley RL. Suppression of B-cell development as a result of selective expansion of donor T cells during the minor H antigen graft-versus-host reaction. Blood. 1993;82(9):2758-66.
442. Baker MB, Riley RL, Podack ER, Levy RB. Graft-versus-host-disease-associated lymphoid hypoplasia and B cell dysfunction is dependent upon donor T cell-mediated Fas-ligand function, but not perforin function. Proc Natl Acad Sci U S A. 1997;94(4):1366-71.
443. Yao Z, Liu Y, Jones J, Strober S. Differences in Bcl-2 expression by T-cell subsets alter their balance after in vivo irradiation to favor CD4+Bcl-2hi NKT cells. European journal of immunology. 2009;39(3):763-75.

444. Hulsdunker J, Ottmuller KJ, Neeff HP, Koyama M, Gao Z, Thomas OS, et al. Neutrophils provide cellular communication between ileum and mesenteric lymph nodes at graft-versus-host disease onset. Blood. 2018;131(16):1858-69.
445. Schwab L, Goroncy L, Palaniyandi S, Gautam S, Triantafyllopoulou A, Mocsai A, et al. Neutrophil granulocytes recruited upon translocation of intestinal bacteria enhance graft-versus-host disease via tissue damage. Nature medicine. 2014;20(6):648-54.
446. Salomao M, Dorritie K, Mapara MY, Sepulveda A. Histopathology of Graft-vs-Host Disease of Gastrointestinal Tract and Liver An Update. American Journal of Clinical Pathology. 2016;145(5):591-603.
447. Lowsky R, Takahashi T, Liu YP, Dejbakhsh-Jones S, Grumet FC, Shizuru JA, et al. Protective Conditioning for Acute Graft-versus-Host Disease. New England Journal of Medicine. 2005;353(13):1321-31.
448. Shulman HM, Cardona DM, Greenson JK, Hingorani S, Horn T, Huber E, et al. HISTOPATHOLOGIC DIAGNOSIS OF CHRONIC GRAFT VERSUS HOST DISEASE: NIH Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-Versus-Host Disease: II. The 2014 Pathology Working Group Report. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2015;21(4):589-603.
449. Krijgsman D, Hokland M, Kuppen PJK. The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach. Frontiers in immunology. 2018;9(367).
450. Goubier A, Vocanson M, Macari C, Poyet G, Herbelin A, Nicolas JF, et al. Invariant NKT cells suppress CD8(+) T-cell-mediated allergic contact dermatitis independently of regulatory CD4(+) T cells. The Journal of investigative dermatology. 2013;133(4):980-7.
451. Terabe M, Matsui S, Noben-Trauth N, Chen H, Watson C, Donaldson DD, et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nature immunology. 2000;1(6):515-20.
452. Fukunaga A, Khaskhely NM, Ma Y, Sreevidya CS, Taguchi K, Nishigori C, et al. Langerhans Cells Serve as Immunoregulatory Cells by Activating NKT Cells. Journal of immunology (Baltimore, Md : 1950). 2010;185(8):4633-40.
453. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. The Journal of experimental medicine. 2007;204(5):995-1001.
454. Monteiro M, Almeida CF, Agua-Doce A, Graca L. Induced IL-17-producing invariant NKT cells require activation in presence of TGF-beta and IL-1beta. Journal of immunology (Baltimore, Md : 1950). 2013;190(2):805-11.
455. Eberl G, Lees R, Smiley ST, Taniguchi M, Grusby MJ, MacDonald HR. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. Journal of immunology (Baltimore, Md : 1950). 1999;162(11):6410-9.
456. Zeng D, Lewis D, Dejbakhsh-Jones S, Lan F, García-Ojeda M, Sibley R, et al. Bone Marrow NK1.1(−) and NK1.1(+) T Cells Reciprocally Regulate Acute Graft versus Host Disease. The Journal of experimental medicine. 1999;189(7):1073-81.
457. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. Testing the NKT cell hypothesis of human IDDM pathogenesis. J Clin Invest. 2002;110(6):793-800.
458. Berzins SP, Cochrane AD, Pellicci DG, Smyth MJ, Godfrey DI. Limited correlation between human thymus and blood NKT cell content revealed by an ontogeny study of paired tissue samples. European journal of immunology. 2005;35(5):1399-407.
459. Helft J, Bottcher J, Chakravarty P, Zelenay S, Huotari J, Schraml BU, et al. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. Immunity. 2015;42(6):1197-211.
460. Park J-Y, Keller H, Sato N, Park J-H. NKT cells require IL-7, not IL-15, for survival and homeostasis (LYM4P.761). The Journal of Immunology. 2014;192(1 Supplement):65.18.

