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Effect of umbilical cord blood regulatory T cells on natural killer cell differentiation and function

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Dissertation for the degree of Doctor of Philosophy

submitted by

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Declaration

I, Isabela Pedroza-Pacheco confirm that the work presented in this thesis is my own.

Where information has been derived by other sources, I confirm that this has been indicated in this thesis.

Isabela Pedroza-Pacheco

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Abstract

Graft versus Host Disease (GvHD) remains one of the main complications after haematopoietic stem cell transplantation (HSCT). Due to their ability to suppress effector cells, $CD4^{+}CD25^{high}Foxp3^{high}$ regulatory T (Treg) cells have been proposed as a cellular therapy to prevent GvHD. However it has been shown that Treg cells can inhibit natural killer (NK) cell functions. NK cells are key effectors of the Graft versus Leukaemia (GvL) effect post-transplant; therefore, it is plausible that a Treg cell therapy may impact on NK cell function and differentiation from haematopoietic stem cells (HSC). This study sought to elucidate the effects of Treg cells on NK cell function and differentiation using umbilical cord blood (CB) as a cell source. Herein, it is confirmed that CB $CD4^{+}CD25^{high}Foxp3^{high}$ Treg cells are fully functional and upon TCR-stimulation express CTLA-4 and LAP, and secrete TGF-β and IL-10. Also, they express receptors associated with trafficking to lymphoid tissues and the bone marrow, which are potential NK cell/Treg cell interaction sites. Furthermore, it is shown that CB Treg cells can suppress CB NK cell functions after TCR-stimulation in steady state but not in the presence of exogenous cytokines. Lastly, in an *in vitro* model of NK cell differentiation, a 90% reduction in total NK cells was observed when TCR-stimulated Treg cells were added at the time when HSC commitment to the NK cell lineage occurs. Interestingly, the few NK cells that developed in these cultures showed normal phenotype, IFN-γ secretion and cytotoxicity. Notably, the addition of human recombinant TGF-β to HSC cultures caused a similar reduction in NK cell differentiation as shown when TCR-stimulated Treg cells were added to HSC cultures. Moreover, the Treg cell-mediated effect was contact-dependent and cytokine competition-independent. Collectively, these results demonstrate for the first time that TCR-stimulated CB Treg cells inhibit NK cell differentiation through TGF-β, providing information for optimisation of the time of delivery for an adoptive Treg cell therapy post-HSCT to prevent GvHD.

Abbreviations

7-AAD	7-Aminoactinomycin D
ADCC	Antibody-dependent cell-mediated cytotoxicity
aGvHD	Acute GvHD
AML	Acute myeloid leukaemia
APC	Antigen-presenting cells
ATG	Anti-thymocyte globulin
ATP5B	ATP synthase 5B
Bcl11b	B-cell lymphoma/leukaemia 11B
BSA	Bovine serum albumin
CB	Umbilical cord blood
CBMC	Umbilical cord blood mononuclear cells
CBT	Umbilical cord blood transplantation
CCR	Chemokine C-C receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein diacetate succinimidyl ester
cGvHD	Chronic GvHD
CLIP	Class II associated invariant peptide
CLP	Common lymphoid progenitors
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitors
CMV	Cytomegalovirus
Cpm	counts per minute
Ct	Cycle threshold
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTL	Cytotoxic T lymphocytes
CXCR	Chemokine C-X-C receptor
c-SMAC	Central supramolecular activation cluster
DAP	DNAX-activating protein
DC	Dendritic cells
E:T	Effector:target

EAE	Experimental autoimmune encephalomyelitis
Eomes	Eomesodermin
ER	Endoplasmic reticulum
FasL	Fas ligand
FBS	Fetal bovine serum
FGL2	Fibrinogen-like protein 2
FHL	Familial haemophagocytic lymphohistiocytosis
Foxp3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
GARP	Glycoprotein A repetitions predominant
GIST	Gastrointestinal stromal tumour
GITR	Glucocorticoid-induced tumour necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Grb2	Growth factor receptor-bound protein 2
GvHD	Graft versus Host Disease
GvI	Graft versus infection
GvL	Graft versus Leukaemia
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
ICAM-1	Intercellular adhesion molecule
Id2	DNA-binding protein inhibitor
IFN	Interferon
IL	Interleukin
iNK	Committed immature NK cell
ION	Ionomycin
IPEX syndrome	immunodysregulation polyendocrinopathy enteropathy X-linked
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
iTreg	*in vitro* expanded Treg cells
KIR	Killer-cell immunoglobulin-like receptor

Lag-3	Lymphocyte activation gene-3
LAMP-1	Lysosomal-asociated membrane protein-1
LAP	Latency-associated peptide
LAT	Linker for activation of T cells
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph nodes
LPS	Lipopolysaccharide
MACPF	Membrane-attack-complex-perforin
MFI	Mean fluorescence intensity
mHAgs	Minor histocompatibility antigens
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
mTEC	Medullary thymic epithelial cells
mTOR	Mammalian target of rapamycin
NCR	Natural cytotoxicity receptor
NK	Natural killer cell
NKG2D	Natural killer group 2D
NKT	Natural killer T cell
Nrp-1	Neuropilin-1
NTAL	Non-T cell activation linker
PAMPs	Pathogen-associated molecular patterns
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositide-3 kinase
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
pTreg	Peripherally derived Treg cell
RIC	Reduced intensity conditioning
p-SMAC	Peripheral supramolecular activation cluster

Rorc	Retinoic acid orphan receptor
SCF	Stem cell factor
SLT	Secondary lymphoid tissue
Tcon	Conventional T cell
TCR	T cell receptor
TF	Transcription factor
Tfh	T follicular helper
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNC	Total nucleated cells
TNF	Tumour necrosis factor
TOP1	Topoisomerase 1
Tox	Thymocyte selection-associated high mobility group box protein
TRAIL	Tumour necrosis factor-related apoptosis-inducing–ligand
Treg	Regulatory T cell
tTreg	Thymus-derived Treg cell
UBC	Ubiquitin C
ULBP	UL16-binding protein
uNK	Uterine NK cells
α-MEM	α–minimum essential medium

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1 Introduction

1.1 The immune system

The immune system is a network of cells and molecules specialised in fighting infections and eliminating non-self and transformed cells. It is divided into two categories, the innate immune system and the adaptive immune system. The innate immune system is the first line of defence against pathogens upon recognition in a non-specific manner. The adaptive immune system, also called the “acquired” immune system, is a second line of defence that consists of cells specialised in mounting an antigen specific immune response against pathogens and generating a memory response (Inaba et al., 1984, Delves and Roitt, 2000a, Delves and Roitt, 2000b).

1.1.1 The innate immune system

The first barrier that a pathogen encounters after the skin is the epithelial surface. This surface is coated with enzymes and mucus that either inhibit attachment of pathogens or have antimicrobial properties (Muller et al., 2005, Johansson et al., 2011) (**Figure 1.1**). For example, intestinal epithelial cells produce defensins and cathelicidins, enzymes that protect mucosal surfaces from bacteria by disrupting their cell walls (Eckmann, 2005).

Moreover, the innate immune response includes molecular components such as the complement system, acute-phase proteins and cytokines, and cellular components such as phagocytic cells (neutrophils, macrophages and dendritic cells (DCs)), pro-inflammatory cells (basophils, mast cells and eosinophils) and cytotoxic cells such as natural killer (NK) cells (Delves and Roitt, 2000a) (**Figure 1.1**).

Neutrophils, macrophages and DCs are phagocytic cells whose primary function is to identify, engulf and destroy microorganisms (Flannagan et al., 2012). These cells recognise pathogen-associated molecular patterns (PAMPs) that are exclusively expressed by pathogens such as bacteria, fungi and parasites via a variety of pattern recognition receptors (PRR) like Toll-like receptors (TLRs) (Flannagan et al., 2012). One example of a PAMP is the bacterial endotoxin lipopolysaccharide (LPS) of gram-negative bacteria that is recognised by TLR4 on phagocytes (Poltorak et al., 1998).

Macrophages are long-lived cells that reside within the parenchyma of tissues (Beutler, 2004). They are the first cells to be recruited to the site of infections that recognise and engulf foreign substances. They also secrete cytokines that recruit other innate and adaptive immune cells (Cavaillon, 1994). Macrophages and DCs are referred to as professional antigen presenting cells (APC). APCs are key in the initiation of the adaptive immune response due to their role in presenting antigens to T cells. Neutrophils are short-lived and are the most abundant cells in inflamed tissues (Summers et al., 2010). Basophils, mast cells, and eosinophils express a high affinity Fc receptor for IgE and produce inflammatory mediators such as histamine, prostaglandins and leukotrienes (Delves and Roitt, 2000a). Moreover, eosinophils can also acquire phagocytic functions upon activation (Delves and Roitt, 2000a). Finally, NK cells recognise tumour or infected-cells through the upregulation of self-proteins and/or the decreased expression of major histocompatibility complex (MHC) class I on target cells (Ljunggren and Karre, 1990, Abbas, 2010). NK cells kill target cells through the release of cytolytic granules that contain perforin and granzyme (Schmidt et al., 1985, Delves and Roitt, 2000a). Additionally, NK cells secrete cytokines such as interferon (IFN)-γ that activate and recruit other cells of the

innate and adaptive immune systems. Since NK cells are one of the main focuses of this study, they will be described in detail in the following sections.

1.1.2 Adaptive immunity

The adaptive immune system is subdivided into humoral immunity and cell-mediated immunity (**Figure 1.1**). B cells are the main effectors of the humoral immune response, being key in the clearance of extracellular pathogens (Abbas, 2010). Following activation, B cells secrete antibodies that mediate pathogen clearance. T cells are responsible for cell-mediated responses recognising antigens presented by APCs (Inaba et al., 1984). Following antigen recognition, T cells undergo activation and can either recruit other cells of the innate and adaptive immune systems by releasing cytokines and/or exert cytotoxicity against pathogen-infected cells, thus targeting intracellular pathogens (Ledbetter et al., 1990, Abbas, 2010).

1.1.2.1 B cells

The main functions of B cells are to produce antibodies that bind to and inactivate viruses and microbial toxins or target them for destruction by other elements of the immune system. In addition, B cells can also act as APCs (Rock et al., 1984, Batista and Harwood, 2009). Following antigenic stimulation through the B cell receptor (MacLennan, 1994, Rajewsky, 1996), B cells undergo clonal expansion and differentiate into plasma cells or memory cells. Plasma cells secrete high-affinity antibodies following affinity maturation, while memory B cells confer long term protection against a possible second challenge with the same antigen (Delves and Roitt, 2000a) . Notably, it has also been reported that B cells can exhibit regulatory properties (Mauri and Ehrenstein, 2008).

1.1.2.2 T cells

T cells recognise antigens through their highly specific T cell receptor (TCR). There are two types of TCRs, γδ receptor and αβ receptor, the latter being the

most commonly expressed while γδ-bearing T cells represent only a small population of T cells (1-5%) (Lanier et al., 1987, Abbas, 2010, Kreslavsky and von Boehmer, 2010). Unlike B cells, T cells do not recognise soluble antigens, instead they interact with specific peptides presented by APCs in association with MHC class I or class II molecules. T cells are categorised according to their expression of the cell surface proteins cluster of differentiation (CD)4 or CD8. $CD4^+$ T cells recognise peptides bound to MHC class II molecules, while $CD8^+$ T cells recognise peptides bound to MHC class I molecules (Abbas, 2010). After activation, naive $CD4^+$ T cells can differentiate into different subsets. These subsets can be categorised into "helper cells" (such as T helper (Th)1, Th2, Th17, and T follicular helper (Tfh) cells) and regulatory T (Treg) cells. Th1 cells generally protect against intracellular pathogens whereas Th2 cells protect against extracellular pathogens (Abbas, 2010). Similarly, Th17 cells target extracellular bacterial and fungal infections (Peck and Mellins, 2010). In contrast, $CD8^+$ T cells are cytotoxic T lymphocytes (CTL) that can directly lyse pathogen-infected or tumour cells (Abbas, 2010). After the expansion contraction phases, whereby the majority of T cells undergo apoptosis, a small proportion of both $CD4^+$ and $CD8^+$ T cells acquire memory phenotypes and are maintained for the purpose of a faster and stronger secondary immune response (Sallusto et al., 1999). The phenotype and functions of Treg cells will be further described in the following sections.

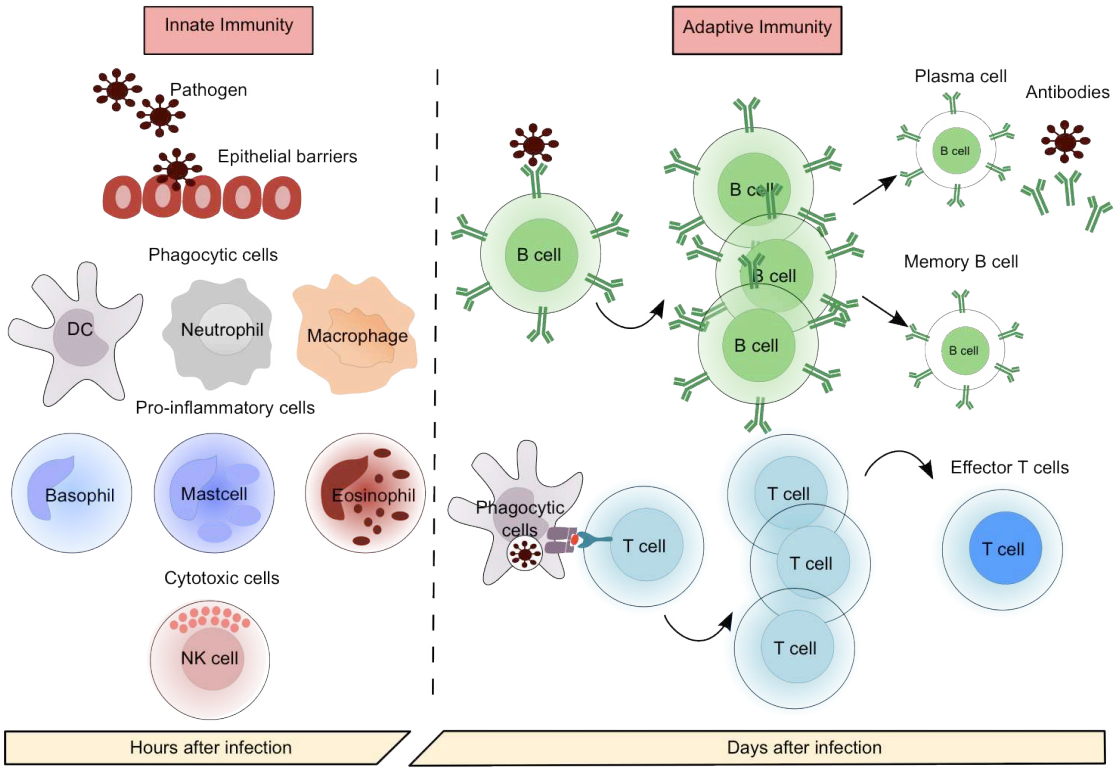


Figure 1.1: Components of the innate and adaptive immune systems. In the first hours after infection, innate immunity provides the first line of defence. Epithelial barriers provide physical and chemical barriers against pathogens whereas phagocytic cells, pro-inflammatory cells and cytotoxic cells eliminate pathogens. If an infection persists, adaptive immune responses mediated by B cells and T cells develop. B cells differentiate into plasma cells characterised by rapid antibody production or memory B cells which confer protection against a possible second challenge with the same antigen. T cells recognise antigens presented by APCs, undergo activation, recruit other cell types and exert cytotoxic functions against pathogen-infected cells.

1.2 The major histocompatibility complex

MHC antigens are highly polymorphic and encode several molecules that play a crucial role in the immune system. The primary role of MHC molecules is to present peptides to T cells allowing them to discriminate between self and non-self antigens. There are three main types of MHC molecules: class I, class II and class III. In humans, MHC molecules are further subdivided into three polymorphic genes: human leukocyte antigen (HLA)-A, -B and -C genes for MHC class I; and HLA-DR, -DQ and -DP genes for MHC class II. MHC class III molecules include genes encoding complement factors and tumour necrosis factor (TNF) (Shaw and Madrigal, 2012). MHC molecules differ with respect to the type of antigens they uptake and to which cells they present these antigens.

MHC class I molecules are expressed by most nucleated cells and platelets and their structure consists of an α chain associated with a β2 microglobulin chain. MHC class I molecules present peptides of 8-10 amino acids in length to CTLs. MHC class II molecules are expressed by a more restricted repertoire of cells, such as B cells, macrophages and DCs. MHC class II molecules present larger peptides of 12-24 amino acids in length to $CD4^+$ T cells and are comprised of two transmembrane glycoproteins, the α and β chains (Abbas, 2010).

1.3 Biology of natural killer cells

NK cells were first described in 1975 as large granular lymphocytes that exhibited natural cytotoxicity against tumour cells without prior activation (Herberman et al., 1975, Kiessling et al., 1975). They were later defined as a separate lymphoid lineage that exhibit cytotoxicity and cytokine-producing functions, capable of lysing tumour cells and cells infected with pathogens such as bacteria, viruses and parasites (Trinchieri, 1989, Biron, 1997, Moretta et al., 2002, Lanier, 2008). NK cells are characterised by the expression of the neural cell adhesion molecule CD56 and the absence of CD3 expression (Robertson and Ritz, 1990). Human NK cells represent 10-15% of peripheral blood (PB) lymphocytes and 15-30% of umbilical cord blood (CB) lymphocytes (Kotylo et al., 1990) but they are also found in the bone marrow (BM), thymus, liver, spleen, placenta and lymph nodes (LN) (Romagnani et al., 2007, Abbas, 2010).

1.3.1 Natural killer cell subsets

NK cells can be subdivided according to their level of CD56 expression and whether or not they express Fcγ receptor III (CD16). They are categorised as $CD56^{bright}CD16^-$ (~10% of the NK cell population in PB and CB) and $CD56^{dim}CD16^+$ NK cells (~90% of NK cells in PB and CB) (Lanier et al., 1986, Cooper et al., 2001a, Luevano et al., 2012a). These subsets differ in functions whereby $CD56^{bright}$ NK cells are cytokine-producing cells and $CD56^{dim}$ NK cells exhibit more cytotoxic functions and express higher levels of CD16 (Robertson and Ritz, 1990, Cooper et al., 2001a).

1.3.1.1 CD56^{bright} natural killer cells

CD56^{bright} NK cells are mostly found in the LN and decidua and represent only 10% of NK cells in PB and in the spleen. CD56^{bright} NK cells are mainly characterised by their capacity to produce and secrete high amounts of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, interleukin (IL)-10, IL-13 and TNF-β (Cooper et al., 2001c), and have low cytolytic functions. However, upon activation with IL-2 or IL-12 they can exert similar cytotoxicity against target cells as CD56^{dim} NK cells (Romagnani et al., 2007). CD56^{bright} NK cells express the high/intermediate affinity IL-2 receptor, which allows proliferation in response to low levels of IL-2 (Caligiuri et al., 1990, Caligiuri et al., 1993).

1.3.1.2 CD56^{dim} natural killer cells

CD56^{dim} NK cells represent 90% of all NK cells and are mostly present in PB. They are fully mature NK cells with high cytotoxic capacity and produce low levels of cytokines (Lanier et al., 1989, De Maria et al., 2011). Importantly, CD56^{dim} NK cells express only intermediate levels of IL-2 receptor, of all affinities; hence their lower proliferative capacity in comparison to CD56^{bright} NK cells (Baume et al., 1992). In addition, CD56^{dim} NK cells express Killer cell immunoglobulin-like receptors (KIR), while CD56^{bright} NK cells do not (Cooper et al., 2001a).

Several authors have demonstrated that CD56^{dim} NK cells are derived from CD56^{bright} NK cells. Using an *in vitro* system of human NK cell differentiation Freud and colleagues observed the transition of CD56^{bright} NK cells to CD56^{dim} NK cells, the latter being the last stage of NK cell differentiation (Freud et al., 2006). Notably, in transplanted patients, donor-derived CD56^{bright} NK cells appear early after engraftment, whereas CD56^{dim} NK cells appear later, and this event is concomitant with a relative decrease in the percentage of CD56^{bright} NK cells (Jacobs et al., 1992, Shilling et al., 2003, Vitale et al., 2004). Another group has confirmed this by analysing telomere length in both subsets (Romagnani et al., 2007). They observed longer telomeres in CD56^{bright} NK

cells than in $CD56^{dim}$ NK cells suggesting that $CD56^{bright}$ NK cells represent an upstream developmental stage of $CD56^{dim}$ NK cells.

1.3.2 Natural killer cell development

Our current understanding of human NK cell differentiation relies mostly on *in vitro* systems. Like many other blood lineages, NK cells derive from $CD34^{+}$ haematopoietic stem cells (HSC) and undergo discrete stages of differentiation. Various tissues have been identified where NK cell differentiation is supported such as the BM (Haller and Wigzell, 1977), thymus (Vosshenrich et al., 2006), liver (Takeda et al., 2005), spleen (Vosshenrich et al., 2005) and secondary lymphoid tissues (SLT) (Freud et al., 2006). However, in adults, the general consensus is that the BM is the main site of NK cell differentiation, since BM damages lead to impaired NK cell development in mice (Seaman et al., 1978, Kumar et al., 1979), whereas dysfunction or absence of other tissues that support NK cell differentiation do not affect NK cell development. This is shown in athymic mice (Herberman et al., 1975) and in individuals with Di George syndrome (Sirianni et al., 1983) that have a dysfunctional thymus, whereby normal numbers of functional NK cells are present. This suggests that the BM is crucial for HSC to commit to the NK cell lineage but that later stages of differentiation can also take place in the aforementioned tissues. How the BM supports NK cell maturation is still under investigation, but it is known that contact between NK cell precursors and BM stromal cells is critical for the production of fully mature NK cells in humans (Miller et al., 1994). The development of *in vitro* systems has considerably advanced our understanding of human NK cell differentiation. HSC from BM (Miller et al., 1994), CB (Grzywacz et al., 2006, Spanholtz et al., 2010) and mobilised PB (Yoon et al., 2010, Zamai et al., 2012) have been used as HSC sources for NK cell production *in vitro*. This has allowed the identification of NK cell intermediates and appropriate culture conditions necessary for NK cell ontogeny.

1.3.2.1 Common lymphoid progenitors

During haematopoiesis, HSC can differentiate into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). According to the traditional model of haematopoiesis (Kondo et al., 1997), CLPs can give rise to B cells, T cells and NK cells but not to myeloid cells. CLPs have been characterised as $Lin^{-}CD34^{+}CD38^{+}CD10^{+}$ cells (Galy et al., 1995). Notably, studies using CB or BM HSC have suggested that the expression of CD7 and CD10 defines NK/T and B cell precursors from CLPs, respectively (Miller et al., 1994, Haddad et al., 2004).

1.3.2.2 Stages of natural killer cell differentiation

In humans, the most accepted model that describes the developmental stages of NK cell differentiation states that CLPs can give rise to mature NK cells in SLT *in vivo* (Freud et al., 2006). In this model, five stages of differentiation have been identified according to CD34, CD117 and CD94 expression. CD34 is mostly expressed by HSC and mesenchymal stem cells (MSC) (Civin et al., 1984). CD117, also known as c-kit or stem cell factor (SCF) receptor, is expressed by half of $CD34^{+}$ cells (Escribano et al., 1998) and CD94 is a C-type lectin receptor, expressed when NK cells undergo maturation (Yu et al., 2010).

In mice, NK/T precursors commit to the NK cell lineage through the expression of the IL-2/IL-15 receptor β subunit (CD122) (Ikawa et al., 1999). The expression of CD122 is fundamental for NK cell development as it confers IL-15 responsiveness, a key cytokine for NK cell differentiation and maturation (Huntington et al., 2009). Hence, the expression of this receptor marks two important steps in NK cell differentiation in mice, whereby a $CD34^{+}CD122^{-}$ progenitor (referred to as a pro-NK cell) gives rise to a $CD34^{-}CD122^{+}$ progenitor (referred to as a pre-NK cell) that can subsequently differentiate into a mature NK cell (Mrozek et al., 1996, Williams et al., 1997, Huntington et al., 2013).

Within human SLT, the first stage of NK cell differentiation (stage 1) is defined by the positive expression of CD34 and CD45RA and the absence of CD117

and CD94. Stage 1 NK cells fit the description of NK/T precursors in mice since they lack CD122 mRNA and express both CLP markers CD7 and CD10 (Freud et al., 2006) (**Figure 1.2**). The second stage of NK cell differentiation within SLT is characterised by the capacity of the cells to respond to soluble IL-15 or IL-2, similarly to what is observed in mice. Stage 2 cells are referred to as pre-NK cells and are defined as $CD34^{+}CD45^{+}CD117^{+}CD94^{-}$ cells. However, this population is still not considered as committed to the NK cell lineage since the expression of CD7 and CD10 persists and differentiation into the T cell lineage can still occur under specific conditions (Freud et al., 2006) (**Figure 1.2**). Unlike pro-NK cells (stage 1) and pre-NK cells (stage 2), stage 3 cells no longer express CD10 and are completely devoid of T cell or DC development potential *in vitro*, thus suggesting that these cells are fully committed to the NK cell lineage. Therefore, stage 3 cells are referred to as committed immature NK (iNK) cells and are defined as $CD34^{-}CD45^{+}CD117^{+}CD94^{-}$ cells. Importantly, a proportion of stage 3 cells express the natural cytotoxicity receptor (NCR) NKp44, involved in NK cell function (**Figure 1.2**). The progression from stage 3 to stage 4 is marked by the positive expression of CD94 and downregulation of CD117. Stage 4 is characterised by numerous phenotypic changes such as expression of the activating NK cell receptors natural killer group 2D (NKG2D) and NKp46, as well as expression of CD122 and perforin, and production of IFN-γ (Freud et al., 2006) (**Figure 1.2**). Due to the similarities in phenotype to $CD56^{bright}$ NK cells, stage 4 cells are referred to as $CD56^{bright}$ cells (Cooper et al., 2001a).

Based on the finding that PB $CD56^{bright}$ NK cells are $CD94^{+}CD16^{+/-}$ and PB $CD56^{dim}$ NK cells are $CD94^{+/-}CD16^{+}$, and that the latter population derives from the former (Cooper et al., 2001a), Freud and Caligiuri reported a fifth stage of NK cell differentiation according to CD94 and CD16 expression on $CD56^{+}$ NK cells (Freud et al., 2006) (**Figure 1.2**). The authors determined three different intermediate stages of differentiation within the SLT defined as $CD56^{+}CD94^{-}CD16^{-}$, $CD56^{+}CD94^{+}CD16^{-}$ and $CD56^{+}CD94^{+}CD16^{+}$, the latter being the most mature NK cell stage. Notably, the expression of KIR, important for NK cell "education", occurs together with the expression of CD16 (Freud et al., 2006). These observations are consistent with other reports showing KIR expression

as a late event during NK cell differentiation *in vivo* and *in vitro* (Valiante et al., 1997, Miller and McCullar, 2001, Sivori et al., 2003). Moreover, these stages have also been observed in a model of differentiation of CB HSC into NK cells *in* vitro (Grzywacz et al., 2006).

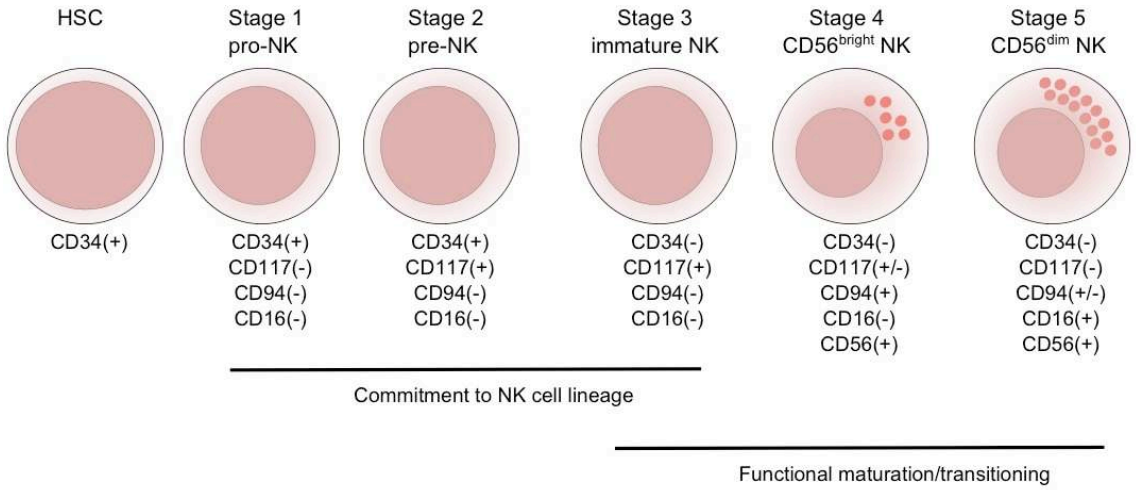


Figure 1.2: NK cell differentiation. Model of NK cell differentiation proposed by Freud and Caligiuri based on the expression of CD34, CD117, CD94, CD56 and CD16. HSC develop into pro-NK cells (stage 1) and subsequently into pre-NK cells (stage 2) acquiring responsiveness to IL-15. From Stage 3-5, HSC acquire full NK cell phenotype and effector functions. The expression of CD16 marks the end of NK cell differentiation.

1.3.2.3 Natural killer cell education or licensing

The engagement of inhibitory receptors on NK cells with self-MHC class I molecules determines whether NK cells will be functional or become hyporesponsive. NK cell "education" has been explained by several models including the "licensing" model (Kim et al., 2005), the "arming/disarming" model (Fernandez et al., 2005, Joncker and Raulet, 2008), the "cis interaction" model (Doucey et al., 2004, Chalifour et al., 2009) and the "rheostat" model (Brodin et al., 2009, Joncker et al., 2009).

The "licensing" model was initially demonstrated in mice by the preferential activation and effector function of NK cells expressing inhibitory receptors for self-MHC class I in MHC congenic mice thus suggesting a selective process of NK cell activation. According to this model, NK cells that do not express inhibitory receptors will be hyporesponsive (Kim et al., 2005) (**Figure 1.3A**). In

contrast, Fernandez and colleagues showed that a subset of NK cells in mice acquires tolerance without expressing self-inhibitory receptors, thus challenging the “licensing” model. The authors proposed the “arming/disarming” model, which suggest that NK cells are fully functional by default. In the absence of inhibitory receptors for self-MHC class I, NK cells become hyporesponsive (Fernandez et al., 2005, Joncker and Raulet, 2008) (**Figure 1.3B**).

The “cis interaction” model suggests that the interaction between inhibitory receptors (Ly49 in mice) in “cis” with ligands on the membrane of the same cell within the immune synapse is sufficient for NK cells to become responsive (Doucey et al., 2004, Chalifour et al., 2009). The evidence for this model is that Ly49 receptors in mice can transmit inhibitory signals even in the absence of engagement but only if present within the immunological synapse (**Figure 1.3C**).

Finally, the “rheostat” model suggests that NK cell responsiveness can be tuned “up” or “down” in a qualitative manner rather than in a binary manner. When NK cells express several inhibitory receptors to self-MHC class I, they exhibit a higher-level of responsiveness, whereas NK cells that express no inhibitory receptors exhibit the lowest level of responsiveness (Brodin et al., 2009, Joncker et al., 2009). This model is applicable to either the licensing model or the education model because an NK cell could be either tuned up (licensing) or down (education) (**Figure 1.3D**). Based on these findings, Brodin and colleagues also proposed a reversible tuning called “the extended rheostat model” (Brodin et al., 2009). The model describes the continuous licensing/disarming of mature NK cells based on the input of the environment (including activating receptors) hence allowing NK cells to adapt depending on conditions such as inflammation, stress or even trafficking.

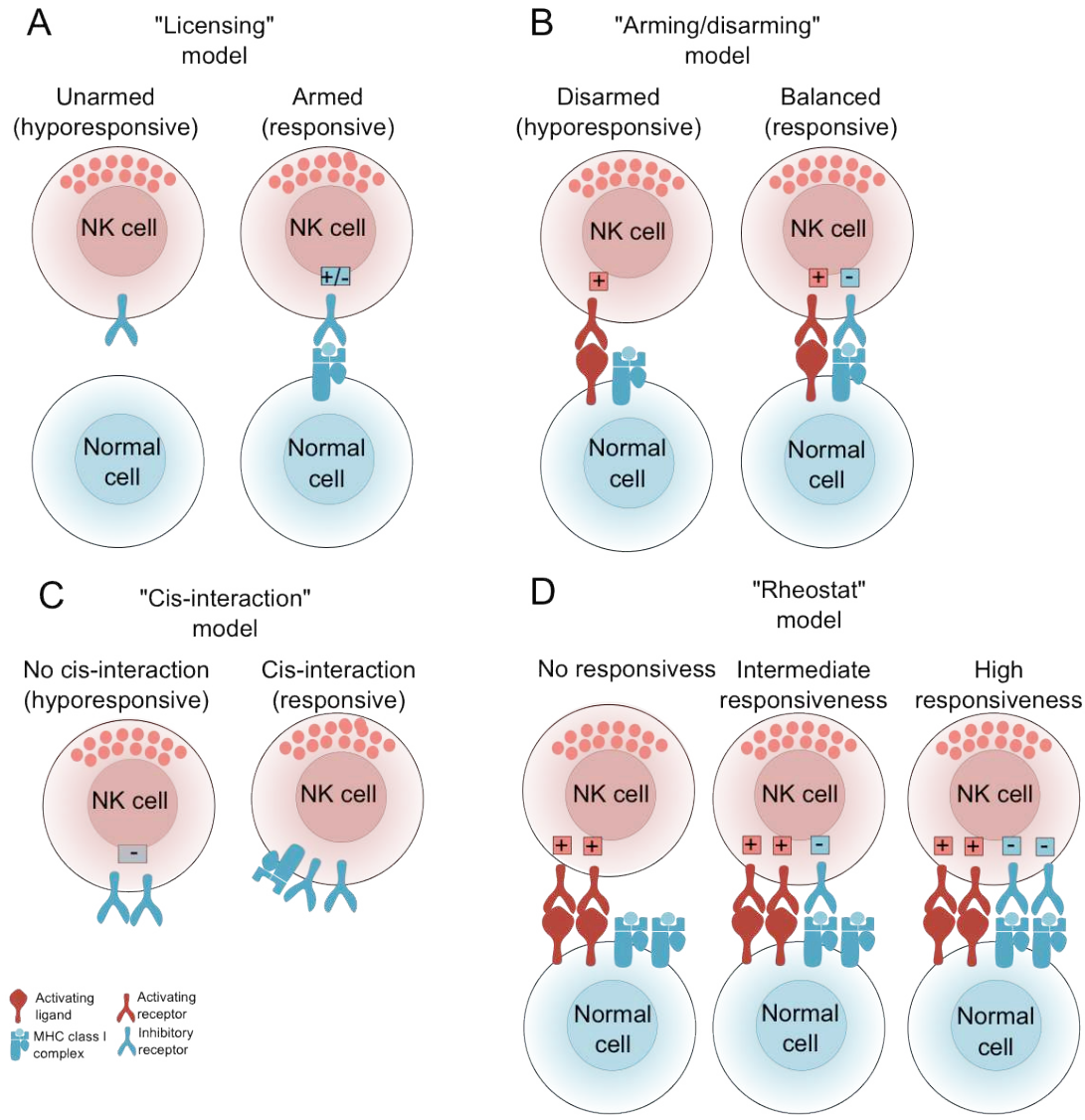


Figure 1.3: Models for NK cell education. In the "licensing" model NK cells are by default unresponsive (A), whereas in the "arming/disarming" model NK cells are activated and rendered tolerant following contact with self-MHC class I molecules (B). The "cis-interaction" model describes the ability of inhibitory receptors to bind to MHC molecules located on the same cell membrane (C). This binding has been suggested to prevent ligand-independent inhibitory signals that can activate NK cells. The "rheostat" model is based on the continuous activation of NK cells rather than an on-off process (D).

1.3.3 Natural killer cell phenotype

1.3.3.1 Natural killer cell recognition

NK cells recognise tumours or pathogen-infected cells through the absence or low expression of MHC class I ("missing-self" theory), or the upregulation of self-proteins ("induced-self" theory") on target cells. Ljunggren and Karre proposed the "missing-self" theory based on observations in syngeneic mice

showing that lymphoma cells deficient in MHC class I, but not lymphoma cells expressing MHC class I, were lysed by NK cells, thus allowing a better understanding of how NK cells detect and lyse target cells (Karre et al., 1986, Ljunggren and Karre, 1990, Karre, 2008). Experiments in humans and mice demonstrated the existence of MHC-specific inhibitory and activating receptors expressed by NK cells (Moretta et al., 1990a, Moretta et al., 1990b, Moretta et al., 1993, Colonna et al., 1999) which led to the description of the "induced-self" model. This model suggests that the activation of NK cells depends on the recognition of "stress" ligands expressed by tumour or pathogen-infected cells by activating receptors on NK cells (**Figure 1.4**).

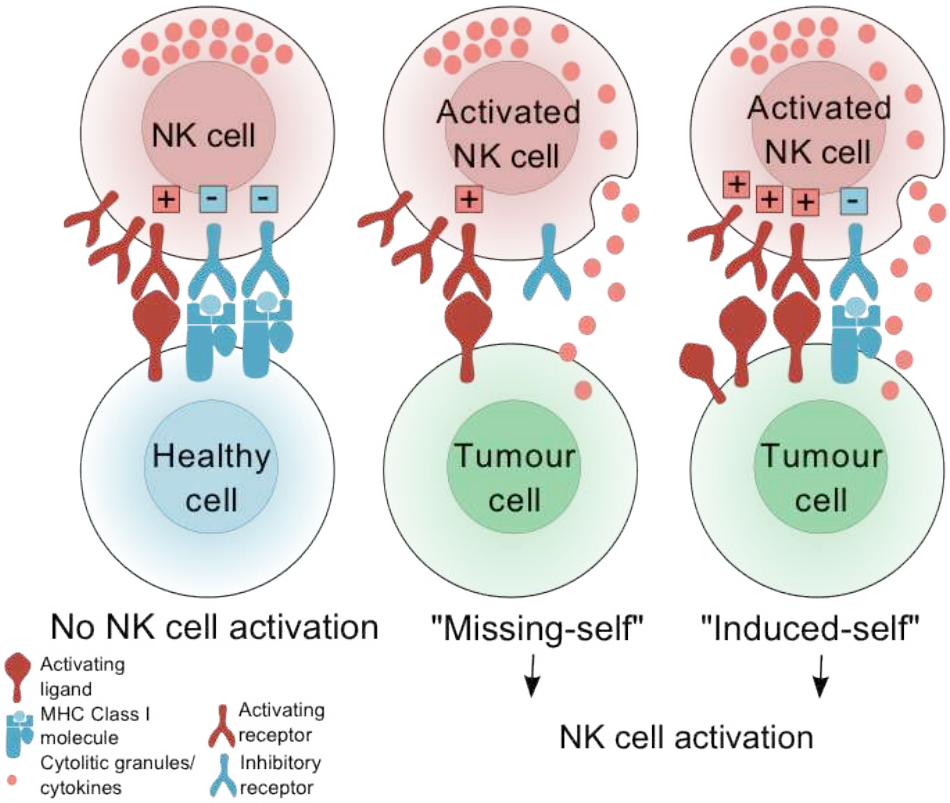


Figure 1.4: NK cell recognition of target cells. NK cells recognise healthy cells and become tolerant through the engagement of self-MHC class I (left). The "missing self" theory consists of the recognition of tumours or pathogen-infected cells that downregulate or don't express MHC class I molecules (middle). The "stress-induced" theory is based on the expression of stress-induced ligands on tumours or pathogen-infected cells. Both "missing-self" and "induced-self" signals trigger NK cell activation.

1.3.3.2 Natural killer cell receptors

1.3.3.2.1 Inhibitory receptors

The expression of inhibitory receptors on NK cells is crucial to discriminate between tumour or pathogen-infected cells and normal healthy cells. In humans, there are two main families of inhibitory receptors recognising MHC class I molecules, the KIR (Wagtmann et al., 1995) and the CD94/NKG2A families (Houchins et al., 1991, Petrie et al., 2008) (**Table 1.1**). Notably, most inhibitory receptors are expressed in a stochastic manner by NK cells (Lanier and Phillips, 1996).

Inhibitory receptors have an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) located within the cytoplasmic tail. ITIMs recruit phosphatases that counteract the signalling cascade initiated by activating receptors. Upon binding, phosphorylation of tyrosines located in the ITIM domain occurs. Subsequently, the protein tyrosine phosphatases SHP-1 and SHP-2, or the inositol phosphatase SHIP are recruited to the cytoplasm. SHP-1 and SHP-2 remove phosphatases from Syk, PLCγ, VAV-1 and CD3ζ whereas SHIP degrades phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3), preventing PKC activation and calcium signalling from the activating signalling cascade (Vely and Vivier, 1997, Long, 1999).

1.3.3.2.2 Activating receptors

NK cells express a variety of activating receptors that synergise to regulate NK cell functions (Bryceson et al., 2006). The NCR family and other receptors such as NKG2D, 2B4, DNAM-1 and CD16, are activating receptors involved in NK cell effector functions (**Table 1.1**). For instance, NKG2D is expressed by “stressed” cells and is crucial for tumour cell rejection (Jinushi et al., 2003), 2B4 is important for the rejection of melanoma cells expressing CD48 (Vaidya et al., 2005), DNAM-1 is involved in the lysis of tumour and infected cells (Gilfillan et al., 2008), and CD16 is responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) (Lanier et al., 1988). Within the NCR family, NKp30 plays a

role in the lysis of immature DCs (Ferlazzo et al., 2002), NKp46 facilitates NK cell lysis of infected and tumour cells (Bottino et al., 2000), and NKp44 is implicated in NK cell lysis (Vitale et al., 1998).

The structure of activating receptors is rather heterogeneous. Some activating receptors signal through immunoreceptor tyrosine-based activating motifs (ITAM) located in the cytoplasmic tail of these receptors, but others like NKG2D use alternative signalling pathways. Upon binding, the ITAM tyrosine residues become phosphorylated by cytoplasmic Src family kinases, which then bind other protein kinases such as Syk and Zap70. Syk and Zap70 phosphorylate transmembrane molecules such as linker for activation of T cells (LAT) or non-T cell activation linker (NTAL) leading to subsequent phosphorylation of several signalling complexes such as phosphatidylinositide-3 kinase (PI3K), phospholipase C (PLC-γ1 and PLC-γ2) and VAV-1, 2 and 3 (Jevremovic et al., 1999). Together, the signals induce cytoskeleton reorganisation that is necessary for cell polarisation and subsequent release of cytolytic granules containing perforin and granzyme.

Other activating receptors such as NKG2D and NCR function through alternative signalling mechanisms using DNAX-activating protein (DAP)-10 and DAP-12, which are signalling subunits associated with ITAMs (Billadeau et al., 2003, Cella et al., 2004). Upon binding, DAP-10 binds to PI3K and growth factor receptor-bound protein 2 (Grb2) initiating a signalling cascade leading to activation. In contrast, DAP-12 and other motifs such as FcεRI and CD3ζ, cause direct phosphorylation of tyrosines within the ITAM motif. The signalling pathways, ligands and receptor families of both inhibitory and activating NK cell receptors are described in **Table 1.1**.

Table 1.1: NK cell receptors in humans. (Moretta et al., 2001, Di Santo, 2006). ULBP: UL16-binding protein, HA; haemagluttinin.

Family	Receptor family	Ligands	Motif	Activating/ inhibitory
KIR	KIR2S, KIR3S	HLA-B-C	ITAM/DAP12	Activating
	KIR2DL, KIR3DL	HLA-A, B, C	ITIM/SHP1-2	Inhibitory
CD94-NKG2	CD94-NKG2A	HLA-E	ITIM/SHP1-2	Inhibitory
	CD94-NKG2C, E	HLA-E	ITAM/DAP12	Activating
NKG2D	NKG2D	MIC-A/-B, ULBP1/2/3/4/5/6	YxM/ DAP10/PI3K	Activating
NCRs	NKp30	BAT-3, HSPG, B7-H6	ITAM/FcγR, CD3ζ, DAP12	Activating
	NKp44	Viral HA	ITAM/FcγR, CD3ζ, DAP12	Activating
	NKp46	Viral HA, HSPG	ITAM/FcγR, CD3ζ, DAP12	Activating
	NKp80	AICL	ITAM/FcγR, CD3ζ, DAP12	Activating
Other receptors	Leukocyte immunoglobulin-like receptor	MHC class I, UL18	ITIM/SHP1-2	Inhibitory
	2B4 (CD244)	CD48	SAP, Fyn	Activating/ Inhibitory
	DNAM-1 (CD226)	CD122, CD155 (Polio virus receptor)	?	Activating
	CD16 (FcγRIII)	FcγRIII	ITAM/FcγR	Activating

1.3.3.3 Natural killer cell functions

Once a target has been recognised, NK cells initiate cytotoxicity through four main mechanisms (Smyth et al., 2002): (i) direct lysis of the infected cell or tumour cell through the release of cytolytic granules containing perforin and granzyme, (ii) via death receptors such as tumour necrosis factor-related apoptosis-inducing–ligand (TRAIL) and Fas ligand (FasL), (iii) via ADCC, or (iv) the activation of other cells through the secretion of IFN-γ or TNF-α (**Figure 1.5**).

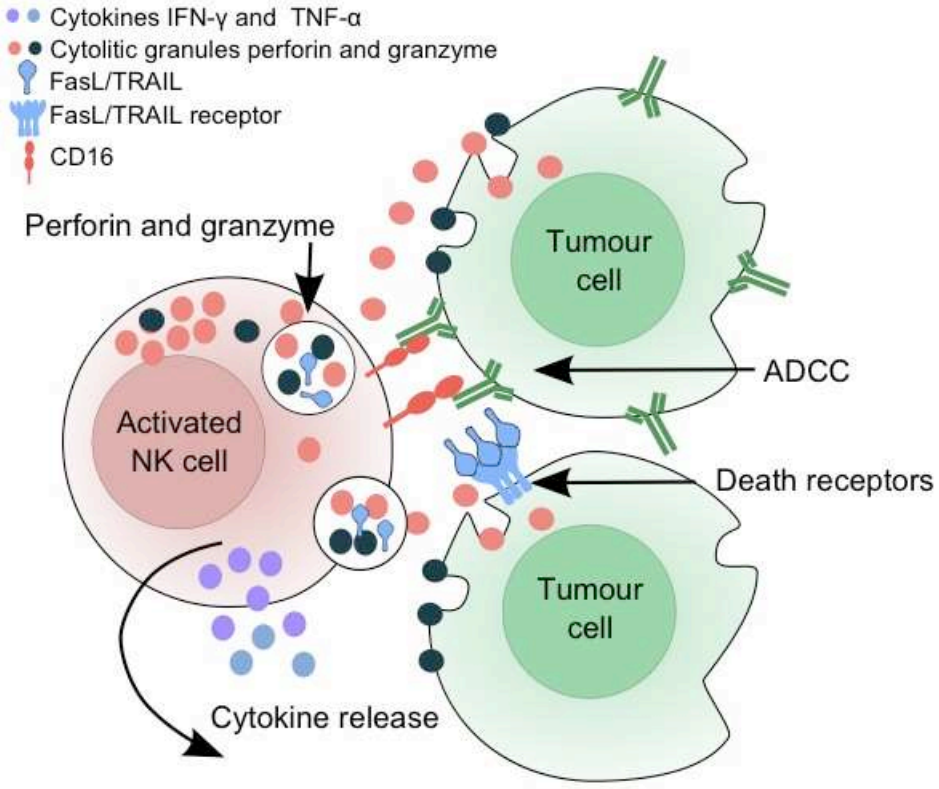


Figure 1.5: NK cell-mediated killing. NK cells recognise malignant or pathogen-infected cells through activating and inhibitory receptors. Once activated, NK cells produce IFN-γ and TNF-α, or mediate cytotoxicity via ADCC, perforin and granzyme release, and expression of death receptors (FasL and TRAIL) that induce target cell apoptosis.

1.3.3.3.1 Perforin and granzyme

Perforin and granzyme-mediated apoptosis is the main cytotoxic pathway used by NK cells to lyse target cells (Shinkai et al., 1988, van den Broek et al., 1995). Perforin is a pore-forming member of the membrane-attack-complex-perforin (MACPF) family of proteins (Podack and Dennert, 1983), whereas granzymes are serine proteases that induce apoptosis in either a caspase dependent

(granzymes B, H, K and M) or caspase-independent manner (granzymes A, B, H, K and M) (Grossman et al., 2003). It has been suggested that all granzymes work together displaying a broad spectrum of activities, thus triggering many pathways at the same time and ensuring apoptosis of target cells. The focus on granzyme B in the majority of NK cell studies is most likely due to the fact that granzymes A and B are the best described granzymes (Krzewski and Coligan, 2012).

For many years, the molecular mechanism of cell death induced by perforin and granzyme remained unresolved; however, new microscopy techniques have allowed a more thorough understanding of the dynamics by which NK cells exert cytotoxicity. Upon NK cell activation, perforin monomers polymerise in a Ca^{2+}-dependent manner to form a range of pores with internal diameters of 120-170 Å on the target cell plasma membrane, thus allowing the entrance of ~50 Å diameter granzyme molecules (Law et al., 2010). Once delivered, target cells undergo membrane repair to reseal the perforin-mediated pores, ultimately limiting osmotic stress and cell lysis. This process was found to take ~80 s (Lopez et al., 2013). Notably, a single NK cell is capable of killing up to ten target cells (Choi and Mitchison, 2013).

It is possible that other molecules present in the lysosomal membrane of NK cells are also involved in this complex mechanism. For instance, recent findings have demonstrated that the lysosomal-asociated membrane protein-1 (LAMP-1, also known as CD107a) is important for perforin trafficking to lytic granules and granule movement and that LAMP-1 silencing by RNA interference blocks granzyme delivery to target cells (Krzewski et al., 2013). Other studies have shown that the glycosylated part of LAMP-1 protects NK cells from enzymes contained within the granules, thus protecting the cellular membrane from self-attack (Fukuda, 1991). LAMP-1 expression has been used as a marker of cytokine production and NK cell-mediated lysis of target cells (Alter et al., 2004). Notably, perforin-deficient mice have reduced capacity to kill syngeneic MHC class I-deficient haematopoietic and epithelial tumour cells (Kagi et al., 1994). In humans, perforin-deficiency leads to familial haemophagocytic

lymphohistiocytosis (FHL) (Stepp et al., 1999), which in some cases cause predisposition to hematologic cancers (Chia et al., 2009).

1.3.3.3.2 Death receptors TRAIL and FasL

Another mechanism by which NK cells can mediate cytotoxicity is via expression of the death receptors TRAIL and FasL that are members of the TNF family. Activated NK cells express FasL and TRAIL in close proximity to cytolytic granules. During granule exocytosis, TRAIL and FasL engage with their cognate ligands, TRAILR-I and -II and Fas (CD95) respectively (Locksley et al., 2001) and induce signalling via the caspase-8 pathway, which leads to apoptosis of target cells.

1.3.3.3.3 Antibody-dependent cell-mediated cytotoxicity

A subset of NK cells express the FcγRIII (CD16), which binds to IgG1 and IgG3 antibody-coated cells. This interaction can overcome inhibitory signals, thus inducing a cytotoxic response (Chan et al., 2012). It has been observed that the co-engagement of CD16 with other activating receptors leads to enhanced NK cell functions (Bryceson et al., 2006). It is important to mention that CD16 is expressed in the final stages of NK cell maturation ($CD56^{dim}CD16^{-}$ NK cells); hence only mature NK cells can mediate killing via ADCC.

1.3.3.3.4 Cytokine secretion

Upon activation, NK cells secrete pro-inflammatory cytokines such as IFN-γ and TNF-α (Biron et al., 1999). IFN-γ production plays a critical role in antiviral defence and can also induce activation of other immune cells such as macrophages, $CD4^{+}$ T cells and $CD8^{+}$ T cells. In addition, IFN-γ enhances antigen presentation by upregulation of MHC class I and class II molecules on DCs. IFN-γ production by NK cells has been observed during mouse cytomegalovirus (CMV) and influenza virus infection (Biron et al., 1999) and it has been shown that IFN-γ induces NK cell effector functions against tumour metastases and sarcoma in mice (Street et al., 2001). Similarly, TNF-α

production by NK cells increases upon infection, in particular during infection with extracellular bacteria such as *S. aureus* (Small et al., 2008). Like IFN-γ, TNF-α can also promote DC maturation, which in turn can activate NK cells via IL-12, providing a feedback loop of activation (Long, 2007).

1.3.4 Regulation of NK cell activation

1.3.4.1 Natural killer cell priming

Despite the classical concept that NK cells do not require pre-activation, it has been found that resting NK cells require an inflammatory context to be fully functional. This was first coined by Bryceson and colleagues who demonstrated that the engagement of NCRs does not trigger lysis by resting NK cells unless they have been previously primed with IL-2 (Bryceson et al., 2006). Moreover, North and colleagues reported that NK cell-resistant tumour cell lines were able to prime resting NK cells to lyse RAJI cells (North et al., 2007), thus suggesting a two-step NK cell activation process consisting of “priming” and “triggering”. It was later found that this “priming” process was KIR independent but CD15-CD2 dependent (Sabry et al., 2011).

Several studies have also confirmed that resting NK cells exhibit low cytotoxicity and cytokine secretion and that these functions are enhanced upon contact with IL-15 trans-presented by DCs (Lucas et al., 2007, Ganal et al., 2012). Trans-presentation of IL-15 by DCs also results in increased translation of perforin and granzyme B (Fehniger et al., 2007). Furthermore, in a MHC class I-deficient mouse ($B2m^{-/-}$) model of CMV infection, all NK cells, regardless of which inhibitory receptors they express, are activated. This suggests that upon infection, NK cell “priming” may be sufficient to break NK cell tolerance in order to clear infections (Sun and Lanier, 2008).

It is also plausible that suppressive cytokines such as transforming growth factor (TGF)-β and/or IL-10 may also play a role in NK cell regulation of activation, since NK cells express the corresponding cognate receptors (Di Santo, 2006). These molecules have been reported to suppress IL-12 and IFN-

γ production, and to block proliferation and cytotoxicity of NK cells (Rook et al., 1986, D'Andrea et al., 1993, Bellone et al., 1995). However, this effect was lost after IL-15 activation of NK cells (Yu et al., 2006).

1.4 Biology of T cells

1.4.1 $CD4^+$ T cell differentiation

T cell precursors originate from HSC in the BM and mature in the thymus. Two major processes occur during T cell differentiation: TCR gene rearrangement and thymic selection. When T cell precursors enter the thymus they interact with peptide-MHC complexes presented by distinct thymic APCs located in different thymic microenvironments. These include cortical thymic epithelial cells, medullary thymic epithelial cells (mTEC) and DCs. This process allows T cell precursors to shape their TCR for antigen recognition. They then undergo a selection process and express surface molecules such as CD4 or CD8. The TCR comprises of αβ chains or γδ chains, combined with four different CD3 subunits (γ, δ, ε, ζ). The TCRα chain is encoded on chromosome 14, whereas the β chain is encoded on chromosome 7. The diversity of the TCR is based on somatic recombination, also known as V(D)J recombination (Chien et al., 1984, Goldrath and Bevan, 1999). Each chain is the result of the rearrangement of multiple copies of V, D and J segments and in the case of the β chain, D segments joined by a C region, during T cell maturation (Goldrath and Bevan, 1999). The thymocyte selection process consists of the deletion of T cells with high affinity for MHC/self-peptide complexes (negative selection), while T cells that exhibit low affinity undergo complete maturation (positive selection).

1.4.2 $CD4^+$ T cell activation

Naive $CD4^+$ T cells recognise peptides associated with MHC class II molecules that are derived from extracellular proteins internalised and processed by APCs. The two chains of the MHC class II molecule assemble in the endoplasmic reticulum (ER) with a third chain, the invariant chain. A portion of

the invariant chain binds to the groove of the MHC class II molecule to prevent peptides and unfolded proteins present in the ER from binding. The invariant chain also guides the transport of the MHC class II molecule out of the ER, through the golgi complex and into the endocytic system where internalised material is trafficked to. Upon acidification of endocytic vesicles, proteases are activated and cleave the invariant chain to give rise to the class II associated invariant peptide (CLIP) that remains bound to the class II molecule. Engulfed proteins are similarly degraded as the vesicle is acidified but the peptides generated cannot bind to the class II molecule due to occupation of the peptide binding groove by CLIP. A specialised MHC class II molecule, HLA-DM, facilitates removal of CLIP and binding of pathogen-derived peptides. The MHC class II molecule is then transported to the cell surface where it can be recognised by the TCR of $CD4^+$ T cells (Weenink and Gautam, 1997). Once engaged, the tyrosine kinase Lck binds to CD4 and phosphorylates the CD3 complex, which triggers downstream signalling pathways (Thomas and Brugge, 1997).

However, complete T cell activation requires co-stimulation by CD28. CD28 acts as a second signal of activation with equal importance to the signal provided by the TCR/CD3 signal (first signal) in the “two signal model of activation” (Ledbetter et al., 1990). Nonetheless, other authors suggest that CD28 acts merely as an enhancer of activation to ensure T cell activation (Diehn et al., 2002, Kane et al., 2002). Particularly at low TCR occupancy, CD28 provides a synergistic signal for naive T cells to effectively activate transcription factors (TF) such as nuclear factor-κB (Kane et al., 2002), nuclear factor of activated T cells (Diehn et al., 2002) and activator protein 1 (Rincon and Flavell, 1994), which are important for cell proliferation and differentiation. Under resting conditions, CD28 is constantly phosphorylated by Lck and dephosphorylated by CD45 leading to a net state of phosphorylation. When APCs and T cells are in contact, CD45 is partially excluded from the synapse to allow constant Lck-mediated phosphorylation of CD28 and subsequent triggering of activation (Evans et al., 2005, Davis and van der Merwe, 2006). Collectively, this downstream signalling provided by TCR and CD28 induces naive T cell proliferation and T cell differentiation into different T cell subsets.

Notably, activation of the TCR in the absence of CD28 co-stimulation leads to T cell anergy (Jenkins and Schwartz, 1987, Harding et al., 1992).

1.4.3 CD4$^+$ T cell subsets

As previously mentioned, CD4$^+$ T cells can differentiate into Th1, Th2, Th17, Tfh and Treg cells (**Figure 1.6**).

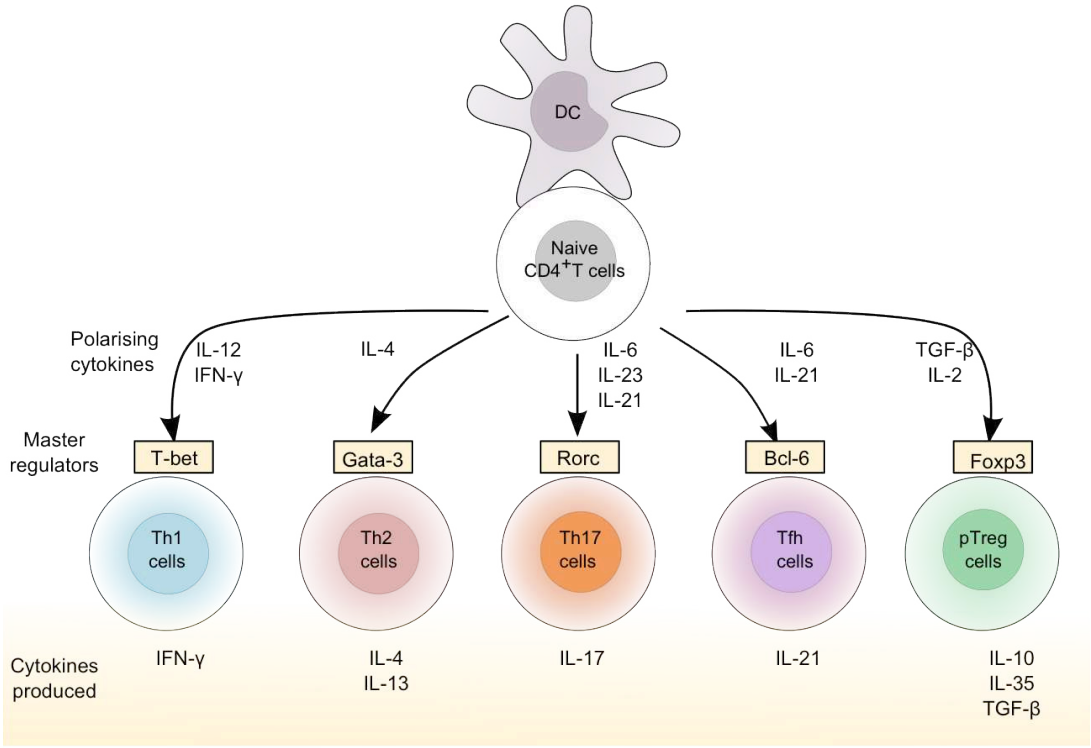


Figure 1.6: CD4$^+$ T cell subsets. Naive CD4$^+$ T cells can differentiate into different subsets based on the polarising cytokines present in the periphery. The environment will trigger transcription of master regulators such as T-bet for Th1 cells, Gata-3 for Th2 cells, Rorc for Th17 cells, Bcl-6 for Tfh cells and Foxp3 for Treg cells. These series of events are required to establish a unique cell type with specific functions.

Th1 differentiation is supported by the cytokines IL-12 and IFN-γ, secreted by APCs and NK cells respectively (Mosmann and Coffman, 1989). The master regulator of Th1 cells is the T-box transcription factor (T-bet), which in turn suppresses Th2 differentiation by blocking the Th2 master regulator Gata-3 (Hwang et al., 2005). The main function of Th1 cells is to activate phagocytic cells and secrete IFN-γ to participate in the generation of CTLs (Abbas, 2010).

IL-4 secretion and the TF Gata-3 are necessary for the differentiation of naive T cells into Th2 cells. Gata-3 suppresses Th1 differentiation by inhibiting expression of the signalling chain of the IL-12 receptor. The main function of Th2 cells is to secrete IL-4, IL-13 and IL-5 which in turn induce production of IgE, and eosinophils and mast cell-mediated immune reactions against helminth infections (Abbas et al., 1996).

Differentiation of naive T cells into Th17 cells requires the transcription of the master regulator retinoic acid orphan receptor (Rorc) (Acosta-Rodriguez et al., 2007) and the presence of IL-6, IL-21, IL-23, and TGF-β. The importance of TGF-β is somewhat controversial, as it seems to act as an indirect inducer of Th17 differentiation (Mangan et al., 2006, Cosmi et al., 2008, Cosmi et al., 2010). Th17 cells secrete IL-17A and IL-17F, which stimulate epithelial cells, endothelial cells and macrophages, and induce the mobilisation, recruitment and activation of neutrophils (Acosta-Rodriguez et al., 2007, Weaver et al., 2007). In addition, Th17 cells can also secrete IL-21, which in turn activates NK cells, CTLs and B cells. The main role of Th17 cells is to confer protection against extracellular bacteria and fungal infections.

Tfh cells were initially described in the germinal centres of human tonsils as cells expressing the chemokine C-X-C receptor (CXCR)5 with the proficiency to help B cells to produce antibodies (Breitfeld et al., 2000, Schaerli et al., 2000). Tfh cells can secrete IL-4 and IFN-γ (Reinhardt et al., 2009) and Bcl-6 acts as a master regulator of Tfh differentiation (Yu et al., 2009).

Treg cells can differentiate in the thymus, (thymus-derived Treg cells (tTreg) cells) or in the periphery (peripherally derived Treg cells (pTreg) cells). pTreg differentiation requires the presence of TGF-β and the expression of the TF forkhead box P3 (Foxp3) (Chen et al., 2003). Since Treg cells are one of the main focuses of this study, they will be described in detail in the following sections.

1.4.4 $CD4^+$ T cell plasticity

Although it was first thought that $CD4^+$ T cell subsets are terminally differentiated, increasing evidence suggests that under certain conditions, they can re-shape their phenotype and function. To understand $CD4^+$ T cell plasticity it is important to first explain the "lineage-specifying model". The paradigm of $CD4^+$ T cell differentiation stems from the assumption that the expression of a single TF ("master regulator") determines the specific $CD4^+$ T cell subset generated. However, recent studies have proposed that $CD4^+$ T cell differentiation instead depends on the interplay between different TFs, referred to as the "lineage-specifying model" (Oestreich and Weinmann, 2012). Thus, this model proposes that Th cells require the co-expression of several TFs to facilitate the expression of the "master regulator" or even re-shape their phenotype and function based on the conditions present in the periphery such as inflammation.

Several studies have confirmed the aforementioned model. Hegazy and colleagues observed the co-expression of T-bet and Gata-3 in Th2 cells upon infection with lymphocytic choriomeningitis virus (LCMV) (Hegazy et al., 2010) and Rudra and colleagues determined Gata-3 as a facilitator of expression of Foxp3 in activated Treg cells in mice (Rudra et al., 2012). Furthermore, it was observed that the transcription of Gata-3 in Treg cells facilitates the control of Th2 responses in the LN, where more pronounced production of effector cytokines such as IL-4, IL-5 and IL-13 was observed (Rudra et al., 2012). It was also found that tTreg cells could lose Foxp3 expression in the absence of TGF-β and start secreting IFN-γ in mice (Komatsu et al., 2009). Moreover, Yang and colleagues demonstrated that in the presence of IL-6 and TCR stimulation, a subset of pTreg cells could downregulate Foxp3 and exhibit increased IL-17 production (Yang et al., 2008). Similarly, subsequent reports in humans demonstrated that only a small population of Treg cells that express CD161 are capable of switching to a Th17 phenotype (Afzali et al., 2013, Pesenacker et al., 2013).

1.4.5 $CD4^+$ T cell tolerance

T cell tolerance is a mechanism that prevents cells from reacting against self-antigens. This mechanism can be divided into central tolerance and peripheral tolerance. Central tolerance occurs in the thymus where T cells with a strong affinity for self-antigens undergo “clonal deletion”. The mTECs are unique thymic APCs that play a crucial role in central tolerance. They present a large number of tissue-specific self-antigens (Derbinski et al., 2001) and express the nuclear factor autoimmune regulator AIRE, which controls the ectopic expression of “tissue-restricted antigens” (Anderson et al., 2002, Gardner et al., 2009). Notably, a small proportion of cells that have strong affinity for self-antigens do not undergo “clonal deletion” but instead undergo “clonal diversion”, which results in the production of self-reactive clones with suppressive capacity, called tTreg cells (Bautista et al., 2009, Leung et al., 2009). Which factors regulate clonal diversion and clonal deletion are still under investigation, however, it has been demonstrated that the absence of IL-2 and TGF-β signalling pathways leads to a complete absence of tTreg cells, thus suggesting their importance for tTreg cell differentiation (Liu et al., 2008c). Additionally, TFs such as NFAT, NF-κB and AP-1 seem to be required for Treg cell differentiation in the thymus (Tai et al., 2005, Mantel et al., 2006).

Peripheral tolerance consists in the suppression or elimination of self-reactive mature T cells in the periphery that have escaped central tolerance. Elimination of autoreactive T cells is achieved by T cell intrinsic mechanisms such as ignorance (Zinkernagel, 1996, Alferink et al., 1998) and anergy (Kurts et al., 1998), or T cell extrinsic mechanisms involving DCs (Janeway, 1992, Kurts et al., 1996) or Treg cells (Sakaguchi et al., 1996).

1.5 Biology of regulatory T cells

Treg cells are involved in the maintenance of immunological self-tolerance and immune homeostasis (Miyara and Sakaguchi, 2011). They have been studied since the early 1970’s and were originally discovered as cells derived from the

thymus capable of suppressing effector cells (Gershon and Kondo, 1970). To date, several subsets of T cells with regulatory functions have been described. Four $CD4^+$ regulatory T cell populations have been reported: the T regulatory 1 cell population that can prevent colitis induced in SCID mice by pathogenic T cells (Groux et al., 1997), the Th3 regulatory T cell subset that results from oral administration of myelin in multiple sclerosis (MS) patients (Fukaura et al., 1996), the Th2 regulatory subset that controls autoreactive T cells in non-obese diabetic mice (Cameron et al., 1997), and the $CD4^+CD25^{high}Foxp3^{high}$ Treg cells that upon depletion lead to severe autoimmune diseases and allergies in humans and mice (Wildin et al., 2001). Herein, the latter are referred to as “Treg cells”.

Some authors have described a $CD8^+$ T cell population that prevents graft rejection by the secretion of IL-10 in mice (Reibke et al., 2006) and a $CD8^+CD28^-$ population that suppresses cytokine production by $CD4^+$ T cells *in vitro* and renders CD28-deficient mice susceptible to experimental autoimmune encephalomyelitis (EAE) upon depletion (Najafian et al., 2003). Furthermore, regulatory populations that are not T cells have been described such as regulatory B cells (Mauri and Ehrenstein, 2008), $CD4^-CD8^-$ double negative T cells (Zhang et al., 2000), natural killer T (NKT) cells (Monteiro et al., 2010) and γδ T cells (Hayday and Tigelaar, 2003).

1.5.1 Regulatory T cells

Based on the seminal work of Sakaguchi and colleagues, Treg cells were first described in mice as $CD4^+CD25^+$ cells (Baecher-Allan et al., 2001, Dieckmann et al., 2001, Jonuleit et al., 2001, Ng et al., 2001, Levings et al., 2002, Taams et al., 2002). Treg cells represent 5-10% of $CD4^+$ T cells in humans and mice (Baecher-Allan et al., 2001, Wing et al., 2002). Later, the Foxp3 transcription factor was identified as the master regulator of Treg cells, since Foxp3-mutant scurfy mice and Foxp3-null mice are devoid of Treg cells, leading to lethal autoimmune syndrome that can be prevented by adoptive transfer of Treg cells (Fontenot et al., 2003, Hori et al., 2003, Khattri et al., 2003). Similarly in

humans, Foxp3 mutations were identified as the cause of human immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (Wildin et al., 2001). Notably, Roncador and colleagues analysed the expression of Foxp3 in $CD4^+CD25^+$ Treg cells in humans and found a direct correlation between CD25 and Foxp3 expression (Roncador et al., 2005).

1.5.1.1 Regulatory T cell subpopulations

There are two main Treg cell populations, tTreg cells and pTreg cells, which exhibit similar phenotypes and are capable of suppressing effector cells (Curotto de Lafaille and Lafaille, 2009); yet differ in stability and epigenetic modifications. The terms tTreg for thymus-derived Treg cells, pTreg for peripherally derived Treg cells and iTreg for induced Treg cells expanded *in vitro* have been used in this thesis based on the new Treg cell nomenclatures agreed at the Third International Conference on Regulatory T cells and Th subsets and clinical applications in human diseases recently held in Shanghai, China (Abbas et al., 2013).

tTreg cells differentiate in the thymus and are able to recognise self-peptides, thus their importance in preventing autoimmunity (Curotto de Lafaille and Lafaille, 2009). Although a proportion of tTreg cells expressing lower levels of Foxp3 are able to switch to an effector phenotype under certain conditions of inflammation, those cells with the highest intensity of expression of Foxp3 are stable after several divisions (Yang et al., 2008, Komatsu et al., 2009). Subsequent studies have shown that this stability is due to Foxp3 demethylation, characteristic of tTreg cells (Polansky et al., 2010). Notably, recent data has demonstrated that the genes for cytotoxic T-lymphocyte antigen-4 (CTLA-4) and Eomes are also demethylated in tTreg cells (Ohkura et al., 2012).

pTreg cells differentiate in the periphery from naive $CD4^+$ T cells. The importance of pTreg cells has been observed during inflammation and in tolerogenic settings. For instance, oral administration of antigens can lead to the development of pTreg cells that display suppressive function in a mouse

model of asthma (Mucida et al., 2005, Curotto de Lafaille et al., 2008). Also, it has been reported that the commensal microbiota directs the development of pTreg cells to induce mucosal tolerance (Round and Mazmanian, 2010, Atarashi et al., 2011, Geuking et al., 2011). Notably, the presence of pTreg cells has been detected in conditions of chronic inflammation such as asthma, colitis (Curotto de Lafaille et al., 2008, Haribhai et al., 2011) and infections with intestinal parasites (Grainger et al., 2010). In the presence of TGF-β and IL-2 (Chen et al., 2003), pTreg cells express Foxp3 and acquire a regulatory phenotype; however, pTreg cells are not as stable as tTreg cells. Upon restimulation in the absence of TGF-β pTreg cells lose Foxp3 expression and their suppressive capacity (Floess et al., 2007). This can be explained by methylation of the Foxp3 promoter (Polansky et al., 2010), which correlates with the lack of stability of these cells.

1.5.1.2 Phenotype of regulatory T cells

Currently, Treg cells are characterised as $CD4^{+}CD25^{high}Foxp3^{high}$ since it has been found that only ~1-2% of $CD25^{+}$ cells with the highest CD25 (Baecher-Allan et al., 2001) and Foxp3 (Gavin et al., 2006, Allan et al., 2007) expression display significant suppressive capacity. Furthermore, Liu and colleagues demonstrated that low expression of the IL-7 receptor α-chain (CD127) directly correlates with high Foxp3 expression (>90%), thus identifying resting Treg cells as $CD4^{+}CD25^{high}CD127^{low}$ without requiring intracellular staining for Foxp3 (Liu et al., 2006). However, upon activation, these markers cannot be used to characterise Treg cells due to upregulation of CD25 and downregulation of CD127 on conventional $CD4^{+}$ T cells (herein referred to as Tcon cells) that have no regulatory function (Mazzucchelli and Durum, 2007). Moreover, other molecules involved in activation, memory, trafficking and function of Treg cells have also been used to define the phenotype of Treg cells providing a more precise characterisation (Sakaguchi et al., 2010). These include CTLA-4 and the latency-associated peptide (LAP) for Treg cell function; L-selectin (CD62L), the chemokines C-C receptors (CCR) 4, 6, and 7, and lack of α4 integrin for trafficking; and CD45RA for naivety.

1.5.2 Regulatory T cell-mediated mechanisms of suppression

Treg cells have been shown to affect or inhibit functions of immune cells such $CD4^+$ and $CD8^+$ T cells (Thornton and Shevach, 2000, Trzonkowski et al., 2004), B cells (Lim et al., 2005), NK cells (Ghiringhelli et al., 2005), DCs (Fallarino et al., 2003), mast cells (Gri et al., 2008) and NKT cells (Hua et al., 2011). Upon stimulation, Treg cells can suppress the proliferation of effector cells with different antigen specificities (Takahashi et al., 1998, Thornton and Shevach, 1998, Tarbell et al., 2004, Yu et al., 2005). Furthermore, Treg cells can suppress target cells directly or indirectly by bystander suppression via interaction with APCs.

1.5.2.1 Direct mechanisms of suppression

There are currently three mechanisms of suppression by which Treg cells can directly suppress the functions of target cells. These include the release of suppressive cytokines, lysis of target cells or IL-2 deprivation.

Treg cells are able to inhibit cell proliferation by the release of suppressive cytokines such as IL-10, TGF-β and IL-35 or expression of galectin-1 (**Figure 1.7A**). Notably, it has also been demonstrated that TGF-β can exert suppression when bound to the membrane of Treg cells, thus requiring cell contact for suppression (Nakamura et al., 2001, Ghiringhelli et al., 2005, Garin et al., 2007). Several authors have elucidated the mechanism by which TGF-β is released from or bound to the surface of Treg cells (**Figure 1.8**). TGF-β is synthesised by Treg cells as a pro-TGF-β precursor, which consists of two 55 kD fragments. The C-terminal homodimer of the pro-TGF-β precursor corresponds to mature TGF-β whereas the N-terminal homodimer contains LAP (**Figure 1.8A**). Mature TGF-β and LAP are non-covalently bound to each other in a complex called latent TGF-β. In this complex, TGF-β is inactive since LAP prevents TGF-β from binding to its receptor (**Figure 1.8B**). Upon activation, LAP undergoes a conformational change or is degraded by proteases allowing TGF-β to bind to its receptor (Annes, 2003, ten Dijke and Arthur, 2007) (**Figure 1.8C**). Importantly, it has been found that Treg cells use a transmembrane

protein called glycoprotein A repetitions predominant (GARP) to bind TGF-β on their cell surface (**Figure 1.8D**). GARP is exclusively expressed upon Treg cell activation and it is thought to function as a receptor for the latent TGF-β complex, thus providing a mechanistic explanation of how Treg cells can bear TGF-β on their surface (Stockis et al., 2009).

Another mechanism by which Treg cells can exert suppressive function is by direct lysis of target cells. (**Figure 1.7B**). Upon *in vitro* TCR-stimulation, human Treg cells upregulate granzyme A and can lyse $CD4^+$ and $CD8^+$ T cells in a perforin-dependent, Fas-FasL independent manner (Grossman et al., 2004a, Grossman et al., 2004b). Notably, lysis of NK cells (Cao et al., 2007) and B cells (Zhao et al., 2006) by Treg cells has also been observed.

Furthermore, Treg cells can suppress effector cells by competition for exogenous IL-2 (**Figure 1.7C**). The first evidence of this mechanism was reported by Pandiyan and colleagues who demonstrated that Treg cells are characterised by the inability to produce IL-2, but in turn exhibit high efficiency binding and degradation of IL-2 via upregulation of the high-affinity IL-2Rα, thus inducing effector "starvation" (Pandiyan et al., 2007). In fact, Treg cells rapidly upregulate IL-2 receptor and at a much faster rate than Tcon cells after priming. Once activated, Tcon cells can override Treg cell-mediated suppression through high production of IL-2.

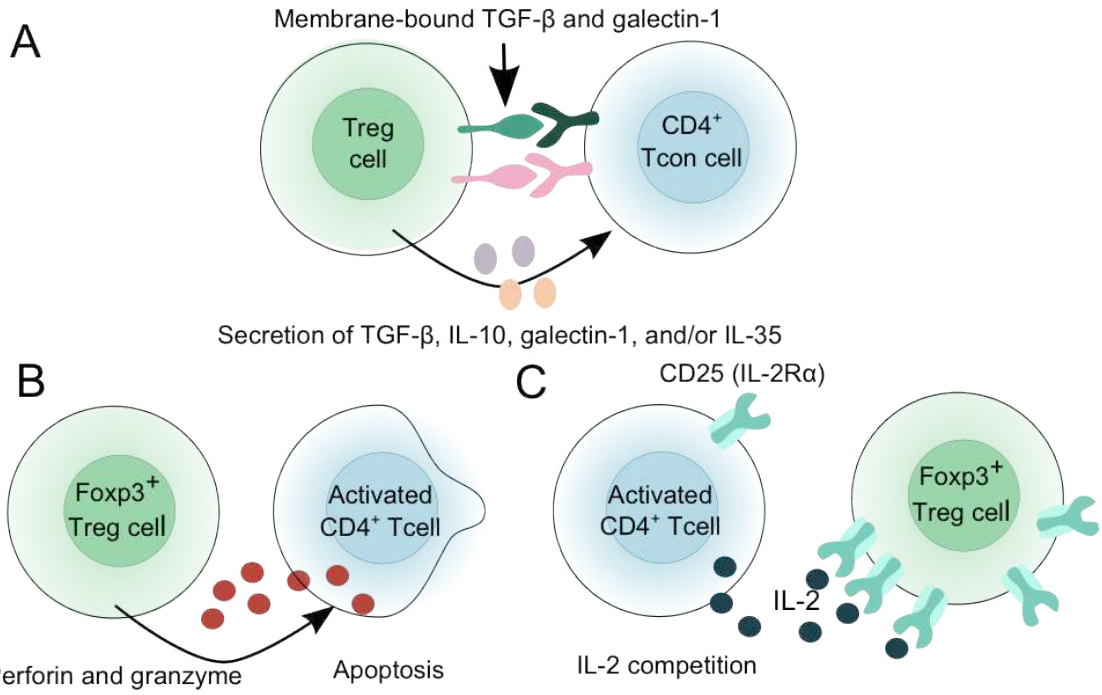


Figure 1.7: Direct mechanisms of Treg cell-mediated suppression. (A) Treg cells suppress target cells through the release of suppressive cytokines such IL-10, IL-35 and IL-10, and/or galectin-1. Galectin-1 and TGF-β are also present as membrane-bound forms. (B) Treg cells secrete perforin and granzyme and induce apoptosis of target cells. (C) IL-2 competition between Treg cells and effector cells leads to cell cycle arrest and apoptosis of target cells.

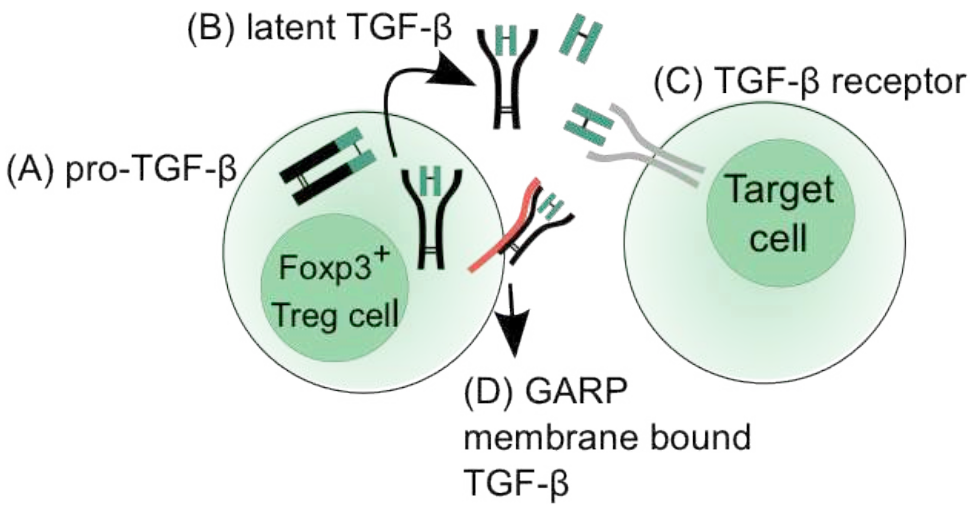


Figure 1.8: TGF-β processing by Treg cells. (A) The pro-TGF-β precursor consists of two fragments: LAP and TGF-β. (B) Mature TGF-β and LAP are non-covalently bound to each other in a complex called latent TGF-β. In this complex, TGF-β is inactive since LAP prevents TGF-β from binding to its receptor. (C) Upon Treg cell activation, the latent TGF-β complex undergoes a conformational change allowing TGF-β to bind to its receptors. (D) The membrane protein GARP binds TGF-β to the cell surface, which represents the membrane-bound form of TGF-β.

1.5.2.2 Indirect mechanisms of suppression

Treg cells can also suppress effector cells indirectly via interaction with APCs, particularly DCs, whereby Treg cells use DCs as bystander suppressors of other effector cells. There are several molecules expressed on the surface of Treg cells that are involved in this mechanism.

CTLA-4 is constitutively expressed in Treg cells and competes against the stimulatory receptor CD28 expressed in Tcon cells for CD80/CD86 ligands in APCs. Upon binding, CTLA-4 can capture CD80/CD86 and internalise them into CTLA-4 expressing cells whereby the ligands are degraded (Thornton et al., 2004a, Qureshi et al., 2011) (**Figure 1.9A**). Lymphocyte activation gene-3 (Lag-3), a CD4 homologue that binds to MHC class II molecules with high affinity, blocks DC maturation and decreases antigen presentation to Tcon cells (Huang et al., 2004) (**Figure 1.9B**). Neuropilin-1 (Nrp-1), a receptor for class III semaphorins, induces prolonged interactions of Treg cells with immature DCs, thus decreasing antigen presentation to Tcon cells (Sarris et al., 2008) (**Figure 1.9D**).

Other molecules such as CD39 (Borsellino et al., 2007), which induces the expression of the immuno-inhibitory molecule adenosine hydrolising ATP or ADP to AMP (**Figure 1.9C**), and fibrinogen-like protein 2 (FGL2), which downregulates DC functions (Shalev et al., 2008) have also been implicated in Treg cell-mediated suppression (**Figure 1.9C**).

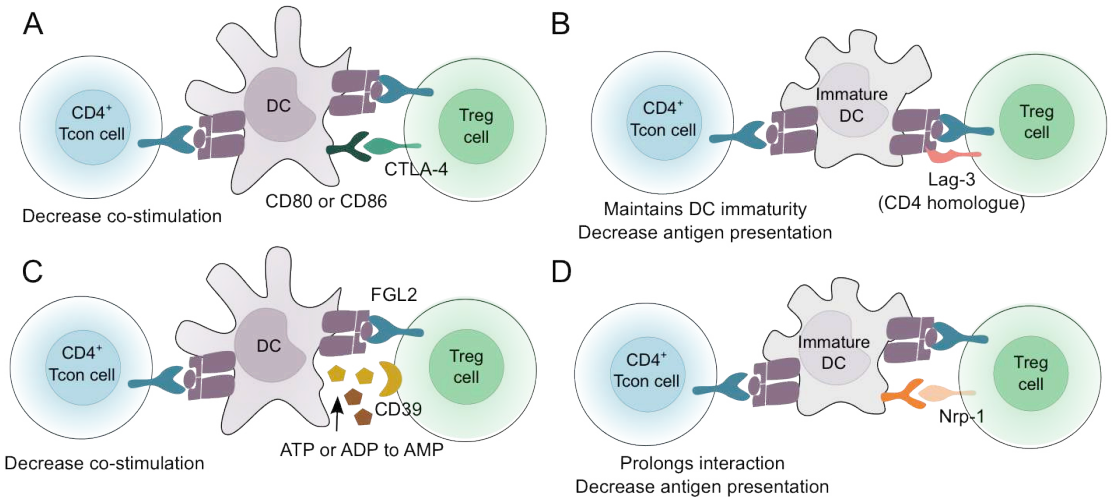


Figure 1.9: Indirect mechanisms of Treg cell-mediated suppression. (A) CTLA-4 on Treg cells bind to CD80 or CD86 expressed on DCs decreasing co-stimulation. (B) Lag-3 expressed on TCR-stimulated Treg cells binds to immature DCs maintaining their state of immaturity and decreasing antigen presentation. (C) CD39, expressed by Treg cells, degrades ATP to ADP and FGL2 dowregulates DC functions. (D) Nrp-1 binds to immature DCs prolonging interaction to Treg cells and decreasing antigen presentation.

1.6 Immunological synapse for T cells and NK cells

The concept of the immunological synapse (IS) was first defined by the studies of Norcross and colleagues in 1984. They described synapsis like organ between T cells and APCs (Norcross, 1984). To date, it is known that IS formation involves a complex orchestered sequence of events with distinct cytoskeletal requirements, that comprises the supramolecular organisation of multiple proteins crucial for cell-cell interactions (i.e. APC:T synapse and NK cells:target synapse). Interestingly, recent studies have further demonstrated that the mechanisms underlying IS formation can vary between cell types (Davis et al., 1999, Batista et al., 2001, Davis and van der Merwe, 2001).

More thorough understanding of the underlying processes of IS formation was gained by simulating T cell:APC IS interactions with a supported lipid bilayer containing agonist MHC peptides and intercellular cell adhesion molecule-1 (ICAM-1). In Th cells, peptide-MHC interactions cluster in a directed manner towards the center of the IS whereby ICAM-1 interacts with its receptor β-2 integrin lymphocyte function-associated antigen 1 (LFA-1). Then, TCR clusters surround LFA-1 clusters, which are located in the central supramolecular

activation cluster (c-SMAC). In the order of minutes, this organisation is reverted in such a way that TCR clusters into the c-SMAC drifting LFA-1 to the peripheral ring, called the peripheral supramolecular activation cluster (p-SMAC) (Monks et al., 1998, Grakoui et al., 1999). In the case of CTLs, TCR clusters are sorrounded by LFA-1 in the first stages and then LFA-1 is segregated into two domains in the c-SMAC, one cointaining the TCR and the other containing the cytolytic granules that are released to the target cell within the IS (Potter et al., 2001, Stinchcombe et al., 2001).

In NK cells, synapse formation was described later by the seminal paper of Davis and colleagues (Davis et al., 1999), which demonstrated that inhibitory KIR receptors induce clustering of HLA-C at the surface of target cells (Davis et al., 1999). In a cytolytic NK cells, SHP-1 initiallly clusters in small areas surrounded by a ring of LFA-1 which later clusters in the p-SMAC. Once the p-SMAC is organised, cytolytic granules cluster in the c-SMAC and released within the IS (Vyas et al., 2002). Notably, the interaction of LFA-1 with ICAM-1 on target cells is required for NK cell cytotoxicity (Helander and Timonen, 1998).

1.7 Lymphocyte trafficking

Lymphocyte trafficking requires the expression of adhesion molecules and chemokine receptors on lymphocytes which directs them to target sites based on a stimulus provided by the milieu (Sackstein, 2005). For example, the integrins $\alpha 4\beta 7$, L-selectin and CCR7 are adhesion molecules and chemokine receptors involved in migration to lymphoid tissues. The chemokine receptors CXCR4 and CXCR7 mediate migration to the BM and the chemokine receptors CCR5, CCR6, CXCR1 and CXCR3 are associated with migration to inflammatory sites (Campbell et al., 2001, Zhang et al., 2009). The reported expression of chemokine receptors and adhesion molecules on Treg cells and NK cells in humans and mice is summarised in **Table 1.2**.

Table 1.2: Comparative table showing expression of chemokine receptors and adhesion molecules on PB NK cells and PB Treg cells. Receptors are clustered in groups associated with lymphoid tissues, BM and inflammatory sites. (1): (Grindebacke et al., 2009), (2): (Taub et al., 1995), (3): (Luevano et al., 2012a), (4): (Redjimi et al., 2012), (5): (Zou et al., 2004), (6): (Zhang et al., 2009), (7): (Eikawa et al., 2010) (8) (Ferlazzo et al., 2004), (9) (Campbell et al., 2001). Analysis was performed on the $CD56^{dim}$ subpopulation unless specified otherwise. +: positive expression, -: negative expression, low: low expression.

Site	Receptor	Ligand	Migration	Treg cell	NK cell
Lymphoid tissues	Integrin α4	VCAM-1	Gut-associated lymphoid tissues	- (PB)[1] +(CB)[1]	+(PB)[2]
	Integrin β7	MadCAM-1	Gut-associated lymphoid tissues	- (PB)[1] +(CB)[1]	Low (PB/CB) [3]
	L-selectin (CD62L)	GlyCAM-1, MadCAM-1, PSGL-1	SLT	+(PB)[1] +(CB)[1]	+, low (PB/CB) [3]
	CCR7	CCL21	SLT	+(PB)[1] +(CB)[1]	+ (PB) only $CD56^{bright}$ NK cells [8]
BM	CXCR4	CXCL12-SDF1	BM recruitment	+(PB)[5]	+ (CB/PB) [3]
	CXCR7	CXCL11, 12	BM recruitment Inflammatory sites	N/D	Low (CB/PB)[3]
Inflammatory sites	CCR5	CCL3-4	Inflammatory sites tumour sites	+(mice)[6]	+(PB) only $CD56^{bright}$ NK cells[9]
	CCR6	MIP-3α	Skin homing	+(mice)[6]	- (CB/PB) [3]
	CXCR1/ IL8-RA	CXCL6 CXCL8	Inflammatory sites	+(PB) [7]	+ (CB/PB) [3]
	CXCR3	CXCL9, 10, 11	Inflammatory sites Involved in integrin activation and cytoskeletal changes Tumour sites	+(mice)[4]	Low (PB) ($CD56^{dim}$ NK cells) + (PB) ($CD56^{bright}$ NK cells)[9]

1.8 Interaction between natural killer cells and regulatory T cells

1.1.1 Regulation of natural killer cell functions by regulatory T cells

The effect of Treg cells on NK cell effector functions such as cytotoxicity, cytokine production and proliferation has been reviewed by us and others (Ralainirina et al., 2006, Zimmer et al., 2008, Pedroza-Pacheco et al., 2013). The mechanisms and conditions under which Treg cells suppress NK cells are summarised in **Table 1.3**. Collectively, these reports agree that Treg cells can

exert suppressive functions (i) in the absence of cytokines, (ii) through expression of membrane bound TGF-β, and (ii) with a minimum ratio between Treg cells and NK cells of 1:5 (**Figure 1.10A**). Notably, Treg cell-mediated suppression of NK cells is reverted in the presence of cytokines such as IL-2, IL-4, IL-7 and supraphysiological doses of IL-12 (**Figure 1.10B**).

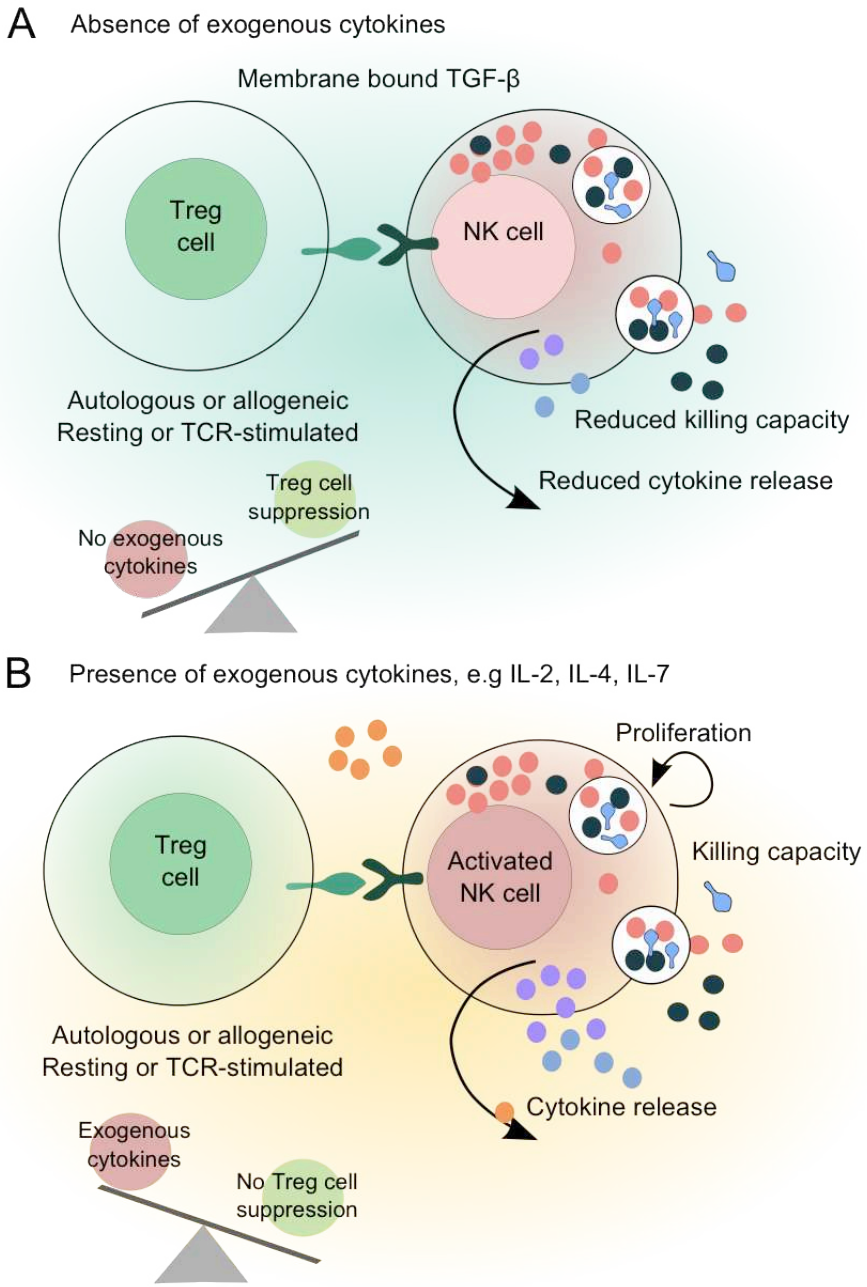


Figure 1.10: Mechanism of suppression of NK cells by Treg cells. (A) Resting Treg cells suppress autologous and allogeneic NK cells through membrane bound TGF-β under resting conditions or when Treg cells are activated with anti-CD3/anti-CD28 and IL-2 or APCs in the absence of cytokines. (B) NK cells can overcome Treg cell-mediated suppression in the presence of exogenous cytokines such as IL-2, IL-4 and IL-7.