461. Brigl M, Tatituri RVV, Watts GFM, Bhowruth V, Leadbetter EA, Barton N, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. The Journal of experimental medicine. 2011;208(6):1163.
462. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12(20 Pt 1):6079-86.
463. Stetson DB, Mohrs M, Reinhardt RL, Baron JL, Wang ZE, Gapin L, et al. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. The Journal of experimental medicine. 2003;198(7):1069-76.
464. Matsuda JL, Gapin L, Baron JL, Sidobre S, Stetson DB, Mohrs M, et al. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. Proc Natl Acad Sci U S A. 2003;100(14):8395-400.
465. Gabrysova L, Wraith DC. Antigenic strength controls the generation of antigen-specific IL-10-secreting T regulatory cells. European journal of immunology. 2010;40(5):1386-95.
466. Gabrysova L, Nicolson KS, Streeter HB, Verhagen J, Sabatos-Peyton CA, Morgan DJ, et al. Negative feedback control of the autoimmune response through antigen-induced differentiation of IL-10-secreting Th1 cells. The Journal of experimental medicine. 2009;206(8):1755-67.
467. Goto M, Murakawa M, Kadoshima-Yamaoka K, Tanaka Y, Nagahira K, Fukuda Y, et al. Murine NKT cells produce Th17 cytokine interleukin-22. Cellular immunology. 2009;254(2):81-4.
468. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. Immunity. 2012;37(2):339-50.
469. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. Blood. 2014;124(5):812-21.
470. Kim EY, Lynch L, Brennan PJ, Cohen NR, Brenner MB. The transcriptional programs of iNKT cells. Seminars in immunology. 2015;27(1):26-32.
471. Lin H, Nieda M, Hutton JF, Rozenkov V, Nicol AJ. Comparative gene expression analysis of NKT cell subpopulations. Journal of leukocyte biology. 2006;80(1):164-73.
472. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. Nature immunology. 2016;17(6):728-39.
473. Moreira-Teixeira L, Resende M, Devergne O, Herbeuval JP, Hermine O, Schneider E, et al. Rapamycin combined with TGF-beta converts human invariant NKT cells into suppressive Foxp3+ regulatory cells. Journal of immunology (Baltimore, Md : 1950). 2012;188(2):624-31.
474. Monteiro M, Almeida CF, Caridade M, Ribot JC, Duarte J, Agua-Doce A, et al. Identification of regulatory Foxp3+ invariant NKT cells induced by TGF-beta. Journal of immunology (Baltimore, Md : 1950). 2010;185(4):2157-63.
475. Moreira-Teixeira L, Resende M, Coffre M, Devergne O, Herbeuval J-P, Hermine O, et al. Proinflammatory Environment Dictates the IL-17–Producing Capacity of Human Invariant NKT Cells. The Journal of Immunology. 2011;186(10):5758-65.
476. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, Iwata M, et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. International immunology. 1998;10(12):1969-80.
477. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. The Journal of experimental medicine. 1998;188(2):287-96.
478. Collison LW, Vignali DAA. In Vitro Treg Suppression Assays. Methods in molecular biology (Clifton, NJ). 2011;707:21-37.

479. Wang C, Liu X, Li Z, Chai Y, Jiang Y, Wang Q, et al. CD8(+)NKT-like cells regulate the immune response by killing antigen-bearing DCs. Scientific reports. 2015;5:14124.
480. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. The Journal of experimental medicine. 1989;170(6):2081-95.
481. Wolk K, Kunz S, Asadullah K, Sabat R. Cutting edge: immune cells as sources and targets of the IL-10 family members? Journal of immunology (Baltimore, Md : 1950). 2002;168(11):5397-402.
482. Grant LR, Yao ZJ, Hedrich CM, Wang F, Moorthy A, Wilson K, et al. Stat4-dependent, T-bet-independent regulation of IL-10 in NK cells. Genes and immunity. 2008;9(4):316-27.
483. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew A, Mostowski HS, et al. Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. Journal of immunology (Baltimore, Md : 1950). 1998;160(6):2637-44.
484. Yanaba K, Bouaziz JD, Matsushita T, Tsubata T, Tedder TF. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. Journal of immunology (Baltimore, Md : 1950). 2009;182(12):7459-72.
485. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nature immunology. 2002;3(10):944-50.
486. Blanco P, Palucka AK, Pascual V, Banchereau J. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine & growth factor reviews. 2008;19(1):41-52.
487. Chomarat P, Rissoan MC, Banchereau J, Miossec P. Interferon gamma inhibits interleukin 10 production by monocytes. The Journal of experimental medicine. 1993;177(2):523-7.
488. Rhodes KA, Andrew EM, Newton DJ, Tramonti D, Carding SR. A subset of IL-10-producing gammadelta T cells protect the liver from Listeria-elicited, CD8(+) T cell-mediated injury. European journal of immunology. 2008;38(8):2274-83.
489. Speiran K, Bailey DP, Fernando J, Macey M, Barnstein B, Kolawole M, et al. Endogenous suppression of mast cell development and survival by IL-4 and IL-10. Journal of leukocyte biology. 2009;85(5):826-36.
490. Sabat R, Grutz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. Cytokine & growth factor reviews. 2010;21(5):331-44.
491. de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. The Journal of experimental medicine. 1991;174(5):1209-20.
492. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. Pillars Article: IL-10 Inhibits Cytokine Production by Activated Macrophages. J. Immunol. 1991. 147: 3815-3822. Journal of immunology (Baltimore, Md : 1950). 2016;197(5):1539-46.
493. Hart PH, Hunt EK, Bonder CS, Watson CJ, Finlay-Jones JJ. Regulation of surface and soluble TNF receptor expression on human monocytes and synovial fluid macrophages by IL-4 and IL-10. Journal of immunology (Baltimore, Md : 1950). 1996;157(8):3672-80.
494. Jenkins JK, Malyak M, Arend WP. The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. Lymphokine and cytokine research. 1994;13(1):47-54.
495. Joyce DA, Gibbons DP, Green P, Steer JH, Feldmann M, Brennan FM. Two inhibitors of pro-inflammatory cytokine release, interleukin-10 and interleukin-4, have contrasting effects on release of soluble p75 tumor necrosis factor receptor by cultured monocytes. European journal of immunology. 1994;24(11):2699-705.
496. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. Journal of immunology (Baltimore, Md : 1950). 1993;150(2):353-60.

497. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. The Journal of experimental medicine. 1996;184(1):19-29.
498. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, et al. IL-10 Is Required for Regulatory T Cells to Mediate Tolerance to Alloantigens In Vivo. The Journal of Immunology. 2001;166(6):3789-96.
499. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nature reviews Cancer. 2012;12(4):252-64.
500. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, et al. Modulation of tryptophan catabolism by regulatory T cells. Nature immunology. 2003;4(12):1206-12.
501. Chen J, Ganguly A, Mucsi AD, Meng J, Yan J, Detampel P, et al. Strong adhesion by regulatory T cells induces dendritic cell cytoskeletal polarization and contact-dependent lethargy. The Journal of experimental medicine. 2017;214(2):327-38.
502. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T Cell Proliferation by Macrophage Tryptophan Catabolism. The Journal of experimental medicine. 1999;189(9):1363-72.
503. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, et al. Treg cells expressing the co-inhibitory molecule TIGIT selectively inhibit pro-inflammatory Th1 and Th17 cell responses. Immunity. 2014;40(4):569-81.
504. Zhang D, Hu W, Xie J, Zhang Y, Zhou B, Liu X, et al. TIGIT-Fc alleviates acute graft-versus-host disease by suppressing CTL activation via promoting the generation of immunoregulatory dendritic cells. Biochimica et biophysica acta. 2018;1864(9 Pt B):3085-98.
505. Huang C-T, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in Regulatory T Cells. Immunity. 2004;21(4):503-13.
506. Polanczyk MJ, Hopke C, Vandenbark AA, Offner H. Treg suppressive activity involves estrogen-dependent expression of programmed death-1 (PD-1). International immunology. 2007;19(3):337-43.
507. Gupta S, Thornley TB, Gao W, Larocca R, Turka LA, Kuchroo VK, et al. Allograft rejection is restrained by short-lived TIM-3+PD-1+Foxp3+ Tregs. The Journal of Clinical Investigation. 2012;122(7):2395-404.
508. Couper KN, Blount DG, Riley EM. IL-10: The Master Regulator of Immunity to Infection. The Journal of Immunology. 2008;180(9):5771-7.
509. Tawara I, Sun Y, Liu C, Toubai T, Nieves E, Evers R, et al. Donor- but not host-derived interleukin-10 contributes to the regulation of experimental graft-versus-host disease. Journal of leukocyte biology. 2012;91(4):667-75.
510. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, et al. IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. Journal of immunology (Baltimore, Md : 1950). 1998;160(5):2059-64.
511. Hassan AT, Dai Z, Konieczny BT, Ring GH, Baddoura FK, Abou-Dahab LH, et al. Regulation of alloantigen-mediated T-cell proliferation by endogenous interferon-gamma: implications for long-term allograft acceptance. Transplantation. 1999;68(1):124-9.