Table 1.3: Effects of Treg cells on NK cell functions in humans and mice. H: humans, M: mouse, Allo: allogeneic, Auto: autologous. Adapted by permission from Macmillan Publishers Ltd: [Cellular and Molecular Immunology] (Pedroza-Pacheco et al., 2013), copyright 2013.

NK cell function	Observations	H/M	NK:T	Type of Treg cells		Mechanism of suppression
Natural cytotoxicity	Inhibited in resting NK cells (Ghiringhelli et al., 2005)	H	1:1	Resting tTreg cells	Allo	Membrane-bound TGF-β
	Not inhibited in resting NK cells if IL-2Rγ-chain specific cytokines are present (Ghiringhelli et al., 2005)	H	1:1	Resting tTreg cells	Allo	N/A
	Inhibited in resting NK cells the presence of APCs (Trzonkowski et al., 2004)	H	1:1	TCR-stimulated tTreg cells	Auto	Not IL-10 mediated
	Inhibited in resting NK cells (Smyth et al., 2006, Lundqvist et al., 2009)	M	1:1	TCR-stimulated tTreg cells	Auto	Membrane-bound TGF-β
	Inhibited in IL-12 activated NK cells (Zhou et al., 2010)	M	1:1	TCR-stimulated iTreg cells	Auto	Membrane-bound TGF-β
	Inhibited in resting NK cells (Sun et al., 2010)	M	1:1	TCR-stimulated tTreg cells	Allo	CD39
	Enhanced in resting and IL-2 activated NK cells in the presence of APCs (Bergmann et al., 2011)	H	1:2	Tumour-specific TCR-stimulated iTreg cells	Auto	Cell-cell contact
Cytokine production	Inhibited if IL-12 present (Ghiringhelli et al., 2005)	H	1:1	Resting tTreg cells	Allo	Membrane-bound TGF-β
	Not inhibited if IL-2/IL-15 present (Ghiringhelli et al., 2005)	H	1:1	Resting tTreg cells	Allo	N/A
	Inhibited in NK cells in the presence of APCs (Trzonkowski et al., 2004)	H	1:1	TCR-stimulated tTreg	Auto	Not IL-10 mediated
	Inhibited in IL-12 activated NK cells (Zhou et al., 2010)	M	1:1	TCR-stimulated iTreg	Auto	Membrane-bound TGF-β
	Inhibited if IL-2 present (Bergmann et al., 2011)	H	1:2	Tumour-specific TCR-stimulated iTreg	Auto	Cell-cell contact
Activating receptors	NKG2D downregulation in resting NK cells (resting Treg cells)(Ghiringhelli et al., 2005)	H	1:1	Resting tTreg cells/ TCR-stimulated tTreg	Allo	Membrane-bound TGF-β
	NKG2D downregulation in resting NK cells (TCR-stimulated Treg cells) (Ghiringhelli et al., 2005)	M	1:1	Resting tTreg cells/ TCR-stimulated tTreg	Allo	Membrane-bound TGF-β
	NKG2D and NKp44 downregulation in IL-2 activated NK cells (Bergmann et al., 2011)	H	1:2	Tumour-specific TCR-stimulated iTreg cells	Auto	Cell-cell contact
Proliferation	Decreased NK cell proliferation enhanced by Tcon cells in presence of APCs (Romagnani et al., 2005)	H	1:1	iTreg and tTreg cells	Auto	N/D

1.8.1 Natural killer cells control regulatory T cell responses

The involvement of NK cells in the control of adaptive immune responses has been observed in chronic viral infections (Welsh and Waggoner, 2013) and infections with *Mycobacterium tuberculosis* (Roy et al., 2008). After infection with LCMV, NK cells kill activated LCMV-specific $CD4^+$ T cells, thus preventing the antiviral $CD4^+$ T cell response (Waggoner et al., 2012) (**Figure 1.11A**).

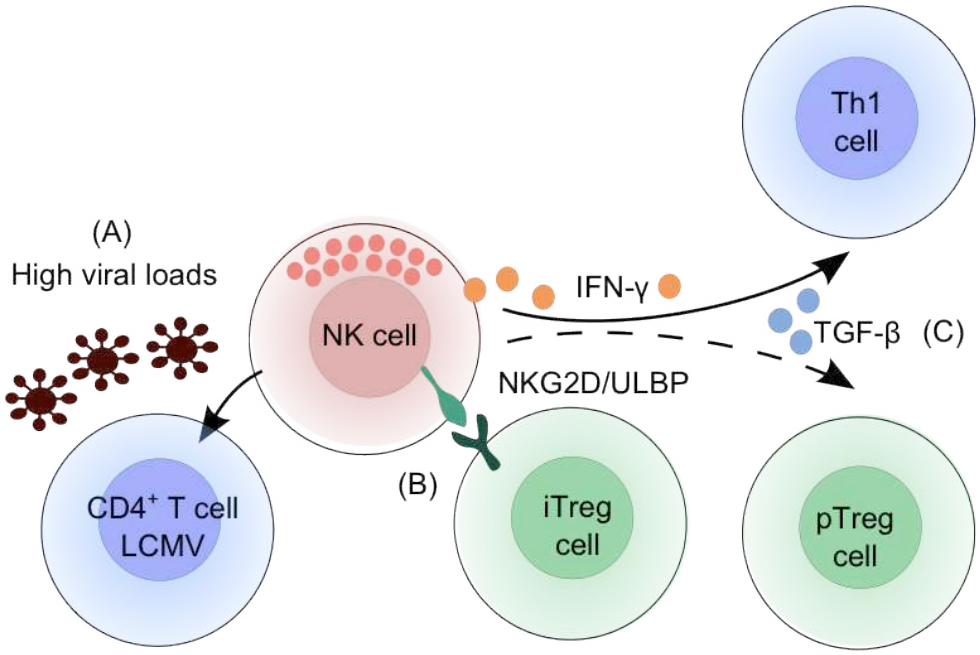


Figure 1.11: NK cells control the adaptive immune response. (A) NK cells kill activated LCMV-infected $CD4^+$ T cells at high viral loads in order to prevent exaggerated $CD4^+$ T cell responses. (B) NK cells inhibit iTreg cell proliferation by the engagement of NKG2D with its cognate ligand ULBP1. (C) NK cells block the proliferation of pTreg cells by secretion of IFN-γ, regardless of the presence of TGF-β, and skew towards Th1 polarisation.

The impact of NK cells on Treg cells has also been studied. Roy and colleagues reported that the presence of NK cells reduced proliferation of pTreg cells but not of tTreg cells in the context of microbial infection (Roy et al., 2008). This effect was reported to be NKG2D mediated (**Figure 1.11B**). Similarly, Brillard and colleagues reported the efficacy of autologous IL-2 activated NK cells to block pTreg cell differentiation (2:1 NK:pTreg cell ratio) in humans and mice via high levels of IFN-γ, polarising T cells towards a Th1 response even in the presence of soluble TGF-β (Brillard et al., 2007) (**Figure 1.11C**). Furthermore,

Chin and colleagues suggested that NK cell depletion led to a significant expansion of tTreg cells favoured by an environment with low levels of IL-6 and high levels of TGF-β in an induced tumour model in mice (Chin et al., 2010). Collectively, these observations demonstrate the capacity of NK cells to control Treg cells under certain conditions.

1.9 Interaction between natural killer cells and regulatory T cells in healthy and pathological conditions

1.9.1 Pregnancy

There is accumulating evidence that the interaction between NK cells and Treg cells is beneficial during pregnancy. This may be due to the requirement for an immunosuppressive environment for the successful implantation of the embryo and tolerance of the embryo by the mother. The uterine endometrium, also called the decidua, is crucial for the development of placental vasculature. Interestingly, 70% of all human decidual lymphocytes are NK cells, defined as uterine or decidual NK (uNK) cells (King et al., 1998). In comparison to PB NK cells, uNK cells are characterised as $CD56^{bright}CD16^{-}CD3^{-}$ cells that express KIR and exhibit low killing capacity despite the fact that they have cytolytic granules (Moffett-King, 2002). Notably, Treg cells are also abundant in the decidua, with a higher frequency of fully functional $CD4^{+}CD25^{bright}$ Treg cells observed in pregnant women as compared to non-pregnant women (Sasaki et al., 2004).

Sasaki and colleagues confirmed the importance of Treg cells in pregnancy by showing reduced frequency of Treg cells in women who have had a spontaneous abortion in comparison to healthy pregnant women (Sasaki et al., 2004). Hsu and colleagues did not observe any difference in the frequency of $CD4^{+}Foxp3^{+}$ Treg cells between healthy pregnant women and women with preeclampsia (Hsu et al., 2012). However, they did observe an impaired systemic expansion of pTreg cells, causing a difference in Treg cell composition.

1.9.2 Cancer

In cancer, NK cells play a crucial role in disease clearance whereas Treg cells are associated with tumour escape; however the interaction between these two cell types in cancer has not yet been investigated. As widely reviewed by Orentas and colleagues, a detectable increase in Treg cell number is observed in various types of cancer where the number of Treg cells inversely correlates with the frequency and function of NK cells (Orentas et al., 2006). In gastrointestinal stromal tumour-bearing (GIST) patients, Ghiringhelli and colleagues detected high Treg cell numbers accompanied with low numbers of NK cells, which exhibited impaired functions (Ghiringhelli et al., 2005). Similar results were observed in colon (Doubrovina et al., 2003) and prostate carcinoma patients (Wu, 2004) whereby downregulation of expression of the activating receptor NKG2D was detected in NK cells in the presence of high levels of TGF-β, possibly secreted by the tumour. Consistently, Betts and colleagues analysed Treg cells before and twelve months after tumour excision in a cohort of patients with colorectal cancer (Betts et al., 2012). The authors observed higher levels of Foxp3 in $CD4^{+}CD25^{high}$ Treg cells in cancer patients compared to healthy controls and showed that after tumour excision Foxp3 levels in Treg cells returned to normal. Moreover, Cai and colleagues showed functional impairment of circulating and intrahepatic NK cells in hepatocellular carcinoma (HCC) patients (Cai et al., 2008). Interestingly, significant reductions in NK cell numbers were observed in tumour regions compared to non-tumour regions in the liver. Moreover, PB NK cells from these patients exhibited reduced killing capacity against K562 target cells and reduced IFN-γ secretion *in vitro,* which was further correlated with a high incidence of $CD4^{+}CD25^{+}$ Treg cells. The addition of Treg cells isolated from HCC patients efficiently inhibited the anti-tumour capacity of autologous NK cells *in vitro*. In fact, further investigations showed that elevated Treg cell numbers are associated with elevated levels of TGF-β (Moo-Young et al., 2009), a mechanism of tumour escape.

1.9.2.1 Regulatory T cell depletion in cancer

Because Treg cells suppress key effector cells of the anti-cancer immune response, many groups focused on depleting them before or after treatment. **Table 1.4** summarises some of the relevant work that has been performed in mice and humans. These studies clearly suggest that the depletion of Treg cells can lead to increased NK cell function and proliferation, which can be further enhanced by IL-2 and possibly IL-15 therapy, although the latter has yet to be investigated.

Table 1.4: Effects of Treg cell depletion in human and mouse cancer. Adapted from (Pedroza-Pacheco et al., 2013). H: humans, M: mice.

NK cell function	Effect	H/M
Natural Cytotoxicity	Increased (2-fold) *(ex-vivo)* (Shimizu et al., 1999, Ghiringhelli et al., 2005)	M
	No effect with CD25 depletion alone, but effect for CD25 depletion + IL-2 (long term-assays) (Hallett et al., 2008)	M
	Enhanced in metastatic lymph node (*ex-vivo*) (Ghiringhelli et al., 2005)	H
	Enhanced in tumour kidney carcinoma *(ex-vivo)* (Ghiringhelli et al., 2005)	H
Proliferation	Increased in spleen *(ex-vivo)* (Ghiringhelli et al., 2005)	M
Tumour clearance	Decreased tumour size (Shimizu et al., 1999, Smyth et al., 2006, Hyka-Nouspikel et al., 2007, Sun et al., 2010)	M
	Increased number of tumour-free mice (Simon et al., 2007)	M
Graft rejection	Increased BM rejection (Barao et al., 2006)	M
Survival	Increased survival when IL-2 infused (Hallett et al., 2008)	M
Tumour regression	Promote tumour regression with the addition of IL-2 (Kottke et al., 2008)	M
Disease remission	Patient skewed towards Graft versus Leukaemia (GvL) instead of Graft versus Host Disease (GvHD) (Maury et al., 2010)	H

1.9.3 T cell regulation of natural killer cells: a third mechanism of natural killer cell tolerance?

In order to prevent spontaneous activation, Treg cells regulate NK cells directly or indirectly via Tcon cells and by cytokine deprivation (IL-2) under homeostatic conditions; hence limiting cytokine availability and thus controlling NK cell activation. This suppression can be reverted by upregulation of CD25 (IL-2 receptor α chain) on NK cells. In particular, IL-12 and IL-18, secreted by macrophages or DCs during inflammation, can upregulate CD25 on NK cells

(Lee et al., 2012) allowing NK cells to compete with Treg cells for IL-2. Other cytokines described to override Treg cell-mediated suppression are IL-4 and IL-7 (Ghiringhelli et al., 2005); however it is currently unknown whether these cytokines can upregulate CD25 on NK cells. These findings highlight the importance of CD25 expression on NK cells as a mechanism to regulate NK cell activation. Two reports confirmed these hypotheses *in vivo* (Gasteiger et al., 2013a, Gasteiger et al., 2013b, Sitrin et al., 2013).

Sitrin and colleagues studied the interaction between NK cells and Treg cells and the importance of IL-2 for Treg cell-mediated NK cell suppression in a BDC2.5/NOD mouse model of type 1 diabetes (Sitrin et al., 2013). This model is ideal of addressing this question since it is characterised by limited IL-2 production by $CD4^+$ T cells in these mice and close proximity between Treg cells and NK cells in the pancreatic islets of Langerhans. Their results confirmed that IL-2 availability is an important mechanism of NK cell regulation mediated by Treg cells. In mice depleted of Treg cells, NK cells upregulate genes involved in NK cell proliferation, cytokine secretion, cytotoxicity and IL-2/Stat5 signalling but downregulate expression of genes involved in TGF-β signalling. These findings suggest that TGF-β or IL-2 consumption is a mechanism of NK cell regulation by Treg cells. However treatment with IL-2 agonists, but not TGF-β, recapitulates the observed effect confirming that IL-2 is the mechanism by which Treg cells regulate NK cells.

In addition, Gasteiger and colleagues showed similar results using $Foxp3^{DTR}$ mice, in which Treg cells can be depleted using diphtheria toxin (Gasteiger et al., 2013b). NK cell cytotoxicity was increased upon depletion of Treg cells, and was decreased by neutralisation with IL-2 mAb. Furthermore, they also found that Treg cells can indirectly regulate NK cells by inhibition of activated $CD4^+$ T cells, as it has been previously described that activated $CD4^+$ T cells can enhance NK cell functions via IL-2 (Fehniger et al., 2003, Bergmann et al., 2011). Collectively, these reports suggest that Treg cells can directly or indirectly regulate NK cells via IL-2 by controlling $CD4^+$ T cells and that upregulation of CD25 on NK cells upon activation with IL-12 and/or IL-18 breaks this suppression. Notably, this mechanism of tolerance has been previously

observed for Treg cell-mediated suppression of $CD4^+$ T cells (Pandiyan et al., 2007, Josefowicz et al., 2012) (**Figure 1.12**).

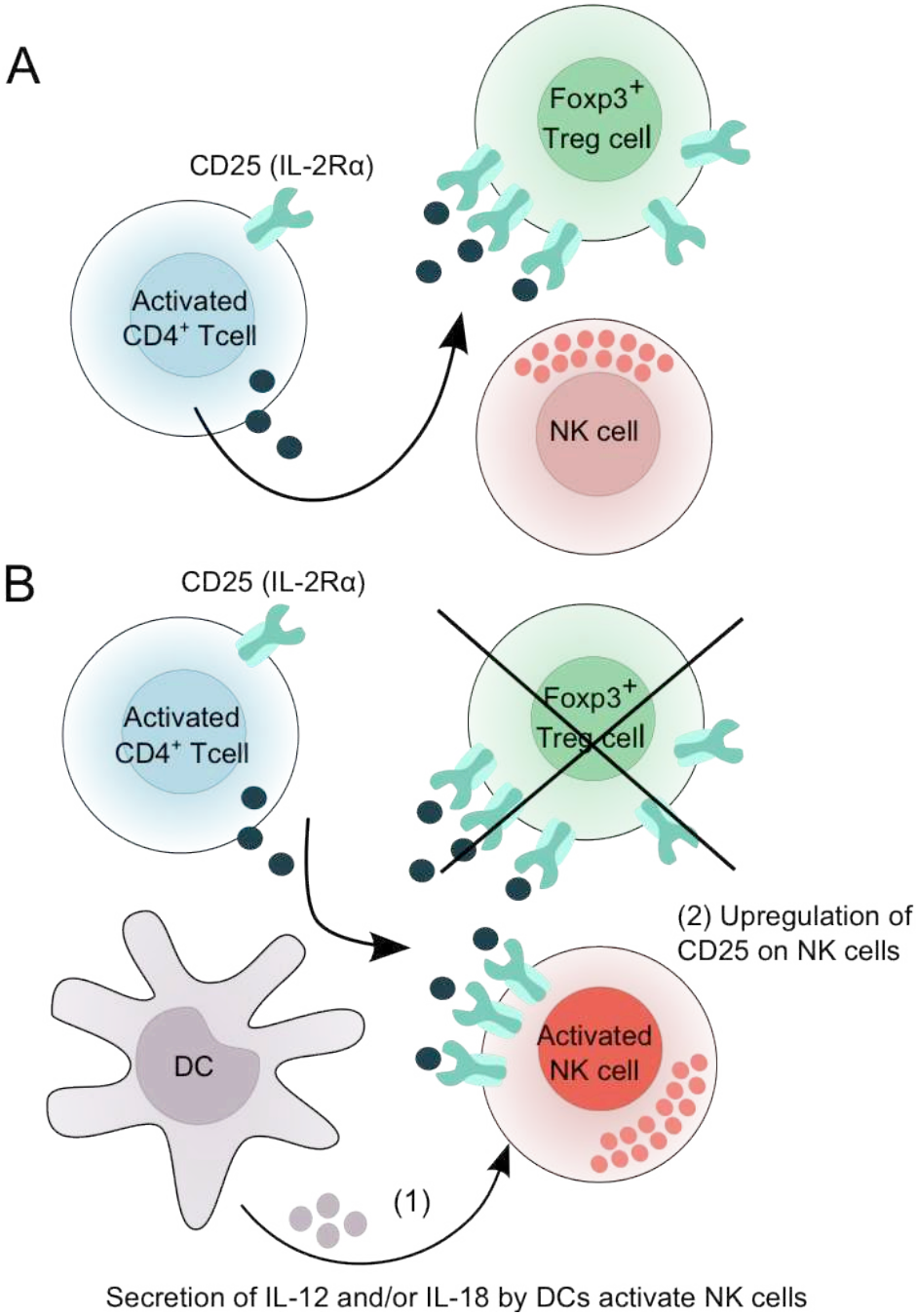


Figure 1.12: Treg cells regulate NK cells via IL-2. (A) CD4 T cells enhance NK cell function by the secretion of IL-2 and Treg cells deprive NK cells of IL-2 to regulate NK cell function. (B) Upon Treg cell depletion, this effect is reverted.

1.10 Haematopoietic stem cell transplantation

1.10.1 Source of haematopoietic stem cells

Haematopoietic stem cell transplantation (HSCT) has been used since the late 1950's to treat haematological malignancies such as leukaemia, anaemia, lymphoma and myeloma (Thomas et al., 1957, Copelan, 2006) and autoimmune diseases such as MS, systemic lupus erythematosus and rheumatoid arthritis (Passweg and Tyndall, 2007). HSC are multipotent cells capable of self-renewal and differentiation into myeloid, lymphoid and erythromegakaryocytic lineages (Copelan, 2006). HSC can be isolated from three different sources: BM, granulocyte colony-stimulating factor (G-CSF) mobilised PB or CB. The donor can be an HLA identical or haploidentical sibling, matched unrelated donor or mismatched donor (Copelan, 2006). Also, HSC can be obtained from the patient and infused after treatment; this is referred to as autologous HSCT. Notably, the choice of the HSC source depends on the type and stage of the disease to treat, age of the patient (adult or paediatric) and availability of HSC.

1.10.1.1 Advantages and disadvantages of umbilical cord blood transplantation

The first CB transplant was successfully performed on a five year-old patient with Fanconi's anaemia in 1988 (Gluckman et al., 1989). The umbilical cord develops from the yolk sac and replaces it by the fifth week of foetal development becoming the source of nutrients for the foetus (Sadler, 2010). The main advantage of CB as compared to other HSC sources is the ease of procurement, the safety for mothers and donors, the reduced likelihood of transmitting infections such as CMV, and the ability to use cryopreserved samples. Currently, more than 30 000 transplants have been reported and more than half a million units have been stored worldwide (Welte et al., 2010, Ballen et al., 2013). Moreover, cord blood transplantation (CBT) is characterised by high overall survival, higher supply rate and higher likelihood of finding a donor compared to BM transplantation (BMT) (70% probability of finding a matched

CB unit in the UK; 2009) (Laughlin et al., 2004, Rocha et al., 2004, Querol et al., 2009). In addition, the requirement for HLA matching is less stringent for CBT than for BMT, ranging between 4-6/6 HLA match (Gluckman, 2012). Notably, CBT is characterised by reduced severity and occurrence of GvHD (Takahashi et al., 2004). However, delayed immune reconstitution (Niehues et al., 2001, Laughlin et al., 2004, Komanduri et al., 2007) and higher risk of infection are the main disadvantages of CBT (Sauter et al., 2011). Another drawback is the requirement of a minimum cell dose of 3 x 10^7 total nucleated cells (TNC)/kg for transplantation, which is why CBT is mostly used for paediatric patients (Ballen et al., 2013). Currently the use of two CB units to achieve the required cell dose has allowed CB to be used for transplantation in adult patients (Barker et al., 2005, Brunstein et al., 2011a). Other approaches to augment cell doses include the use of expansion techniques in which HSC from CB are cultured with cytokines or stromal cells (Delaney et al., 2010), or the delivery of CB cells via intra-BM injection which has been associated with better engraftment and lower incidence of GvHD (Davies et al., 2008, Frassoni et al., 2010).

1.10.2 Complications after haematopoietic stem cell transplantation

Major progresses have been made since the first transplant was performed to minimise the likelihood of graft failure since the first transplant was performed. For example the introduction of reduced intensity conditioning (RIC) regimes prior to HSCT decreases tissue damages (Gomez-Almaguer et al., 2008, Schub et al., 2011, Gratwohl and Carreras, 2012). However, delayed immune reconstitution, risk of infection and GvHD are still complications for transplanted patients.

1.10.2.1 Delayed immune reconstitution

A main concern after transplantation is the profound and long-lasting immunodeficiency, which can lead to severe post-transplant infections, relapse and secondary malignancies (Mackall et al., 2009). There are several factors that determine the timing of immune reconstitution, such as the HSC source and the conditioning regimen used. For instance, the addition of T cell depleting

agents (ATG, Campath) to reduce GvHD has been associated with delayed immune reconstitution.

A study by Davies and colleagues using a cohort of 24 BM-transplanted patients showed that normal levels of NK cells, B cells and T cells are reached within the first three months post-transplantation (Davies et al., 2008). Similarly, in patients with haploidentical HSCT, $CD8^+$ T cells and NK cells also reconstitute within the first three months post-transplantation, but B cells and $CD4^+$ T cells take up to six and eighteen months to recover respectively (Jacobson et al., 2012). Moreover, CBT recipients are reported to experience long-lasting post-transplant deficiency in adaptive immunity but early recovery of NK cells. Komanduri and colleagues analysed immune reconstitution in 32 CBT patients under RIC conditioning regimens and observed late T cell recovery of up to one year but early reconstitution of both NK cells and B cells in the first months after transplantation (Komanduri et al., 2007) (**Figure 1.13**). Similar levels of immune reconstitution have been observed in a cohort of 42 patients who received double CBT (Jacobson et al., 2012). Overall, the prolonged immune reconstitution observed, particularly in CBT, leads to higher susceptibility to infection.

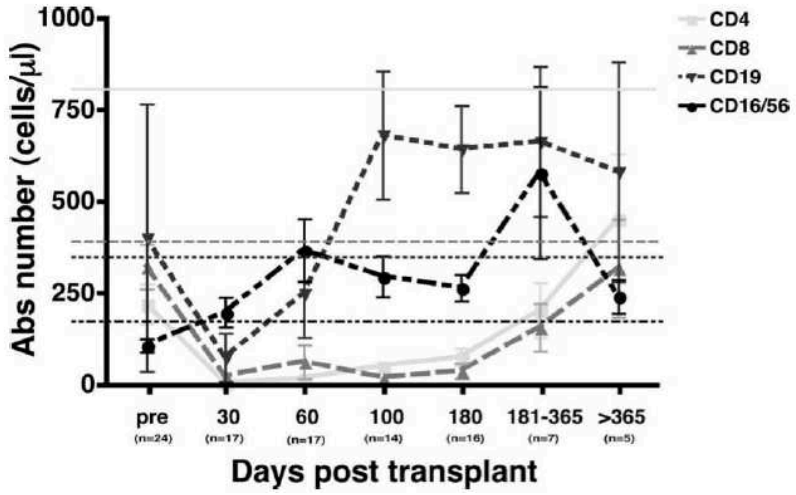


Figure 1.13: Immune reconstitution post-CBT. Cell count of T cells ($CD3^+CD4^+$ and $CD3^+CD8^+$), B cells ($CD19^+$) and NK cells ($CD16^+$ and/or $CD56^+$) post-CBT. Patients underwent RIC conditioning. Horizontal lines depict normal values obtained from a healthy HSC transplant donor. Figure from (Komanduri et al., 2007).

1.10.2.2 Risk of infection

Infections are one of the main causes of mortality post-HSCT. The degree of susceptibility to infection depends on various factors such as the type of transplantation, source of HSC, conditioning regimen used, degree of histocompatibility and type of GvHD prophylaxis used such as T cell depleting agents, which render patients susceptible to a number of opportunistic infections (Junghanss et al., 2002). During the first 30 days post-HSCT, patients undergo a phase of neutropenia. At this stage, gram-negative and gram-positive bacteria, Candida spp. and Herpes Simplex Virus cause the principal infections observed. Once engraftment is established, there is a high incidence of CMV, Adenovirus and Epstein Barr Virus infection (from 30-100 days) and finally after 100 days, occurrence of Varicella Zoster Virus and Pneumocystitis jiroveci are most common (Ninin et al., 2001, Tomblyn et al., 2009). The importance of NK cells in the immune response to infections in the context of HSCT was first observed in transplanted patients with decreased NK cell function, since they exhibited exacerbated viral infections (Quinnan et al., 1982). Notably, other authors have demonstrated that KIR2DS2 and KIR2DS4 expression is associated with reduced CMV reactivation in haploidentical donors (Zaia et al., 2009, Gallez-Hawkins et al., 2011) and that a correlation exists between a higher number of KIR activating receptors present on NK cells and a lower susceptibility to CMV reactivation (Sobecks et al., 2011).

1.10.2.3 Graft versus Host Disease

GvHD is one of the main challenges after HSCT causing high incidence of mortality. It involves the allogeneic recognition of recipient tissues by donor T cells caused by HLA mismatches between the donor and the patient (Ferrara et al., 2009), or in the case of fully matched transplants, GvHD is caused by minor histocompatibility antigens (mHAgs) (Voogt et al., 1988). According to the National Institute of Health classification, GvHD can be subdivided into acute GvHD (aGvHD) that occurs within the first 100 days post-transplantation and chronic GvHD (cGvHD) that occurs after 100 days (Filipovich et al., 2005, Griffith et al., 2008).

1.10.2.3.1 Minor histocompatibility antigens

Following HLA-matched HSCT, peptides that arise from polymorphic self-proteins can direct donor T cell immune responses. These polymorphic peptides are designated as mHAgs and are expressed on the cell surface of HLA class I or class II molecules (Goulmy, 1996). mHAgs can be encoded by sex chromosomes, autosomes, or mitochondrial DNA. The first clinical results demonstrated the relevance of mHAgs in GvHD showing a direct correlation of the minor HA-1 antigen mismatch with severity of GvHD. (Goulmy et al., 1996). Furthermore, in a cohort of T cell depleted HLA-matched HSCT patients, mismatches for the ubiquitous Y chromosome-derived mhAgs resulted in higher incidence of GvHD as compared to autosomal-derived mHAgs (Hobo et al., 2013).

1.10.2.3.2 Pathways of allorecognition

To date, three main pathways of allogeneic recognition have been described. The “direct pathway” describes the recognition of alloantigens presented by recipient APCs by donor T cells (**Figure 1.14A**). There are currently two theories to explain the molecular mechanisms behind this pathway. The “high determinant density” model (Smith et al., 1997) identifies alloantigens as the primary source of allorecognition. This model assumes that every MHC molecule serves as a ligand, hence providing high cell surface antigen density as compared to the density of the complex of a specific peptide with a MHC molecule. On the other hand, “the multiple binary complex” model considers the peptide as the main source of allorecognition. This model predicts that different bound peptides will be recognised by different alloreactive T cells, thus stimulating a large number of T cells (Matzinger and Bevan, 1977). The “indirect pathway” describes how donor T cells recognise recipient alloantigens presented by donor APCs (**Figure 1.14B**). Herrera and colleagues described a third model of allorecognition whereby donor APCs are able to uptake intact recipient MHC/peptide complexes from recipient APCs and present them to donor T cells. This is referred to as the “semi-direct pathway” or “cross-

dressing" (Herrera et al., 2004) (Smyth et al., 2008) (**Figure 1.14C**). As a result of this transfer, a single donor APCs is able to present allopeptides via self-MHC class II molecules to $CD4^+$ T cells (indirect pathway) and simultaneously prime T cells by an acquired MHC molecule (direct pathway). The fundamental role of the semi-direct pathway relies on the capability to stimulate both $CD4^+$ and $CD8^+$ T cells and create a $CD8^+$ alloreactive memory effector T cell population, thus linking together the direct and indirect pathways. In the context of HSCT, the indirect and semi-direct pathways are more vigorous since donors APCs generally present minor histocompatibility antigens to donor T cells (Kishimoto et al., 2004).

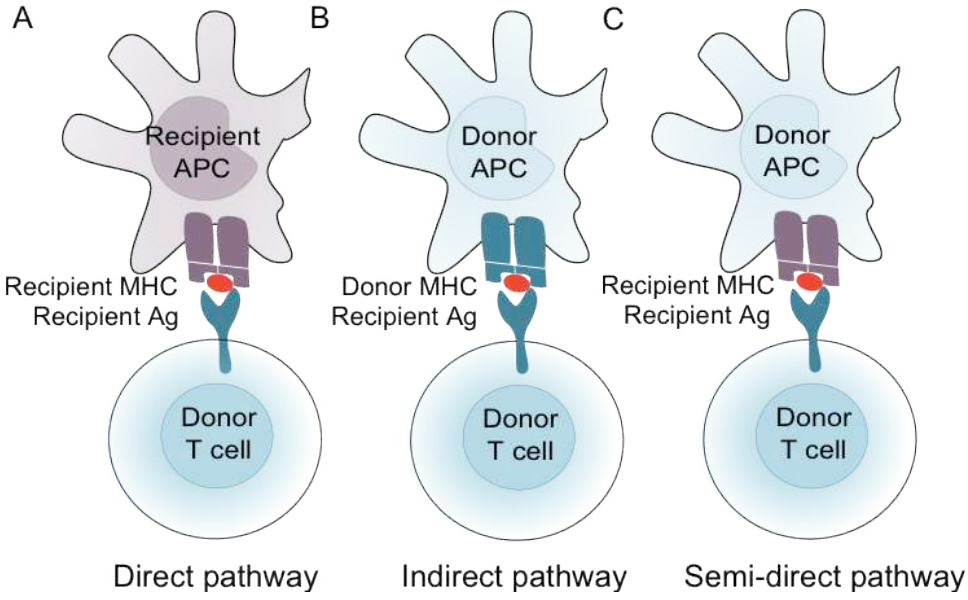


Figure 1.14: $CD4^+$ T cell allorecognition pathways. Donor $CD4^+$ T cells recognise recipient antigens presented by recipient APCs (direct pathway) (A) or by donor APCs (indirect pathway) (B). Donor APCs are able to uptake intact recipient MHC/peptide complexes and present them to donor $CD4^+$ T cells (C).

1.10.2.3.3 Acute Graft versus Host Disease

aGvHD occurs in ~40% of all allogeneic HSCT recipients and is characterised by damages to the skin, upper gastrointestinal tract and liver. It can be divided into three sequential phases (Ferrara et al., 2009) (**Figure 1.15**). Phase I (conditioning phase) consists of tissue damages caused by the conditioning regimen, which leads to activation of host APCs. Activated host APCs then activate donor T cells leading to clonal expansion (Phase II or afferent phase).

Phase III, also called the efferent phase, comprises of the activation of cellular and inflammatory effectors such as CTLs, NK cells, neutrophils and the secretion of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1 and nitric oxide causing organ damages (Ferrara et al., 2009).

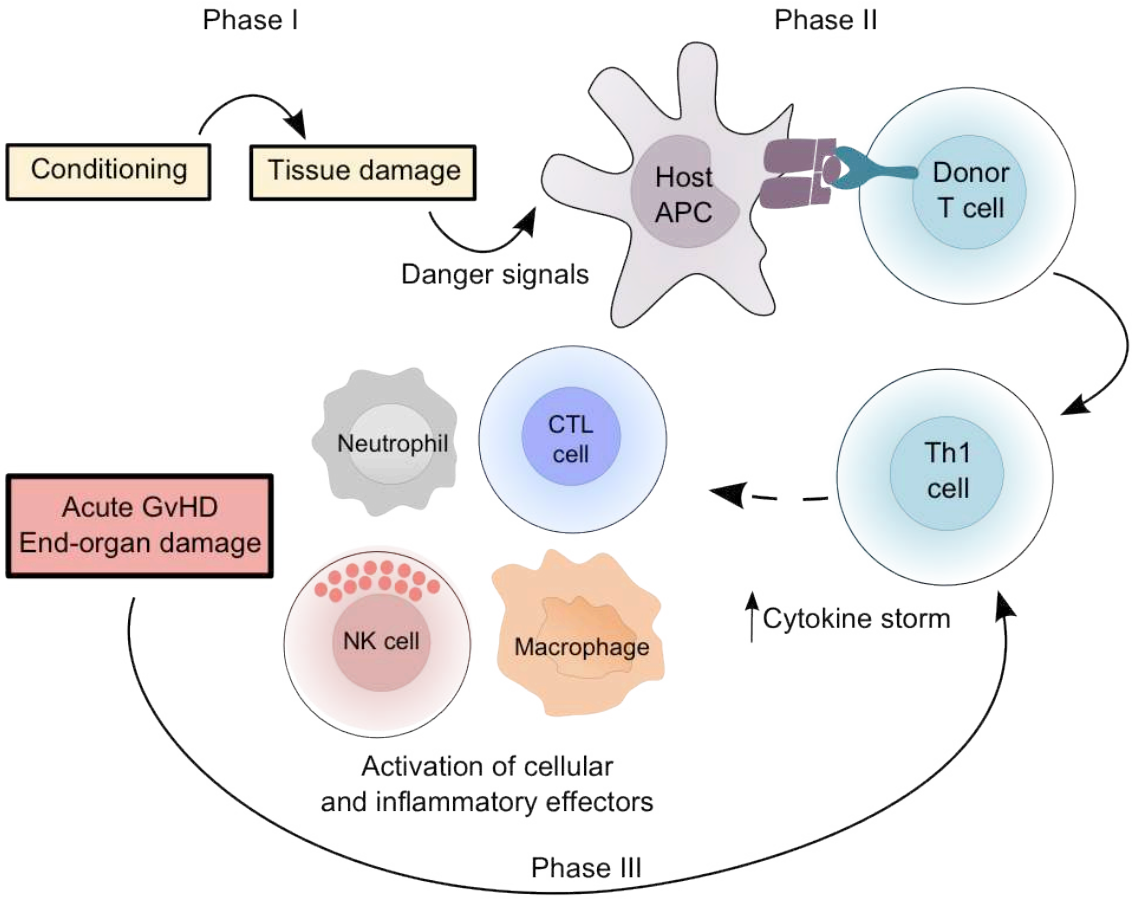


Figure 1.15: Pathophysiology of aGvHD. (Phase I) Conditioning regimen causes tissue damages, which in turn causes release of danger signals that activate host APCs. (Phase II) Activated host APCs present alloantigens to donor T cells leading to clonal expansion. (Phase III) Activation of cellular and inflammatory effectors and cytokine storm cause end-stage organ damages.

1.10.2.3.4 Chronic Graft versus Host Disease

In contrast to aGvHD, the pathophysiology of cGvHD is less understood. cGvHD occurs in 50% of haploidentical HSCT and 70% of matched unrelated HSCT. It is characterised by damages to nails, eyes, mouth, lungs, kidneys, heart, gastrointestinal tract and BM. cGvHD severity is graded according to the NIH global scoring which considers the number of affected sites/organs and their severity (Apperley and Masszi, 2012). To date, various factors

characteristic of the development of cGvHD have been identified (Blazar et al., 2012). Thymus failure, which can be caused by the conditioning regimen or aGvHD, leads to reduced negative selection of alloreactive $CD4^+$ T cells. Also, macrophage activation and release of cytokines such as IL-2, IL-10 and TGF-β is caused by polarisation towards a Th2 response. Subsequently, production of the aforementioned cytokines induces the activation of tissue fibroblasts that in turn cause tissue fibrosis. Low numbers of Treg cells lead to loss of immune tolerance and uncontrolled autoreactive responses. Finally, the dysregulation of B cells induces the formation of autoreactive B cells (Blazar et al., 2012).

1.10.2.3.5 Therapies for prevention or treatment of graft versus host disease

The use of steroids and extra-corporeal photopheresis (irradiation of white blood cells through ultraviolet light) are the most commonly chosen lines of treatment to control GvHD (Ferrara et al., 2009). However, prolonged treatment with steroids leads to long-term mortality rates of 90% (Apperley and Masszi, 2012). New approaches have been proposed as alternatives to steroids; yet all these therapies are still being assessed in clinical trials (**Figure 1.16**). For instance, the use of mammalian target of rapamycin (mTOR) and hystone deacetylase (HDAC) inhibitors such as sirolimus (Phase II and III) (Shin et al., 2011) and vorinostat (Phase II) (Choi and Reddy, 2011) respectively, decrease Th1 cell numbers and/or inhibit APCs. In patients with steroid-refractory aGvHD, antibodies such as alemtuzumab (Phase I-IV) that deplete T cells can reduce the severity of the disease (Gomez-Almaguer et al., 2008, Schub et al., 2011). Furthermore, the use of cellular therapies has recently been proposed to modulate or prevent GvHD. Under current investigation is the use of Treg cells to promote tolerance (Phase I-II) (Brunstein et al., 2011b, Di Ianni et al., 2011), MSC infusion to modulate the functions of B cells, T cells and NK cells (Phase I-III) (Kebriaei and Robinson, 2011b, Kebriaei and Robinson, 2011a), and infusion of donor T cells containing a caspase 9 suicide gene to delete alloreactive T cells (Phase I) (Di Stasi et al., 2011).

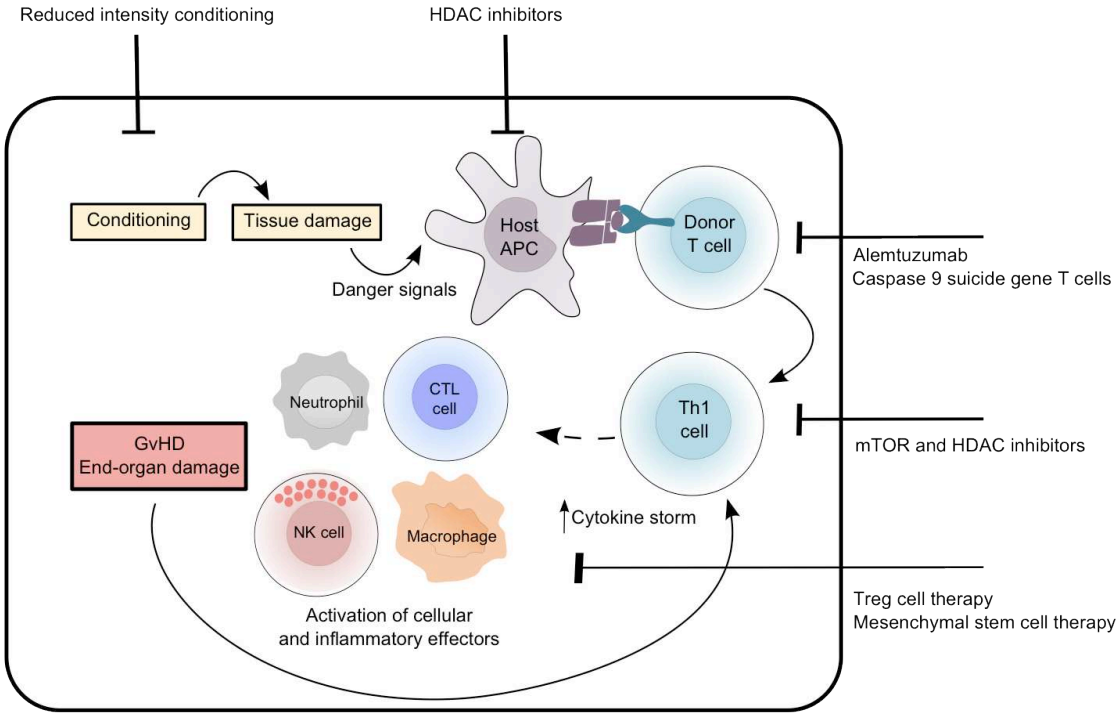


Figure 1.16: Alternative therapies to prevent or modulate GvHD. RIC regimens decrease tissue damages, HDAC and mTOR inhibitors block host APC activation and/or decrease Th1 cell numbers, whereas adoptive cellular therapies such as Treg cells and MSC suppress cellular and inflammatory effectors. The use of these therapies alone or in combination may provide an alternative treatment to steroids for GvHD.

1.10.3 Graft versus Leukaemia effect

In 1979, Weiden and colleagues observed an anti-leukaemic effect of GvHD in human recipients of allogeneic BM grafts (Weiden et al., 1979). Subsequently, Apperley and colleagues observed that chronic myeloid leukaemia (CML) patients transplanted with T cell depleted grafts exhibited lower GvHD but higher relapse rate, thus suggesting that T cells are crucial for disease clearance (Apperley et al., 1988). Two years later, Mackinnon and colleagues confirmed these findings by studying the *in vitro* capacity of donor allogeneic LAK^+ and $CD3^+$ cells to kill recipient CML cells but not healthy cells. This occurred in a cell-contact dependent manner and confirmed that T cells and NK cells can exert effector functions against leukemic cells (Mackinnon et al., 1990a, Mackinnon et al., 1990b), hence establishing the concept of GvL or graft versus tumour (GvT).

Furthermore, there is evidence that NK cells can mediate GvL effects in HSCT. In haploidentical transplants for acute myeloid leukaemia (AML), Ruggeri and colleagues demonstrated that donor versus recipient NK cell alloreactivity reduced the risk of relapse whilst improving engraftment and protecting against GvHD (Ruggeri et al., 2002). To date, many investigators have focused on the optimal conditions to tip the balance between GvHD and GvL.

1.11 Regulatory T cell therapy

In view of their ability to induce tolerance, Treg cells have been proposed as an adoptive therapy to prevent or modulate GvHD after HSCT or to treat autoimmune diseases. This idea was first based on the work of Sakaguchi and colleagues who observed that Treg cells from naive mice prevented skin graft rejection in nude mice infused with Tcon cells (Sakaguchi et al., 1995). In the context of HSCT, preclinical and clinical studies have demonstrated the safety of this therapy in transplanted patients but the potential impact of Treg cells on GvL and infections is still controversial (Trenado, 2003, Maury et al., 2010, Brunstein et al., 2013). Furthermore, the use of polyclonal or antigen-specific Treg cells is still debatable. For instance, Trenado and colleagues have demonstrated in transplanted mice that recipient specific Treg cells can control GvHD and promote immune reconstitution better than third party polyclonal Treg cells (Trenado, 2003). However, other authors favour the use of polyclonal Treg cells due to their higher capacity for expansion that would allow reaching Treg cell doses in adult patients (Hoffmann et al., 2004, Brunstein et al., 2010).

In humans, Trzonkowski and colleagues determined for the first time that the infusion of expanded Treg cells could control cGvHD and allowed withdrawal of steroid treatment (Trzonkowski et al., 2009). Later, the safety of infusion of expanded CB Treg cells in adult patients that received double CBT was demonstrated (Brunstein et al., 2011b). Notably, Treg cell-treated patients exhibited reduced incidence of aGvHD (61% to 41%) in comparison to historical CBT patients. However, the efficacy of prevention of GvHD could not be demonstrated due to the co-administration of GvHD prophylaxis. It is also

important to mention that Treg cells could only be detected in the patients up to fourteen days after infusion, which may suggest reduced persistence possibly due to exhaustion following *in vitro* expansion prior to infusion. In another phase I clinical trial, Di Ianni and colleagues demonstrated for the first time that freshly isolated donor Treg cells are able to counteract the potential GvHD that is induced by the infusion of a high number of Tcon cells in haploidentical transplanted patients (Di Ianni et al., 2011). Finally, Edinger and Hoffmann demonstrated no adverse effects of the administration of Treg cells in a cohort of nine patients at high risk of relapse, thus demonstrating safety and feasibility of Treg cell infusion (Edinger and Hoffmann, 2011).

There is substantial evidence that some autoimmune diseases are associated with Treg cell dysfunction such as type 1 diabetes (Kukreja et al., 2002), IPEX (Wildin et al., 2001), Sjongren's syndrome (Liu et al., 2008b), systemic lupus erythematosus (Liu et al., 2004) and MS (Haas et al., 2005, Huan et al., 2005, Liu et al., 2008a). Therefore, the use of Treg cells as an adoptive cell therapy to treat autoimmune diseases has been tested in mice. In a mouse model of diabetes, the adoptive transfer of antigen specific-Treg cells can prevent the disease (Szanya et al., 2002, Tang et al., 2004, Tarbell et al., 2007). Moreover, prior infusion of antigen-specific Treg cells in a mouse model of MS (EAE) can confer protection against the disease (Olivares-Villagomez et al., 1998). To date, the adoptive transfer of Treg cells in humans for the control of autoimmune diseases has not yet been tested.