512. Durgan K, Ali M, Warner P, Latchman YE. Targeting NKT cells and PD-L1 pathway results in augmented anti-tumor responses in a melanoma model. Cancer immunology, immunotherapy : CII. 2011;60(4):547-58.
513. Trujillo-Ocampo A, Cho HW, Herrmann AC, Ruiz-Vazquez W, Thornton AB, He H, et al. Rapid ex vivo expansion of highly enriched human invariant natural killer T cells via single antigenic stimulation for cell therapy to prevent graft-versus-host disease. Cytotherapy. 2018;20(8):1089-101.
514. Schmid H, Schneidawind C, Jahnke S, Kettemann F, Secker KA, Duerr-Stoerzer S, et al. Culture-Expanded Human Invariant Natural Killer T Cells Suppress T-Cell Alloreactivity and Eradicate Leukemia. Frontiers in immunology. 2018;9:1817.

515. Billiau A. Interferon-gamma: biology and role in pathogenesis. Advances in immunology. 1996;62:61-130.
516. Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, et al. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. The Journal of experimental medicine. 2005;202:1627-33.
517. Barthlott T, Moncrieffe H, Veldhoen M, Atkins CJ, Christensen J, O'Garra A, et al. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. International immunology. 2005;17(3):279-88.
518. Sojka DK, Hughson A, Sukiennicki TL, Fowell DJ. Early kinetic window of target T cell susceptibility to CD25+ regulatory T cell activity. Journal of immunology (Baltimore, Md : 1950). 2005;175(11):7274-80.
519. Bauché D, Joyce-Shaikh B, Jain R, Grein J, Ku KS, Blumenschein WM, et al. LAG3⁺ Regulatory T Cells Restrain Interleukin-23-Producing CX3CR1⁺ Gut-Resident Macrophages during Group 3 Innate Lymphoid Cell-Driven Colitis. Immunity. 2018;49(2):342-52.e5.
520. Sun H, Gao W, Pan W, Zhang Q, Wang G, Feng D, et al. Tim3(+) Foxp3 (+) Treg Cells Are Potent Inhibitors of Effector T Cells and Are Suppressed in Rheumatoid Arthritis. Inflammation. 2017;40(4):1342-50.
521. Liu Z, McMichael EL, Shayan G, Li J, Chen K, Srivastava R, et al. Novel Effector Phenotype of Tim-3(+) Regulatory T Cells Leads to Enhanced Suppressive Function in Head and Neck Cancer Patients. Clinical cancer research : an official journal of the American Association for Cancer Research. 2018;24(18):4529-38.
522. Heng TS, Painter MW. The Immunological Genome Project: networks of gene expression in immune cells. Nature immunology. 2008;9(10):1091-4.
523. Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178 dependent and is correlated with antigenic potency. Journal of immunology (Baltimore, Md : 1950). 2010;185(5):2721-9.
524. Lan F, Zeng D, Higuchi M, Huie P, Higgins JP, Strober S. Predominance of NK1.1⁺TCRαβ⁺ or DX5⁺TCRαβ⁺ T Cells in Mice Conditioned with Fractionated Lymphoid Irradiation Protects Against Graft-Versus-Host Disease: "Natural Suppressor" Cells. The Journal of Immunology. 2001;167(4):2087-96.
525. Pillai AB, George TI, Dutt S, Strober S. Host natural killer T cells induce an interleukin-4-dependent expansion of donor CD4+CD25+Foxp3+ T regulatory cells that protects against graft-versus-host disease. Blood. 2009;113(18):4458-67.
526. Messina G, Giaccone L, Festuccia M, Irrera G, Scortechini I, Sorasio R, et al. Multicenter experience using total lymphoid irradiation and antithymocyte globulin as conditioning for allografting in hematological malignancies. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2012;18(10):1600-7.
527. Hashimoto D, Asakura S, Miyake S, Yamamura T, Van Kaer L, Liu C, et al. Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells. The Journal of Immunology. 2005;174(1):551-6.
528. Kuns RD, Morris ES, Macdonald KP, Markey KA, Morris HM, Raffelt NC, et al. Invariant natural killer T cell-natural killer cell interactions dictate transplantation outcome after alpha-galactosylceramide administration. Blood. 2009;113(23):5999-6010.
529. Hsieh CS, Macatonia SE, O'Garra A, Murphy KM. T cell genetic background determines default T helper phenotype development in vitro. The Journal of experimental medicine. 1995;181(2):713-21.
530. Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. The Journal of experimental medicine. 1989;169(1):59-72.

531. R Mosmann T, Cherwinski H, W Bond M, Giedlin MA, L Coffman R. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins1986. 2348-57 p.
532. Kernan NA, Bordignon C, Heller G, Cunningham I, Castro-Malaspina H, Shank B, et al. Graft failure after T-cell-depleted human leukocyte antigen identical marrow transplants for leukemia: I. Analysis of risk factors and results of secondary transplants. Blood. 1989;74(6):2227-36.
533. Pillai AB, George TI, Dutt S, Teo P, Strober S. Host NKT cells can prevent graft-versus-host disease and permit graft antitumor activity after bone marrow transplantation. Journal of immunology (Baltimore, Md : 1950). 2007;178(10):6242-51.
534. Kohrt HE, Turnbull BB, Heydari K, Shizuru JA, Laport GG, Miklos DB, et al. TLI and ATG conditioning with low risk of graft-versus-host disease retains antitumor reactions after allogeneic hematopoietic cell transplantation from related and unrelated donors. Blood. 2009;114(5):1099-109.
535. Haraguchi K, Takahashi T, Matsumoto A, Asai T, Kanda Y, Kurokawa M, et al. Host-residual invariant NK T cells attenuate graft-versus-host immunity. Journal of immunology (Baltimore, Md : 1950). 2005;175(2):1320-8.
536. Li J, Scott P, Farrell JP. In vivo alterations in cytokine production following interleukin-12 (IL-12) and anti-IL-4 antibody treatment of CB6F1 mice with chronic cutaneous leishmaniasis. Infection and immunity. 1996;64(12):5248-54.
537. Strong BSI, Newkold TJ, Lee AE, Turner LE, Alhajjat AM, Heusel JW, et al. Extrinsic allospecific signals of hematopoietic origin dictate iNKT cell lineage-fate decisions during development. Scientific reports. 2016;6:28837.
538. Markey KA, MacDonald KP, Hill GR. The biology of graft-versus-host disease: experimental systems instructing clinical practice. Blood. 2014;124(3):354-62.
539. Herrera C, Torres A, Garcia-Castellano JM, Roman J, Martin C, Serrano J, et al. Prevention of graft-versus-host disease in high risk patients by depletion of CD4+ and reduction of CD8+ lymphocytes in the marrow graft. Bone Marrow Transplant. 1999;23(5):443-50.
540. Nimer SD, Giorgi J, Gajewski JL, Ku N, Schiller GJ, Lee K, et al. Selective depletion of CD8+ cells for prevention of graft-versus-host disease after bone marrow transplantation. A randomized controlled trial. Transplantation. 1994;57(1):82-7.
541. Amir AL, Hagedoorn RS, van Luxemburg-Heijs SA, Marijt EW, Kruisselbrink AB, Frederik Falkenburg JH, et al. Identification of a coordinated CD8 and CD4 T cell response directed against mismatched HLA Class I causing severe acute graft-versus-host disease. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2012;18(2):210-9.
542. Koyama M, Hill GR. Alloantigen presentation and graft-versus-host disease: fuel for the fire. Blood. 2016;127(24):2963-70.
543. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M. Methods for detection, isolation and culture of mouse and human invariant NKT cells. Nature protocols. 2008;3(1):70-8.
544. O'Reilly V, Zeng SG, Bricard G, Atzberger A, Hogan AE, Jackson J, et al. Distinct and overlapping effector functions of expanded human CD4+, CD8alpha+ and CD4-CD8alpha- invariant natural killer T cells. PloS one. 2011;6(12):e28648.
545. Iyoda T, Ushida M, Kimura Y, Minamino K, Hayuka A, Yokohata S, et al. Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation. International immunology. 2010;22(11):905-13.
546. Heinrichs J, Bastian D, Veerapathran A, Anasetti C, Betts B, Yu X-Z. Regulatory T-Cell Therapy for Graft-versus-host Disease. Journal of immunology research and therapy. 2016;1(1):1-14.
547. Nguyen VH, Zeiser R, Dasilva DL, Chang DS, Beilhack A, Contag CH, et al. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. Blood. 2007;109(6):2649-56.

548. Li J, Heinrichs J, Haarberg K, Semple K, Veerapathran A, Liu C, et al. HY-Specific Induced Regulatory T Cells Display High Specificity and Efficacy in the Prevention of Acute Graft-versus-Host Disease. Journal of immunology (Baltimore, Md : 1950). 2015;195(2):717-25.
549. Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, et al. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. Gastroenterology. 2000;119(1):119-28.
550. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, et al. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice. Inflammatory bowel diseases. 2005;11(1):35-41.
551. Heller F, Fuss IJ, Nieuwenhuis EE, Blumberg RS, Strober W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity. 2002;17(5):629-38.
552. Beyaz S, Kim JH, Pinello L, Xifaras ME, Hu Y, Huang J, et al. The histone demethylase UTX regulates the lineage-specific epigenetic program of invariant natural killer T cells. Nature immunology. 2017;18(2):184-95.