1.12 Aims of the study

GvHD is one of the main challenges of allogeneic HSCT. Given their ability to suppress effector cells, $CD4^{+}CD25^{high}Foxp3^{high}$ Treg cells have been proposed as an attractive cellular therapy to prevent GvHD. However, Treg cells are also capable of suppressing NK cells, key effectors of the GvL effect (Ruggeri et al., 2002) and crucial for anti-viral responses (Quinnan et al., 1982). Currently, our understanding of the effect of Treg cells on NK cells in HSCT is poor compared to other settings such as pregnancy and cancer. Notably, preclinical data show

discrepancies as whether Treg cells can impair or induce GvL. Also, to my knowledge, no data is available on the impact of Treg cells on NK cell differentiation.

This thesis focuses on the effects of Treg cells on NK cell differentiation and functions in the setting of CBT. CB was selected as a model since NK cell reconstitution occurs early on after transplantation and NK cells constitute most of the lymphocytes in the circulation after CBT (Komanduri et al., 2007). These NK cells are capable of killing leukaemia cells *ex-vivo* (Beziat et al., 2009) and it is likely that they mediate the GvL effect observed in the first months after CBT. Moreover, the safe use of CB Treg cells as an adoptive therapy in double CBT patients has recently been demonstrated in a Phase I clinical trial, however issues such as higher susceptibility to viral reactivation still requires further investigation (Brunstein et al., 2011b, Brunstein et al., 2013). Therefore, the use of CB Treg cells as an adoptive therapy to prevent GvHD in CBT should be carefully reviewed as it could severely compromise NK cell functions.

The specific aims of this study are:

1. To determine the homing properties of CB Treg cells as compared to PB Treg cells and propose potential sites where CB Treg cells may interact with CB NK cells (Chapter 3).
2. To describe the phenotype and functions of CB Treg cells (Chapter 3).
3. To investigate the particular conditions under which CB Treg cells may impair CB NK cell effector functions (Chapter 4).
4. To determine whether CB NK cells can lyse CB Treg cells (Chapter 4).
5. To investigate the effects of CB Treg cells on NK cell differentiation *in vitro* (Chapter 5) and if any, to elucidate by which mechanism(s) Treg cells mediate these effects (Chapter 6).

2 Materials and methods

2.1 Human samples

CB units were obtained from the Programa Concordia Banc de Sang i Teixits, Barcelona, Spain, and the Anthony Nolan Cord Blood Bank, Nottingham, UK, with prior consent and ethical committee approval. Samples were collected using routine banking procedures into a CB donation bag containing citrate-phosphate-dextrose anticoagulant buffer. Adult PB samples were collected from healthy adults with prior written consent. To prevent coagulation, 2 IU/ml heparin sodium sulfate (Sigma, UK) was added to collection tubes. All samples were processed within 36 h of collection.

2.2 Mononuclear cell isolation

CB and PB samples were diluted 1:1 with RPMI 1640 (Lonza, Belgium) supplemented with 0.63% trisodium citrate (Sigma, USA) and 0.05 μM β-mercaptoethanol (Transport media). Mononuclear cells were separated by density gradient centrifugation at 2 000 rpm for 30 min (without break) using Ficoll-Paque PLUS (GE Healthcare, Sweden) for CB samples and Lympholyte-H (Cedarlane, USA) for PB samples. The mononuclear cell layer was collected and washed twice with RPMI 1640 (1 500 rpm for 10 min). When required, red blood cell lysis was performed using 3 ml of 1X BD PharmLyse Buffer (BD Biosciences, UK) for 4 min at 37 °C (water bath) followed by an extra washing

step. Cell counts were performed with two different dye exclusion methods: trypan blue (Sigma, UK) to determine cell viability and Turk's to determine the number of nucleated cells. The later was used for magnetic cell isolation purposes.

2.3 Isolation of primary cells using magnetic column separation

Treg cells, NK cells and HSC were isolated using a magnetic column based-cell separation system. All incubation times and reagents were maintained at 4° C to avoid antibody capping.

2.3.1 Regulatory T cell isolation

Treg cells were purified from peripheral blood mononuclear cells (PBMC) and umbilical cord blood mononuclear cells (CBMC) using the $CD4^+CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotec, Germany). PB Treg cells were isolated according to the manufacturer's protocol, whilst a published protocol optimised to isolate CB Treg cells was used for CBMCs (Figueroa-Tentori et al., 2008). The purity of PB and CB Treg cells was assessed by flow cytometry by gating on $CD4^+CD25^{high}CD127^{low}$ cells (Seddiki et al., 2006a) (**Figure 2.1**). Purity ranged between 69 and 85% from total lymphocytes with a Treg cell recovery of 0.5 to 1.2%.

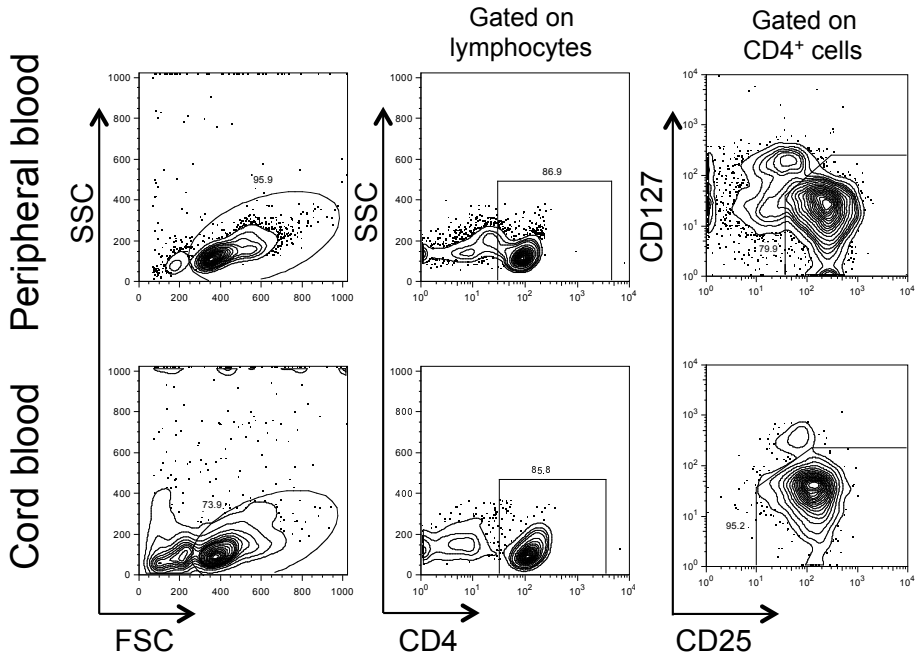


Figure 2.1: Gating strategy to determine CB and PB Treg cell purity. Flow cytometric analysis of isolated CB and PB Treg cells showing surface expression of CD4, CD25 and CD127 receptors. Plots are representative of 62 (CB Treg cells) and nine (PB Treg cells) independent experiments.

2.3.2 Natural killer cell isolation

CB NK cells were purified from CBMCs by negative selection using the NK cell isolation kit (Miltenyi Biotec, Germany) following the manufacturer's recommendations. An additional granulocyte depletion step prior to isolation was required due to significant granulocyte contamination in CB samples that could not be removed after negative selection. The removal of granulocytes was performed by immunodensity depletion during the density gradient centrifugation using Ficoll. The quantity of 5 µl/ml RosetteSep Human Granulocyte Depletion cocktail (Stem Cell Technologies, France) was added to CB samples, incubated at 25 °C (room temperature) for 15 min on a platform shaker model str6 (Stuart Scientific, UK), and then diluted 1:1 with transport media. Ficoll layering was performed as described in Section 2.2.

The purity of NK cells, identified as $CD56^{+}CD3^{-}$ cells, ranged between 89 and 97% from total lymphocytes with an NK cell recovery of 10-15% (**Figure 2.2**). From the total $CD56^{+}$ cell population, 7-12% were $CD56^{bright}$ NK cells (**Figure 2.2D.1**) and 88-93% were $CD56^{dim}$ NK cells (**Figure 2.2D.2**).

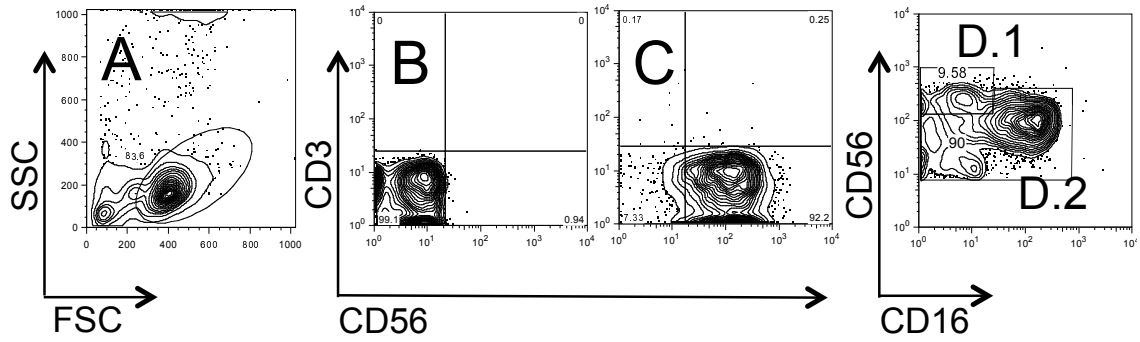


Figure 2.2: Gating strategy to determine CB NK cell isolation purity. Flow cytometric analysis of isolated CB NK cells showing surface expression of CD3, CD56, and CD16. Gated lymphocytes (A), gated lymphocytes unstained as negative control (B), expression of $CD56^{+}$ cells on isolated NK cells (C), $CD56^{bright}$ subpopulation percentage (D.1), and $CD56^{dim}$ subpopulation percentage (D.2). Plots are representative of ten independent experiments.

2.3.3 Haematopoietic stem cell isolation

HSC were purified by positive selection from CBMCs using the CD34 MicroBead Kit (Miltenyi Biotec, Germany). CBMCs were isolated using a modified published protocol (Jaatinen and Laine, 2007). In order to prevent non-specific binding, 20% human AB serum (Lonza, Belgium) was added during the labelling step. Purity of isolated HSC was analysed following the International Society of Hematotherapy and Graft Engineering (ISHAGE)-single platform guidelines to enumerate $CD34^{+}$ cells (Allan et al., 2002) (**Figure 2.3**). Purity ranged between 90% and 98% with a HSC recovery of 0.5-1% from total lymphocytes.

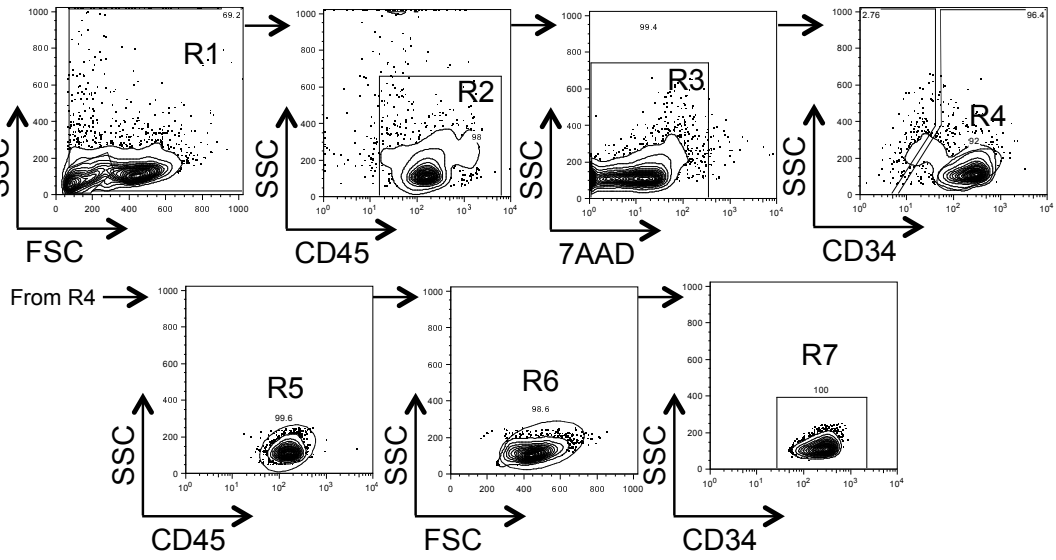


Figure 2.3: Sequential ISHAGE gating strategy to determine CB HSC isolation purity. CB HSC viability and purity were assessed by flow cytometry through the respective expression of CD45, 7-Aminoactinomycin D (7-AAD) and CD34 following single-platform ISHAGE guidelines. R1: Non-debris, R2: $CD45^{+}$gating, R3: Live cells, R4: Live $CD34^{+}$ cells, R5: Gating for $CD45^{low}SSC^{low}$ SC, R6: Gating to exclude granulocytes, R7: $CD34^{+}$ cells. Purity was calculated as follows: (CD34/7-AAD)*100 [total cells]. Data is representative of nine independent experiments.

2.4 Cell lines

The embryonic liver cell line EL08.1D2 was kindly provided by Dr Robert Oostendorp (Technical University, Munich). This cell line is reported to support NK cell differentiation *in vitro* (Oostendorp et al., 2002, McCullar et al., 2008).
The cell line K562 was established from a 53-year old female patient with CML. K562 carries the Philadelphia chromosome and does not express MHC class I.

2.5 Freezing, storing and thawing of isolated cells

For cryostorage, isolated cells and cell lines were resuspended in heat inactivated fetal bovine serum (FBS) (Lonza, Belgium) containing 10% dimethyl sulfoxide (Sigma, UK) (freezing media). FBS heat inactivation was performed in a water bath at 56 °C for 35 min and immediately placed at -20 °C to prevent protein degradation. Cells were stored in cryotube vials (Nunc, Denmark) at a concentration of $1x10^6$ cells/ml (primary cells), $2x10^6$ cells/ml (EL08.1D2) and $10x10^6$ cells/ml (K562) in 1 ml final volume. Cryovials containing the cell

suspension were then placed at -70 °C in freezing containers (Sigma, UK) that will allow a cooling rate gradient of 1 °C/min. After 24 h, cells were transferred to liquid nitrogen for long-term storage.

For thawing, primary cells were thawed according to a published protocol optimised for high cell recovery of CB samples (manual washing) (Rodriguez et al., 2004). Cell lines were fast-thawed in a 37 °C water bath for 2 min, resuspended in 50 ml RPMI 1640 containing 10% heat inactivated FBS and washed. Cell counts with trypan blue were performed for all samples.

2.6 Cell culture

All cultures were incubated at 37 °C, 5% CO_2 and 96% humidity, except for the EL08.1D2 feeder layer cells, which was incubated at 32 °C.

2.6.1 Cell culture conditions for regulatory T cell analysis

To study the phenotype and function of Treg cells, cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, 0.05 mM β-mercaptoethanol (Life Technologies, UK), and 1% penicillin/streptomycin (Lonza, Belgium), henceforth referred to as “complete media”. For proliferation and survival, 600 IU/ml (CB) or 100 IU/ml (PB) of IL-2 (Prospec, Israel) were added to the culture. A concentration of 50 000 Treg cells/well was plated in 96-well round-bottom plates (Sarstedt, Germany) in 200 µl final volume. When expression of homing receptors was analysed, FBS was replaced by 1% Fatty acid-free bovine serum albumin (BSA) (Sigma, UK) to avoid non-specific migration.

When required, Treg cells were activated by TCR-stimulation. Concentrations for maximal TCR stimulation levels with anti-CD3 and anti-CD28 were optimised in the laboratory by Dr Richard Duggleby. Treg cells were stimulated in two different ways depending on the level of stimulation required. Soluble anti-CD3 provides a gradual T cell stimulation whereas plate bound anti-CD3 promotes a

strong T cell stimulation in a short period of time with less variability between samples (van Lier et al., 1989) (**Figure 2.4**).

For soluble anti-CD3-mediated TCR stimulation 4 µg/ml (CB) or 10 µg/ml (PB) anti-CD3 (clone HIT3a, BD Biosciences, Belgium) and 8 µg/ml (CB) or 10 µg/ml (PB) anti-CD28 (clone CD28.2, BD Biosciences, Belgium) were added to the complete media. For plate bound-mediated TCR stimulation only CB Treg cells were used. Plates were pre-coated for 2-12 h with 10 µg/ml anti-CD3 in 1X phosphate buffered saline solution (PBS) (Lonza, Belgium) at 37 °C and then washed with PBS. Treg cells were resuspended in complete media supplemented with 10 µg/ml anti-CD28. Activation of Treg cells was measured by flow cytometry by analysis of surface expression of CD69, CTLA-4, glucocorticoid-induced tumour necrosis factor receptor (GITR) and LAP.

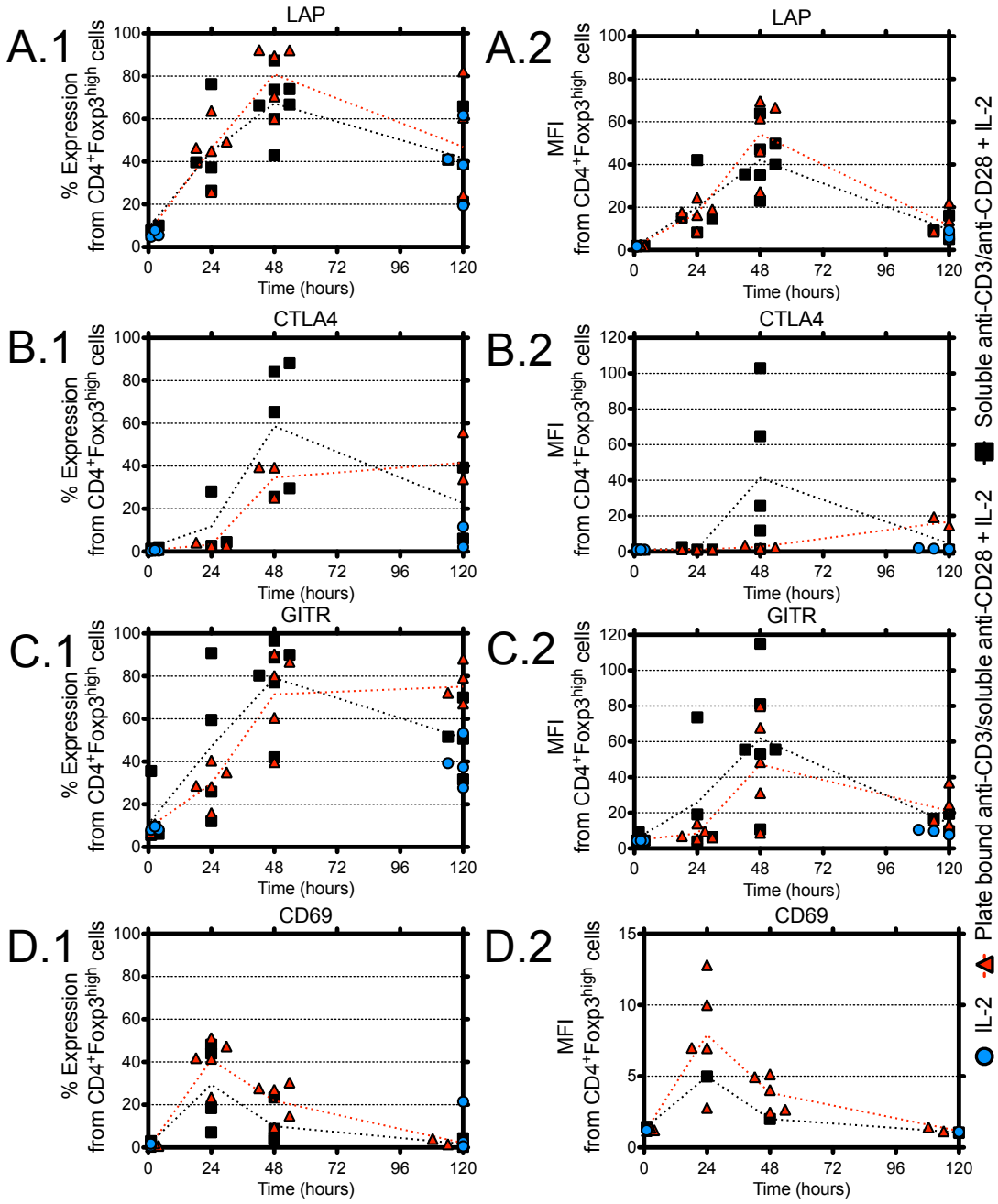


Figure 2.4: Comparative study of CB Treg cell activation using soluble and plate bound anti-CD3. Flow cytometric analysis of frequency (left) and mean fluorescence intensity (MFI) (right) of LAP (A.1-A.2), GITR (B.1-B.2), CTLA-4 (C.1-C.2) and CD69 (D.1-D.2) on CB Treg cells activated with soluble anti-CD3/CD28 and IL-2 (black squares) or plate bound anti-CD3, soluble anti-CD28 and IL-2 (red triangles), or IL-2 (blue circles). 600 IU/ml of IL-2 were added for CB Treg cells. Activation and function levels were analysed at 1, 24, 48 and 120 h, except for IL-2 (blue circles), which was analysed at 0 and 120 h. The lines represent medians. n=3-5.

2.6.2 Cell culture conditions for regulatory T cell-natural killer cell interactions

To assess the effect of Treg cells on NK cells, different combinations of cell activation methods for both cell types were tested. **Table 2.1** summarises the culture conditions under which these experiments were performed. Complete media was used for all combinations and cultures were performed in 96-well round-bottom plates. Media was changed by hemi-depletion every 48 h. In these experiments, IL-2 treatment does not cause significant activation but is essential for the survival and proliferation of CB cells. In all protocols, CB NK cells and CB Treg cells were used, except for Protocol A (**Table 2.1**), where NK cells and Treg cells from PB were used. Ratios of 1:1 (NK cells:Treg cells) and 4:1 were used for these assays. NK cell concentration was always kept constant as 200 000 cells/well in 200 µl final volume.

Table 2.1: Culture conditions for NK cell and Treg cell co-cultures.* NK cells without Treg cells were used as controls in all conditions.

Protocol	NK cell/ Treg cell	NK cells (pre co-culture)	Treg cells (pre co-culture)	Co-culture conditions*
A	Resting/ Resting	N/A	N/A	No cytokines for 4 h.
B	Resting/ Resting	N/A	N/A	1 000 IU/ml IL-2 for 24 h.
C	Resting/ TCR-stimulated	N/A	N/A	1 000 IU/ml IL-2, plate bound anti-CD3/soluble anti-CD28 for 24 or 48 h.
D	Activated/ Resting	20 ng/ml IL-15 (Peprotech, USA) for 40 h	1 000 IU/ml IL-2	Washed cells. No cytokines for 4 h.
E	Activated/ TCR-stimulated	20 ng/ml IL-15 for 40 h	1 000 IU/ml IL-2, plate bound anti-CD3/soluble anti-CD28	Washed cells. No cytokines for 4 h.

2.6.3 Cell culture conditions for natural killer cell differentiation-regulatory T cell interaction

The embryonic liver cell line EL08.1D2 was cultured according to a published protocol (Grzywacz et al., 2006) in gelatinised flasks (Sarstedt, Germany) of 25, 75 or 150 cm^2 at 32 °C, 5% CO_2 and 96% humidity. The amount of 0.1% Bovine

gelatine (Sigma, UK) in PBS was used to coat the flasks for 15-30 min at 37 °C and then washed with PBS. Cells were then resuspended at a concentration of 4 000 cells/cm^2 in basic medium: 40.5% α–minimum essential medium (α-MEM)(Lonza, Belgium), 50% myelocult (Stem cell Technologies, France), 7.5% FBS, 50 μM β-mercaptoethanol, 2 000 μM GlutaMAX (Life Technologies, UK), 1% penicillin/streptomycin and 1 μM hydrocortisone (Sigma, UK) containing 20% of conditioned media (0.2 μm filtered supernatant from previous cultures).

Once the cells reached 95% confluence, the supernatant was removed. Cells were washed with PBS and then trypsin (Life Technologies, UK) was added for 4 min at 37 °C to detach the cells. Once the cells were detached, they were washed with complete media, centrifuged at 1 200 rpm for 5 min and counted with Trypan blue. Cells were irradiated with 30 Gy (3 000 rad) for 7 min, resuspended in basic media at a concentration of 20 000 cells/well in gelatin-coated (50 μl) 96-well flat-bottom plates (Sarstedt, Germany) and left overnight to allow cell adhesion. The following day, basic media was removed by flicking.

Based on the published protocol by Grzywacz and colleagues and further optimisation protocol by Luevano and colleagues (Grzywacz et al., 2006) (Luevano et al., Plos One, under revision), a concentration of 500 frozen-thawed CD34$^+$ HSC per well (final volume of 200 μl) were added to the monolayer of EL08.1D2 in NK cell media, which consists of Ham F12 (Lonza, Belgium) plus Dulbecco modified Eagle medium (Lonza, Belgium) (1:2 ratio) with 20% heat-inactivated AB serum, 50 μM ethanolamine (Sigma, UK), 20 mg/l ascorbic acid (Sigma, UK), 5 μg/l sodium selenite (Sigma, UK) and 1% penicillin/streptomycin. AB serum was heat inactivated the same way as FBS, as described in Section 2.5. This media was kept at 4 °C for up to one month. On weeks 1-3, 10 ng/ml IL-15, 5 ng/ml IL-3 (only weeks 1 and 2), 20 ng/ml IL-7, 20 ng/ml c-kit ligand (SCF) and 10 ng/ml Flt3 ligand were added. For weeks 4-5 (starting day 21), only IL-15 was added at a concentration of 50 ng/ml. All cytokines were purchased from Prospec, Israel, except for IL-3 (R&D, UK). The culture was maintained for 35 days and underwent weekly hemi-depletion of fresh media with the cytokines' schedule described above.

In order to assess the effect of CB Treg cells on NK cell differentiation, different cultures were designed as shown in **Table 2.2.** Treg cells were added at key time points of HSC cultures when transition from one stage of differentiation to another occurs. Resting Treg cells were isolated and immediately added to the cultures while TCR-stimulated Treg cells were pre-cultured for 24 h with plate bound anti-CD3 and soluble anti-CD28, as mentioned in Section 2.6.1. After stimulation, the cells were washed and then added to the cultures. The ratio of NK cells:Treg cells was 4:1 in all co-cultures except when Treg cells were added at day 2 when a ratio of 1:1 was used. Different CB samples were used for each culture. Treg cell activation was assessed by flow cytometry by analysing the expression of GITR and LAP.

Table 2.2: Condition used for NK cell differentiation cultures.

Culture	Day of Treg cell addition (resting or TCR-stimulated)	Ratio HSC: Treg cell
1 (HSC)	No Treg cells were added	N/A
2 (HSC+Treg(D2))	Day 2	1:1
3 (HSC+Treg(D9))	Day 9	4:1
4 (HSC+Treg(D16))	Day 16	4:1
5 (HSC+Treg(D23))	Day 23	4:1
6 (HSC+Treg(D30))	Day 30	4:1

2.7 Flow cytometry

Characterisation and function of Treg cells, NK cells and HSC was performed by flow cytometry using a FACSCalibur (BD, UK). Data analysis was performed with FlowJo software (Treestar Inc., USA).

2.7.1 Cell surface staining

Cells were stained for cell surface markers using directly conjugated antibodies at pre-titrated concentrations (**Table 2.3**). Stainings were performed using 25 000-100 000 cells resuspended in 50 µl PBS with 5% FBS (FACS buffer) in 96-well V-bottom plates (Nunc, Denmark) for 15 min at 4 °C (except for CXCR4 and CXCR7, which were incubated for 45 min following the manufacturer's recommendations). Cells were then washed with FACS buffer and analysed by flow cytometry.

Table 2.3: Antibodies used for the assessment of Treg cells, NK cells, HSC and HSC-differentiated NK cell phenotype.

Antibody	Clone	Company	Description	Dilution	Fluorochrome	Use
7-AAD		BD Pharmingen	7-Aminoactinomycin D	1/100		Apoptosis
Annexin V		BD Pharmingen	Phospholipid binding protein	1/50	FITC	Early apoptosis
CCR5	45531	R&D Systems	Chemokine receptor	1/10	PE	Migration
CCR6	53103	R&D Systems	Chemokine receptor	1/10	PE	Migration
CCR7	150503	R&D Systems	Chemokine receptor	1/10	PE	Migration
CD3	SK7	BD Pharmingen	TCR receptor complex	1/20	PerCP	T cell phenotype
CD4	MEM-241	Immunotools	Co-receptor MHC class II	1/100	FITC	T cell phenotype
CD4	SK3	BD Pharmingen	Co-receptor MHC class II	1/50	PE	T cell phenotype
CD4	SK3	BD Pharmingen	Co-receptor MHC class II	1/25	PerCP	T cell phenotype
CD5	L17F12	BD Pharmingen	Type 1, transmembrane protein	1/50	APC	Lymphoid lineage
CD7	Ebio124-1d1	BD Pharmingen	Type I transmembrane protein	1/20	PE	Lymphoid lineage
CD8	SK1	BD Pharmingen	TCR co-receptor	1/50	FITC	CD8 T cell phenotype
CD10	CB-CALLA	eBioscience	Membrane metallo-endopeptidase	1/5	PE	Myeloid lineage
CD11a (LFA-1)	HI111	BD Pharmingen	Cell adhesion molecule	1/100	FITC	Cell interactions
CD16	3G8	BD Pharmingen	FcγRIII	1/5	FITC	NK cell phenotype/ function
CD16	3G8	BD Pharmingen	FcγRIII	1/600	PE-Cy5	NK cell phenotype/ function
CD19	467	BD Pharmingen	B-lymphocyte antigen receptor	1/50	PE	B cell phenotype
CD25	2A3	BD Pharmingen	IL-2α chain receptor	1/17	PE	Treg cell phenotype
CD25	2A3	BD Pharmingen	IL-2α chain receptor	1/20	APC	Treg cell phenotype
CD33	HIM3-4	BD Pharmingen	Trans-membrane receptor	1/5	FITC	Myeloid lineage
CD34	581	BD Pharmingen	Cell surface glycoprotein	1/15	FITC	SC phenotype
CD34	581	BD Pharmingen	Cell surface glycoprotein	1/10	APC	SC phenotype
CD45	HI30	BD Pharmingen	Leukocyte common antigen protein	1/10	FITC	Leukocytes
CD45	HI30	BD Pharmingen	Leukocyte common antigen protein	1/10	APC	Leukocytes
CD45RA	HI100	eBioscience	CD45 isoform	1/100	FITC	Naive cells

Antibody	Clone	Company	Description	Dilution	Fluorochrome	Use
CD45RA	HI100	eBioscience	Protein tyrosine phosphatase rec.	1/100	PE	Naive cells
CD49d (α4-integrin)	9F10	eBioscience	Integrin protein	1/50	PE	Homing
CD56	B159	BD Pharmingen	Isoform of NCAM	1/20	PE	NK cell phenotype
CD56	53G8	BD Pharmingen	Isoform of NCAM	1/10	PE-Cy5	NK cell phenotype
CD56	B159	BD Pharmingen	Isoform of NCAM	1/10	APC	NK cell phenotype
CD62L (L-selectin)	DREG56	eBioscience	Cell adhesion molecule	1/50	FITC	Homing
CD69	L78	BD Pharmingen	Early activation	1/50	PerCP	Treg/NK cells
CD94	HP-3D9	BD Pharmingen	Killer cell lectin-like receptor	1/100	FITC	NK cell precursor
CD95	DX2	BD Pharmingen	FAS receptor	1/10	FITC	Death receptor
CD117	104D2	BD Pharmingen	Mast/SC growth factor receptor	1/100	PE	NK cell precursor
CD127	eBioRDR5	eBioscience	IL-7α chain receptor	1/25	FITC	Treg cell phenotype
CD127	hiL-7R-M21	BD Pharmingen	IL-7α chain receptor	1/50	PE	Treg cell phenotype
CD226(DNAM-1)	DX11	BD Pharmingen	DNAX accessory molecule-1	1/5	FITC	NK cell function
CD244(2B4)	eBioPP35	eBioscience	NK cell receptor 2B4	1/20	FITC	NK cell function
CTLA-4	BNI3	BD Pharmingen	Cytotoxic T-lymphocyte antigen-4	1/25	PE	Treg cell function
CXCR1	42705	R&D Systems	Chemokine receptor	1/10	PE	Migration
CXCR3	IC6	BD Pharmingen	Chemokine receptor	1/5	APC	Migration
CXCR4	I2G5	R&D Systems	Chemokine receptor	1/5	PE	Migration
CXCR7	358426	R&D Systems	Chemokine receptor	1/100	PE	Migration
GITR	110416	R&D Systems	Glucocorticoid-induced TNFR	1/25	FITC	Treg cell function
LAP	27232	R&D Systems	Latency-associated peptide	1/10	PE	Treg cell function
LAMP-1 (CD107a)	H4A3	BD Pharmingen	NK cell degranulation marker	1/40	FITC	NK cell function
NKG2D	BAT221	MACS	Activating receptor	1/10	PE	NK cell function
NKp30	P30-15	BD Pharmingen	NCR	1/100	PE	NK cell function
NKp44	P44-8	Biolegend	NCR	1/10	APC	NK cell function
NKp46	9E2/NKp46	BD Pharmingen	NCR	1/50	APC	NK cell function
β7 integrin	FIB540	eBioscience	Integrin protein	1/100	FITC	Homing

2.7.1.1 Cell viability

Cell viability was assessed using two markers: Annexin V, which is specific for phosphatidylserine expressed on the surface of apoptotic cells, and 7-AAD, which binds to double stranded DNA exposed once the cell membrane has been compromised. This combination allows the identification of three populations: live cells (Annexin V^-/7-AAD^-), early apoptotic cells (Annexin V^+/7-AAD^-) and late apoptotic or necrotic cells (Annexin V^+/7-AAD^+). **Figure 2.5** shows the gating strategy used for all co-cultures to determine the viability of each cell type.

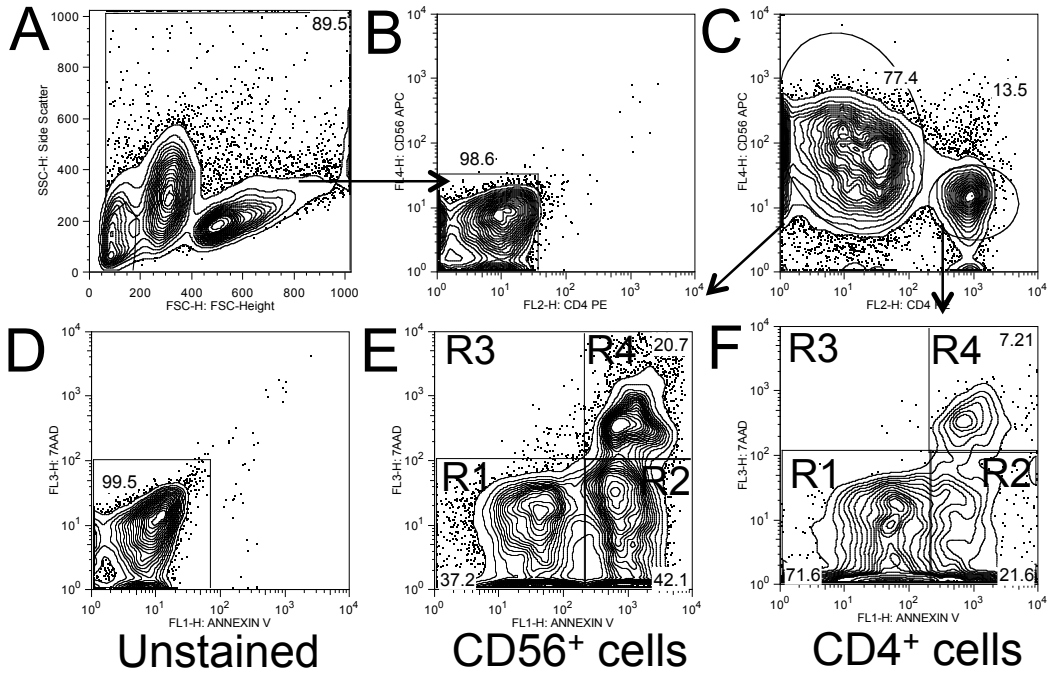


Figure 2.5: 7-AAD/Annexin V gating strategy used to assess cell viability. CB NK cell and CB Treg cell viability was assessed by flow cytometry through the expression of CD4, CD56, 7-AAD, and Annexin V. (A) Non-debris gate, (B) CD4 and CD56 unstained negative control, (C) CD4 and CD56 gating from the non-debris gate, (D) 7-AAD/Annexin V unstained negative control, E. $CD56^+$ cell viability, (F) $CD4^+$ cell viability. Region R1: Live cells; Region R2: Early apoptotic cells; Region R3 and R4: Late apoptotic and necrotic cells. Data is representative of 9 independent experiments.

To assess viability of specific cell populations, cells were labelled with CD4 for Treg cells and CD56 for NK cells (as described in the previous section). Cells were then resuspended in 1X Annexin V binding buffer (BD Pharmingen, UK). 7-AAD and Annexin V were added 10 min before analysis.

2.7.2 Intranuclear staining protocol

A minimum of 50 000 cells were stained using the Foxp3 Staining Buffer Kit (eBioscience, USA) following the manufacturer's recommendations. The clone PCH101 (PE, eBioscience; 1/20 dilution) was used in this study.

2.7.3 Cell sorting for NK cell differentiation

Resting or TCR-stimulated Treg cells were added at day 9 of HSC cultures at a ratio of 1:4 (Treg cell:HSC) and co-cultured until day 12 or day 35 of differentiation. A total of 4-20x10^6 cells were labelled with CD4-PE, clone SK3 (BD Biosciences, UK) and then sorted using a MoFlow cell sorter (Beckman Coulter). Sorted HSC were either cultured with irradiated EL08.1D2 feeder layer cells (protocol described in Section 2.6.3) or RNA was extracted for gene expression analysis. Purity was assessed by flow cytometry and ranged between 90.3% and 100% (n=21).

2.8 Functional assays

2.8.1 *In vitro* proliferation assays

To determine cell proliferation, cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the Cell Trace CFSE cell proliferation kit (Life Technologies, UK), which covalently labels long-lived intracellular molecules with high fluorescence intensity. The fluorescent signal halves every cell division, which makes it a reliable and easy technique to follow cell proliferation that can be detected by flow cytometry. Two different protocols were used for Treg cells and NK cells.

Treg cells were labelled following an optimised protocol for low T cell numbers (Quah et al., 2007). A titration of CFSE concentration was performed with 2x10^6 and 0.25x10^6 Treg cells activated with Dynabeads human T-activator CD3/CD28 (Life Technologies, UK). It was found that a 2.5 mM CFSE concentration gave the best division peaks after 96 h (**Figure 2.6**). Cells were

cultured as described in Section 2.6.2. Analysis by flow cytometry was performed at 0, 24, 48 and 120 h after CFSE addition.

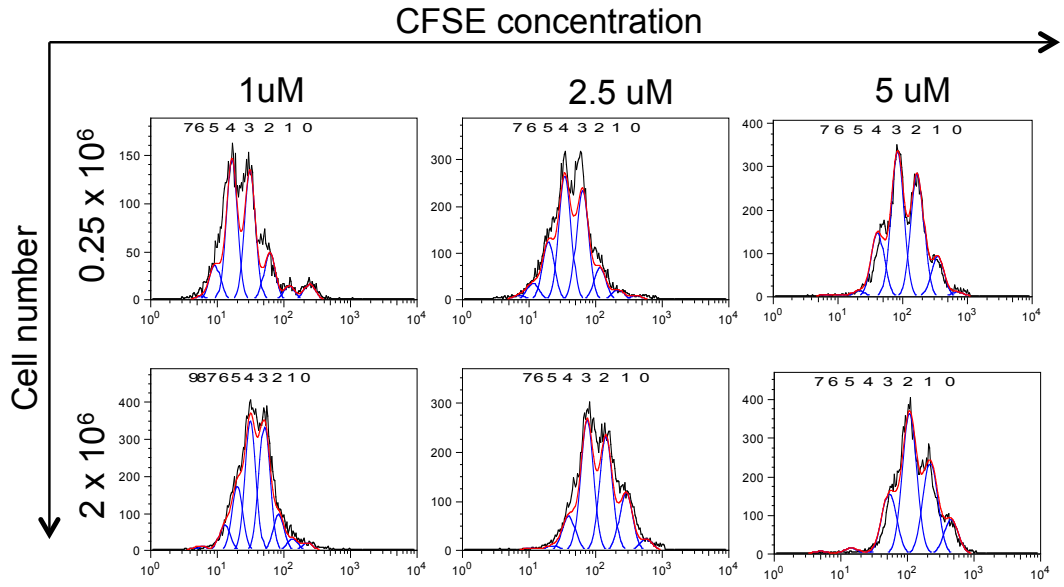


Figure 2.6: CFSE titration for low Treg cell numbers. $2x10^6$ and $0.25x10^6$ CB Treg cells were labelled with different CFSE concentrations and activated with Dynal Beads at 1:1 ratio (Treg cells:beads). Cell proliferation was measured by flow cytometry after three days. The red line depicts the overall proliferation profile and the blue lines are individual histograms for each cell division. The numbers show percentages of cells in each generation (0-8). Representative sample of three different experiments.

For NK cells, a different protocol was followed. NK cells ($1x10^6$) were resuspended in 1 ml PBS without FBS and 2 µM CFSE was added (previously reconstituted in DMSO). Cells were incubated for 10 min at 37 °C, washed twice with chilled complete media, counted and resuspended as described in Section 2.6.2 except for Section 2.6.2 Protocol E, where cells were pre-activated, washed and then CFSE-labelled.

2.8.2 *In vitro* Treg cell suppression assay

CB Treg cell suppression was measured by thymidine assay and CFSE assay (described in the previous section) according to a published study (Seddiki et al., 2006b). Both assays were performed in 96-well round-bottom plates with RPMI 1640 supplemented with 5% heat inactivated FBS, 0.05 mM β-mercaptoethanol, 1% penicillin/streptomycin and 1 µg/ml anti-CD3, clone HIT3a, in a final volume of 200 µl. All wells contained 50 000 CFSE-labelled or non-labelled autologous effector cells (CBMCs or non-$CD4^+CD25^{high}$ CB cells),

referred to as “responders” and 100 000 PBMCs irradiated at 30 Gy (3 000 rad) for 7 min, as APCs. Treg cells, referred to as “suppressors”, were added at a final suppressor:responder ratio of 0:1, 1:1 (50 000 cells), 1:4 (12 500 cells), and 1:10 (5 000 cells). Non-$CD4^+$ T cells were added to the cultures instead of Treg cells as negative controls of suppression.

For CFSE assays, CFSE-labelled responders were analysed after 72 h by flow cytometry. Results were expressed using the division index, which is the average number of cell divisions that a cell has undergone.

For thymidine assays, the cultures were pulsed with 1µCi ^{3}H-Thymidine (GE Healthcare, Sweden) and harvested 16-18 h later in a Microbeta filterMate-96 harvester (Perkin Elmer, UK). Triplicates were carried out in all conditions. Cell proliferation was then measured in a liquid scintillation counter 1 450 Microbeta (Perkin Elmer, UK). Results were expressed in counts per minute (cpm), which is the measurement of particle emission rates that directly correlates with proliferation. Percentage of suppression was calculated using the following equation:

$$\%\, Suppression = \left(\frac{cpm\ target}{cpm\ control} - 1\right)(100)$$

2.8.3 Chromium release assay

To determine the suppressive effect of Treg cells on cytotoxicity of NK cells chromium release assays were performed. This assay measures NK cell cytolytic activity against a leukaemia cell line, K562.

K562 cells were washed with PBS and labelled with 100 µCi/1x10^6 cells ^{51}-Chromium (Perkin Elmer, UK) for 45 min at 37 °C. Cells were then washed twice with PBS and resuspended in complete media at a concentration of 5 000 cells/100 µl. Treg cell and NK cell co-cultures (**Table 2.1** and **Table 2.2)** were adjusted to reach ratios of NK cells (effector):K562 (target) of 1:1, 5:1 and 10:1, with a constant K562 cell number of 5 000 cells in 100 µl final volume. When there was a limited cell number, the ratios of NK cells: K562 of 1:1 and 5:1 were

excluded. As a positive control, 100 µl of 0.1% Triton X-100 (VWR International, UK) were added to K562 cells. After 4 h incubation at 37 °C, cells were centrifuged at 1 200 rpm for 4 min and 30 µl of supernatants were transferred to 96-well sample plates (Perkin Elmer, UK) and left overnight to dry. The following day, 30 µl Optiphase Supermix scintillation solution (Wallac, UK) was added and chromium release was measured in a liquid scintillation counter 1 450 Microbeta. All conditions were performed in triplicate and results were expressed in percentage of lysis to represent target lysis by NK cells. The percentage of specific lysis by NK cells was calculated using the following equation:

$$\%\, Specific\ lysis = \left(\frac{cpm\ experimental\ release - cpm\ spontaneous\ release}{cpm\ maximum\ release - cpm\ spontaneous\ release}\right)(100)$$

2.8.4 NK cell degranulation assay

The surface expression of CD107a (LAMP-1) is a marker of NK cell degranulation, which can be easily detected by flow cytometry. After culture (**Table 2.1** and **Table 2.2**), NK cells were stimulated or not with K562 cells (1:1 ratio; NK cell:K562), or 100 ng/ml Phorbol myristate acetate (PMA) (Sigma, UK) and 1 µg/ml Ionomycin (ION) (Sigma, UK) for 2 h at 37 °C, 5% CO_2 as a positive control. Cells were washed and blocked for 15 min with 10% mouse serum in PBS. After blocking, cells were washed and stained with anti-CD56, anti-CD3 and anti-CD16 in 2% FBS, and 2 mM EDTA in PBS (Paisley, UK) ("staining buffer") for 15 min at 4 °C. After another wash with staining buffer, cells were stained with anti-CD107a or isotype control for 45 min at 4 °C, washed and analysed by flow cytometry.

2.8.5 Analysis of phosphorylation of Smad2/3 and Stat3

TGF-β and IL-10 signalling pathways were analysed through the phosphorylation of Smad2 and Smad3 (for TGF- β) and Stat3 (for IL-10), key molecules in the corresponding signalling cascades. The intracellular expression of phosphorylated Smad2/3 and phosphorylated Stat3 was analysed

using the BD Phosflow kit: PE mouse anti-human Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (clone 072-670, BD Biosciences, UK), and PE mouse anti-human Stat3 (pY705) (clone 4-P/Stat3, BD Biosciences, UK). Before staining, cultured HSC were washed and resuspended in RPMI 1640 for 2 h. This was done to reduce basal phosphorylation caused by high levels of FBS or AB serum. Cell viability in the absence of serum was confirmed using 7-AAD showing no difference between the conditions tested, **Figure 2.7**.