553. Hayakawa Y, Takeda K, Yagita H, Van Kaer L, Saiki I, Okumura K. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. Journal of immunology (Baltimore, Md : 1950). 2001;166(10):6012-8.
554. Liao C-M, Zimmer MI, Wang C-R. The Functions of Type I and Type II Natural Killer T (NKT) Cells in Inflammatory Bowel Diseases. Inflammatory bowel diseases. 2013;19(6):1330-8.
555. Sáez de Guinoa J, Jimeno R, Gaya M, Kipling D, Garzón MJ, Dunn-Walters D, et al. CD1d-mediated lipid presentation by CD11c(+) cells regulates intestinal homeostasis. The EMBO journal. 2018;37(5):e97537.
556. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, et al. Protective mucosal immunity mediated by epithelial CD1d and IL-10. Nature. 2014;509(7501):497-502.
557. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal Microbes Affect Phenotypes and Functions of Invariant Natural Killer T Cells in Mice. Gastroenterology. 2012;143(2):418-28.
558. Shibolet O, Kalish Y, Klein A, Alper R, Zolotarov L, Thalenfeld B, et al. Adoptive transfer of ex vivo immune-programmed NKT lymphocytes alleviates immune-mediated colitis. Journal of leukocyte biology. 2004;75(1):76-86.
559. CORRY RJ, WINN HJ, RUSSELL PS. PRIMARILY VASCULARIZED ALLOGRAFTS OF HEARTS IN MICE: THE ROLE OF H-2D, H-2K, AND NON-H-2 ANTIGENS IN REJECTION. Transplantation. 1973;16(4):343-50.
560. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. The Journal of experimental medicine. 1990;171(1):307-14.
561. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. Nature immunology. 2006;7(6):652-62.
562. Brown K, Sacks SH, Wong W. Extensive and bidirectional transfer of major histocompatibility complex class II molecules between donor and recipient cells in vivo following solid organ transplantation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2008;22(11):3776-84.
563. Diana J, Lehuen A. NKT cells: friend or foe during viral infections? European journal of immunology. 2009;39(12):3283-91.
564. Fletcher MT, Baxter AG. Clinical application of NKT cell biology in type I (autoimmune) diabetes mellitus. Immunology and cell biology. 2009;87(4):315-23.

565. Lehuen A, Lantz O, Beaudoin L, Laloux V, Carnaud C, Bendelac A, et al. Overexpression of natural killer T cells protects Valpha14- Jalpha281 transgenic nonobese diabetic mice against diabetes. The Journal of experimental medicine. 1998;188(10):1831-9.
566. Wang B, Geng YB, Wang CR. CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. The Journal of experimental medicine. 2001;194(3):313-20.
567. Beaudoin L, Laloux V, Novak J, Lucas B, Lehuen A. NKT cells inhibit the onset of diabetes by impairing the development of pathogenic T cells specific for pancreatic beta cells. Immunity. 2002;17(6):725-36.
568. Cain JA, Smith JA, Ondr JK, Wang B, Katz JD. NKT cells and IFN-gamma establish the regulatory environment for the control of diabetogenic T cells in the nonobese diabetic mouse. Journal of immunology (Baltimore, Md : 1950). 2006;176(3):1645-54.
569. Wang J, Cho S, Ueno A, Cheng L, Xu BY, Desrosiers MD, et al. Ligand-dependent induction of noninflammatory dendritic cells by anergic invariant NKT cells minimizes autoimmune inflammation. Journal of immunology (Baltimore, Md : 1950). 2008;181(4):2438-45.
570. Griseri T, Beaudoin L, Novak J, Mars LT, Lepault F, Liblau R, et al. Invariant NKT cells exacerbate type 1 diabetes induced by CD8 T cells. Journal of immunology (Baltimore, Md : 1950). 2005;175(4):2091-101.
571. Saez de Guinoa J, Jimeno R, Farhadi N, Jervis PJ, Cox LR, Besra GS, et al. CD1d-mediated activation of group 3 innate lymphoid cells drives IL-22 production. EMBO Reports. 2017;18(1):39-47.
572. Ericsson AC, Davis JW, Spollen W, Bivens N, Givan S, Hagan CE, et al. Effects of Vendor and Genetic Background on the Composition of the Fecal Microbiota of Inbred Mice. PloS one. 2015;10(2):e0116704.
573. Parker KD, Albeke SE, Gigley JP, Goldstein AM, Ward NL. Microbiome Composition in Both Wild-Type and Disease Model Mice Is Heavily Influenced by Mouse Facility. Frontiers in Microbiology. 2018;9:1598.
574. Dorozynska I, Majewska-Szczepanik M, Marcinska K, Szczepanik M. Partial depletion of natural gut flora by antibiotic aggravates collagen induced arthritis (CIA) in mice. Pharmacological reports : PR. 2014;66(2):250-5.
575. Weber D, Jenq RR, Peled JU, Taur Y, Hiergeist A, Koestler J, et al. Microbiota Disruption Induced by Early Use of Broad-Spectrum Antibiotics Is an Independent Risk Factor of Outcome after Allogeneic Stem Cell Transplantation. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2017;23(5):845-52.
576. Shono Y, Docampo MD, Peled JU, Perobelli SM, Velardi E, Tsai JJ, et al. Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. Science translational medicine. 2016;8(339):339ra71.
577. Simms-Waldrip TR, Sunkersett G, Coughlin LA, Savani MR, Arana C, Kim J, et al. Antibiotic-Induced Depletion of Anti-inflammatory Clostridia Is Associated with the Development of Graft-versus-Host Disease in Pediatric Stem Cell Transplantation Patients. Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation. 2017;23(5):820-9.
578. Peled JU, Gomes A, Buchan ML, Stein-Thoeringer CK, Slingerland JB, Slingerland AE, et al. Intestinal Microbiota Injury during Allo-Hsct Is Generalizable across Transplantation Centers and Is Associated with Increased Mortality, Broad-Spectrum Antibiotics, and Decreased Calorie Intake. Blood. 2017;130(Suppl 1):750-.
579. Han L, Jin H, Zhou L, Zhang X, Fan Z, Dai M, et al. Intestinal Microbiota at Engraftment Influence Acute Graft-Versus-Host Disease via the Treg/Th17 Balance in Allo-HSCT Recipients. Frontiers in immunology. 2018;9:669.
580. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. The Journal of experimental medicine. 2012;209(5):903-11.

581. De Giorgi L, Sorini C, Cosorich I, Ferrarese R, Canducci F, Falcone M. Increased iNKT17 Cell Frequency in the Intestine of Non-Obese Diabetic Mice Correlates With High Bacterioidales and Low Clostridiales Abundance. Frontiers in immunology. 2018;9:1752.
582. Bedard M, Salio M, Cerundolo V. Harnessing the Power of Invariant Natural Killer T Cells in Cancer Immunotherapy. Frontiers in immunology. 2017;8(1829).
583. Lam PY, Nissen MD, Mattarollo SR. Invariant Natural Killer T Cells in Immune Regulation of Blood Cancers: Harnessing Their Potential in Immunotherapies. Frontiers in immunology. 2017;8:1355.
584. Markey KA, Burman AC, Banovic T, Kuns RD, Raffelt NC, Rowe V, et al. Soluble lymphotoxin is an important effector molecule in GVHD and GVL. Blood. 2010;115(1):122-32.
585. Matte CC, Cormier J, Anderson BE, Athanasiadis I, Liu J, Emerson SG, et al. Graft-versus-leukemia in a retrovirally induced murine CML model: mechanisms of T-cell killing. Blood. 2004;103(11):4353-61.
586. Morris ES, MacDonald KP, Rowe V, Banovic T, Kuns RD, Don AL, et al. NKT cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. J Clin Invest. 2005;115(11):3093-103.
587. Nair S, Dhodapkar MV. Natural Killer T Cells in Cancer Immunotherapy. Frontiers in immunology. 2017;8:1178.
588. Metelitsa LS. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. Clinical immunology (Orlando, Fla). 2011;140(2):119-29.
589. Schneidawind D, Pierini A, Negrin RS. Regulatory T cells and natural killer T cells for modulation of GVHD following allogeneic hematopoietic cell transplantation. Blood. 2013;122(18):3116-21.
590. Hourd P, Chandra A, Medcalf N, Williams DJ. Regulatory challenges for the manufacture and scale-out of autologous cell therapies. StemBook. Cambridge (MA): Harvard Stem Cell Institute

591. Wu J, Rostami MR, Cadavid Olaya DP, Tzanakakis ES. Oxygen transport and stem cell aggregation in stirred-suspension bioreactor cultures. PloS one. 2014;9(7):e102486.
592. Prado-Lopez S, Conesa A, Arminan A, Martinez-Losa M, Escobedo-Lucea C, Gandia C, et al. Hypoxia promotes efficient differentiation of human embryonic stem cells to functional endothelium. Stem cells (Dayton, Ohio). 2010;28(3):407-18.
593. Mason C, Manzotti E. Regen: the industry responsible for cell-based therapies. Regenerative medicine. 2009;4(6):783-5.
594. Thomas RJ, Hourd PC, Williams DJ. Application of process quality engineering techniques to improve the understanding of the in vitro processing of stem cells for therapeutic use. Journal of biotechnology. 2008;136(3-4):148-55.