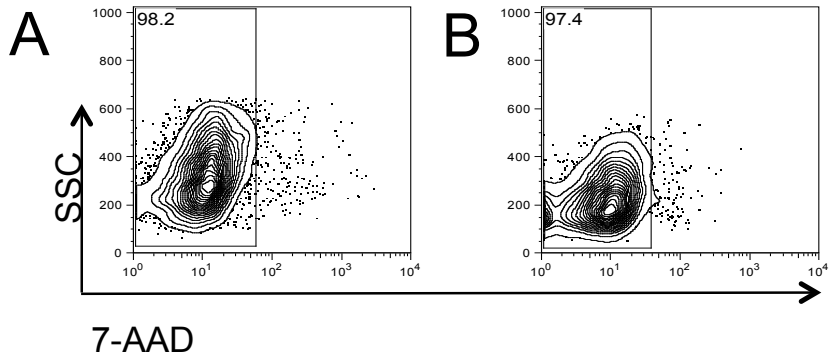


Figure 2.7: HSC viability in absence of serum. HSC were washed and resuspended in NK media (A) or RPMI 1640 (B) for 2 h. Viability was measured by flow cytometry by assessing staining with 7-AAD on $CD56^+$ gated cells. Data is representative of three independent experiments.

After the 2 h incubation, HSC were cultured in the presence or absence of Treg cells at a ratio of 1:4 (Treg cell:HSC) in RPMI 1640. HSC treated with 10 ng/ml human recombinant TGF-β (BD Biosciences, UK) and 50 ng/ml human recombinant IL-10 (Prospec, Israel) were used as positive controls. After 15 min, cells were resuspended in 100 µl BD Cytofix buffer (BD Biosciences, UK) and incubated at 37 °C for 15 min. The latest was pre-warmed in a 37 °C water bath for 10 min before use. Cells were then centrifuged at 1800 rpm for 5 min and supernatants were removed. 150 µl Perm buffer (BD Biosciences, UK) was added and incubated for 30 min at 4 °C. Cells were washed twice with 100 µl Stain buffer (BD Biosciences, UK) and resuspended in the same buffer (50 µl). The cells were stained with anti-CD4-APC (clone RPA-T4), washed and then stained with Smad2/3 or Stat3 at room temperature in the dark. For Smad phosphorylation, 1/10 dilution of anti-Smad2/3 PE or 1/10 dilution of mouse IgG1-PE isotype control (clone MOPC-21) were used whereas 1/5 dilution of anti-Stat3 PE or 1/5 dilution of mouse IgG2a isotype (clone MOPC-173) were

used for Stat phosphorylation. Cells were then washed with 200 µl Stain buffer, resuspended in the same buffer and immediately analysed by flow cytometry.

2.8.6 Addition of recombinant human TGF-β and/or recombinant human IL-10 to HSC cultures

To emulate how Treg cells suppress NK cell differentiation, human recombinant TGF-β and/or IL-10 were added to NK cell differentiation cultures (described in Section 2.6.3). Different concentrations of TGF-β and/or IL-10 were added every week after day 9 of culture. Cell viability was measured using 7-AAD.

2.8.7 Transwell suppression assays

To determine whether Treg cells inhibit NK cell differentiation in a cell contact dependent manner, HSC/Treg cell co-cultures were performed using HTC Transwell 96 Systems 0.4µm polycarbonate membrane plates (Corning, USA). These plates consist of two chambers separated by a 0.4µm porous membrane that allows exchange of soluble molecules between cells but prevents cell contact. The lower compartment was coated with 100 µl of 0.1% Bovine gelatine in PBS for 15-30 min at 37 °C, and then washed with PBS. Then, a concentration of 20 000 irradiated EL08.1D2 feeder layer cells/well (cultured and resuspended as described in Section 2.6.3) were added in a final volume of 275 µl basic media and incubated at 32 °C, 5% CO_2 and 96% humidity overnight. The following day, the basic media was removed and the Transwell insert was then placed in the receiver plate. Frozen HSC were thawed, resuspended in NK media (described in Section 2.6.3) at a concentration of 500 cells/well in a 275 µl final volume and added to the lower compartment. Resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures in the upper compartment. Treg cells were resuspended in NK media and their cell number was adjusted as to have a ratio of 1:4 (Treg cells:HSC) in a 75 µl final volume. Cultures underwent weekly hemi-depletion (described in Section 2.6.3) via the basolateral access port.

2.9 Enzyme linked immunosorbent assay

IFN-γ, TGF-β, TNF-α and IL-10 secretion from all culture conditions were measured using the corresponding human Ready-set-go kits (eBioscience, USA) according to manufacturer's protocols. Supernatants were collected from cultures with constant cell numbers: 200 000 cells for NK cells and 50 000 cells for Treg cells. Cell numbers in HSC cultures varied through time but was kept consistent between samples. All assays were performed with non-stimulated samples, except for IFN-γ, where K562 cells (1:1 ratio; NK cell:K562) and 100 ng/ml PMA + 1 µg/ml ION (positive control) were used. Supernatants were stored at -20 °C until analysed. Samples were read using the BioTek Reader (BioTek USA) using KC Junior software (BioTek, USA).

2.10 Molecular biology techniques

2.10.1 RNA extraction

RNA extraction was performed using the RNeasy mini kit (Qiagen, UK) following the manufacturer's recommendations. To prevent RNase contamination, RNase Zap (Life Technologies, UK) was used to clean all materials and surfaces. Once extracted, RNA concentration was measured using the NanoDrop ND-1 000 spectrophotometer (Thermo Scientific, USA) and stored at -70 °C until used.

2.10.2 Reverse transcription

Complementary DNA (cDNA) was obtained using the Superscript III Reverse Transcriptase (Life Technologies, UK). An amount of 2 µl RNA at 100 ng/µl, 7 µl sterile distilled water, 2 µl Random Primers 600 µg/ml (Promega, USA), and 1 µl 25 mM dNTPS (25 mM each nucleotide) (Bioline, USA) were added and incubated in an Eppendorf Mastercycle thermocycler (Eppendorf, USA). First, the mixture was heated at 65 °C for 5 min and placed on ice for 5 min (optimal annealing temperature of primers). Then, 4 µl Buffer 5X, 2 µl DTT (100 mM) and 1 µl recombinant RNasin ribonuclease inhibitor 40 U/µl (Roche,

Switzerland) were added to the mixture. The mixture was then incubated at 25 °C for 10 min, followed by 42 °C for 2 min. Then, 1 µl Superscript III Reverse transcriptase 200 U/µl (Life Technologies, UK) was added and the mixture was incubated at 42 °C for 50 min and 70 °C for 15 min. Finally, the cDNA was resuspended in 40 µl sterile distilled water and stored at -20 °C. Samples were used within three days.

2.10.3 Real time PCR

To assess gene expression, the Precision 2X qPCR Master Mix with Low ROX SYBRgreen (Primer Design, UK) was used. Optimal housekeeping genes were selected using a geNorm kit (Primer Design, UK) in the laboratory by Martha Luevano. ATP synthase 5B (ATP5B), ubiquitin C (UBC) and topoisomerase 1 (TOP1) genes exhibited the best stability among the twelve genes tested; therefore they were used as reference genes throughout all experiments. Primers were chosen from published studies (**Table 2.4**) and primer concentrations and the PCR program standardised (not depicted). All primers were used at a concentration of 300 nM, except for TGF-β, IL-10, Foxp3, and Rorc at 900 nM. The PCR program was set as follows for all primers: 50 °C for 2 min, 95 °C for 10 min and 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. To assure specificity, a dissociation stage was added after each reaction: 1 cycle of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. Results are presented as relative expression (cycle threshold (ct) target gene/ ct reference gene), where ct is defined as the number of cycles required for the fluorescent value to cross the threshold or baseline level of expression. Hence, the higher the ratio, the lower the amount of messenger RNA (mRNA) of the gene of interest.

Table 2.4: Primer sequences used for real time PCR. Information about sequences, product lengths (base pairs) and relevant reference are given. PL: product length, Id2: DNA-binding protein inhibitor, Tox: thymocyte selection-associated high mobility group box protein, Irf-2: interferon regulatory factor-2, Bcl11b: B-cell lymphoma/leukaemia 11B, Eomes: Eomesodermin. All primers were purchased from Sigma, UK.

Gene		Sequence 5'-3'	PL	Reference
Bcl11b	Fwd	CTCTCACCCACGAAAGGCAT	137	(Pinho et al., 2012)
	Rev	GCACGCAGAGGTGAAGTGAT		
E4bp4	Fwd	CCAAGGGCCCCATCCATTC	315	(Vacca et al., 2011)
	Rev	GATGCCAGTGCTCCGATTTG		
Eomes	Fwd	ACTGGTTCCCACTGGATGAG	160	(Hertoghs et al., 2010)
	Rev	CCACGCCATCCTCTGTAACT		
Foxp3	Fwd	CACCTGGCTGGGAAAATGG	63	(Sun et al., 2012)
	Rev	GGAGCCCTTGTCGGATGAT		
Gata-3	Fwd	AGCACAGAAGGCAGGGAGTGT	148	(Pinho et al., 2012)
	Rev	TTCGCTTGGGCTTAATGAGGGGC		
Helios	Fwd	ACACCTCAGGACCCATTCTG	129	(Cai et al., 2009)
	Rev	TCCATGCTGACATTCTGGAG		
Id2	Fwd	CGGATATCAGCATCCTGTCC	100	(Cupedo et al., 2009)
	Rev	TCATGAACACCGCTTATTCAG		
IL-10	Fwd	TGAGAACAGCTGCACCCACT	164	(Sun et al., 2012)
	Rev	GGCAACCCAGGTAACCCTTA		
IFN-γ	Fwd	CCAGGACCCATATGTAAAAG	144	(Gober et al., 2008)
	Rev	TGGCTCTGCATTATTTTTC		
Irf-2	Fwd	CCTATGCAGAAAGCGAAACGACTGA	122	(Pinho et al., 2012)
	Rev	TCGAGTCCCCATGTTGCTGAGGT		
Pu.1	Fwd	TGTTACAGGCGTGCAAAATGGAAGG	104	(Bonadies et al., 2010)
	Rev	CTCGTGCGTTTGGCGTTGGTATAGA		
Rorc	Fwd	AGTCGGAAGGCAAGATCAGA	192	(Ortega et al., 2009)
	Rev	CAAGAGAGGTTCTGGGCAAG		
T-bet	Fwd	GGATGCGCCAGGAAGTTTCA	149	(Pinho et al., 2012)
	Rev	CTCTGGCTCTCCGTCGTTCA		
Tgf-β	Fwd	GACTACTACGCCAAGGAGGTCA	88	(White et al., 2010)
	Rev	TGCTGTGTGTACTCTGCTTGAAC		
Tox	Fwd	TATGTGCCAGCCAGCCAGTCCTA	92	(Pinho et al., 2012)
	Rev	TGGTCTGGGAGGGAAGGAGGAGTAA		

2.11 Statistics

Statistical analysis and graphs were prepared using PRISM v.5 (GraphPad Software,USA). Mann-Whitney non-parametric test or paired t-test was applied. Degree of significance was determined as p value<0.05.

3 Umbilical cord blood regulatory T cell phenotype and function

3.1 Introduction

$CD4^+CD25^{high}Foxp3^{high}$ Treg cells are a subtype of $CD4^+$ T cells that primarily regulates other immune cell types including T cells (Takahashi et al., 1998, Thornton and Shevach, 1998, Thornton and Shevach, 2000, Trzonkowski et al., 2004), B cells (Lim et al., 2005) and NK cells (Ghiringhelli et al., 2005). Because of their function, the use of Treg cells as an adoptive therapy has been proposed to reduce exacerbated reactions such as GvHD post-HSCT. However, characterisation of Treg cells, activation state, purity of isolations, cell doses, functionality and stability of Treg cells after infusion are parameters that still need further investigation to optimise a Treg cell therapy for GvHD.

PB and CB have been proposed as potential cell sources for a Treg cell therapy. In particular, CB Treg cells have become more attractive than PB Treg cells due to their naivety and better capacity to maintain Foxp3 expression, two characteristics that allow them to maintain robust suppressive capacity and stability after expansion (Hoffmann et al., 2006). In addition, it has also been reported that CB Treg cells are more resistant to apoptosis than PB Treg cells (Miyara et al., 2009). In terms of function of CB Treg cells, some groups have demonstrated their capacity to suppress effector cells *in vitro* (Wing et al., 2005, Seddiki et al., 2006b), while others have shown that they are not suppressive (Wing et al., 2003, Thornton et al., 2004b, Chang et al., 2005, Fujimaki et al.,

2008). The discrepancies observed between these studies could be due to the use of different culture conditions such as the choice of stimulus (soluble anti-CD3 or IL-2), and/or the presence and source of APCs used.

The coordinated action of chemokines secreted by target tissues mediates lymphocyte migration via cognate receptors (Moser and Loetscher, 2001). Human PB Treg cells express receptors that can mediate migration to lymphoid tissues (Grindebacke et al., 2009), inflammatory sites (Zhang et al., 2009), tumour sites (Redjimi et al., 2012) and BM (Zou et al., 2004). However little information is available about the homing properties of CB Treg cells (Grindebacke et al., 2009).

This chapter investigates the phenotype and suppressive function of both resting and TCR-stimulated CB Treg cells. The aim is to identify differences between resting and TCR-stimulated CB Treg cells and to determine the conditions under which CB Treg cells are suppressive *in vitro*. Moreover, the trafficking repertoire of CB Treg cells in comparison to PB Treg cells was analysed under different conditions to understand their capacity to migrate to tissues where tolerance is required and to identify potential sites of NK cell/Treg cell interaction.

3.2 Assessment of umbilical cord blood regulatory T cell purity and phenotype after isolation

3.2.1 Phenotype of umbilical cord blood and peripheral blood regulatory T cells after isolation: a comparative study

The purity of Treg cell isolations from CB and PB was assessed by flow cytometry using a published protocol (Figueroa-Tentori et al., 2008). The expression of CD4, CD25 and CD127 was analysed in accordance with a published Treg cell gating strategy (Liu et al., 2006), which demonstrates that low expression of the IL-7 receptor α-chain (CD127) directly correlates with $Foxp3^{high}$ cells (>90%), thus identifying Treg cells as $CD4^{+}CD25^{high}CD127^{low}$ (**Figure 3.1**) without requiring intracellular staining for Foxp3.

In this study, the isolation purity of Treg cells from $CD4^{+}$ T cells was lower for PB than CB (~73.9% and ~90.10% respectively, p=0.0009) (**Figure 3.1A**). This difference in purity can be explained by the presence of memory Tcon cells in PB that express CD25 (**Figure 3.1C.2**), but are absent in CB (**Figure 3.1C.1**). Furthermore, as it was aimed to co-culture CB Treg cells with CB NK cells, the frequency of Treg cells from total lymphocytes was also evaluated. Consistently, it was found that CB Treg cells exhibited higher purity in comparison to PB Treg cells with a median of 76.95% and 56.75% respectively (p=0.0002) (**Figure 3.1B)**. Notably, recovery rates of Treg cells were comparable from both cell sources (~0.5-1%).

Foxp3 is the signature marker of Treg cells. For this reason, the difference in density of expression of Foxp3 was analysed between CB and PB Treg cells. Interestingly, $CD4^{+}CD25^{high}CD127^{low}$ CB Treg cells showed lower Foxp3 expression than PB Treg cells (MFI of 155 and 350, respectively), which is consistent with previously reported data (Milward et al., 2013) (**Figure 3.1D.1-D.2**).

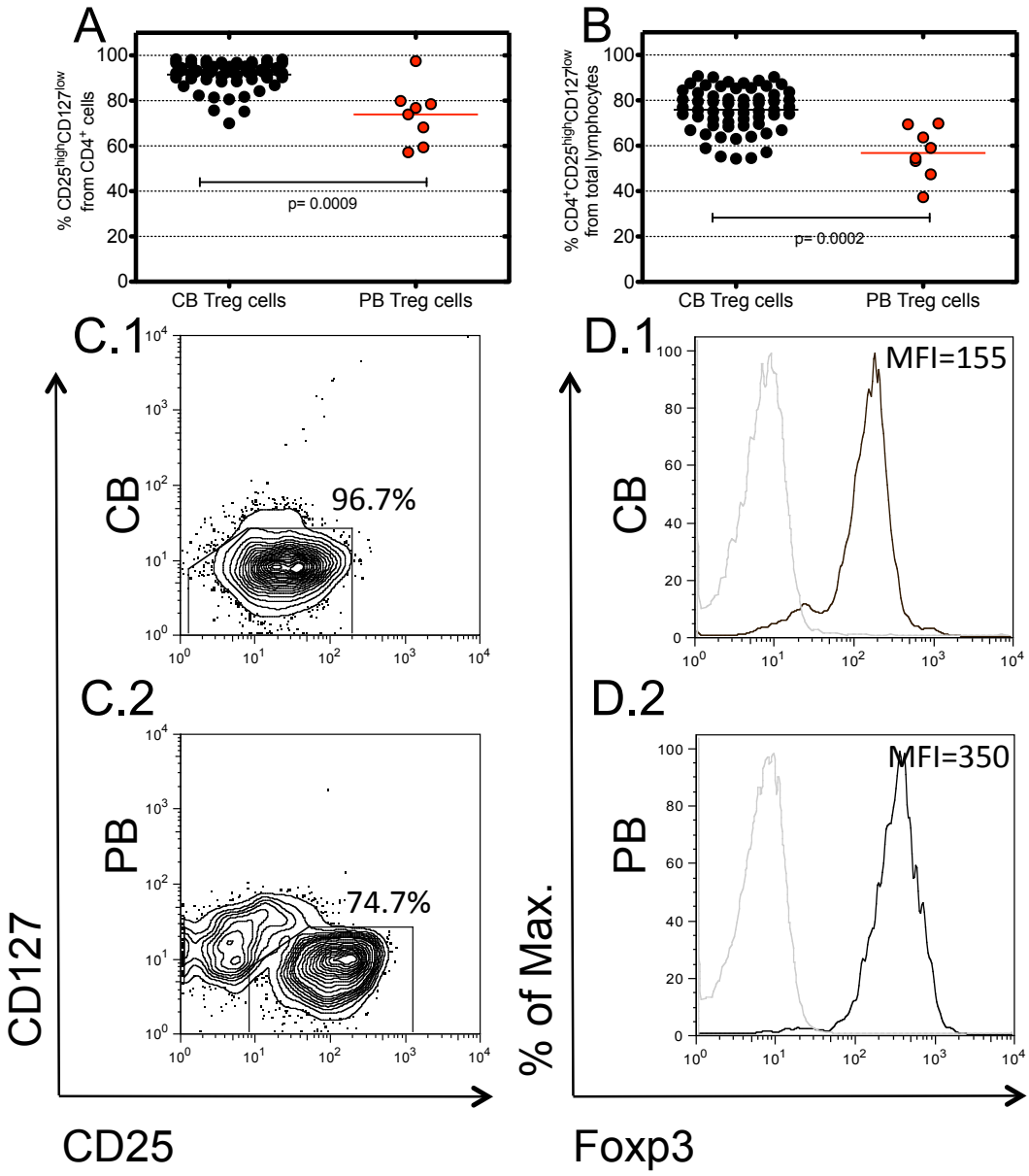


Figure 3.1: Purity and phenotypic comparison of resting CB Treg cells and resting PB Treg cells. (A) Assessment of purity of CB and PB Treg cells when gated on $CD4^+$ cells. (B) Treg cell purity gated on total lymphocytes. n=62(CB) and n=9 (PB). (C.1-C.2) Flow cytometric analysis of CD25 and CD127 expression on CB and PB Treg cells, gated on $CD4^+$ T cells. (D.1-D.2) Foxp3 MFI on $CD4^+CD25^{high}CD127^{low}$ Treg cells. Grey line: unstained. Data is representative of nine independent experiments.

To further assess the phenotype of CB Treg cells, the expression of LAP, which is associated with Treg cell function was evaluated. LAP is a molecule that remains non-covalently bound to TGF-β in a complex called latent TGF-β (Khalil, 1999). Upon TCR-stimulation, LAP either undergoes conformational change or is degraded (Annes, 2003, ten Dijke and Arthur, 2007), thus allowing TGF-β release from the complex and activation. Hence, LAP expression can be

directly correlated with the availability of TGF-β in its soluble form or membrane bound on Treg cells (Stockis et al., 2009).

In resting conditions, CB and PB Treg cells expressed LAP at a similar level (<10% LAP-positive Treg cells) (**Figure 3.2A**); however, CB Treg cells had lower LAP density than PB Treg cells (MFI of 1.78 and 4.44 respectively, p=0.03) (**Figure 3.2B**). Upon TCR-stimulation, CB and PB Treg cells had similar LAP expression in frequency (~80%) and density (MFI of 40), however LAP expression started to decline after 48 h stimulation, particularly for CB Treg cells (**Figure 3.2A-B**). These findings are consistent with published data, which report <4% LAP expression on resting PB Treg cells and <60% on TCR-stimulated PB Treg cells (Stockis et al., 2009).

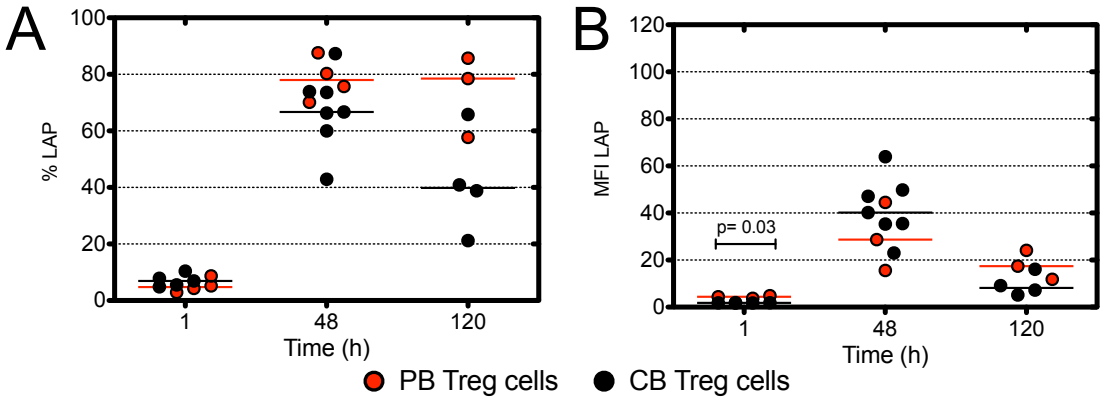


Figure 3.2: Expression of LAP on Treg cells. Percentages (A) and MFI (B) of LAP expression as analysed by flow cytometry for CB and PB Treg cells after a "resting step" of 1 h or after TCR-stimulation for 48 and 120 h with soluble anti-CD3/soluble anti-CD28 and 600 IU/ml (for CB Treg cells) or 100 IU/ml (for PB Treg cells) IL-2. Cells were gated on $CD4^{+}CD25^{high}CD127^{low}$ when resting and $CD4^{+}Foxp3^{high}$ when TCR-stimulated. n=3-7.

3.2.2 TGF-β and IL-10 production by umbilical cord blood regulatory T cells

TGF-β and IL-10 have been reported to be involved in Treg cell suppressive function (Annacker et al., 2001, Oida et al., 2006). As it has been suggested that Treg cells require TCR-stimulation to be suppressive (Thornton and Shevach, 1998, Takahashi et al., 2000), the production of these suppressive molecules by CB Treg cells was evaluated when resting and upon TCR-

stimulation. CB Treg cells were isolated and cultured in the presence of IL-2 (resting) or IL-2 and plate bound anti-CD3/soluble anti-CD28 (TCR-stimulated). Notably, the concentration of IL-2 used in these experiments did not cause significant activation but was essential for the survival and proliferation of CB Treg cells (Section 2.6.1). Supernatants were collected every 24 h over five days and analysed by ELISA to measure secreted TGF-β and IL-10.

Resting and TCR-stimulated CB Treg cells secreted high levels of TGF-β, with levels remaining constant over the period studied (**Figure 3.3A**). Conversely, whilst resting CB Treg cells secreted low amounts of IL-10, TCR-stimulated CB Treg cells secreted increasing amounts of IL-10 with the duration of stimulation (**Figure 3.3B**).

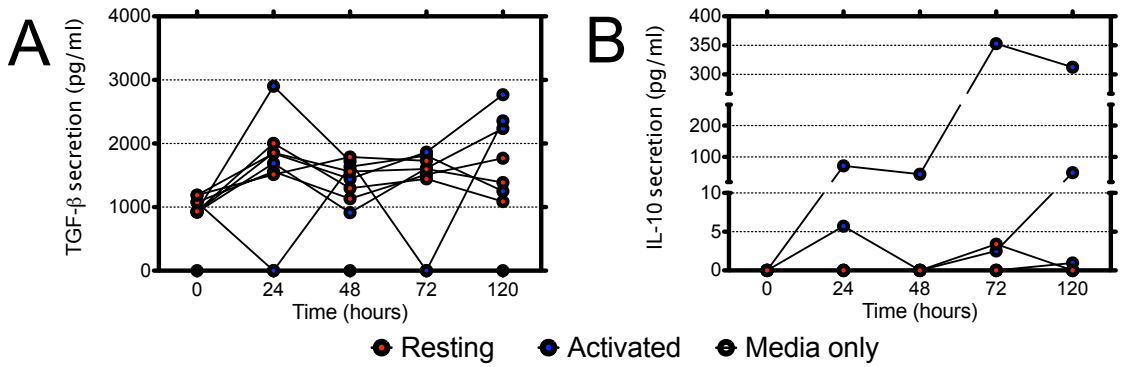


Figure 3.3: Assessment of IL-10 and TGF-β secretion by CB Treg cells. CB Treg cells were cultured with 1 000 IU/ml IL-2 (resting) or with 1 000 IU/ml IL-2 and plate bound anti-CD3/soluble anti-CD28 (TCR-stimulated). Supernatants were collected at 0, 24, 48, 72 and 120 h. TGF-β (A) and IL-10 (B) secretion in supernatants was analysed by ELISA. Complete media was used as negative control. n=4.

3.3 Umbilical cord blood regulatory T cells display a naive phenotype upon TCR-stimulation

CB has been identified as a potential source of Treg cells for adoptive therapy because the majority of CB Treg cells exhibit a naive phenotype ($CD45RA^+$). This phenotype in PB has been associated with a homogenous population of Treg cells with higher Foxp3 expression, proliferative rate and suppressive capacity after expansion (Hoffmann et al., 2006). To evaluate whether CB Treg cells maintained naivety upon TCR-stimulation, the expression of markers associated with naivety on resting and TCR-stimulated CB Treg cells was

assessed. For the latter, the TCR-stimulation protocol described in Chapter 2 (plate bound anti-CD3/soluble anti-CD28 and IL-2) was used to assess whether CB Treg cells switch from a naive to a memory phenotype ($CD45RA^-$ cells) after stimulation. It was found that the majority of resting and TCR-stimulated CB Treg cells express CD45RA after stimulation and therefore have a naive phenotype (**Figure 3.4A**) with no difference in MFI over the period studied (**Figure 3.4B**).

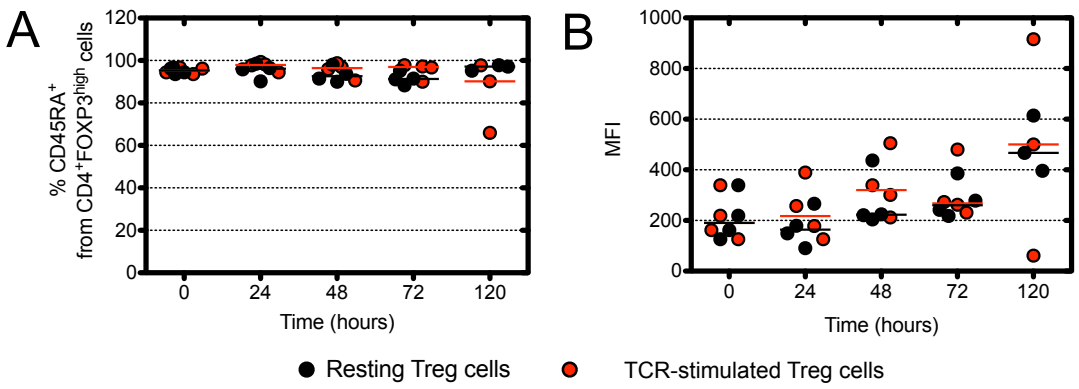


Figure 3.4: Assessment of CD45RA expression on CB Treg cells. Flow cytometric analysis of CD45RA expression as percentages (A) and MFI (B) on resting (black) and TCR-stimulated (red) CB Treg cells (gated on $CD4^+Foxp3^{high}$). CB Treg cells were TCR-stimulated using plate bound anti-CD3/soluble anti-CD28 and IL-2 (1 000 IU/ml). The lines represent medians. n=3-4.

CCR7 and L-selectin, homing receptors associated with migration to SLT, are expressed on naive but not on memory T cells, suggesting the use of these markers as a complementary strategy to identify naive T cells (Sallusto et al., 1999). Notably, the expression of these receptors on naive PB Treg cells has also been reported (Ermann et al., 2005, Valmori et al., 2005). Therefore, to fully evaluate whether CB Treg cells maintain a naive phenotype upon TCR-stimulation, the expression of these two receptors on resting and TCR-stimulated CB Treg cells was assessed. Interestingly, constant and high levels of expression of these two markers were observed on CB Treg cells in resting conditions and upon TCR-stimulation with a median of ~95% expression for L-selectin and 96% expression for CCR7 over the period studied (**Figure 3.5**). Collectively, these results suggest that CB Treg cells maintain a naïve phenotype for up to 120 h upon TCR-stimulation, under the conditions used in this study.

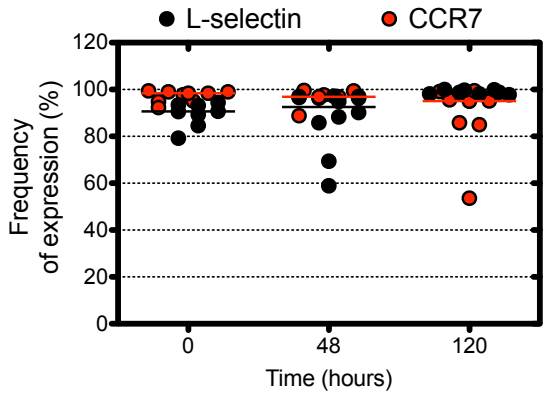


Figure 3.5: L-selectin and CCR7 expression on CB Treg cells. Flow cytometric analysis of L-selectin (black) and CCR7 (red) expression on TCR-stimulated CB Treg cells (gated on $CD4^{+}Foxp3^{high}$). CB Treg cells were TCR-stimulated using plate bound anti-CD3/soluble anti-CD28 and IL-2 (1 000 IU/ml). The lines represent medians. n=8-10.

3.4 Umbilical cord blood regulatory T cells proliferate upon TCR-stimulation

Recent studies from Miyara and colleagues (Miyara et al., 2009) have demonstrated that PB Treg cells proliferate upon TCR-stimulation and exposure to IL-2 and/or Tcon cells (source of IL-2). In the present study, all CB Treg cells express CD45RA. Therefore, the ability of Treg cells to proliferate in the presence of high levels of IL-2 and TCR-stimulation was investigated. Proliferation was analysed using CFSE, which can be used to measure the number of divisions a cell has undergone (Lyons and Parish, 1994). CB Treg cells proliferate upon TCR-stimulation and underwent one division after 24 h, and up to five divisions after 120 h (**Figure 3.6**). This suggests that CB Treg cells are responsive and can proliferate upon stimulation.

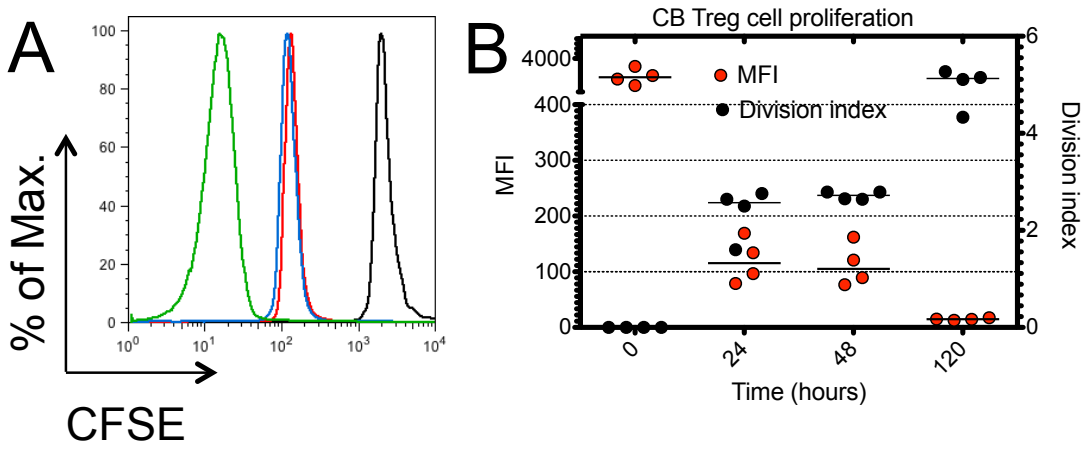


Figure 3.6: CB Treg cell proliferation upon TCR-stimulation. CFSE labelled CB Treg cells were TCR-stimulated with plate bound anti-CD3/soluble anti-CD28 and IL-2 (1 000 IU/ml), and analysed by flow cytometry at 0 (black), 24 (red), 48 (blue) and 120 h (green). Cells were gated on $CD4^+Foxp3^{high}$ cells. (A) Representative histogram plots. Data is representative of four independent experiments, (B) MFI and division index. Cells were gated on $CD4^+Foxp3^{high}$ cells. The lines represent medians. n=4.

3.5 Umbilical cord blood regulatory T cells are suppressive *in vitro*

It is currently not clear whether CB Treg cells are suppressive because of contradictory results between studies performed by different groups. This could be explained by the difference in the conditions used in the reported suppressive assays (i.e. source of responders, stimuli, and/or presence and source of APCs). In this study, the suppressive capacity of CB Treg cells was assessed *in vitro* based on a method developed by Seddiki and colleagues (Seddiki et al., 2006b). For this method, CB Treg cells are co-cultured with autologous CB $CD4^+$ Tcon cells and irradiated PBMCs as APCs, in the presence of soluble anti-CD3. Proliferation was measured by titriated thymidine (^3H) incorporation after 72 h culture. Overall, CB Treg cells were capable of suppressing proliferation of CB $CD4^+$ Tcon by ~60% at a 1:1 Treg cell:Tcon cell ratio (p=0.009) and ~40% at a 1:4 ratio (p=0.01), but showed no significant suppressive capacity at a 1:10 ratio (**Figure 3.7A-B**).

The impact of CB Treg cells on the proliferation of CB $CD4^+$ Tcon cells was also assessed by CFSE dilution. After isolation, $CD4^+$ Tcon cells were labeled with CFSE and co-cultured with CB Treg cells, irradiated PBMCs and soluble anti-

CD3. Proliferation was measured at 72 h by flow cytometry. CB Treg cells suppressed the proliferation of CB $CD4^+$ Tcon cells when co-cultured at a 1:1 ratio reducing the number of divisions by 55% (p=0.02) and by ~20% at a 1:4 ratio (p=0.01) (**Figure 3.7C**). Hence, both assays demonstrated the suppressive capacity of CB Treg cells when a Treg cell:Tcon cell ratio of a minimum of 1:4 was used.

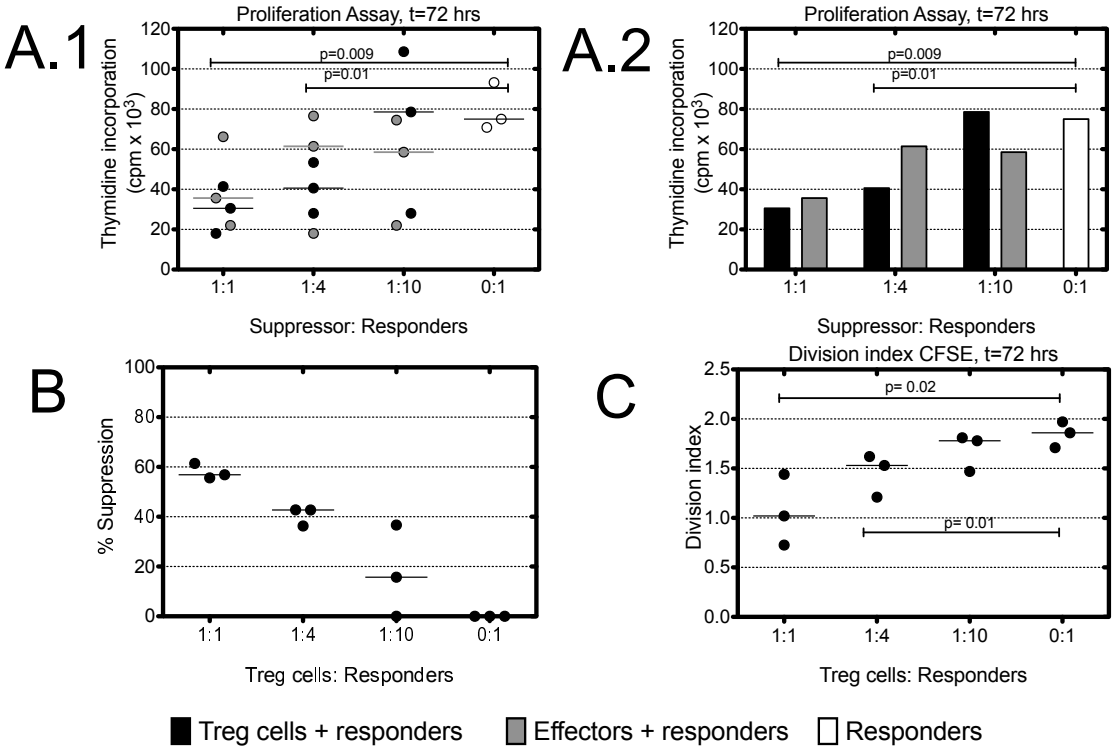


Figure 3.7: Suppression of proliferation of Tcon cells by CB Treg cells *in vitro*. (A.1) Suppression of proliferation by thymidine assay in cpm at 72 h. (A.2) Summary plot of A.1 at 72 h. (B) Percentage of suppression of Treg cells over Tcon cells. ([(cpm target/cpm control)-1]*100). (C) Suppression of proliferation of CFSE labelled "responders" ($CD4^+CD25^-$) at 72 h. Treg cells ($CD4^+CD25^{high}CD127^{low}$) or Tcon cells ($CD4^+CD25^-$), referred to as "suppressors" were cultured at different ratios indicated on the x-axis. Irradiated PBMCs were used as APCs and 1 µg/ml soluble anti-CD3 as stimulus. The lines represent medians of three independent experiments.

3.6 Trafficking repertoire of umbilical cord blood and peripheral blood regulatory T cells under resting conditions and upon TCR-stimulation

Grindebacke and colleagues described the expression of homing receptors such as α4 and β7 integrins, CCR7 and L-selectin to be crucial for CB Treg cells to traffic to SLT and the gut, both of which are major sites of interaction

between the immune system and pathogens during early life (Grindebacke et al., 2009). To my knowledge, no information is available on the expression of receptors involved in trafficking of CB Treg cells to other tissues such as the BM and inflammatory sites, which are potential tissues in which the interaction between Treg cells and NK cells could take place.

Analysis of the trafficking repertoire of CB and PB Treg cells has been extended to include CXCR4 and CXCR7 expression (homing to the BM) as it has been shown that the BM is a significant reservoir and priming site of $CD4^+$ Tcon (Feuerer et al., 2001, Feuerer et al., 2003) and Treg cells (Zou et al., 2004), and the main site of NK cell differentiation. In addition, the expression of CCR5 (Zhang et al., 2009), CCR6 (Yamazaki et al., 2008), CXCR1 (Eikawa et al., 2010) and CXCR3 (Duffner et al., 2003) on Treg cells was also assessed as these markers are associated with migration to inflammatory sites under pathological conditions and to target organs in GvHD (Wysocki et al., 2005).

Since TCR-stimulation is a factor that changes the migratory repertoire of Treg cells (Ding et al., 2012), the expression of the aforementioned receptors was analysed in resting and TCR-stimulated conditions. This analysis sought to provide a comparison of the migration repertoire of CB and PB Treg cells in order to correlate the results from this study with results reported in the literature for PB Treg cells.

3.6.1 Expression of homing receptors on regulatory T cells under resting conditions

Freshly isolated CB Treg cells expressed all the aforementioned markers (**Figure 3.8**). β7 integrin expression was higher on CB Treg cells compared to PB Treg cells (p=0.004) (**Figure 3.8A**). Conversely, L-selectin (p=0.008), CCR5 (p=0.05), CCR6 (p=0.05), CXCR1 (p=0.04) and CXCR3 (p=0.01) expression was lower on CB Treg cells than on PB Treg cells (**Figure 3.8A**). Regarding MFI, only the inflammation-associated markers, CCR5 (p=0.05), CCR6 (p=0.05) and CXCR1 (p=0.05), showed higher density of expression on PB Treg cells (**Figure 3.8B**). These results suggest that resting CB Treg cells display a different trafficking and migration repertoire than resting PB Treg cells.

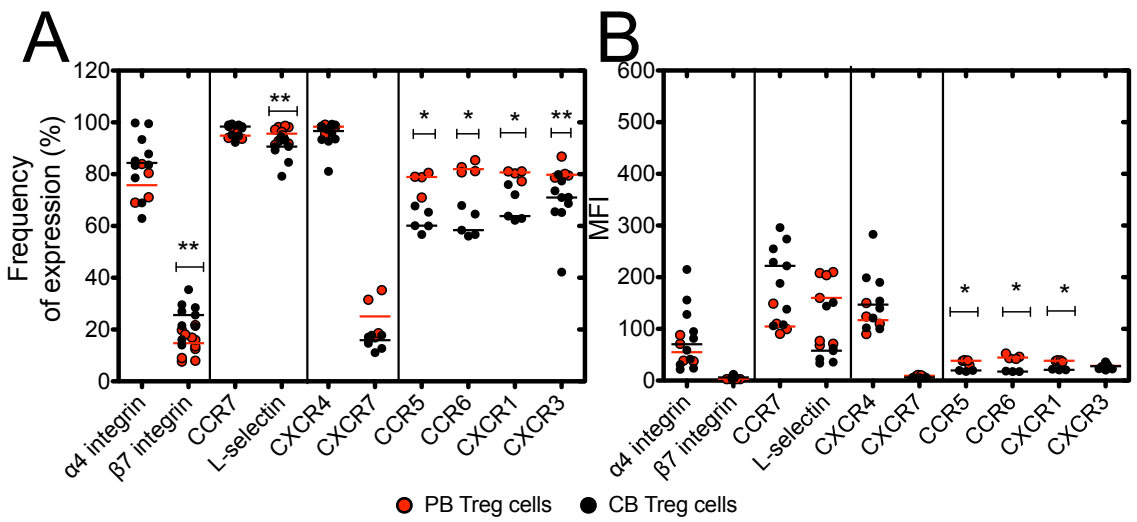


Figure 3.8: Expression of surface markers involved in homing and migration of freshly isolated CB and PB Treg cells. Flow cytometric analysis of migration receptors on CB Treg cells (black) and PB Treg cells (red). Data is presented as frequency of expression (A) and MFI (B) from $CD4^{+}CD25^{high}CD127^{low}$ gated Treg cells. Cells were isolated and cultured for 1 h with RPMI and 1% BSA without fatty acids prior staining. The lines represent medians. *, p value ≤ 0.05, **, p value ≤ 0.01. n= 3-10.

3.6.2 Expression of homing receptors on regulatory T cells upon TCR-stimulation

To evaluate whether CB Treg cells exhibit a similar phenotype to PB Treg cells upon TCR-stimulation, CB and PB Treg cells were TCR-stimulated with soluble anti-CD3/soluble anti-CD28 and IL-2 and then their trafficking repertoire was analysed by flow cytometry at two different time points (**Figure 3.9**).

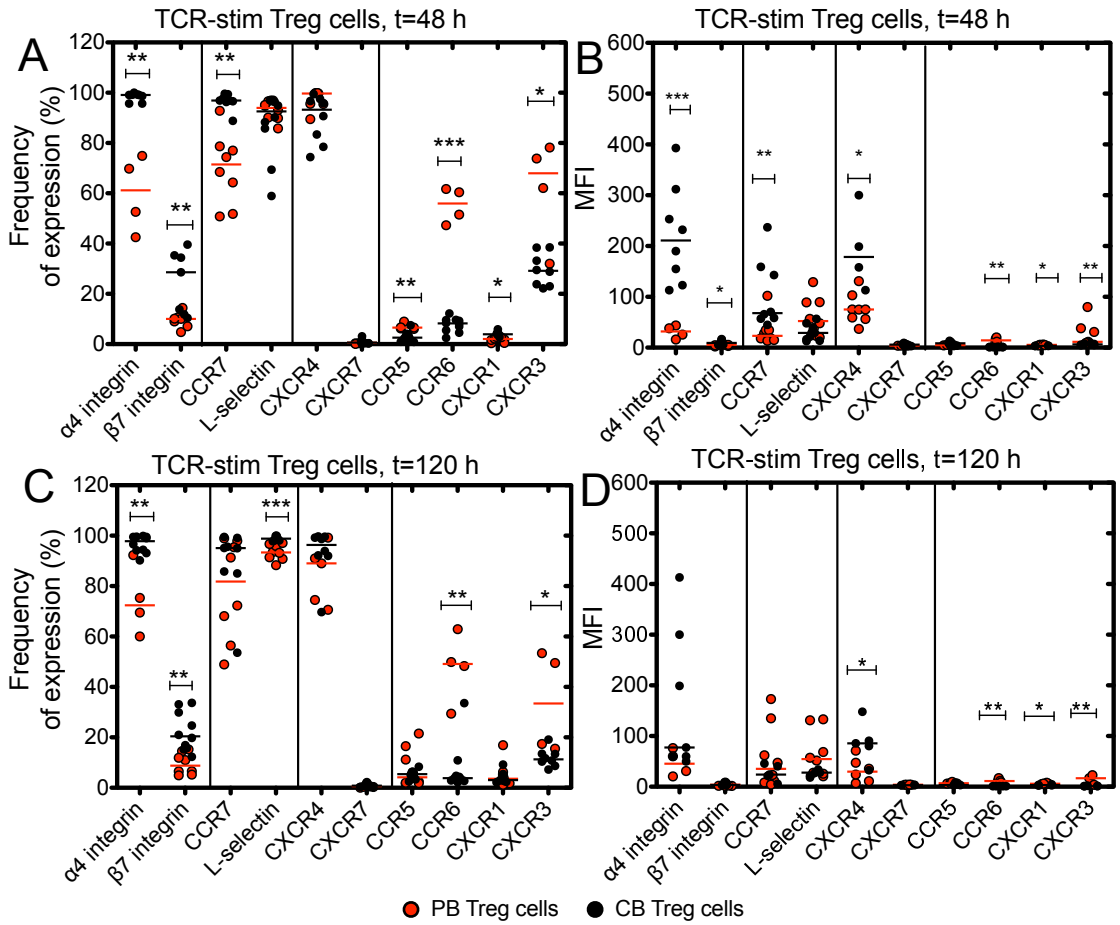


Figure 3.9: Expression of markers involved in homing and migration on CB and PB Treg cells upon TCR-stimulation. Flow cytometric analysis of migration receptors on CB Treg cells (black) and PB Treg cells (red) activated with soluble anti-CD3/soluble anti-CD28 and IL-2 (600 IU/ml for CB Treg cells and 100 IU/ml for PB Treg cells) after 48 and 120 h stimulation. Data represent frequency of expression (A and C) and MFI (B and D) from $CD4^{+}Foxp3^{high}$ gated Treg cells. n= 3-10. The lines represent medians. *, p value ≤ 0.05, **, p value ≤ 0.01, *** p value ≤ 0.001.

Upon TCR-stimulation, the expression of α4 and β7 integrins was significantly higher on CB Treg cells after 48 h stimulation (p=0.002 and p=0.007, respectively) (**Figure 3.9A**) and 120 h stimulation (p=0.004 and p=0.002, respectively) (**Figure 3.9C**). Likewise, CCR7 expression was higher on CB Treg cells after 48 h stimulation (p=0.003) (**Figure 3.9A**), however CB and PB Treg cells showed similar CCR7 expression after 120 h stimulation (**Figure 3.9C**). In addition, CB and PB Treg cells expressed high levels of L-selectin (~95%) over the period studied (**Figure 3.9A,C**). Importantly, CXCR4 expression was constant on CB and PB Treg cells, suggesting that Treg cells have the capacity to migrate to the BM where NK cell differentiation and maturation occurs (**Figure 3.9C**). Lastly, lower expression of the receptors associated with migration to inflammation sites was observed on CB Treg cells in comparison to

PB Treg cells: CCR5 after 48 h stimulation (p=0.008) (**Figure 3.9A**), CCR6 after 48 and 120 h stimulation (p=0.0007 and p=0.005, respectively) (**Figure 3.9A,C**), and CXCR3 after 48 and 120 h stimulation (p=0.02 and p=0.01, respectively) (**Figure 3.9A,C**).

As for the density of expression (MFI), integrins α4 (p=0.007) and β7 (p=0.02), CCR7 (p=0.003) and CXCR4 (p=0.02) showed higher levels on CB Treg cells than PB Treg cells after 48 h stimulation (**Figure 3.9B**); however, only CXCR4 (p=0.02) had higher expression levels after 120 h stimulation (**Figure 3.9D**). In contrast, CCR6 (p value t_{48h}=0.006, p value t_{120h}=0.01), CXCR1 (p value $t_{48h, 120h}$=0.02) and CXCR3 expression (p value t_{48h}=0.001, p value t_{120h}=0.01) was higher on PB Treg cells than on CB Treg cells at both time points analysed (**Figure 3.9B,D**).

3.6.3 Summary of homing and migration receptor repertoire on resting and TCR-stimulated regulatory T cells

To summarise, three different patterns of expression of homing and trafficking receptors on CB Treg cells were detected (**Figure 3.10A**): one group of markers that were constitutively expressed (integrins α4 and β7, L-selectin, CCR7 and CXCR4), one group of markers that were highly expressed under resting conditions but whose expression was greatly reduced upon TCR-stimulation (CCR5, CCR6, CXCR1 and CXCR3) and a group of markers that showed low level of expression, which decreased over the period studied (CXCR7).

The same trend was observed for PB Treg cells except that the markers whose expression that decreased upon TCR-stimulation can be further subdivided into two groups; those that showed a pronounced reduction in expression (CXCR1 and CCR5) and those that showed a modest decrease in expression (CCR7, CXCR3, CCR6, and α4 and β7 integrins). Moreover, PB Treg cells constitutively expressed CXCR4 and L-selectin, whereas CXCR7 was expressed at a low level and its expression decreased over the period studied (**Figure 3.10B**).

Finally, the expression of markers associated with activation of CB and PB Tregs cells after TCR-stimulation was evaluated (**Figure 3.10A-B**). CD69 expression by T cells appears early after stimulation. Once expressed, CD69 functions as a co-stimulatory molecule for T cell stimulation and proliferation (Ziegler et al., 1994). In this study, CD69 expression was observed on CB and PB Treg cells after 48 h stimulation but then decreased after 120 h (**Figure 3.10A-B**). It is noteworthy that this receptor is an early marker of activation, which could explain the low expression at the time points analysed. It is possible that the peak of expression for this marker is earlier than 48 h but this was not analysed.

The expression of GITR is also induced on Treg cells after stimulation (Allan et al., 2007) and acts as a co-stimulatory receptor for T cell stimulation (Ronchetti et al., 2004). In this study, high expression of GITR was observed after TCR-stimulation for both CB and PB Treg cells, 48 h being the time point where GITR expression was the highest (**Figure 3.10A-B**).

Another marker of activation is CTLA-4, which is a negative regulator of T cell activation, expressed after T cell stimulation. Hence, besides its function as an indirect mediator of suppression of Treg cells via DCs, it can also be used to analyse the activation status of Treg cells (Yamazaki et al., 2003, Allan et al., 2007). In this study, CTLA-4 was also upregulated after 48 h TCR-stimulation for both CB and PB Treg cells (**Figure 3.10A-B**).

Finally, the expression of LAP as an activation marker was also analysed due to its specific upregulation on Treg cells upon TCR-stimulation (Stockis et al., 2009). Similarly to GITR and CTLA-4, the highest expression of this receptor was observed after 48 h TCR-stimulation (**Figure 3.10A-B**). Collectively, the expression of these four receptors demonstrates that both CB and PB Treg cells are activated following 48 h TCR-stimulation.

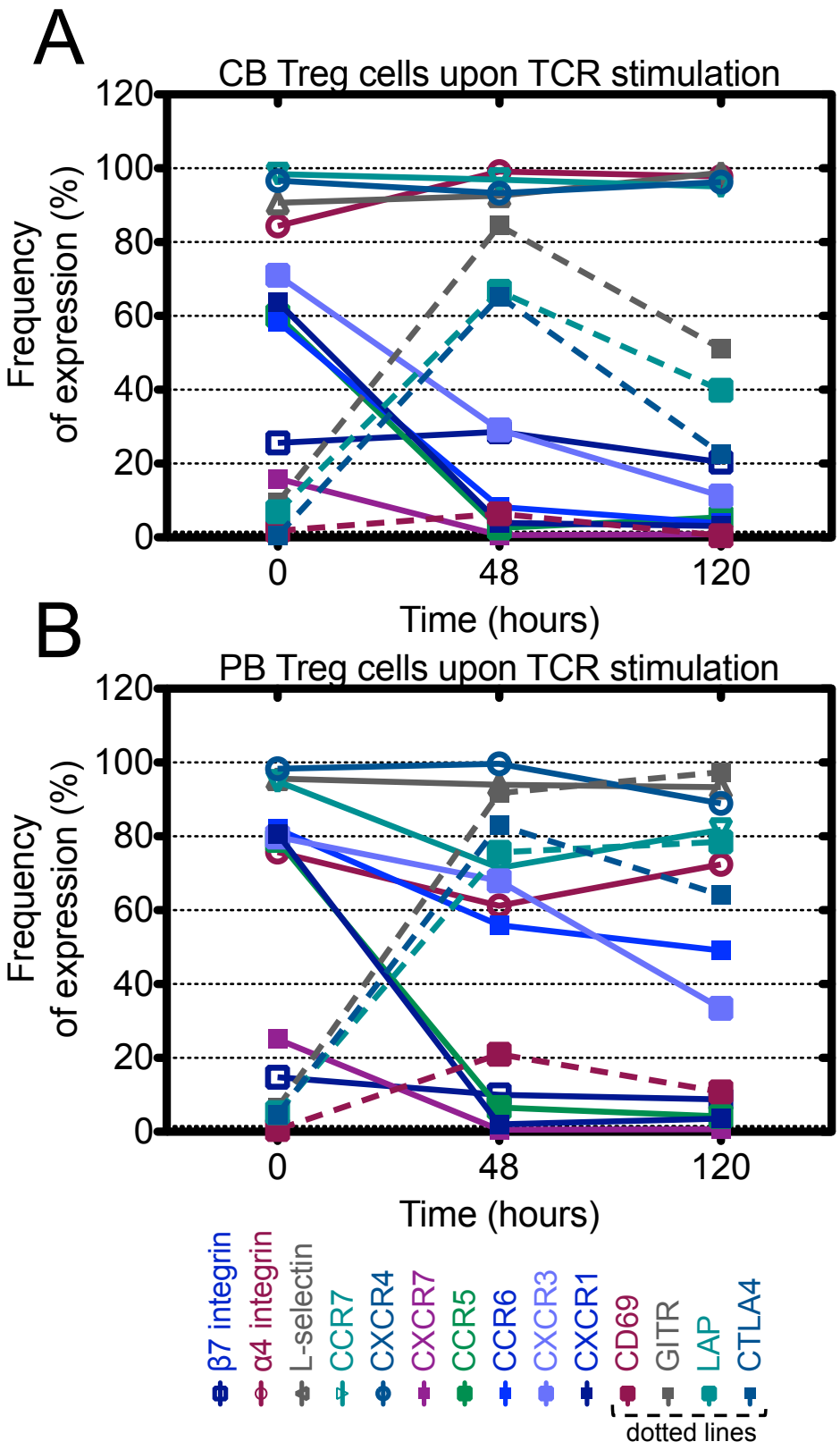


Figure 3.10: Kinetics of expression of homing and activation markers on CB and PB Treg cells. Flow cytometric analysis of surface markers involved in homing and activation of CB Treg cells (A) and PB Treg cells (B) at different time points post-activation. Treg cells were gated on $CD4^{+}Foxp3^{high}$ except for time=1h, in which cells were gated on $CD4^{+}CD25^{high}CD127^{low}$. The lines represent medians. n=5-10.

3.7 Discussion

The use of CB as a source of Treg cells for cell therapy has become more attractive. This is due to the fact that CB Treg cells are a more homogeneous population with profound suppressive capacity and ability to express Foxp3 after expansion (Hoffmann et al., 2006). In recent years, major progress has been made to evaluate the safety of using CB Treg cells in the clinic (Brunstein et al., 2011b). However complications such as a lack of Treg cell persistence and maintenance of tolerance *in vivo* require a thorough understanding of the optimal conditions under which CB Treg cells suppress and of the homing properties that these cells exhibit in order to reach target tissues *in vivo*. Hence, this chapter evaluated the phenotype of resting and TCR-stimulated CB Treg cells and analysed their suppressive capacity against $CD4^+$ Tcon cells. In addition, a panel of ten homing and chemokine receptors were analysed for the purpose of identifying potential NK cell/Treg cell interaction sites.

Low purity and yield of Treg cell isolations are still major challenges for Treg cell-based adoptive therapies. Importantly, it has been found that because of differences in T cell phenotype between CB and PB, it is possible to isolate Treg cells with higher purity from CB using magnetic column based-cell separation systems (Bresatz et al., 2007, Figueroa-Tentori et al., 2008, Milward et al., 2013). When PB Treg cells are isolated using a $CD4^+CD25^+$ selection, a population of $CD25^+$ memory T cells is co-purified with PB Treg cells, whereas CB Treg cells isolated using the same method show a clear delineation between $CD25^-$ Tcon cells and $CD25^{high}$ Treg cells. This study confirms that isolated CB Treg cells are of higher purity than PB Treg cells with comparable recovery percentages of ~0.5-1%.

The phenotype of CB Treg cells was assessed by analysing the expression of Foxp3, LAP, and by the secretion IL-10 of and TGF-β, which are both crucial for Treg cell-mediated suppression. Lower density of Foxp3 expression was found in CB Treg cells than in PB Treg cells, which is consistent with previous studies (Miyara et al., 2009, Milward et al., 2013). Miyara and colleagues observed that

$CD45RA^+$ naive PB Treg cells, despite their lower Foxp3 density, maintain high levels of Foxp3 after expansion and thus provide stability and potential plasticity, as compared to mature Treg cells (Miyara et al., 2009). Furthermore, similarly to PB Treg cells, CB Treg cells exhibited high levels of LAP after 48 h stimulation with a dramatic decrease after 120 h stimulation, which is in contradiction with what has been described by Milward and colleagues for CB Treg cells (Milward et al., 2013). The expression of this marker directly correlates with the availability of TGF-β (Stockis et al., 2009) and with a more suppressive Treg cell population. Notably, $CD4^+CD25^+LAP^+$ cells have been found to be more suppressive *in vitro* and *in vivo* when compared to $CD4^+CD25^+LAP^-$ Treg cells (Chen et al., 2008). In addition, it was found that CB Treg cells secrete TGF-β and IL-10 upon stimulation, which is consistent with published data for CB Treg cells where a different stimulation method was used (Godfrey et al., 2005). The expression of LAP on Treg cells has also been associated with the secretion of TGF-β and IL-10 in mice (Chen et al., 2008).

As previously mentioned, some studies have highlighted $CD45RA^+CD4^+Foxp3^+$ Treg cells as a more beneficial population to consider for cell therapy as compared to memory Treg cells (Hoffmann et al., 2006, Miyara et al., 2009). The results presented here show that the majority of CB Treg cells are positive for CD45RA under resting conditions, which is consistent with published data (Milward et al., 2013). However, it was uncertain whether CB Treg cells could still maintain a naive phenotype upon TCR-stimulation. Interestingly, after 120 h of TCR-stimulation with plate bound anti-CD3/soluble anti-CD28 and high concentrations of IL-2, the expression of CD45RA, L-selectin and CCR7 remained high on CB Treg cells. This suggests that over the period studied and under the conditions of stimulation used, CB Treg cells maintain a naive phenotype. Notably, several authors have reported the importance of CCR7 and L-selectin for Treg cell-mediated suppression. For instance, naive Treg cells from knockout CCR7 mice have impaired suppressive capacity (Menning et al., 2007), while the L-selectin subset of $CD4^+CD25^+$ Treg cells show more potency to protect against acute GvHD (Ermann et al., 2005) and to control diabetes (Szanya et al., 2002). Hence, It would be interesting to study longer time points and other markers associated with maturation such as CD45RO

(memory marker) in order to determine when and under which conditions the switch between naive and memory phenotype for CB Treg cells occurs after TCR-stimulation.

Furthermore, TCR-stimulation and addition of exogenous IL-2 induced proliferation of CB Treg cells in this study, which is in contradiction with the results presented by Miyara and colleagues using $CD45RA^+$ PB Treg cells (Miyara et al., 2009). However, unresponsiveness of naive Treg cells might be caused by the suppression of memory Treg cells also present in the culture from PB, or alternatively by the death of memory Treg cells. In addition, Li and colleagues analysed the importance of exogenous IL-2 for CB Treg cell proliferation (Li et al., 2005). They observed that TCR-stimulated CB Treg cells do not proliferate unless IL-2 is present as this cytokine can revert Treg cells from an anergic state. The results presented here are in agreement with this study, highlighting the importance of IL-2 for CB Treg cell proliferation.

Importantly, CB Treg cells were able to suppress Tcon cells in the presence of adult APCs and polyclonal stimulation (plate bound anti-CD3) with a minimum ratio of 1:4 (Treg cells:$CD4^+$ Tcon cells). This threshold of cell ratio is consistent with what has been reported by Brunstein and colleagues who also showed this Treg cell:Tcon cell ratio to be suppressive in their study (Brunstein et al., 2011b).

The expression of homing and chemokine receptors is crucial for Treg cells to reach specific tissues to exert suppression. Resting and TCR-stimulated CB Treg cells expressed markers that are associated with homing to secondary lymphoid organs (CCR7 and L-selectin) and to the gut (integrin α4 and β7). The latter favours the hypothesis of Grindebacke and colleagues (Grindebacke et al., 2009) who described the importance of the gut as the primary site of antigen exposure for T cells in early life where the first exposure to maternal microbiota occurs (Kelly et al., 2007). As for markers associated with inflammation, both CB and PB Treg cells showed the same expression patterns, with the exception that PB Treg cells maintained a higher frequency of these markers over the period studied. These results suggest that PB Treg cells may have a higher

capacity to migrate to inflammatory sites, but this requires further investigation. Notably, CB Treg cells express CXCR4, regardless of their stimulation state. This is consistent with published data that showed high frequencies of PB Treg cells in the BM, thus suggesting the BM as a reservoir for Treg cells (Zou et al., 2004). Hence, CB Treg cells express receptors associated with homing to the BM, LN, tumour and inflammatory sites.

It has been reported that resting CB NK cells express markers associated with migration to the BM (CXCR4), inflammatory sites (CXCR1 and CXCR3) and the gut (α4 integrin), but expressed low levels of markers associated with migration to LN (CCR7 and L-selectin) (Luevano et al., 2012a)(Alnabhan et al, unpublished data). This data is consistent with reports in mice which demonstrate that under certain conditions NK cells can lyse tumour cells in the LN (Chen et al., 2005, Berahovich et al., 2006, Garrod et al., 2007) and migrate to tumour sites via upregulation of CXCR3 (Walser et al., 2007). Hence, given the comparable patterns of homing receptor expression of CB NK cells and Treg cells, this study proposes that the BM, LN (under certain conditions), tumour and inflammatory sites are potential locations of Treg cell/NK cell interaction.

Overall, the data presented in this chapter suggests that CB Treg cells are a regulatory population with a naive phenotype that exhibit *in vitro* suppressive capacity. The ideal cell ratio for CB Treg cells to suppress was 1:4 (Treg cells:Tcon cells), which was used for subsequent suppression assays using NK cells presented in chapters 4, 5 and 6. Finally, it was demonstrated that both CB Treg cells and CB NK cells express markers associated with migration to the BM, LN (under certain conditions) and inflammatory sites, suggesting that interaction between these cells may take place in the aforementioned tissues.

4 Effect of umbilical cord blood regulatory T cells on natural killer cell phenotype and function

4.1 Introduction

The interaction between NK cells and Treg cells has been described during pregnancy as well as in pathological conditions such as viral infections, autoimmune diseases and cancer. Therefore, the work of several groups has focused on understanding the importance of this interaction using *in vitro* studies using human cells (Ghiringhelli et al., 2005, Romagnani et al., 2005, Bergmann et al., 2011) and in mice (Ghiringhelli et al., 2005, Smyth et al., 2006, Ralainirina et al., 2007, Zimmer et al., 2008, Lundqvist et al., 2009, Pedroza-Pacheco et al., 2013). Notably, it has been shown that Treg cells can inhibit NK cell functions such as natural cytotoxicity and cytokine production (Trzonkowski et al., 2004, Ghiringhelli et al., 2005, Smyth et al., 2006, Lundqvist et al., 2009, Sun et al., 2010, Zhou et al., 2010, Bergmann et al., 2011), can downregulate receptors involved in NK cell cytotoxicity such as NKG2D (Ghiringhelli et al., 2005, Bergmann et al., 2011) and NKp44 (Bergmann et al., 2011), and can inhibit NK cell proliferation (Romagnani et al., 2005, Kim et al., 2007) (**Table 1.3**). Similarly to what has been described for the effect of Treg cells on $CD4^+$ Tcon cells, all these studies identified three specific conditions that are required for Treg cell-mediated suppression of NK cells: (i) absence of cytokines, (ii) a

minimum ratio of Treg cells per target cell (in this case NK cells), (iii) and TCR-stimulation of Treg cells (Godfrey et al., 2005, Pandiyan et al., 2007).

One of the theories by which Treg cells can mediate suppression is by the depletion of cytokines. In order to prevent NK cell activation, human Treg cells can deprive NK cells of cytokines such as IL-2 under homeostatic conditions (Ghiringhelli et al., 2005). This suppression can be overcome during inflammation by the presence of exogenous IL-2, IL-4, IL-7 (Ghiringhelli et al., 2005), IL-12 or IL-18 (Lee et al., 2012). Gasteiger and colleagues have recently confirmed that this mechanism of cytokine deprivation also occurs in mice (Gasteiger et al., 2013a, Gasteiger et al., 2013b). They observed that Treg cells can regulate the activation of NK cells by controlling the availability of IL-2, however under inflammatory conditions such as acute infection, NK cells could overcome this inhibition. Concurrently, Sitrin and colleagues observed similar results in a mouse model of diabetes suggesting a general mechanism of Treg cell-mediated NK cell regulation, which depends on environmental signals (Sitrin et al., 2013).

Treg cell-mediated suppression is also regulated by the ratio of Treg cells to target cells. The optimal Treg cell dose required for suppression of Tcon cells in patients is still under investigation (Brunstein et al., 2011b, Di Ianni et al., 2011), however *in vitro* assays show that a minimum ratio of 1:5 (Treg cells:Tcon cells) is necessary for suppression to occur (Seddiki et al., 2006b, Brunstein et al., 2011b). The ratio between Treg cells and NK cells used in suppression assays (Ghiringhelli et al., 2005, Sun et al., 2010, Bergmann et al., 2011) is similar to those reported between $CD4^+$ Tcon cells and Treg cells (Seddiki et al., 2006a, Brunstein et al., 2011b), ranging *in vitro* between ratios of 1:5 and 1:1 (Treg cells:$CD4^+$ Tcon cells or NK cells).

Finally, TCR stimulation is essential for Treg cells to suppress (Thornton and Shevach, 2000). Treg cells can suppress NK cell functions directly by sequestering IL-2 (Sitrin et al., 2013), via TGF-β (Ghiringhelli et al., 2005), or indirectly through the control of $CD4^+$ Tcon cells (Romagnani et al., 2005). It is also important to mention that, once stimulated, Treg cells can suppress any

cell type regardless of antigen specificity (Thornton and Shevach, 2000). However, Ghiringhelli and colleagues have shown that PB Treg cells can also inhibit effector functions of NK cells in the absence of TCR-stimulation, thus challenging the concept of TCR-stimulation as a requirement for suppression (Ghiringhelli et al., 2005). These authors observed that membrane-bound TGF-β is sufficient to inhibit the function of resting NK cells.

Regulation of Treg cell suppression by NK cells has also been reported. In humans and mice, Brillard and colleagues reported that the efficacy of autologous IL-2 activated NK cells to block pTreg cell differentiation depends on high levels of IFN-γ, which favours the development of a Th1 response regardless of the presence of soluble TGF-β (Brillard et al., 2007). This is in agreement with the observations of Beriou and colleagues, who proposed that inflammation might induce Treg cells to lose their suppressive capacity and to produce IL-17, which is a pro-inflammatory cytokine (Beriou et al., 2009). In addition, Roy and colleagues reported reduced pTreg cell proliferation, but not tTreg cell proliferation, in the presence of NK cells in the context of microbial infections (Roy et al., 2008). Furthermore, increased numbers of tTreg cells were observed in a mouse model of prostate carcinoma following NK cell depletion (Chin et al., 2010).

To date, no studies have assessed the impact of the interaction between Treg cells and NK cells using CB as a cell source. Given that CBT is being increasingly used (Ballen et al., 2013), it is important to evaluate the impact of the interaction between Treg cells and NK cells in this context.

This chapter aims to investigate whether CB Treg cells can suppress CB NK cell functions in a non-APC dependent *in vitro* system. First, the ability of CB Treg cells to suppress PB NK cells was assessed using the protocol from Ghiringhelli and colleagues (Ghiringhelli et al., 2005), as a comparative study. Next, it was determined whether CB Treg cells require prior stimulation to exert suppression of NK cells and if this suppression is dependent on the activation status of NK cells (i.e. resting or activated NK cells). Also, the impact of CB NK cells on CB Treg cell viability was assessed. Finally, to determine whether

mismatched Treg cells enhance or diminish the effect of Treg cells on NK cells the aforementioned experiments were performed with both autologous and allogeneic CB Treg cells.

4.2 Peripheral blood natural killer cells are suppressed by peripheral blood regulatory T cells but not umbilical cord blood regulatory T cells

The ability of Treg cells to regulate NK cell cytotoxicity has been observed in humans and mice. As previously mentioned, Ghiringhelli and colleagues reported suppression of PB NK cells by PB Treg cells *in vitro* (Ghiringhelli et al., 2005). They showed ~85% reduction in killing of MHC class I-deficient K562 cells by NK cells in the presence of PB Treg cells at a 1:4 Treg to NK cell ratio. As shown in the previous chapter and by Godfrey and colleagues, CB Treg cells are capable of suppressing CB $CD4^+$ Tcon cells (Godfrey et al., 2005), but their effect on NK cells was not known. Therefore, the effects of CB and PB Treg cells on PB NK cell functions were assessed using an *in vitro* NK cell cytotoxicity assay adapted from Ghiringhelli and colleagues (Ghiringhelli et al., 2005). Freshly isolated autologous and allogeneic CB and PB Treg cells were cultured at a ratio of 1:1 and 1:4 with PB NK cells for 4 h without cytokines. The effect of Treg cells on NK cell cytotoxicity was measured by assessing the capacity of NK cells to kill K562 cells in the presence or absence of Treg cells.

A reduction in NK cell killing of K562 cells was observed when autologous and allogeneic PB Treg cells were co-cultured with PB NK cells (~40% at both ratios; 1:1 and 1:4) (**Figure 4.1A-B**). However, statistical significance was only detected when autologous PB Treg cells were cultured with PB NK cells at a 1:1 ratio ($p=0.03$). These results are similar to those reported by Ghiringhelli and colleagues (Ghiringhelli et al., 2005) with the exception that in the present study no significant difference was observed when PB Treg cells were co-cultured with PB NK cells at a 1:4 ratio. This could be explained by the high variability between Treg samples used in this study, as Treg cells from one sample had no effect on the specific NK cell lysis of K562 cells, whereas Treg cells from the other three samples inhibited NK cell function by 20-40%. In contrast to PB Treg cells, allogeneic CB Treg cells were unable to suppress PB NK cells at any of the ratios tested under the experimental conditions used (**Figure 4.1A-B**).

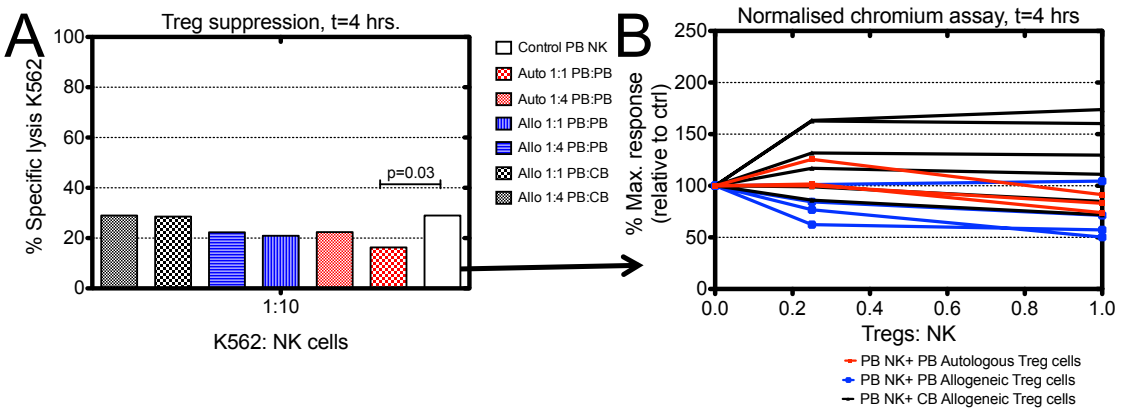


Figure 4.1: Inhibition of PB NK cell cytolytic activity by PB Treg cells but not by CB Treg cells *in vitro*. (A) Freshly isolated PB NK cells were incubated alone or in the presence of autologous or allogeneic CB or PB Treg cells at 1:1 or 1:4 ratios (Treg cells:NK cells) for 4 h before incubation with ^{51}Cr-labelled K562 cells at a 10:1 effector:target (E:T) ratio (x-axis). No cytokines were added to these cultures. (B) Normalisation of % specific lysis to PB NK cell response. The bars represent medians of 3-6 independent experiments.

4.3 Effect of resting and TCR-stimulated umbilical cord blood regulatory T cells on resting umbilical cord blood natural killer cells

Given that CB Treg cells require prior stimulation with IL-2 to be functional (Li et al., 2005), CB Treg cell-mediated suppression of CB NK cell functions was assessed in the presence of IL-2 using a combination of different assays. These included assessment of NK cell natural cytotoxicity by chromium release assay, viability, expression of activating receptors and adhesion markers required for NK cell killing, proliferation and IFN-γ secretion. In these experiments, the concentration of IL-2 used caused no significant Treg cell or NK cell activation but was essential for the survival and proliferation of CB cells.

4.3.1 Natural killer cell natural cytotoxicity

Natural cytotoxicity of CB NK cells was assessed by measuring lysis of K562 cells in the presence of IL-2. Resting CB NK cells were cultured in the presence or absence of resting or TCR-stimulated Treg cells (autologous or allogeneic) for 24 (referred to as resting Treg cell:NK cell co-cultures) or 48 h (referred to as TCR-stimulated Treg cell:NK cell co-cultures) in the presence of 1 000 IU/ml IL-2 at a 1:4 ratio (Treg cells:NK cells). TCR-stimulation of CB Treg cells was achieved by the addition of plate bound anti-CD3/soluble anti-CD28, hereafter

referred to as “TCR-stimulated Treg cells” in this chapter. Controls (NK cells in the absence of Treg cells) were treated the same as other cultures to ensure that plate bound anti-CD3 had no effect on resting CB NK cells. K562 cells (targets) were co-cultured with NK cells at E:T ratios as indicated.

No significant suppression of NK cell natural cytotoxicity by resting or TCR-stimulated Treg cells was observed in the presence of exogenous IL-2 (**Figure 4.2**). A similar level of K562 lysis by resting NK cells was observed in the presence or absence of autologous or allogeneic resting Treg cells (**Figure 4.2A.1, A.2).** The specific lysis mediated by resting NK cells ranged between 5 and 15% for all ratios tested (1:1, 5:1 and 10:1 E:T ratio). Treg cell numbers were maintained constant with a ratio of 1:4 Treg cells:NK cells. Consistently, similar results were observed when TCR-stimulated CB Treg cells were co-cultured in the presence of IL-2 (**Figure 4.2B1, B.2**). Collectively, for all conditions and ratios tested (resting NK cells ± autologous or allogeneic Treg cells), no statistically significant difference in NK cell lysis of K562 cells was observed between the different groups. In line with previous studies by Ghiringhelli and colleagues, it appears as though the presence of exogenous IL-2 overrides the ability of CB Treg cells to suppress NK cell function (Ghiringhelli et al., 2005).

However, it is noteworthy that the killing capacity of CB NK cells was relatively low in four out of six of these experiments (up to 15% at the highest E:T ratio; 10:1). This may suggest that activated CB NK cells should be used instead of resting CB NK cells for these experiments.

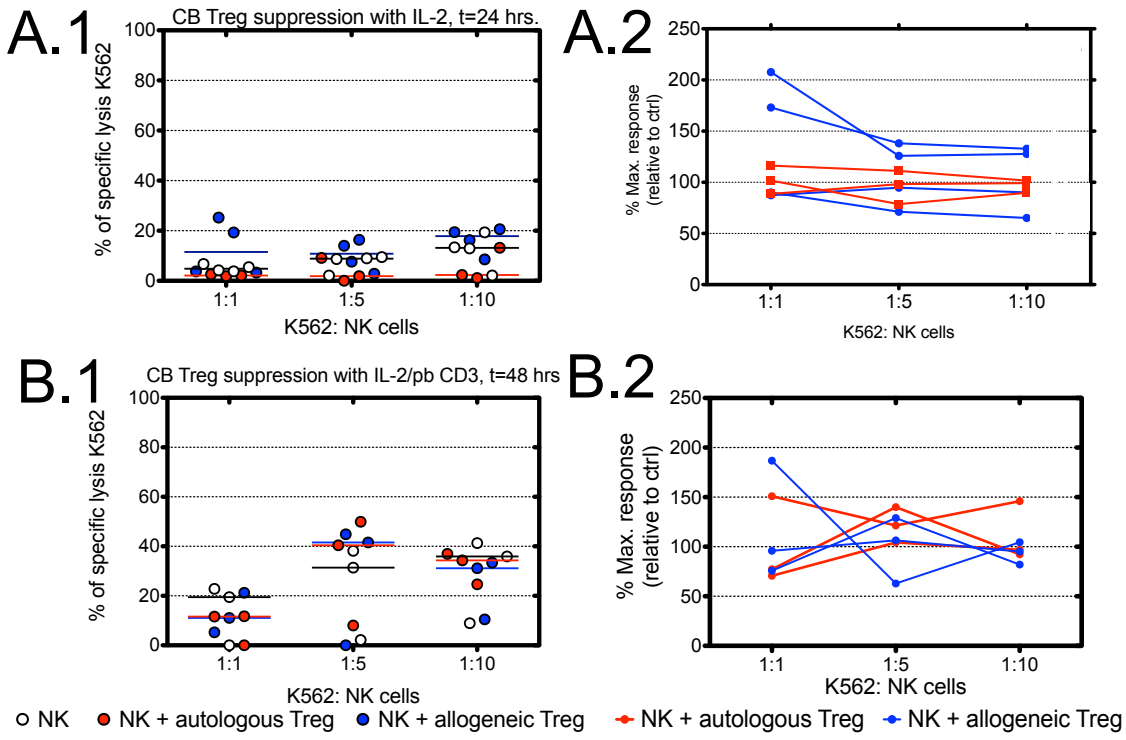


Figure 4.2: CB Treg cells do not suppress NK cell natural cytotoxicity in the presence of IL-2. Percent specific lysis of K562 cells by NK cells (alone: black) at different E:T ratios in the presence or absence of autologous or allogeneic CB Treg cells. CB NK cells were cultured with resting CB Treg cells (1 000 IU/ml IL-2) (A.1-A.2) or TCR-stimulated CB Treg cells (plate bound anti-CD3/soluble anti-CD28 and 1 000 IU/ml IL-2) (B.1-B.2) for 24 or 48 h prior to the assay. Percentage of maximal response was measured relative to NK cells alone as controls. The lines represent the medians of 3-4 independent experiments.

4.3.2 Natural killer cell viability

Several authors have reported the ability of TCR-stimulated Treg cells to kill autologous cells through perforin and granzyme in humans and in mice (Grossman et al., 2004a, Gondek et al., 2005, Zhao et al., 2006, Daniel et al., 2013). Furthermore, Cao and colleagues (Cao et al., 2007) observed in a tumour mouse model that Treg cells promote tumour escape by the lysis of autologous NK cells and $CD8^+$ T cells. However, it is not known whether CB Treg cells can also lyse autologous CB NK cells. To evaluate this, NK cells were cultured in the presence or absence of resting or TCR-stimulated Treg cells (autologous or allogeneic) at a 1:4 ratio (Treg cells:NK cells) with exogenous IL-2. NK cell viability was assessed using 7-AAD and Annexin V by flow cytometry. The gating strategy used is described in Section 2.7.1.1.

Resting or TCR-stimulated CB Treg cells (autologous and allogeneic) had no effect on NK cell viability when compared to NK cells alone (**Figure 4.3A-B**), suggesting that under these conditions (i.e. in the presence of IL-2) CB Treg cells do not impair CB NK cell viability.

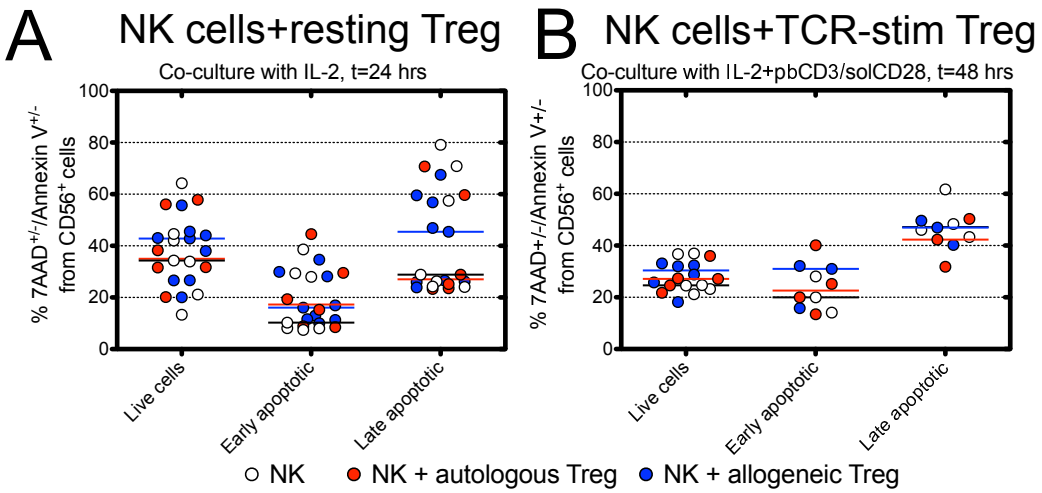


Figure 4.3: Viability of resting NK cells following co-culture with IL-2 and Treg cells. CB NK cell viability was assessed by flow cytometry using CD56, 7-AAD and Annexin V. Resting CB NK cells were co-cultured with resting CB Treg cells (A) or plate bound anti-CD3/soluble anti-CD28 TCR-stimulated CB Treg cells (B). A ratio of 1:4 (Treg cell:NK cells) was used. Autologous and allogeneic CB Treg cells were analysed in both conditions. Co-cultures were performed for 24 or 48 h in the presence of 1 000 IU/ml IL-2. The lines represent the medians. n=3-9.

4.3.3 Expression of receptors involved in natural killer cell killing capacity

4.3.3.1 Activating and inhibitory receptors

The integration of signals provided by activating and inhibitory receptors upon the detection of infected cells or tumours determines NK cell function (Pegram et al., 2011). Hence, changes in the expression of these receptors may cause impairment of NK cell effector functions, as observed in individuals with prostate cancer (Wu et al., 2004) and colon carcinoma (Doubrovina et al., 2003). Importantly, several authors have demonstrated downregulation of the activating receptors NKG2D and NKp44 on NK cells in the presence of Treg cells in humans and mice (Ghiringhelli et al., 2005, Bergmann et al., 2011). Therefore, the ability of CB Treg cells to regulate the expression of the following

activating receptors involved in NK cell killing capacity was assessed: CD16 (a mediator of ADCC (Lanier et al., 1988)); DNAM-1 (involved in lysis of tumour cells and infected cells, necessary for actin cytoskeletal rearrangement and critical for NK cell function (Gilfillan et al., 2008)); NKG2D (critical for the response to cellular stress (Raulet, 2003)); NKp30 (implicated in NK cell-mediated apoptosis and killing of immature DCs (Ferlazzo et al., 2002, Byrd et al., 2007)); NKp46 (facilitates NK cell lysis of infected cells (Bottino et al., 2000)); and 2B4 (co-receptor and CD2 family member that also plays a role as an inhibitory receptor (Sivori et al., 2000)).

Resting or TCR-stimulated CB Treg cells (autologous or allogeneic) were added to CB NK cells at a 1:4 ratio (Treg cells:NK cells) and receptor expression was analysed by flow cytometry after 0, 4 and 24 h co-culture. When resting NK cells were co-cultured with resting CB Treg cells (autologous or allogeneic), a reduced expression of CD16 (**Figure 4.4A.1**), NKp46 (**Figure 4.4C.1**), DNAM-1 (**Figure 4.5B.1**), NKG2D (**Figure 4.5A.1**) and NKp30 (**Figure 4.4B.1**) was noted on NK cells, whereas no effect was observed on the expression of 2B4 (**Figure 4.5C.1**). In addition, CD16 (**Figure 4.4A.1**) was significantly downregulated by 75% on resting NK cells ($p=0.03$) after 4 h co-culture with resting allogeneic Treg cells, while a non-significant trend towards reduced CD16 expression was observed in co-cultures with resting autologous Treg cells (~75%; $p=0.06$). However, CD16 expression on NK cells recovered after 24 h, thus suggesting a transient Treg cell effect. NKp46 was also downregulated on NK cells (**Figure 4.4C.1)** when co-cultured with resting allogeneic ($p=0.002$) or autologous ($p=0.002$) Treg cells for 4 h, however the effect was lost after 24 h co-culture (autologous $p=0.09$, allogeneic $p=0.04$).

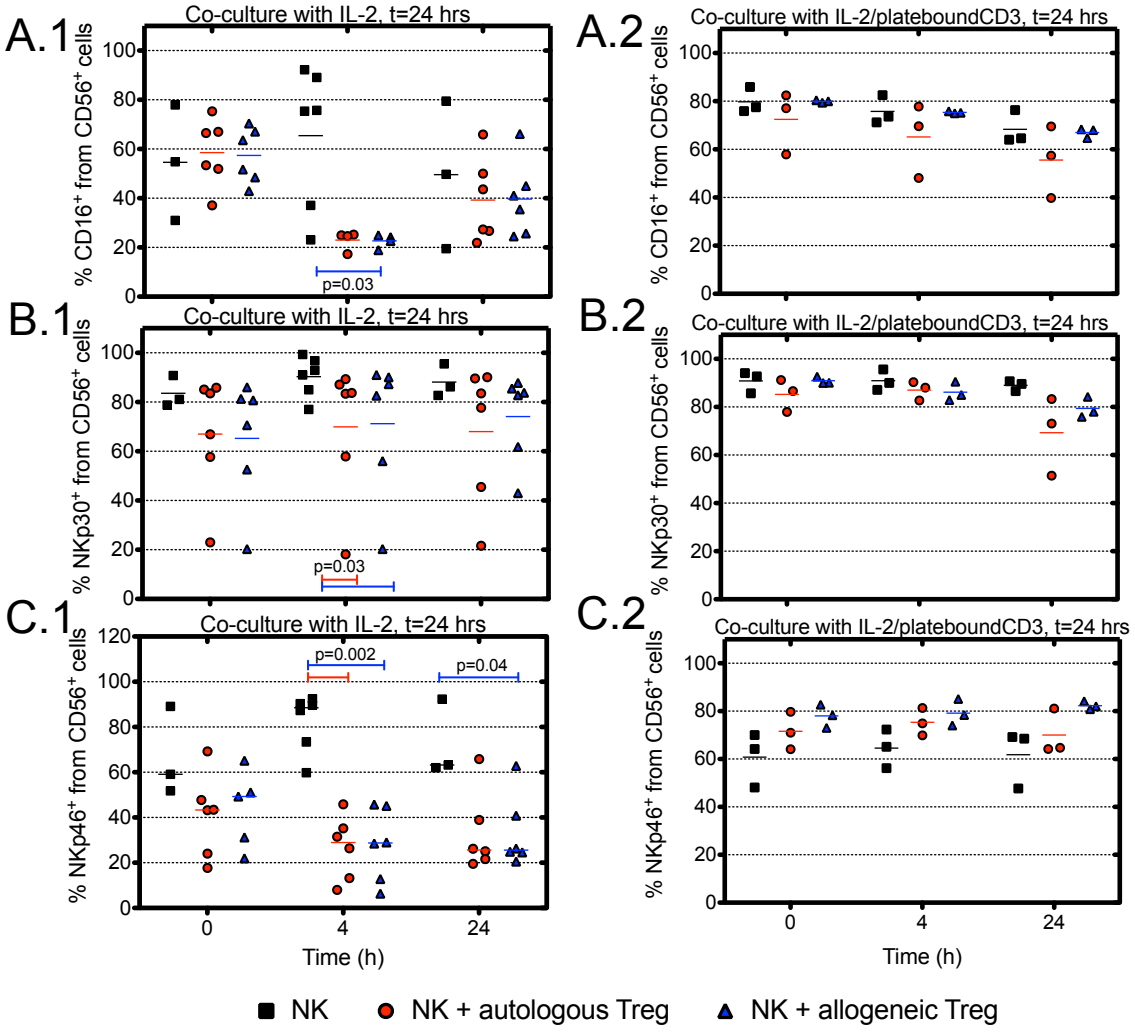


Figure 4.4: NK cell expression of CD16, NKp30 and NKp46 when co-cultured with IL-2 and Treg cells. Flow cytometric analysis of CD16 expression (A.1-A.2), NKp30 expression (B.1-B.2) and NKp46 expression (C.1-C.2) on NK cells. All analysis was performed on gated $CD56^+$ cells. Left panel: Resting CB NK cells with resting Treg cells; Right panel: Resting CB NK cells with TCR-stimulated CB Treg cells (plate bound anti-CD3/soluble anti-CD28). A ratio of 1:4 (Treg cell:NK cells) was used. Co-cultures and controls were analysed at 0, 4 and 24 h in the presence of 1 000 IU/ml IL-2. The lines represent medians. n=3-9.

Similarly, an effect on DNAM-1 expression by NK cells was observed immediately after addition of Treg cells at 0 h with a 50 % reduction in expression when resting CB NK cells were co-cultured with autologous (p=0.04) or allogeneic (p=0.07) resting CB Treg cells. After 4 h co-culture, this effect was still detected for both co-cultures with autologous and allogeneic resting Treg cells (p=0.01) but was not significantly different after 24 h (**Figure 4.5B.1).** The effect of resting CB Treg cells on NKG2D expression was not as pronounced as for other NK cell receptors when resting NK cells were co-cultured with resting CB Treg cells (autologous or allogeneic) (**Figure 4.5A.1**). A ~15 % reduction in

NKG2D expression was observed on resting CB NK cells in the co-cultures with autologous (p=0.01) or allogeneic (p=0.01) resting CB Treg cells after 4 h. NKG2D was further downregulated (~20%) on NK cells after 24 h co-culture in the presence of CB Treg cells (autologous p=0.04, allogeneic p=0.02).

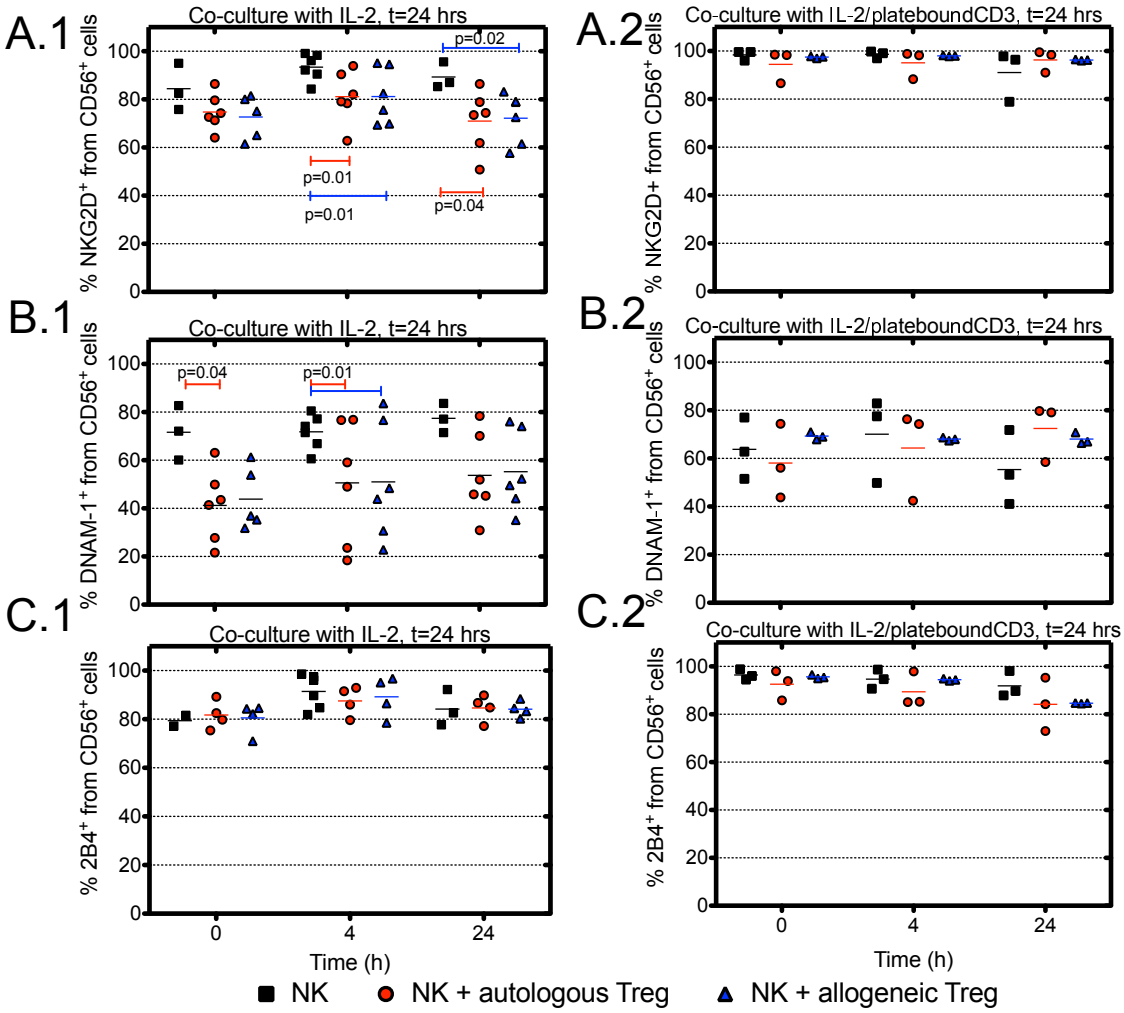


Figure 4.5: NK cell expression of NKG2D, DNAM-1 and 2B4 following co-culture with IL-2 and Treg cells. Flow cytometric analysis of NKG2D expression (A.1-A.2), DNAM-1 expression (B.1-B.2) and 2B4 expression (C.1-C.2) on NK cells. All analysis was performed on gated $CD56^+$ cells. Left panel: Resting CB NK cells with resting Treg cells; Right panel: Resting CB NK cells with plate bound anti-CD3/soluble anti-CD28 TCR-stimulated CB Treg cells. A ratio of 1:4 (Treg cell:NK cells) was used. Co-cultures were analysed at 0, 4 and 24 h in the presence of 1 000 IU/ml IL-2. n=3-9.