595. Bravery CA, Carmen J, Fong T, Oprea W, Hoogendoorn KH, Woda J, et al. Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. Cytotherapy. 2013;15(1):9-19.
596. Williams DJ, Thomas RJ, Hourd PC, Chandra A, Ratcliffe E, Liu Y, et al. Precision manufacturing for clinical-quality regenerative medicines. Philosophical transactions Series A, Mathematical, physical, and engineering sciences. 2012;370(1973):3924-49.
597. Thomas RJ, Chandra A, Liu Y, Hourd PC, Conway PP, Williams DJ. Manufacture of a human mesenchymal stem cell population using an automated cell culture platform. Cytotechnology. 2007;55(1):31-9.
598. Thomas RJ, Anderson D, Chandra A, Smith NM, Young LE, Williams D, et al. Automated, scalable culture of human embryonic stem cells in feeder-free conditions. Biotechnology and bioengineering. 2009;102(6):1636-44.
599. Thomas RJ, Hope AD, Hourd P, Baradez M, Miljan EA, Sinden JD, et al. Automated, serum-free production of CTX0E03: a therapeutic clinical grade human neural stem cell line. Biotechnology letters. 2009;31(8):1167-72.

600. Liu Y, Hourd P, Chandra A, Williams DJ. Human cell culture process capability: a comparison of manual and automated production. Journal of Tissue Engineering and Regenerative Medicine. 2010;4(1):45-54.
601. Heathman TR, Nienow AW, McCall MJ, Coopman K, Kara B, Hewitt CJ. The translation of cell-based therapies: clinical landscape and manufacturing challenges. Regenerative medicine. 2015;10(1):49-64.
602. Mesoblast International S, Quintiles I, Mesoblast L. A Prospective Study of Remestemcel-L, Ex-vivo Cultured Adult Human Mesenchymal Stromal Cells, for the Treatment of Pediatric Patients Who Have Failed to Respond to Steroid Treatment for Acute GVHD 2018 [updated January. Available from: https://ClinicalTrials.gov/show/NCT02336230.
603. Sullivan K. Thomas' Hematopoietic Cell Transplantation. 2004.
604. Anassi E, Ndefo UA. Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer. P & T : a peer-reviewed journal for formulary management. 2011;36(4):197-202.
605. Agency EM. European Public Assessment Report: Provenge

2013 [Available from: www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002513/human_med_001680.jsp&mid=WC0b01ac058001d124.
606. Exley MA, Hou R, Shaulov A, Tonti E, Dellabona P, Casorati G, et al. Selective activation, expansion, and monitoring of human iNKT cells with a monoclonal antibody specific for the TCR alpha-chain CDR3 loop. European journal of immunology. 2008;38(6):1756-66.
607. Exley MA, Wilson B, Balk SP. Isolation and Functional Use of Human NKT Cells. Current Protocols in Immunology. 2010;90(1):14.1.1-.1.7.
608. Rogers PR, Matsumoto A, Naidenko O, Kronenberg M, Mikayama T, Kato S. Expansion of human Valpha24+ NKT cells by repeated stimulation with KRN7000. J Immunol Methods. 2004;285(2):197-214.
609. Moreno M, Molling JW, von Mensdorff-Pouilly S, Verheijen RH, von Blomberg BM, van den Eertwegh AJ, et al. In vitro expanded human invariant natural killer T-cells promote functional activity of natural killer cells. Clinical immunology (Orlando, Fla). 2008;129(1):145-54.
610. Bernin H, Fehling H, Marggraff C, Tannich E, Lotter H. The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. Medical microbiology and immunology. 2016;205(4):321-32.
611. Hou R, Goloubeva O, Neuberg DS, Strominger JL, Wilson SB. Interleukin-12 and interleukin-2-induced invariant natural killer T-cell cytokine secretion and perforin expression independent of T-cell receptor activation. Immunology. 2003;110(1):30-7.
612. Delhove JMKM, Qasim W. Genome-Edited T Cell Therapies. Current Stem Cell Reports. 2017;3(2):124-36.
613. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. Journal of immunology (Baltimore, Md : 1950). 2001;167(7):4046-50.
614. Tian G, Courtney AN, Jena B, Heczey A, Liu D, Marinova E, et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. The Journal of Clinical Investigation. 2016;126(6):2341-55.
615. Rotolo A, Caputo VS, Holubova M, Baxan N, Dubois O, Chaudhry MS, et al. Enhanced Anti-lymphoma Activity of CAR19-iNKT Cells Underpinned by Dual CD19 and CD1d Targeting. Cancer cell. 2018;34(4):596-610.e11.