Notably, no effect was observed on the expression of any of the receptors involved in NK cell cytotoxicity for any of the co-cultures with TCR-stimulated Treg cells (**Figure 4.4A.2, B.2, C.2** and **Figure 4.5A.2, B.2, C.2**). Collectively, these results suggest that co-culture with resting but not TCR-stimulated CB Treg cells reduces the expression of the NK cell activating receptors NKG2D,

DNAM-1, CD16, NKp30 and NKp46, yet this effect seems to be transient, as no effect or reduced effects were observed after 24 h co-culture, except for NKG2D, which remained reduced on resting NK cells.

4.3.3.2 LFA-1 expression

The interaction of LFA-1 with its ligand intercellular adhesion molecule (ICAM-1) on target cells is required for NK cell cytotoxicity (Helander and Timonen, 1998). Importantly, the absence of this interaction, first observed in β-2 integrin-deficient leukocyte adhesion deficiency patients (Timonen et al., 1988), and later observed using *in vitro* LFA-1 blocking assays in the context of viral infections (Barber et al., 2004), causes impaired NK cell cytotoxicity.

Because of the importance of this adhesion molecule for NK cell functions, it was proposed that Treg cells suppress NK cells by inducing LFA-1 down-regulation on NK cells. However, no effect was observed on the frequency of LFA-1$^+$ NK cells (**Figure 4.6A**) or MFI for any of the conditions studied after 24 h co-culture of NK cells with Treg cells (**Figure 4.6B**). This suggests that Treg cells do not impair the LFA-1 pathway.

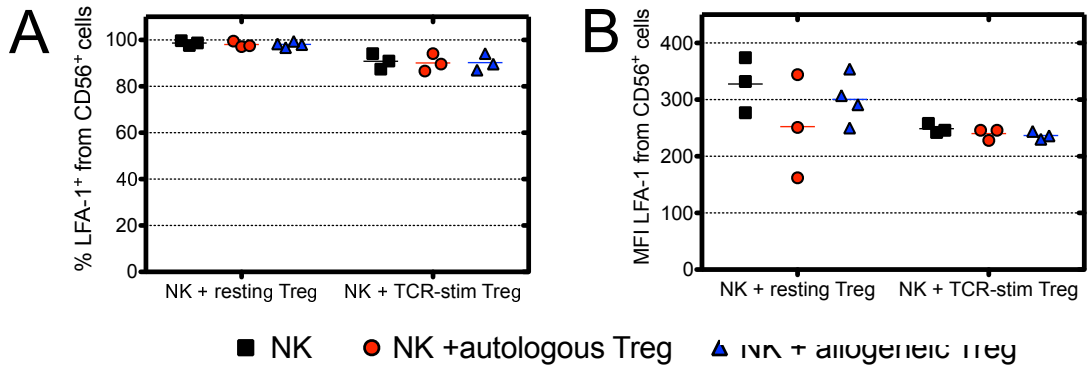


Figure 4.6: NK cell expression of LFA-1 when co-cultured in the presence or absence of CB Treg cells. Flow cytometric analysis of LFA-1 on NK cells. Frequency of expression (A) and MFI (B) on CD56$^+$ gated cells in the presence or absence of resting or TCR-stimulated (plate bound anti-CD3/soluble anti-CD28) CB Treg cells (autologous or allogeneic) at a ratio of 1:4 (Treg cells: NK cells) in the presence of 1 000 IU/ml IL-2. Co-cultures were analysed at 24 h. The lines represent medians. n=3-4.

4.3.4 Natural killer cell proliferation

Increased proliferation of human NK cells *in vitro* has been observed following interaction of NK cells with autologous Tcon cells ($CD4^+CD25^-Foxp3^-$) and plasmacytoid DCs; however this effect was completely abrogated by the addition of TCR-stimulated $CD4^+CD25^{high}$ Treg cells (Romagnani et al., 2005). This suggests that Treg cells can indirectly control NK cell proliferation through suppression of $CD4^+$ Tcon cells.

Based on these findings and considering the presence of ~2.5% $CD4^+$ Tcon cells in the co-culture system used in this study, the ability of CB Treg cells to suppress CB NK cell proliferation in a similar manner was evaluated. CB NK cells were labelled with CFSE, a fluorescent dye that halves in intensity at every cell division (Lyons and Parish, 1994). CFSE-labelled NK cells were then co-cultured in the presence or absence of resting or TCR-stimulated CB Treg cells (autologous or allogeneic). Co-cultured cells were analysed by flow cytometry at 0, 24, 48 and 96 h, when resting CB Treg cells were added, and at 0, 24 and 48 h when TCR-stimulated CB Treg cells were added. TCR-stimulated Treg/NK cell co-cultures were not analysed at 96 h due to low cell numbers.

Resting CB Treg cells (**Figure 4.7A, C**) and TCR-stimulated CB Treg cells (**Figure 4.7B, D**) had no effect on CB NK cell proliferation under any of the conditions tested. This might be due to the presence of exogenous IL-2 that could bypass Treg cell suppression or the fact that inhibition of NK cell proliferation by Treg cells could only be detected in co-cultures where NK cells, Tcon cells and Treg cells are present in the system (Romagnani et al., 2005), but not in co-cultures of NK cells with Treg cells in the absence of Tcon cells, as examined in this study.

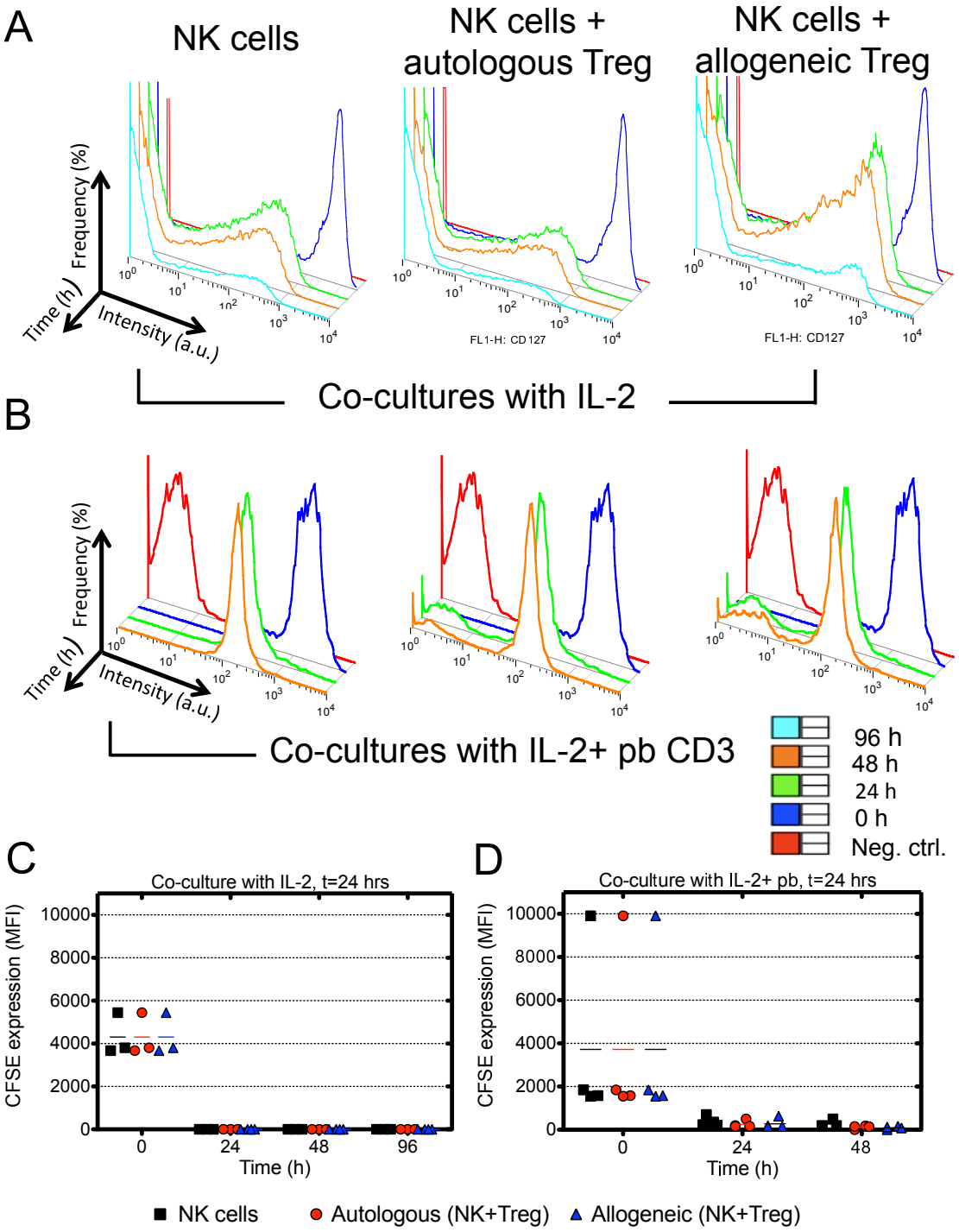


Figure 4.7: NK cell proliferation in the presence or absence of CB Treg cells and exogenous IL-2. CB NK cell proliferation was assessed by flow cytometry at 0, 24, 48 and 96 h after co-culture using CFSE-labelled NK cells. Autologous and allogeneic CB Treg cells were added at a 1:4 ratio (Treg cell:NK cells) in the presence of 1 000 IU/ml IL-2 (for resting CB Treg cells) (A) or 1 000 IU/ml IL-2, plate bound anti-CD3 and soluble anti-CD28 (for TCR-stimulated Treg cells) (B). Data is representative of 4 independent experiments. (C and D) MFI at different time points for resting CB NK cells + resting CB Treg cells (C) or resting CB NK cells + TCR-stimulated CB Treg cells (D). The lines represent medians. n=3-4.

4.3.5 Interferon-γ secretion

Treg cell-mediated suppression of NK cells can also be assessed by measuring the secretion of cytokines such as IFN-γ. IFN-γ is secreted by NK cells upon interaction with pathogen-infected cells or following tumour recognition, and is involved in differentiation, proliferation and recruitment of other immune cells. Several authors have observed a decrease in IFN-γ production by NK cells in the presence of Treg cells (Trzonkowski et al., 2004, Ghiringhelli et al., 2005, Zhou et al., 2010, Bergmann et al., 2011), hence, it is plausible that CB Treg cells decrease CB NK cell-mediated IFN-γ secretion. In this study, resting or TCR-stimulated CB Treg cells (autologous or allogeneic) were cultured with CB NK cells for 24 h in the presence of IL-2 and then stimulated for 2 h with or without K562 cells at a 1:1 ratio or with PMA/ION as a positive control.

Overall, resting or TCR-stimulated CB Treg cells did not affect NK cell-mediated IFN-γ production. Similar amounts of IFN-γ secretion by resting CB NK cells were observed when cultured alone or in the presence of resting CB Treg cells (0-300 pg/ml for unstimulated NK cells, ~300 pg/ml for K562 stimulated NK cells, ~3 500 pg/ml for PMA/ION stimulated NK cells) (**Figure 4.8A).** Likewise, no difference in IFN-γ secretion was observed when TCR-stimulated CB Treg cells were added to NK cell cultures (**Figure 4.8B**), which suggests that under these particular conditions, CB Treg cells are unable to impair IFN-γ secretion by NK cells.

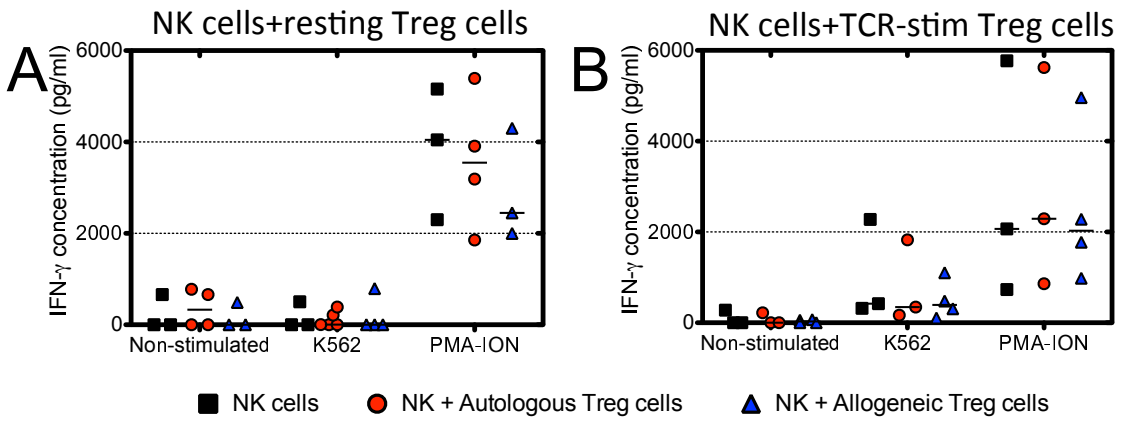


Figure 4.8: IFN-γ secretion by NK cells in the presence or absence of CB Treg cells and exogenous IL-2. CB NK cells were cultured in the presence or absence of resting Treg cells (A) or TCR-stimulated CB Treg cells (B) and 1 000 IU/ml IL-2 at a 1:4 ratio (Treg cells:NK cells). For TCR-stimulation, Treg cells were incubated with plate bound anti-CD3/soluble anti-CD28. After 24 h co-culture, cells were incubated with or without K562 cells (1:1 ratio; NK cells:K562 cells), or PMA/ION for 2 h. Supernatants were collected and IFN-γ secretion analysed by ELISA. The lines represent medians. n=3-4.

4.4 Effect of resting or TCR-stimulated umbilical cord blood regulatory T cells on activated umbilical cord blood natural killer cells

In the previous section, the inability of freshly isolated CB Treg cells to suppress resting PB NK cell functions as compared to PB Treg cells was demonstrated. This could be explained by the immature phenotype of CB Treg cells and/or a requirement for IL-2 for them to suppress (Godfrey et al., 2005). The data presented shows that in the presence of IL-2, CB Treg cells, regardless of their activation state, do not impair effector functions of resting NK cells. These observations suggest that either IL-2 allows NK cells to bypass suppression by CB Treg cells, as observed for PB Treg cells by Ghiringhelli and colleagues (Ghiringhelli et al., 2005), or that CB NK cell activation may be a requirement for CB Treg cell-mediated suppression as it has been observed that resting CB NK cells exhibit an immature phenotype (Luevano et al., 2012a). Therefore, the effect of CB Treg cells on activated NK cell functions in the absence of cytokines was investigated.

To evaluate this, an optimised CB NK cell activation protocol was first required. Cytokines such as IL-2, IL-12, IL-15 and IL-18 play a crucial role in NK cell

differentiation, proliferation, activation and function (Cooper et al., 2002, Gracie et al., 2003, Becknell and Caligiuri, 2005). In order to determine which cytokine optimally activates CB NK cells, a comparative study of these four cytokines was performed whereby CB NK cell function and activation was measured by chromium release assay and flow cytometry respectively. Concentration of cytokines and incubation times were chosen according to published data for PB NK cells except for IL-2, in which data for CB NK cells was available (Condiotti et al., 2001, Luevano et al., 2012a) (**Table 4.1**).

Table 4.1: Published cytokine concentrations and incubation times used for NK cell activation with IL-2, IL-12, IL-15, and IL-18.

Cytokine	Concentration	Time of incubation (h)	Reference
IL-2	1 000 IU/ml	120 h	(Condiotti et al., 2001, Luevano et al., 2012a)
IL-12	10 ng/ml	40 h	(Cooper et al., 2001b)
IL-15	20 ng/ml	120 h	(Chiossone et al., 2007)
IL-18	100 ng/ml	40 h	(Agaugue et al., 2008)

Among the four cytokines tested, treatment of CB NK cells with IL-15 induced the highest NK cell cytotoxicity against K562 cells reaching levels of ~40% killing for all ratios tested at 40 h (**Figure 4.9A**) and 120 h (**Figure 4.9B**). The levels of cytotoxicity observed were three-fold higher than those observed for resting CB NK cells in Section 4.3.1.

To further investigate the effect of IL-15 on CB NK cells after 40 h incubation, NK cell activation was assessed by measuring the expression of the early activation marker CD69 and of NKp44, exclusively expressed upon NK cell activation (Borrego et al., 1993, Vitale et al., 1998). After 40 h IL-15 treatment, 95-100% of NK cells expressed both surface markers (**Figure 4.10B).** The 40 h time point was selected as it allows evaluation of Treg cell/NK cell co-cultures with autologous TCR-stimulated CB Treg cells, as both NK cells and Treg cells can be isolated from the same CB sample and activated simultaneously prior to co-culture. Hence, NK cells were activated with 20 ng/ml IL-15 for 40 h and are referred to here as “activated NK cells”.

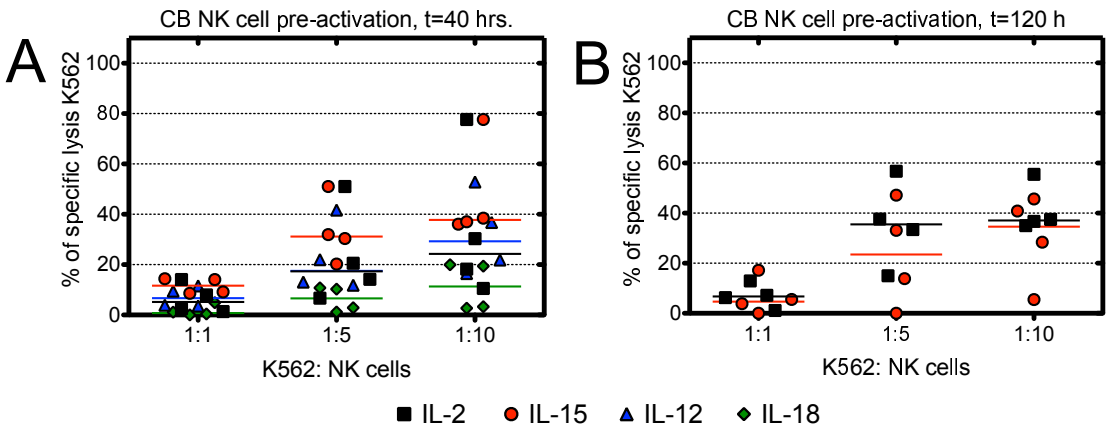


Figure 4.9: Cytolytic activity of CB NK cells activated with IL-2, IL-12, IL-15 or IL-18. CB NK cells were activated with 1 000 IU/ml IL-2, 20 ng/ml IL-15, 10 ng/ml IL-12 or 100 ng/ml IL-18 for 40 h (all cytokines) (A), and 40 and 120 h (IL-2 and IL-15) (B). NK cells were cultured with ^{51}Cr-labelled K562 cells at different E:T ratios for 4 h. Results are represented as means of triplicate wells. Percentage of specific lysis was determined by the following equation: % lysis= [(experimental release - spontaneous release)/(maximum release - spontaneous release)]*100. n=4.

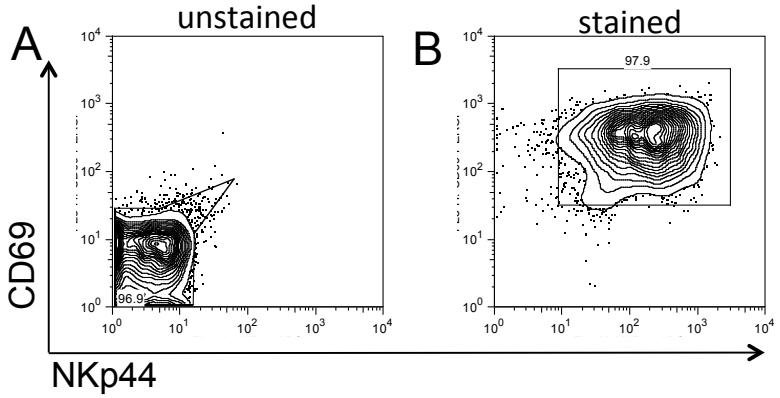


Figure 4.10: Expression of CD69 and NKp44 on IL-15 activated NK cells. CD69 and NKp44 expression was analysed by flow cytometry after 40 h incubation with 20 ng/ml IL-15. (A) Unstained CB NK cells after 40 h of IL-15 activation. (B) Expression of CD69 and NKp44 on CB NK cells after 40 h IL-15 activation. Data is representative of 10 independent experiments.

4.4.1 Natural killer cell natural cytotoxicity

4.4.1.1 *In vitro* cytolytic activity

To test whether CB Treg cells have an effect on the cytotoxicity of activated CB NK cells chromium release assays were performed. NK cells and resting or TCR-stimulated Treg cells (autologous or allogeneic) were cultured separately for 40 h. NK cells were activated with 20 ng/ml IL-15, whereas resting and TCR-stimulated CB Treg cells were activated with 1 000 IU/ml IL-2 or 1 000 IU/ml IL-

2 + plate bound anti-CD3/soluble anti-CD28 respectively. Subsequently, resting or TCR-stimulated CB Treg cells were washed and co-cultured with NK cells at a 1:1 or 1:4 ratios for 4 h in the absence of cytokines. Killing of K562 cells by NK cells from these co-cultures was then assessed by chromium release assay.

Resting allogeneic CB Treg cells reduced NK cell cytotoxicity by ~20% at a ratio of 1:1 (**Figure 4.11A.1,A.2**), whereas NK cell cytotoxicity was not affected when the Treg:NK cell ratio was of 1:4, suggesting that high numbers of resting Treg cells are required for suppression to occur. Furthermore, no consistent effect was observed when autologous resting CB Treg cells were added to activated CB NK cells at any ratio tested, as only one out of three samples exhibited Treg cell-mediated suppression.

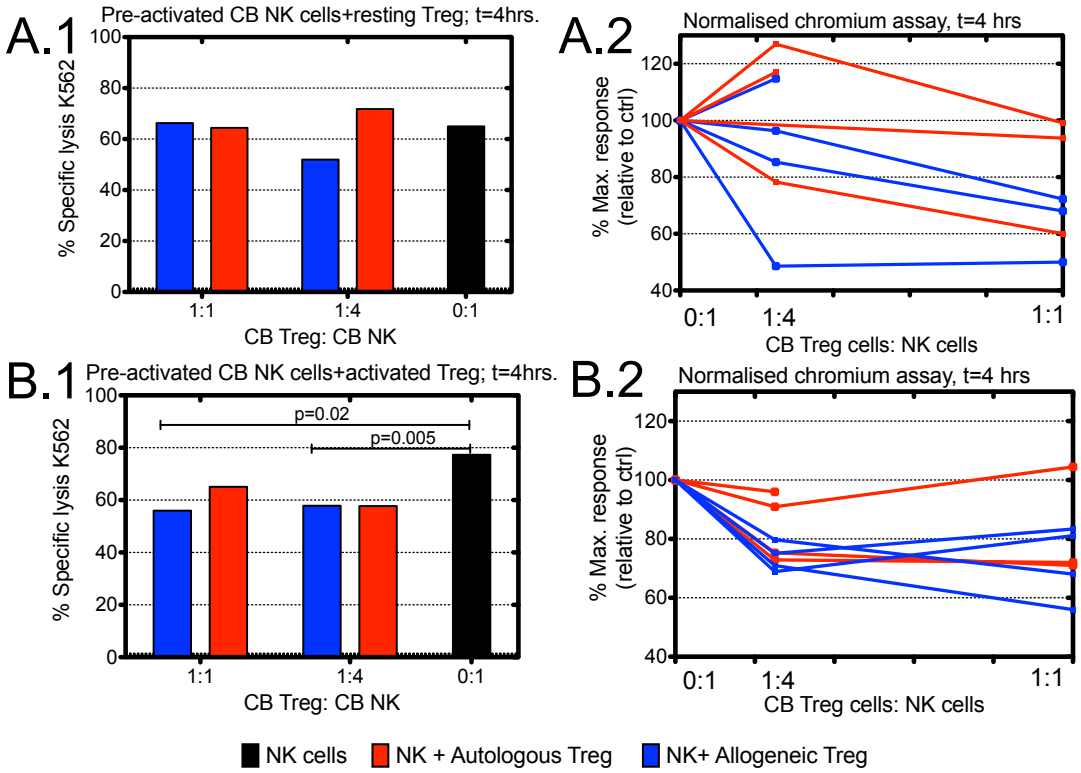


Figure 4.11: Suppression of IL-15 activated NK cell cytotoxicity by resting or TCR-stimulated CB Treg cells. CB NK cells and CB Treg cells were activated separately for 40 h, washed and co-cultured at different ratios (x-axis) for 4 h with ^{51}Cr-labelled K562 cells at a fixed E:T ratio of 10:1. CB NK cells were activated with 20 ng/ml IL-15. Autologous or allogeneic CB Treg cells were cultured with 1 000 IU/ml IL-2 (resting) or 1 000 IU/ml IL-2, plate bound anti-CD3/soluble anti-CD28 (TCR-stimulated) (Protocol E). Data is presented as percent specific lysis or normalised to controls (NK cells only) for co-cultures with resting Treg cells (A.1, A.2) or TCR-stimulated Treg cells (B.1, B.2). Results of the chromium release assay are represented as means of triplicate wells. Percentage of specific lysis was calculated using the following equation: % lysis = [(experimental release-spontaneous release)/(maximum release-spontaneous release)]*100. The values in the graphs represent the medians. n=4.

Conversely, both autologous and allogeneic TCR-stimulated CB Treg cells inhibited cytotoxicity of activated NK cells at a 1:1 ratio with ~19% (p=ns) and ~30% reduction (p=0.02), respectively (**Figure 4.11B.1,B.2)**. Furthermore, this effect was still observed when CB Treg cell numbers were reduced to a 1:4 ratio (~10% reduction for autologous CB Treg cells (p=ns) and ~20% reduction for allogeneic CB Treg cell co-cultures (p=0.005)). In summary, these results demonstrated that CB Treg cells require TCR-stimulation to exert suppression against NK cells.

4.4.1.2 Degranulation assay

The expression of CD107a (LAMP-1) by NK cells correlates with degranulation, as this protein is exclusively expressed when vesicle membranes fuse with the cell membrane to release cytolytic granules containing perforin and granzyme (Alter et al., 2004). TCR-stimulated Treg cells reduced NK cell cytotoxicity (Section 1.4.1.1), but whether or not Treg cells affect the number of degranulating NK cells was not known. As suppression of activated CB NK cells by TCR-stimulated but not resting Treg cells was observed, the following sections focus on TCR-stimulated Treg cells only.

CD107a expression was assessed on activated NK cells previously cultured alone or in the presence of TCR-stimulated CB Treg cells (autologous and allogeneic) for 4 h in the absence of cytokines. NK cells were incubated with either K562 cells at a ratio of 1:1 or PMA/ION.

A trend in reduction of CD107a expression by CB NK cells was detected for both autologous and allogeneic conditions when data was normalised to controls (NK cells only) (**Figure 4.12A-B**). Firstly, the addition of CB Treg cells to the NK cell cultures resulted in a decrease of background staining for CD107a. Activated NK cells co-cultured with autologous TCR-stimulated CB Treg cells showed a ~30% reduction in CD107a expression (relative to the controls; NK cells), whereas a ~70% reduction in CD107a expression on activated CB NK cells was observed when allogeneic TCR-stimulated Treg cells were present. Secondly, in the presence of K562 cells or PMA/ION stimulation,

activated NK cells were affected in the same manner showing 10-20% reduction in CD107a expression for both autologous and allogeneic conditions, relative to the controls (NK cell alone). However none of these differences were statistically significant.

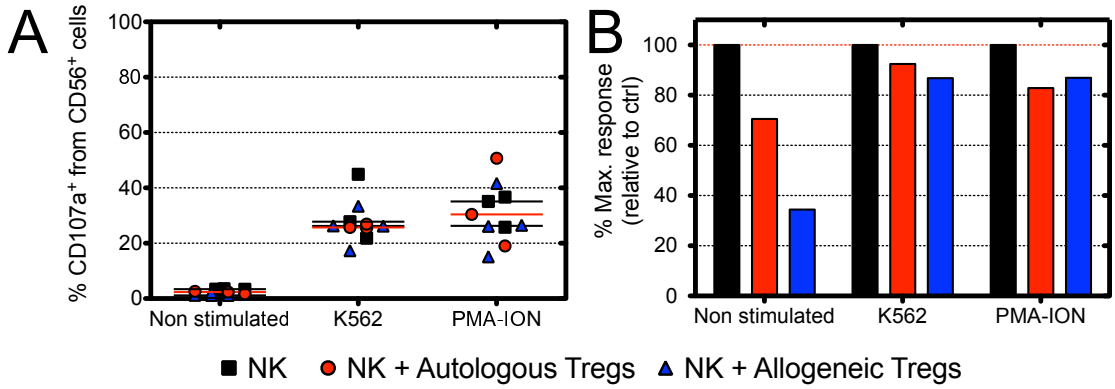


Figure 4.12: CD107a expression on IL-15 activated CB NK cells in the presence of TCR-stimulated CB Treg cells. IL-15 activated NK cells were cultured with TCR-stimulated Treg cells at 1:4 ratio (Treg cell:NK cells) for 4 h (Protocol E), and then incubated with or without K562 cells (1:1 ratio) or PMA/ION for 2 h. Cells were analysed by flow cytometry for the expression of CD107a on NK cells. (A) Percentage of $CD56^+$ cells expressing CD107a in response to different stimuli. (B) Normalised data to controls (NK cells alone). The lines represent medians. n=3-4.

4.4.2 Natural killer cell and regulatory T cell viability

As previously mentioned, TCR-stimulated Treg cells are able to kill autologous cells, including NK cells, through perforin and granzyme (Grossman et al., 2004a, Gondek et al., 2005, Zhao et al., 2006, Daniel et al., 2013) Therefore, the viability of activated NK cells, in the presence or absence of TCR-stimulated CB Treg cells (autologous or allogeneic) was assessed using 7-AAD and Annexin V, as described in Section 2.7.1.1.

In the 4 h co-cultures, the viability of activated NK cells remained constant showing similar levels of live (~20%), early apoptotic (~40%) and late apoptotic (~40%) cell populations in both controls (activated CB NK cells only) or co-cultures (activated CB NK cells + autologous or allogeneic TCR-stimulated CB Treg cells) (**Figure 4.13**). This suggests that under the conditions tested no

impairment in viability of activated NK cells by TCR-stimulated Treg cells is detected.

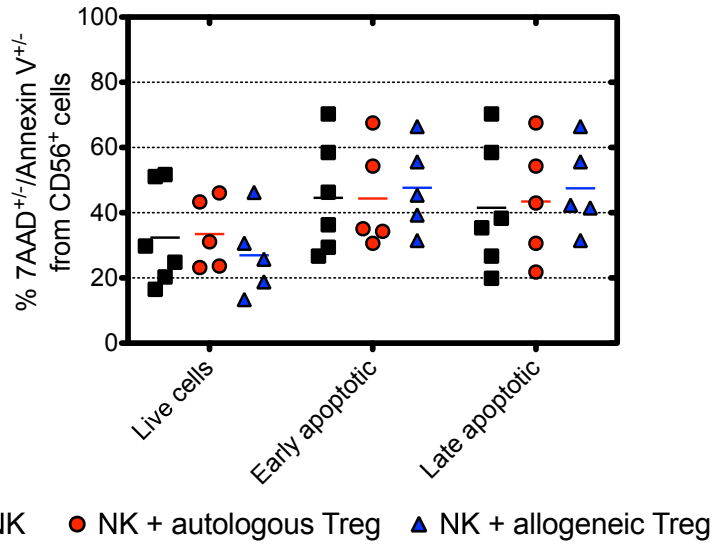


Figure 4.13: Viability of IL-15 activated NK cells cultured with or without autologous or allogeneic Treg cells. IL-15 activated NK cells were cultured with TCR-stimulated CB Treg cells (plate bound anti-CD3/soluble anti-CD28), either autologous or allogeneic, at 1:4 ratio (Treg cell:NK cells) for 4 h (Protocol E)**.** NK cell viability was assessed by flow cytometry using 7-AAD and Annexin V on $CD56^+$ gated cells. The lines represent the medians. n=3-6.

4.4.3 Expression of receptors involved in natural killer cell killing capacity

4.4.3.1 Activating and inhibitory receptors

Upregulation of the transmembrane receptor NKG2D on NK cells following contact with Tcon cells or *in vitro* expanded Treg (iTreg) cells can be abrogated by the addition of Treg cells (Bergmann et al., 2011). Therefore, the ability of TCR-stimulated Treg cells to directly downregulate activating receptors involved in NK cell killing was assessed as described in Section 4.3.3. CB NK cells and CB Treg cells (autologous or allogeneic) were activated separately, washed and co-cultured in the absence of cytokines for 4 h. The expression of CD16, NKp30, DNAM-1, NKG2D, 2B4 and NKp46 on NK cells was analysed by flow cytometry. TCR-stimulated Treg cells (autologous and allogeneic) did not affect expression (**Figure 4.14A**) or MFI (**Figure 4.14B**) of any of the surface makers studied.

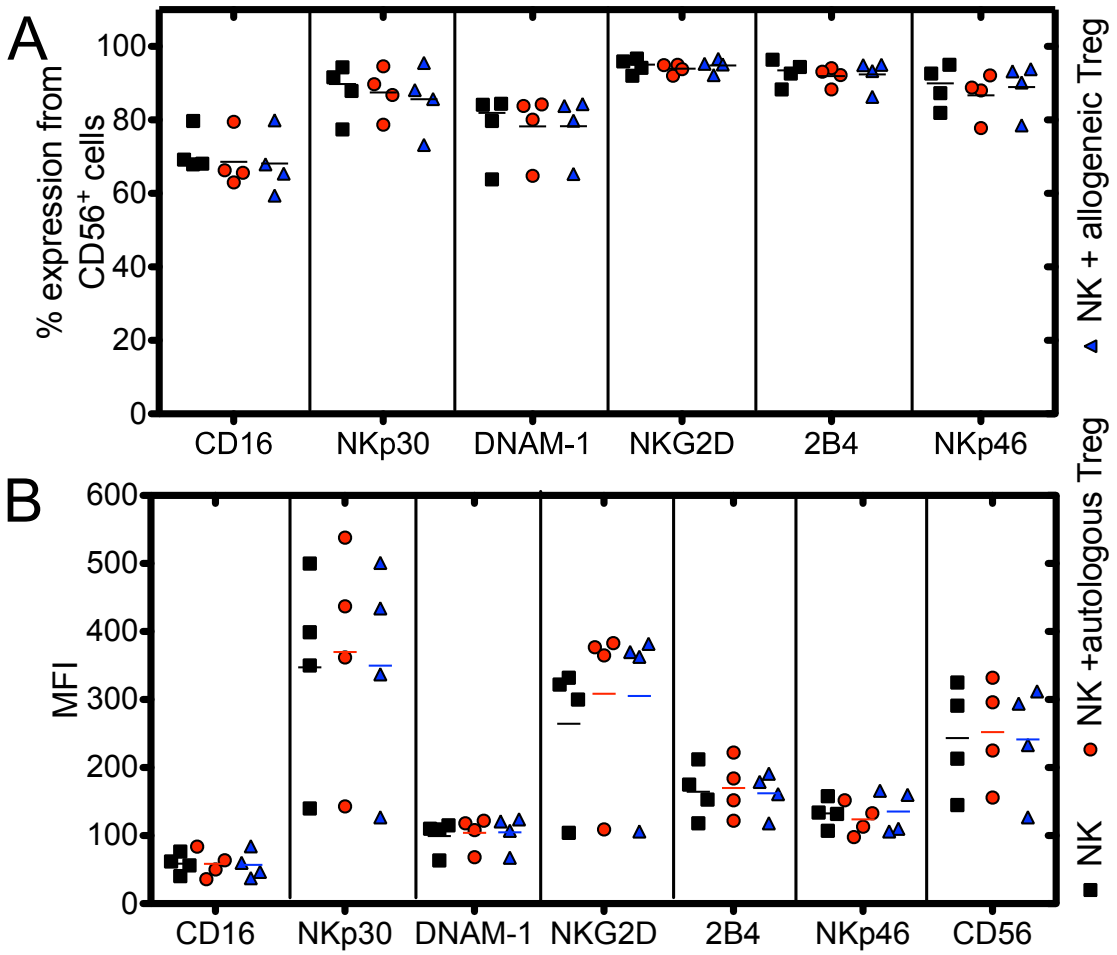


Figure 4.14: Expression of receptors involved in NK cell killing capacity on IL-15 activated NK cells in the presence of TCR-stimulated Treg cells. IL-15 activated NK cells were cultured with autologous or allogeneic TCR-stimulated Treg cells (plate bound anti-CD3/soluble anti-CD28) at a 1:4 ratio (Treg cell:NK cells) for 4 h (Protocol E). The expression of CD16, NKp30, NKp46, NKG2D, DNAM-1 and 2B4 on NK cells (gated on $CD56^+$ cells) was assessed by flow cytometry. The data is shown as percentage of NK cells expressing the corresponding receptors (A) and MFI (B). The lines represent medians. n=4

4.4.3.2 LFA-1 expression

As previously mentioned, the interaction of LFA-1 with ICAM-1 on target cells is required for NK cell cytotoxicity (Helander and Timonen, 1998, Barber et al., 2004). CB NK cells and CB Treg cells (autologous or allogeneic) were activated separately, washed and co-cultured in the absence of cytokines. After 4 h, the expression of LFA-1 on NK cells was assessed by flow cytometry. Similarly to the results observed when using resting CB NK cells co-cultured with CB Treg cells, LFA-1 expression (**Figure 4.15A**) and MFI (**Figure 4.15B**) on activated

CB NK cells was not affected when co-cultured with TCR-stimulated CB Treg cells (autologous or allogeneic) in the absence of cytokines.

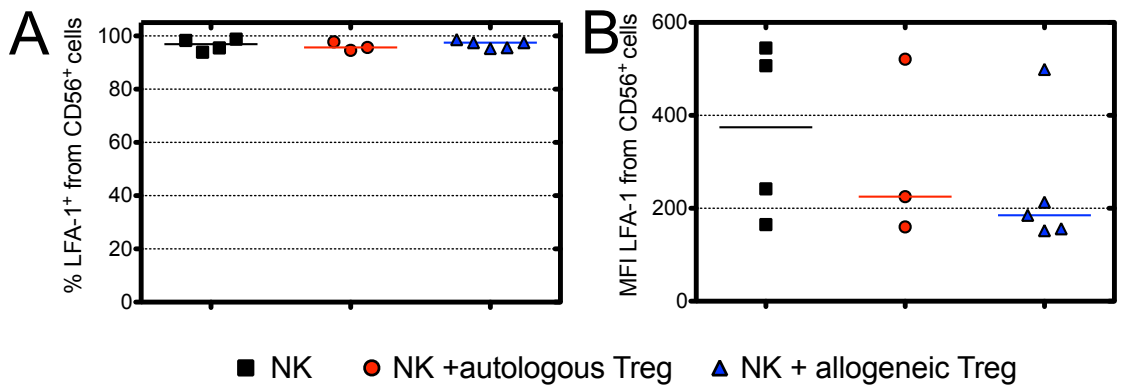


Figure 4.15: LFA-1 expression on NK cells after IL-15 activation when co-cultured with TCR-stimulated Treg cells. Flow cytometric analysis of LFA-1 on NK cells (gated on $CD56^+$ cells) in the presence or absence of TCR-stimulated CB Treg cells (autologous or allogeneic) at a ratio of 1:4 (Treg cells:NK cells). Co-cultures were analysed at 4 h in the absence of exogenous cytokines (Protocol E). The data is shown as percentage of NK cells expressing LFA-1 (A) and MFI (B). The lines represent medians. n=3-4.

4.4.4 Natural killer cell proliferation

As previously mentioned, increased proliferation of NK cells *in vitro* has been observed when NK cells interact with autologous Tcon cells ($CD4^+CD25^-Foxp3^-$) and plasmacytoid DCs in humans (Romagnani et al., 2005); however this effect was completely abrogated with the addition of TCR-stimulated $CD4^+CD25^{high}$ Treg cells. The data shown in Section 4.3.4 demonstrated no effect on the proliferation of resting NK cells when cultured with either resting or TCR-stimulated CB Treg cells. Here, it was evaluated whether proliferation of activated CB NK cells could be affected by the presence of TCR-stimulated CB Treg cells.

To detect differences in NK cell proliferation, CB NK cells were activated with 20 ng/ml IL-15 for 40 h, CFSE labelled and then co-cultured for 4 h with autologous or allogeneic TCR-stimulated CB Treg cells. CB NK cell proliferation was assessed by flow cytometry at 0, 4 and 24 h after labelling. No differences were observed between NK cells alone or NK cells co-cultured with autologous or allogeneic TCR-stimulated CB Treg cells (**Figure 4.16**). Overall, these results

suggest that Treg cells require the presence of $CD4^+$ Tcon cells and/or DCs to decrease NK cell proliferation.

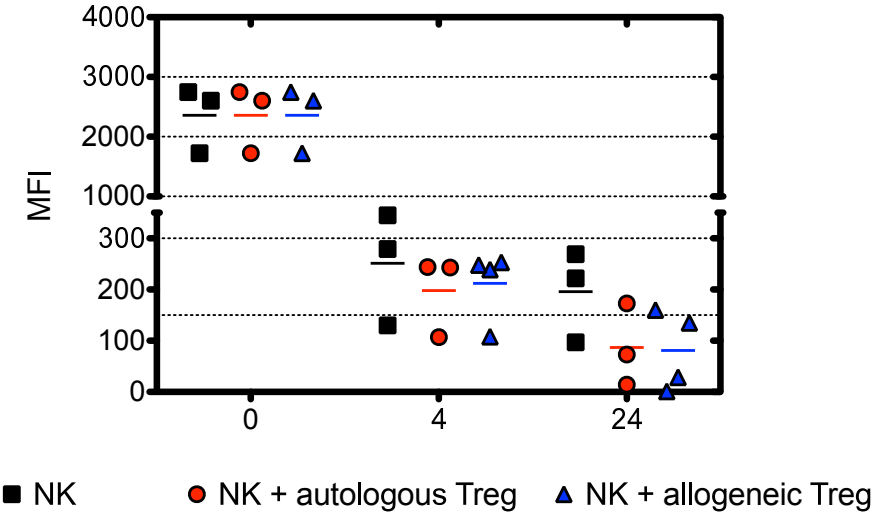


Figure 4.16: Proliferation of IL-15 activated NK cells in the presence of TCR-stimulated CB Treg cells. IL-15 activated NK cells were cultured with autologous or allogeneic pre-stimulated Treg cells (plate bound anti-CD3/soluble anti-CD28) at a 1:4 ratio (Treg cell:NK cells) for 4 h (Protocol E). CB NK cell proliferation was assessed by flow cytometry using CFSE-labelled NK cells at 0, 4 and 24 h after co-culture and data is presented as MFI values. The lines represent the medians. n=3-4.

4.4.5 Interferon-γ secretion

In Section 4.3.5, the inability of CB Treg cells to impair IFN-γ secretion by NK cells in the presence of IL-2, regardless of their stimulation state, was shown. Hence, the ability of TCR-stimulated CB Treg cells to impair IFN-γ secretion by activated CB NK cells in the absence of IL-2 was assessed. In this study, TCR-stimulated CB Treg cells (autologous or allogeneic) were cultured with activated CB NK cells for 4 h and stimulated for 2 h with or without K562 cells at a 1:1 ratio or with PMA/ION.

TCR-stimulated CB Treg cells augmented IFN-γ secretion by activated NK cells in the absence of cytokines (**Figure 4.17**). When activated CB NK cells were stimulated with K562 cells, secretion levels of IFN-γ were increased by two-fold in the presence of allogeneic TCR-stimulated CB Treg cells ($p=0.05$), but not with autologous TCR-stimulated CB Treg cells. Consistently, IFN-γ secretion by PMA/ION-stimulated CB NK cells was significantly different to controls ($p=0.05$)

when co-cultured with allogeneic TCR-stimulated CB Treg cells (~2 500 pg/ml and 10 000 pg/ml respectively). These findings suggest that CB Treg cells, regardless of their stimulation state, may increase IFN-γ secretion by activated NK cells. Notably, it seems that allogeneic CB TCR-stimulated Treg cells may enhance this effect.

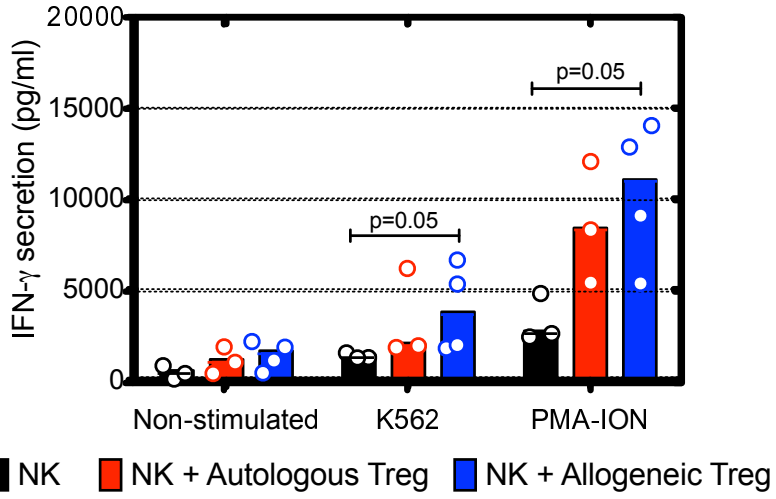


Figure 4.17: Effect of TCR-stimulated CB Treg cells on IFN-γ secretion by IL-15 activated NK cells. IFN-γ secretion by IL-15 activated NK cells cultured with autologous or allogeneic TCR-stimulated Treg cells (plate bound anti-CD3/soluble anti-CD28) at 1:4 ratio (Treg cell:NK cells) for 4 h (Protocol E), and then incubated with or without K562 cells (1:1 ratio) or PMA/ION for 2 h. Supernatants were analysed by ELISA. Results are represented as means of duplicate wells. The lines represent medians. n=4.

4.5 Effect of umbilical cord blood natural killer cells on umbilical cord blood regulatory T cells

The regulation of the adaptive immune response by activated NK cells has been reported after transplantation and in certain pathological conditions such as autoimmune diseases and with viral and bacterial infection (Crome et al., 2013). NK cell-mediated control of proliferation or lysis of $CD8^+$ and $CD4^+$ T cells (including Treg cells) as a mechanism to control adaptive immune responses has been described in humans and mice (Brillard et al., 2007, Roy et al., 2008, Chin et al., 2010). Regulation of the T cell immune response by NK cells can be through cytokine production such as IFN-γ (Lee et al., 2009b), TNF-α (Nie et al., 2013) and IL-10 (Lee et al., 2009b), secretion of perforin (Bielekova et al., 2006) and/or engagement of activating receptors such as NKG2D and NKp46 (Roy et al., 2008, Noval Rivas et al., 2010), LFA-1 (Nielsen

et al., 2012) or the death receptor FAS (Noval Rivas et al., 2010). In particular, Roy and colleagues demonstrated that during infection with *Mycobacterium tuberculosis* NK cells could control the proliferation of TCR-stimulated pTreg cells *in vitro* but not of tTreg cells (Roy et al., 2008).

To date, no information is available on the effect of CB NK cells on CB Treg cells in this context. For this reason, CB Treg cell viability, proliferation and expression of Fas in the presence of CB NK cells was assessed.

4.5.1 Frequency and viability of umbilical cord blood regulatory T cells

To evaluate whether CB NK cells could compromise CB Treg cell viability and frequency, autologous and allogeneic CB Treg cells were cultured with CB NK cells under different conditions (**Table 4.2**). Viability of both cell types was measured using 7-AAD and Annexin V by flow cytometry, as previously described in Section 2.7.1.1.

Table 4.2: Culture conditions for NK and Treg cell co-cultures to measure cell viability. *NK cells or Treg cells cultured alone were used as controls in all conditions.

Condition	NK cells (pre-culture)	Treg cells (pre-culture)	Treg cell/NK cell co-culture conditions*
24 h resting	N/A	N/A	1 000 IU/ml IL-2 for 24 h
24 h together	N/A	N/A	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 24 h
72 h together	N/A	N/A	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 72 h
24 h separated+24 h together	1 000 IU/ml IL-2 for 24 h	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 24 h	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 24 h
48 h separated+24 h together	1 000 IU/ml IL-2 for 48 h	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 48 h	1 000 IU/ml IL-2 + plate bound anti-CD3/soluble anti-CD28 for 24 h

The frequencies of both cell types were identical to the controls in all studied conditions (**Figure 4.18A-B**), suggesting that there is no effect of CB NK cells on CB Treg cell frequency and vice-versa. However, Treg cell viability was considerably affected in the 72 h co-cultures with activated CB NK cells (**72 h tog**). The frequency of live TCR-stimulated CB Treg cells (Annexin V^-/7-AAD^- cells) decreased from ~70% to ~20% (**Figure 4.18C**) accompanied with an increase of ~2% to ~50% of late apoptotic cells (Annexin V^+/7-AAD^+ cells) (**Figure 4.18G**), whereas the proportion of early apoptotic TCR-stimulated CB Treg cells (Annexin V^+/7-AAD^- cells) remained relatively constant at ~30% (**Figure 4.18E**). Conversely, activated CB NK cell viability was not impaired at any time point (**Figure 4.18D,F,H).** Importantly, these results are in agreement with published data in humans and mice (Brillard et al., 2007, Roy et al., 2008), as only activated but not resting NK cells (either autologous or allogeneic) were able to affect TCR-stimulated CB Treg cell viability.

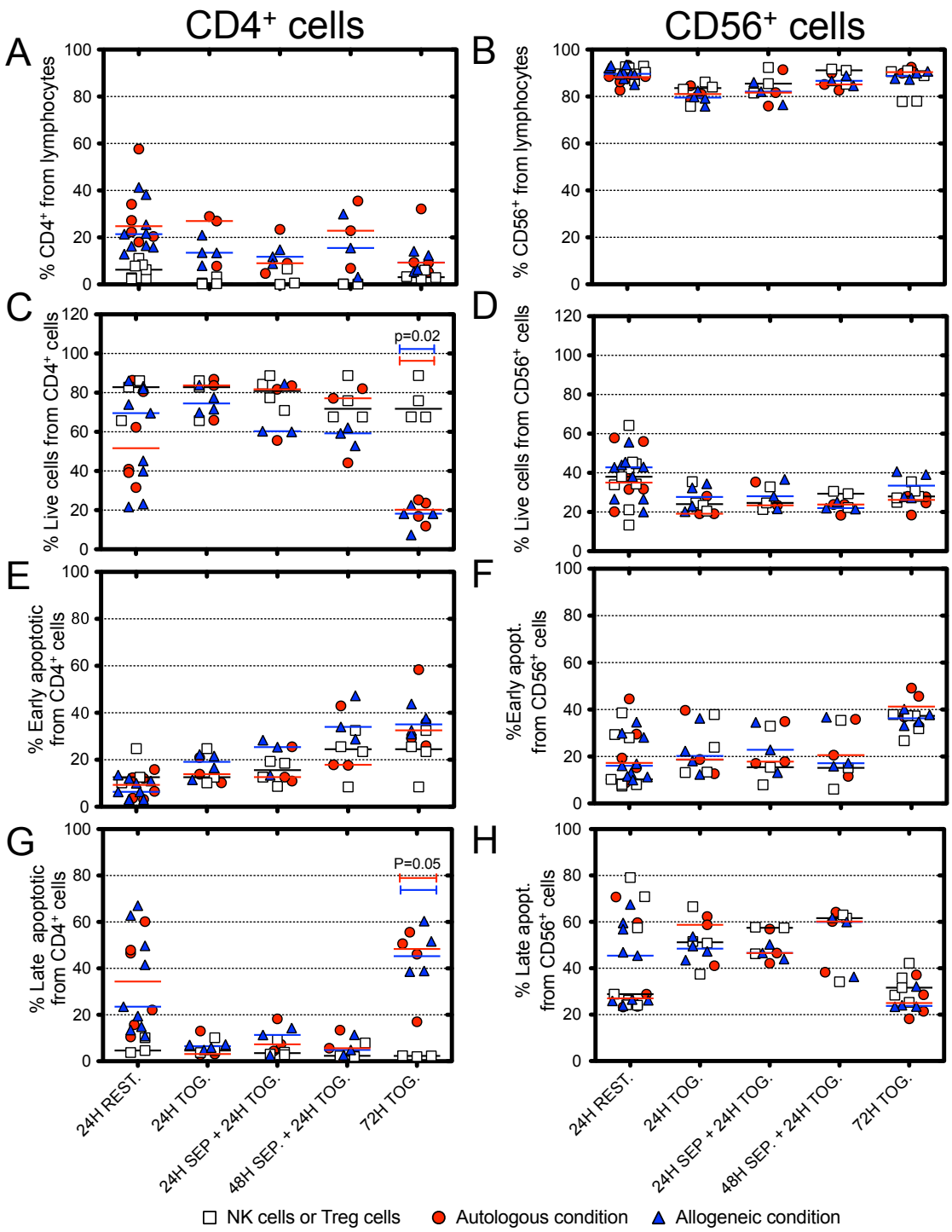


Figure 4.18: Frequency and viability of CB Treg cells and NK cells after co-culture in the presence of exogenous IL-2. Resting or TCR-stimulated CB Treg cells were cultured with CB NK cells in the presence of 1 000 IU/ml IL-2. Data is presented as $CD4^+$ (A) and $CD56^+$ (B) total cell percentages gated on lymphocytes. Viability was analysed by flow cytometry using CD4, CD56, 7-AAD and Annexin V. $CD4^+$ (C) and $CD56^+$ (D) live cell percentages are gated on 7-$AAD^-$$AnnexinV^-$ cells. $CD4^+$ (E) and $CD56^+$ (F) early apoptotic percentages are gated on 7-AAD^- $AnnexinV^+$ cells. $CD4^+$ (G) and $CD56^+$ (H) late apoptotic percentages are gated on 7-$AAD^+$$AnnexinV^+$ cells. The lines are represented as medians. n=3-9. Tog.: cultured together, sep: cultured separatelly, rest: resting.

4.5.2 Regulatory T cell proliferation

Autologous activated NK cells are able to inhibit the conversion of $CD4^+$ Tcon cells to pTreg cells in humans and mice under pathological conditions (Brillard et al., 2007, Roy et al., 2008); however no information is available on the effect of CB-derived NK cells on CB Treg cells. Hence, CFSE-labeled CB Treg cells were cultured in the presence of autologous or allogeneic CB NK cells upon TCR stimulation and treatment with exogenous IL-2. The proliferation of TCR-stimulated Treg cells alone or in co-culture with NK cells was analysed by flow cytometry at 0, 24, 48 and 72 h and presented as MFI. CB NK cells did not affect CB Treg cell proliferation upon TCR stimulation (**Figure 4.19**), which is consistent with the results shown in **Figure 4.19A; left panel.**

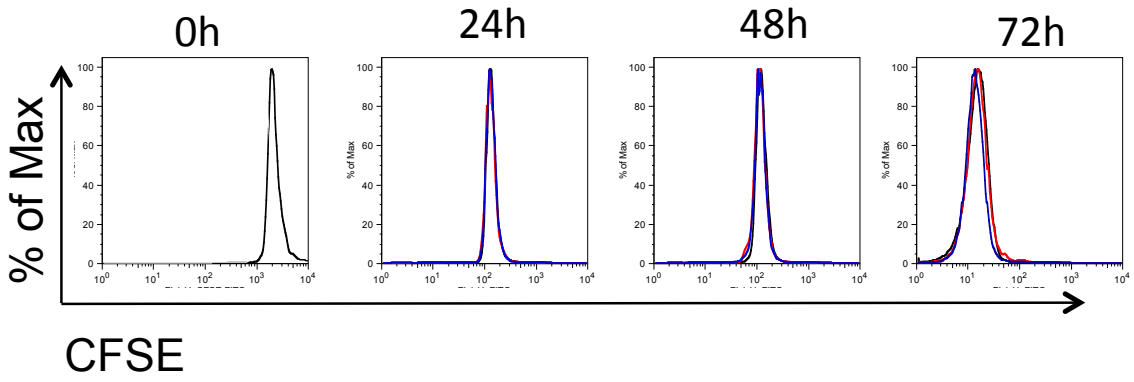


Figure 4.19: Effect of CB NK cells on CB Treg cell proliferation. CB Treg cells were CFSE-labelled and cultured alone (black) or with autologous (red) or allogeneic (blue) CB NK cells in the presence of 1 000 IU/ml IL-2 and plate bound anti-CD3/soluble anti-CD28. Cells were analysed by flow cytometry at 0, 24, 48 and 72 h. Representative example of 4 independent experiments.

4.5.3 Fas expression on regulatory T cells

In view of the effect of autologous and allogeneic activated CB NK cells on the viability of TCR-stimulated CB Treg cells observed in this study, expression of the death receptor Fas (CD95) on CB Treg cells was determined, in line with data reported for $CD4^+$ T cells (Noval Rivas et al., 2010). Fas expression was assessed by flow cytometry on resting and TCR-stimulated CB Treg cells at 0, 24, 48 and 72 h.

Interestingly, Fas expression was reduced on both resting and TCR-stimulated Treg cells (**Figure 4.20**); however only resting CB Treg cells showed significant downregulation of Fas expression after 48 and 72 h culture ($p_{48h,72h}$=0.02) (**Figure 4.20A**). Consistently, Fas MFI on resting CB Treg cells also decreased from ~60 arbitrary units (a.u.) to ~20 a.u. after 24, 48 and 72 h culture ($p_{24h, 48h, 72h}$=0.02) (**Figure 4.20B**). The fact that Fas expression is maintained on TCR-stimulated CB Treg cells could explain their higher susceptibility to NK cell lysis as compared to resting CB Treg cells. However, further studies will need to be performed to (i) assess how NK cells are causing Treg cell lysis, (ii) to determine if Fas is the main cognate receptor that promotes Treg cell lysis, and (iii) to determine whether other molecules such as perforin and granzyme could also play a role in Treg cell lysis by NK cells.

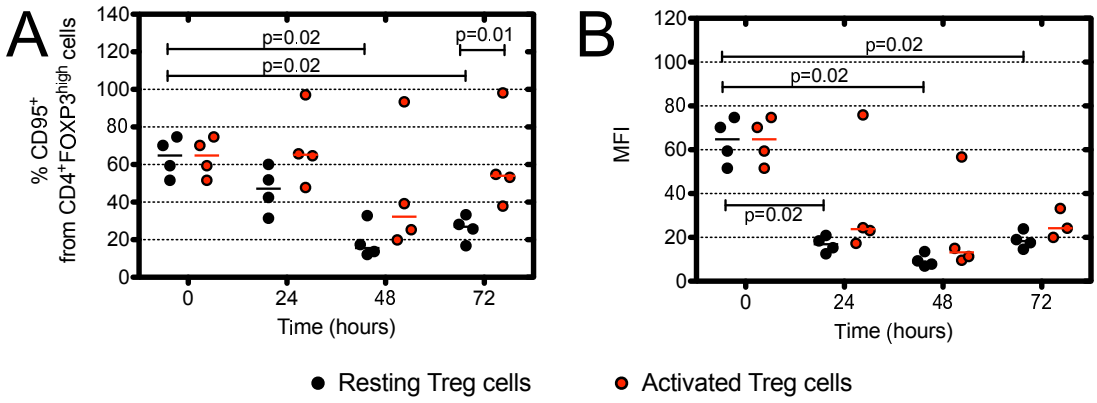


Figure 4.20: Fas expression on resting and TCR-stimulated CB Treg cells. CB Treg cells were cultured with 1 000 IU/ml IL-2 (resting) or 1 000 IU/ml IL-2 and plate bound anti-CD3/soluble anti-CD28 (activated) and analysed by flow cytometry after 0, 24, 48 and 72 h culture. Data is presented as percentage of Fas ($CD95^+$) cells (A) and MFI (B) from CB Treg cells gated on $CD4^+Foxp3^{high}$ cells. The lines are represented as medians. n=4.

4.6 Discussion

The potential to use NK cells as an adoptive cell therapy for cancer has driven considerable investigation towards understanding the mechanisms of tolerance that prevent NK cell spontaneous activation. To date, there are several theories that explain NK cell tolerance or regulation of NK cell activation. Firstly, NK cell "education" occurs by control of the functional development of NK cells by MHC class I molecules. The engagement of inhibitory receptors on NK cells by self-MHC class I molecules determines whether NK cells will be functional or whether they will become hyporesponsive following stimulation, thus being able to distinguish self from non-self. NK cell "education" has been explained by several models, the "licensing" model (Kim et al., 2005), the "arming/disarming" model (Fernandez et al., 2005, Joncker and Raulet, 2008), the "cis interaction" model (Doucey et al., 2004, Chalifour et al., 2009) and the "rheostat" model (Brodin et al., 2009, Joncker et al., 2009) as described in Section 1.3.2.3. Secondly, another process by which NK cell activation is regulated is referred to as NK cell priming. This process describes how NK cells require appropriate "priming" and "triggering" signals provided by cytokines or other cells to be fully functional (Lucas et al., 2007, North et al., 2007, Ganal et al., 2012). Furthermore, it has recently been proposed that Treg cells can also regulate NK cell activation through IL-2 (Gasteiger et al., 2013a, Gasteiger et al., 2013b, Sitrin et al., 2013). Under homeostatic conditions, Treg cells can limit the availability of IL-2 indirectly, via Tcon cell exhaustion, or directly by competition for soluble IL-2. However, under inflammatory conditions, it has been shown that NK cells upregulate CD25 and compete with Treg cells for IL-2 allowing NK cells to override Treg cell-mediated suppression during inflammation.

No information is available on the effects of CB Treg cells on CB NK cell effector functions. As previously mentioned, PB Treg cell-mediated NK cell suppression requires three conditions: an adequate cell-to-cell ratio between Treg cells and NK cells, absence of cytokines and TCR-stimulation. In this study, the following conditions were assessed: two different cell ratios (1:1 and

1:4, NK cells:Treg cells), presence or absence of IL-2, resting or TCR-stimulated Treg cells, and autologous or allogeneic conditions.

In this chapter, the impact of Treg cells (resting or TCR-stimulated, autologous or allogeneic) on resting or activated NK cells was assessed by the analysis of NK cell functions: cytotoxicity, cytokine production, expression of activating receptors, viability and proliferation. A comparison of the presented results with the literature is shown in **Table 4.3**, followed by discussion of each NK cell function.

Table 4.3: Comparison of the results presented in this study with existing published studies. Only reported studies that show direct suppression are presented.

NK cell function	PB studies (direct studies)	This study (CB)
Natural cytotoxicity	• Inhibited by resting Treg cells (membrane-bound TGF-β) • Not inhibited if IL-2 present	• Not inhibited when freshly isolated NK cells and resting Treg cells were used • Inhibited when activated NK cells and TCR-stimulated Treg cells were used in absence of IL-2 • Not inhibited when resting NK cells and resting or TCR-stimulated Treg cells were used in presence of IL-2
Cytokine Production (IFN-γ)	• Downregulated if IL-12 present • Not downregulated if IL-2/IL-15 present	• Not downregulated on resting NK cells in the presence of resting Treg cells • Not downregulated on resting NK cells in the presence of resting or TCR-stimulated Treg cells and in the presence of IL-2 • Enhanced cytokine production by IL-15 activated NK cells in absence of IL-2
Activating Receptors	• NKG2D downregulated in the presence of resting Treg cells	• NKG2D, DNAM-1, NKp30 and NKp46 and CD16 downregulated on NK cells in the presence of resting CB Treg cells
LFA-1	• N/D	• No effect
Viability	• N/D (only studies *in vivo*)	• No effect
Proliferation	• N/D (only studies with Tcon cells and APCs)	• No effect

Because of the findings of Ghiringhelli and colleagues who show that resting PB Treg cells inhibit PB NK cell effector functions, the ability of CB Treg cells to suppress CB NK cells in the same manner was assessed (Ghiringhelli et al., 2005). In contrast to PB Treg cells, freshly isolated allogeneic CB Treg cells failed to suppress PB NK cells in the absence of cytokines. This could be due to the immaturity of Treg cells, which require IL-2 stimulation to be functional (Godfrey et al., 2005). However, neither resting nor TCR-stimulated CB Treg cells abrogated resting CB NK cell functions in the presence of exogenous IL-2. This could be explained by the presence of IL-2 which may override Treg cell-mediated suppression (Ghiringhelli et al., 2005) and/or the need for CB NK-cell activation, since CB NK cells have an immature phenotype (Luevano et al., 2012a). Hence, a requirement for activation of CB NK cells and “steady state conditions” (i.e. no cytokines) was established. Another explanation for these differences could be that CB Treg cells exhibit different levels of expression of membrane-bound TGF-β as compared to PB Treg cells. Expression of membrane-bound TGF-β is the mechanism by which resting PB Treg cells suppress resting PB NK cells without the need for TCR-stimulation (Ghiringhelli et al., 2005), but this requires further investigation for CB Treg cells.

TCR-stimulated CB Treg cells abrogated CB NK cell cytotoxicity *in vitro*, when NK cells were activated with IL-15. In line with these results, a decrease in the frequency of activated CB NK cells that degranulate when stimulated with K562 cells or PMA/ION was also observed in the presence of TCR-stimulated CB Treg cells, but these observations showed no statistical significance. This might suggest that mature NK cells are more susceptible to Treg cell-mediated suppression, which is in agreement with the results from Sungur and colleagues, who observed a preferential expansion of licensed NK cells over unlicensed NK cells during infection with mouse CMV when Treg cells were depleted (Sungur et al., 2013). Based on the assumption that the degree of licensing correlates with NK cell maturation, it may be possible that Treg cells could selectively suppress specific NK cell subpopulations, however further experiments would be required to assess this concept.

Next, the ability of CB Treg cells to impair NK cell viability was assessed. According to what is reported in the literature, there is no clear evidence that Treg cells can lyse NK cells. While several authors have suggested NK cell (Cao et al., 2007), T cell (Grossman et al., 2004a) and B cell (Zhao et al., 2006) lysis by autologous TCR-stimulated Treg cells via perforin and/or granzyme A or B in mice, Balaji and colleagues suggest that cytotoxic cells such as CTL and NK cells are resistant to killing via perforin and granzyme due to the expression of cathepsin B, a mechanism that prevents self-destruction when degranulation occurs (Balaji et al., 2002). The data presented in this chapter support the argument of Balaji and colleagues since no effect on CB NK cell viability was observed for any of the conditions tested (i.e. activation status, allogeneic Treg cells, presence of cytokines). One explanation for this could be that CB Treg cells exhibit a different pattern of expression of granzyme A as compared to PB Treg cells. Thus it would be interesting to evaluate granzyme A expression by CB Treg cells as compared to PB Treg cells.

The integration of signals provided by activating and inhibitory receptors upon detection of infected cells or tumours determines NK cell function (Pegram et al., 2011). Hence, changes in the expression of these receptors may cause impairment of NK cell effector functions, as observed in individuals with prostate (Wu et al., 2004) and colon (Doubrovina et al., 2003) carcinoma. For instance, upregulation of NKG2D and NKp44 on NK cells is observed when they are co-cultured with $CD4^+$ Tcon cells; however this effect is reverted by the addition of Treg cells (Bergmann et al., 2011), suggesting a potential regulation of NK cell activation by T cells. Furthermore, NKG2D downregulation on resting NK cells by PB Treg cells can also be detected in the absence of cytokines (Ghiringhelli et al., 2005).

The results presented in this study are in line with what is reported by Ghiringhelli and colleagues, as downregulation of NKG2D, but also CD16, NKp30, NKp46 and DNAM-1 was detected on resting CB NK cells when co-cultured with resting (allogeneic and autologous) CB Treg cells. However this effect seems to be transient as statistical significance decreased over the period studied. These observations can be explained by the previously

mentioned study from Ghiringhelli and colleagues, who observed similar results for resting PB cells and detected membrane-bound TGF-β as the main mechanism of suppression (Ghiringhelli et al., 2005). Hence, it would be interesting to further analyse the expression of membrane-bound TGF-β by resting CB Treg cells and perform blocking assays to further elucidate whether CB Treg cells use this mechanism to decrease the expression of the aforementioned NK cell receptors. Notably, TCR-stimulated CB Treg cells did not affect the phenotype of activated NK cells in the absence of cytokines. This could be accounted for the potential differences in expression of membrane-bound TGF-β between resting and TCR-stimulated Treg cells as it has been described that resting Treg cells exhibit higher expression of membrane-bound TGF-β than TCR-stimulated Treg cells (Jonuleit and Schmitt, 2003). Another receptor that may have critical implications in NK cell degranulation is LFA-1; however, in this study the LFA-1 pathway was not impaired in NK cells following co-culture with Treg cells.

As previously mentioned, human Treg cells can abrogate $CD4^+$ Tcon cell-mediated NK cell proliferation (Romagnani et al., 2005). Here, no impairment of NK cell proliferation in the co-cultures with CB Treg cells (autologous or allogeneic) was observed, regardless of their stimulation status and the presence of exogenous cytokines. A possible explanation for this might be that Treg cells only impair NK cell proliferation via $CD4^+$ Tcon cells, which are barely present in this experimental system (<2.5% of total cells in co-culture). Thus, it would be interesting to assess whether CB Treg cells can inhibit NK cell proliferation in the presence of Tcon cells.

Treg cell-mediated suppression of NK cells can also be assessed by the measurement of cytokine secretion such as IFN-γ. Several authors have observed a decrease in IFN-γ production by NK cells in the presence of Treg cells (Trzonkowski et al., 2004, Ghiringhelli et al., 2005, Zhou et al., 2010, Bergmann et al., 2011). Hence, it is plausible that CB Treg cells could also decrease IFN-γ secretion by CB NK cells. In this study, CB Treg cells (autologous or allogeneic) did not decrease NK cell-mediated IFN-γ secretion by resting CB Treg cells in presence of IL-2. However, an enhanced production

of IFN-γ by activated CB NK cells was detected when NK cells were cultured with TCR-stimulated CB Treg cells in the absence of IL-2. Notably, this is the only experiment in which a difference between the effects of autologous and allogeneic CB Treg cells on CB NK cells was observed suggesting that MHC mismatch may be involved in this effect. This may have therapeutic implications, for example in transplantation, as NK cell-mediated control of infections is crucial for overall survival (Gallez-Hawkins et al., 2011), but this requires further investigation.

NK cells are able to lyse T cells under specific conditions, such as during viral infection, in autoimmune diseases and in the context of transplantation. During CMV infection in mice, NK cells can restrict T cell responses by killing infected T cells (Andrews et al., 2010). Furthermore, Roy and colleagues have demonstrated that human activated NK cells can lyse iTreg cells but not freshly isolated Treg cells in a model of *Mycobacterium tuberculosis* infection (Roy et al., 2008). To date, it was unknown whether CB NK cells can lyse CB Treg cells. The results presented in this study indicate that resting CB NK cells are unable to lyse resting CB Treg cells whereas IL-2 activated NK cells can severely compromise both autologous and allogeneic TCR-stimulated Treg cell viability *in vitro*. The reason why CB NK cells failed to kill CB Treg cells in less than 40 h can be explained by the observations presented in this study on CB NK cell activation with different cytokines, as IL-2 mediated activation of CB NK cells required longer times of activation (>40 h) for efficient killing capacity and activation (Luevano et al., 2012a).

Furthermore, the expression of Fas (CD95) on TCR-stimulated CB Treg cells may suggest that Fas represents a mechanism of recognition of Treg cells by activated CB NK cells, as observed by Noval Rivas and colleagues (Noval Rivas et al., 2010). However, other molecules such as perforin and granzyme (Yamaji et al., 2012) could also play a role. Further studies such as blocking of Fas and analysis of perforin and granzyme expression in CB Treg cells in the presence of activated CB NK cells would need to be performed to fully elucidate how CB NK cells lyse TCR-stimulated CB Treg cells. These results may have therapeutic implications, particularly in HSCT, as NK cells activated by the

cytokine storm evident in most post-HSCT patients after radiotherapy, could compromise the persistence of adoptive Treg cells when used as a therapy to prevent GvHD.

The data presented in this chapter demonstrates that TCR-stimulated CB Treg cells inhibit NK cell lysis in the absence of cytokines, whereas the presence of IL-2, crucial during inflammation, may revert this effect. Also, unlike PB Treg cells (Ghiringhelli et al., 2005), CB Treg cells require TCR-stimulation to impact on NK cell effector functions. Notably, TCR-stimulated CB Treg cells enhanced IFN-γ secretion but decreased NK cell cytotoxicity against K562 cells by activated NK cells. This finding may suggest that TCR-stimulated CB Treg cells have different effects on NK cell subpopulations, as $CD56^{bright}$ NK cells are primary NK cell cytokine producers whereas $CD56^{dim}$ NK cells are mostly cytotoxic (Lanier et al., 1989). However, further experiments with sorted NK cell subpopulations are required to prove this concept. Hence, the theory of NK cell regulation by IL-2 recently proposed by Kerdiles and colleagues may apply as well to CB Treg cell/NK cell interactions, as no effect was observed in the presence of IL-2; however further investigation is required to confirm this hypothesis. This study presents the optimal conditions under which both CB Treg cells and CB NK cells compromise each other's functions, thus providing information for the optimisation of an adoptive CB Treg cell therapy.

5 Effect of umbilical cord blood regulatory T cells on natural killer cell differentiation

5.1 Introduction

Preclinical and clinical studies suggest that the use of Treg cells to prevent or modulate GvHD in transplanted patients is safe (Trzonkowski et al., 2009, Brunstein et al., 2011b, Di Ianni et al., 2011, Edinger and Hoffmann, 2011), but the potential impact of Treg cells on GvL and Graft versus Infection (GvI) is still controversial (Trenado, 2003, Maury et al., 2010, Brunstein et al., 2013). This is important because after CBT, NK cells are the first lymphocytes to reconstitute (Beziat et al., 2009) and for several months are the only effector cells that are present in the blood of transplanted patients that can provide GvL and GvI.

Given that the clinical trials that assessed the use of Treg cells as a therapy for GvHD focused on clinical safety, limited data on the effects of Treg cells on immune reconstitution have been reported. For instance, Brunstein and colleagues assessed whether CB Treg cells can prevent GvHD in 23 double CBT patients and provide some evidence that Treg cells may impair GvI (Brunstein et al., 2011b). In this study, higher susceptibility to early viral reactivation was observed within 30 days after transplantation in Treg cell-treated patients as compared to historical controls (Brunstein et al., 2013). In

addition, Di Ianni and colleagues demonstrated that adoptive transfer of freshly isolated donor Treg cells counteracted the potential GvHD induced by megadoses of donor Tcon cells in 28 patients receiving a haploidentical graft. When compared to a cohort of 152 patients, Treg cell-treated patients exhibited higher immune reconstitution and improved immunity to opportunistic infections, thus suggesting that under these particular conditions, NK cells may not be impaired.

Likewise, there is discrepancy in results obtained from preclinical studies that analysed the impact of Treg cells on GvL and immune responses to viral infections. For instance, Nguyen and colleagues observed that co-infusion of donor Treg cells with donor Tcon cells in a mouse model of GvHD after BMT enhanced immune reconstitution and GvI while preventing GvHD-induced damage to the thymus and SLT (Nguyen et al., 2007). However, whether adoptively transferred Treg cells can compromise NK cell-mediated GvL is still debatable. Whilst the clearance of A20 leukaemia cells (GvL effect) was observed in BM-transplanted Balb/c mice in the presence of recipient-alloantigen-specific Treg cells, Treg cells compromised the GvL effect when a different mouse strain and a different tumour cell line was used (Trenado, 2003).

As Treg cells can suppress NK cell function *in vitro* (Ghiringhelli et al., 2005, Ralainirina et al., 2006) and *in vivo* (Sun et al., 2010, Zhou et al., 2010), it is possible that adoptive transfer of Treg cells could also impair NK cell differentiation in transplanted patients and thus severely compromise NK cell-mediated GvL and GvI. This chapter aims to investigate whether CB Treg cells can impair NK cell differentiation *in vitro* and if so, to determine at which stages of NK cell differentiation and at what particular time point of NK cell differentiation this potential suppression occurs.

5.2 Effect of regulatory T cells on natural killer cell differentiation

Only Romagnani and colleagues have studied the impact of Treg cells on numbers of mature NK cells *in vitro* (Romagnani et al., 2005). They observed increased proliferation of human PB NK cells following interaction with autologous Tcon cells ($CD4^+CD25^-Foxp3^-$) and plasmacytoid DCs; however this effect was completely abrogated by the addition of TCR-stimulated $CD4^+CD25^{high}$ Treg cells. Currently, no information is available on the effect of Treg cells on NK cell differentiation. To evaluate this effect, an established *in vitro* model of NK cell differentiation was used (Grzywacz et al., 2006) (Luevano et al., Plos One, under revision). This model consists of the culture of CB $CD34^+$ HSC for 35 days in the presence of EL08.1D2 feeder layer cells and cytokines such as IL-3, IL-7, IL-15, SCF and Flt3 ligand to induce NK cell differentiation. This model is ideal to analyse the potential effects of CB Treg cells on NK cell differentiation as HSC only differentiate into NK cells (and no other cell type) under the conditions used in this system (**Figure 5.1**).

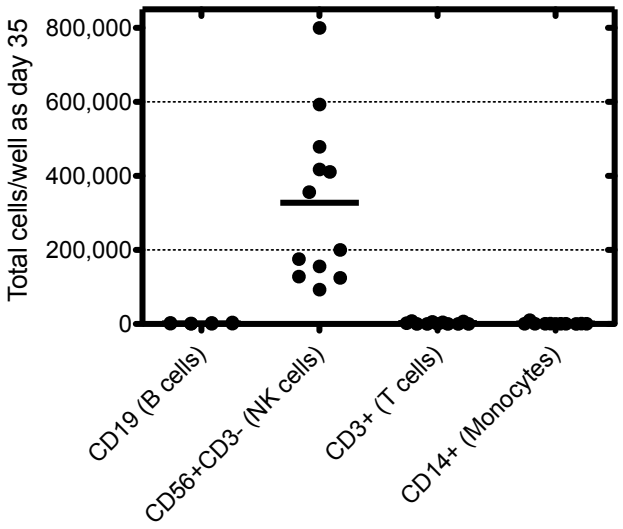


Figure 5.1: Cell types present at day 35 of HSC cultures. Flow cytometric analysis of B cells ($CD19^+$), NK cells ($CD56^+CD3^-$), T cells ($CD3^+$) and monocytes ($CD14^+$) at day 35 of HSC cultures. The lines represent medians. Cell counts were calculated from total cell numbers and the cell ratios determined by flow cytometry. n=8-12.

Allogeneic CB Treg cells, either resting or TCR-stimulated, were added at five key time points during HSC cultures where the transition from one differentiation stage to another occurs (**Figure 5.2**). TCR-stimulated CB Treg cells were activated with plate bound anti-CD3/soluble anti-CD28 and 1 000 IU/ml IL-2 for 24 h and washed before addition to HSC cultures.

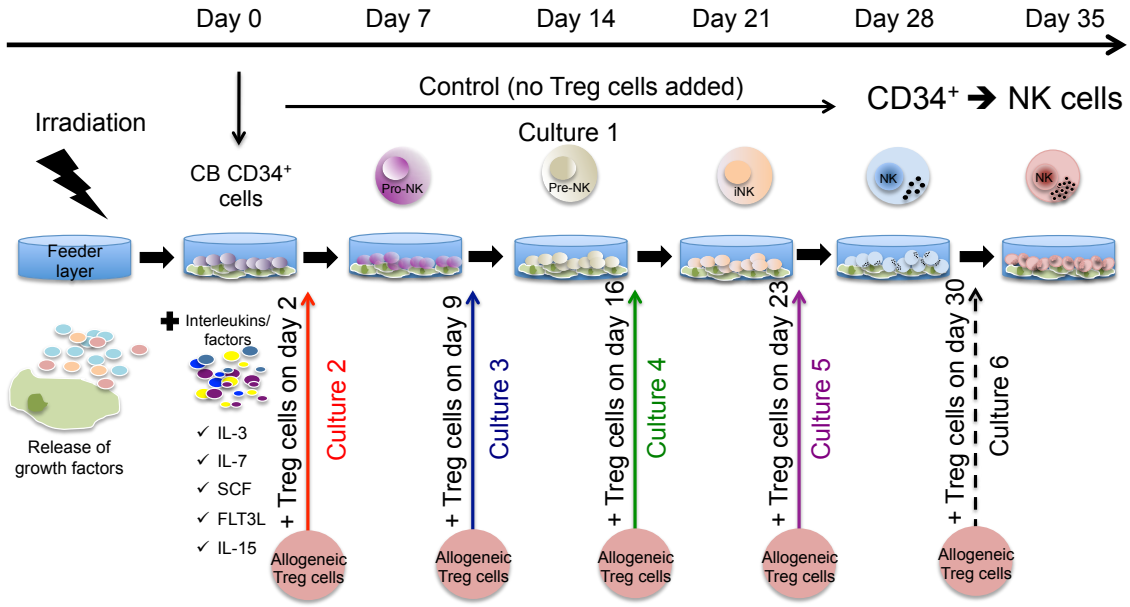


Figure 5.2: Experimental design to assess a potential effect of Treg cells on HSC cultures. HSC ± resting or TCR-stimulated CB Treg cells were cultured in the presence of irradiated EL08.1D2 cells and cytokines for 35 days. CB Treg cells were added to HSC cultures at a ratio of 1:4 (Treg cells:NK cells), except when CB Treg cells were added at day 2, where a ratio of 1:1 was used. TCR-stimulated CB Treg cells were activated with plate bound anti-CD3/soluble anti-CD28 and 1 000 IU/ml IL-2 for 24 h and washed before addition to HSC cultures. Figure adapted by permission from Macmillan publisher Ltd: [Cellular and Molecular Immunology] (Luevano et al., 2012b), copyright 2012.

The impact of CB Treg cells on NK cell differentiation was assessed by measuring the total NK cell number at the end of HSC cultures at day 35, which was calculated from the NK cell frequency determined by flow cytometry and total cell number per well. It was found that resting CB Treg cells do not have an effect on NK cell numbers, regardless of the time point at which they were added. In addition, expansion rates in all resting Treg cell/HSC co-cultures were similar to HSC cultures (~300 000 NK cells; 600-fold to 1 000-fold expansion) (**Figure 5.3A.1**). However, when TCR-stimulated CB Treg cells were added, a significant reduction in NK cell numbers was observed, except when TCR-stimulated CB Treg cells were added at day 2 of culture (**Figure 5.3A.2**).

Remarkably, the strongest effect was observed when TCR-stimulated CB Treg cells were added at day 9 of culture with 90% reduction in NK cell numbers observed at day 35 of culture (p=0.0006). This is the time point at which HSC commitment to the NK cell lineage occurs (Luevano et al., Plos One, under revision). Notably, the effect of TCR-stimulated Treg cells on NK cell numbers was reduced when Treg cells were added at later stages of NK cell differentiation and maturation. A 50% reduction in NK cell numbers was observed when TCR-stimulated CB Treg cells were added at day 16 of culture (p=0.0012) and a 40% reduction at days 23 (p=0.0025) and 30 (p=0.0480) of culture.

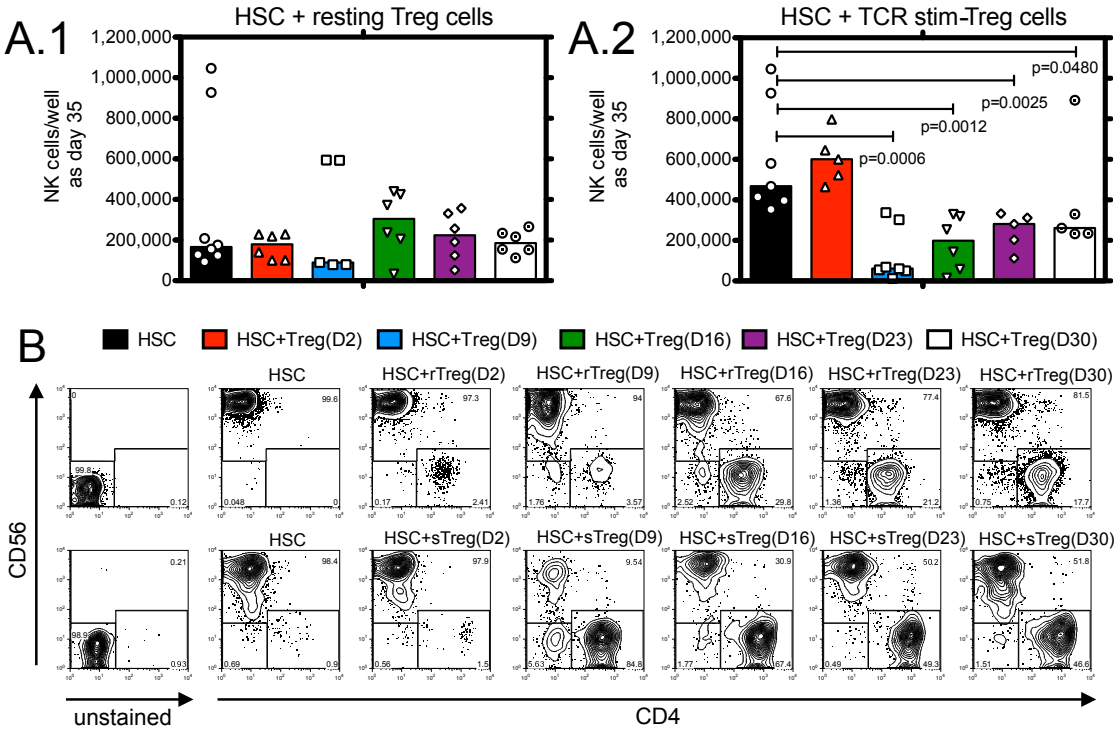


Figure 5.3: Allogeneic TCR-stimulated CB Treg cells but not resting CB Treg cells inhibit NK cell differentiation from CD34$^+$ cells. CD34$^+$ cells were cultured in the presence or absence of allogeneic resting or TCR-stimulated CB Treg cells added at day 2, 9, 16, 23 or 30 of HSC cultures at a ratio of 1:4 (Treg cells:HSC). Total NK cell count at day 35 of differentiation ± resting CB Treg cells (A.1) or TCR-stimulated CB Treg cells (A.2). The lines represent medians. n=5-8. (B) Representative flow cytometric analysis of NK cells (CD56$^+$) and Treg cells (CD4$^+$) from all cultures at day 35 of differentiation. Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell number per well. rTreg: resting CB Treg cells, sTreg: TCR-stimulated Treg cells.

As this model of HSC differentiation does not give rise to T cells (**Figure 5.1**) it can be assumed that all the CD4$^+$ T cells observed at day 35 of culture were

derived from Treg cells that were added to the cultures at the indicated time points (**Figure 5.3B**).

In all Treg cell/HSC co-cultures, only Treg cells and NK cells were observed, thus suggesting that CB Treg cells do not favour the differentiation of HSC into another cell lineage. Notably, it seems that TCR-stimulated CB Treg cells can respond to the cytokines present in the cultures and proliferate.

5.3 Effect of regulatory T cells on viability of $CD56^+$ natural killer cells

Several groups have reported that Treg cells can affect the viability of target cells such as T cells, B cells and more recently HSC (Grossman et al., 2004a, Zhao et al., 2006, Fujisaki et al., 2011). Therefore, it was next examined whether CB Treg cells could impair the viability of HSC in culture. Allogeneic resting or TCR-stimulated CB Treg cells were added at five key time points of HSC cultures (as described in **Figure 5.1**), and the viability of $CD45^+$ cells, NK cells and Treg cells was analysed by flow cytometry using 7-AAD.

A similar frequency of $CD45^+$ viable cells was detected in control cultures and for HSC cultured in the presence of resting Treg cells (**Figure 5.4A**) or TCR-stimulated CB Treg cells (**Figure 5.4B**) with an average of 90% live cells ($7\text{-}AAD^-$ cells). Similarly, neither resting (**Figure 5.5A**) nor TCR-stimulated CB Treg cells (**Figure 5.5B**) affected NK cell viability, with all cultures showing similar percentages of viable NK cells to controls.

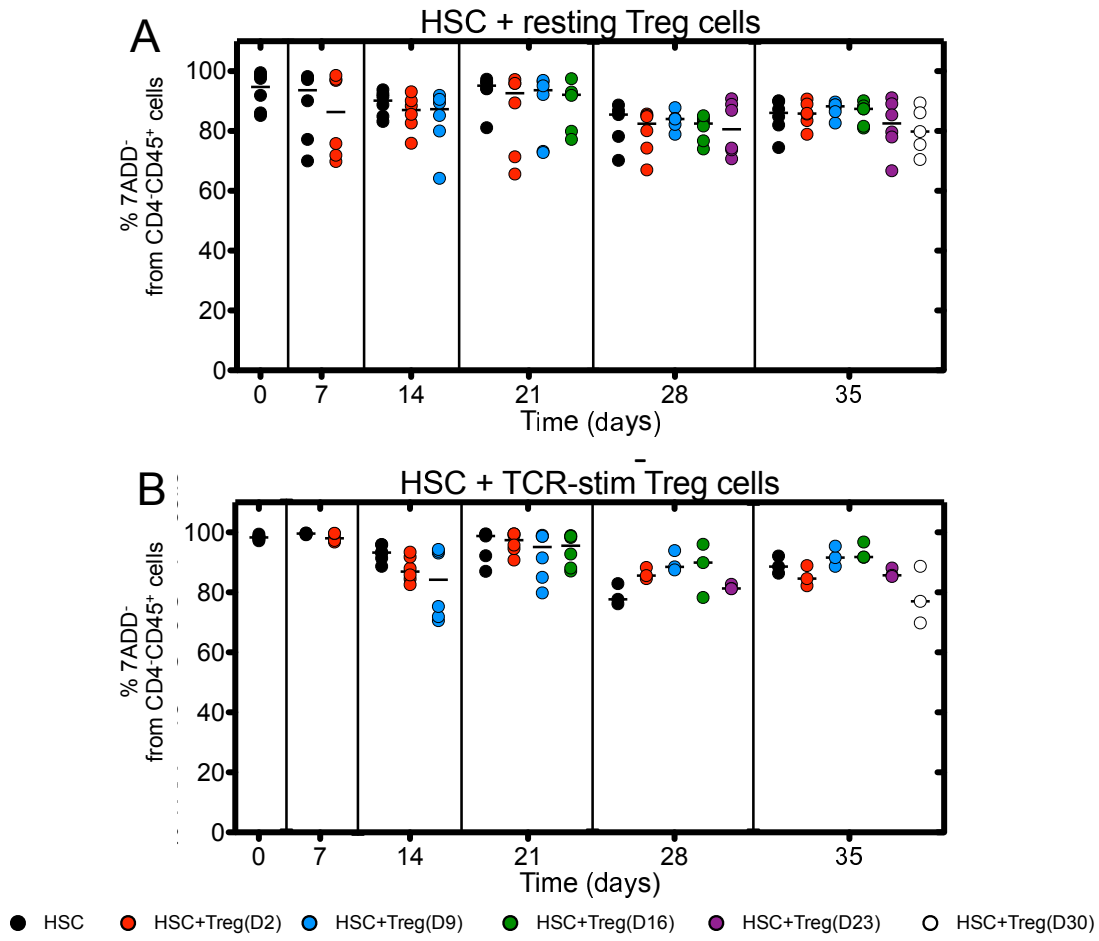


Figure 5.4: Viability of CD45$^+$ cells in HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. Viability of CD45$^+$ cells was assessed by flow cytometry using CD4, CD45 and 7-AAD. HSC were cultured in the presence or absence of allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B). Treg cells were added at key time points of differentiation. The lines represent medians. n=6-8.

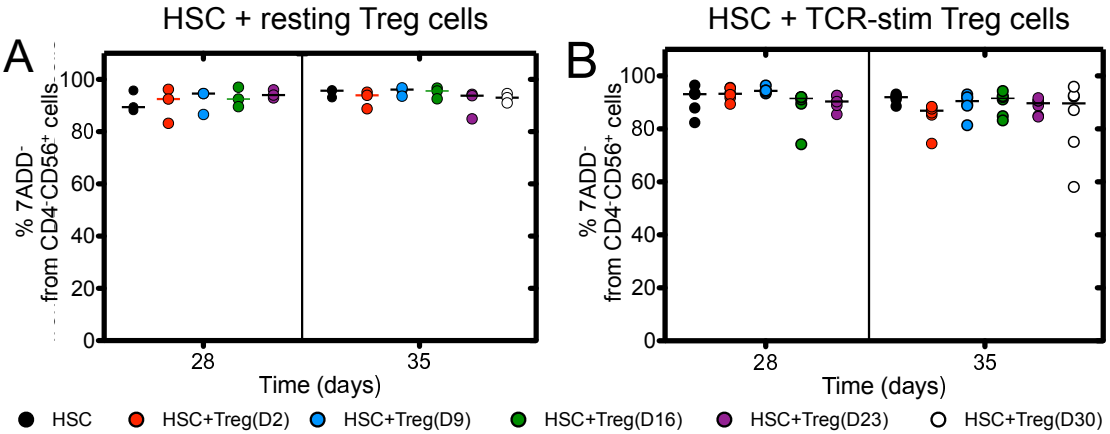


Figure 5.5: Viability of CD56$^+$ cells in HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. NK cell viability was assessed by flow cytometry using CD4, CD45, CD56 and 7-AAD. HSC were cultured in the presence or absence of allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B). The lines represent medians. n=3-6.

Imamichi and colleagues have suggested that IL-15 is a potent inducer of tTreg cell proliferation (Imamichi et al., 2008); however it is unknown whether CB Treg cells are able to persist and proliferate under the conditions used in this model of NK cell differentiation. Hence, resting or TCR-stimulated CB Treg cell viability was assessed in all cultures by flow cytometry using 7-AAD and by gating on $CD4^+$ T cells. Resting (**Figure 5.6A**) and TCR-stimulated CB Treg cells (**Figure 5.6B**) were viable at all time points of HSC cultures analysed. The frequency of viable resting CB Treg cells was more variable between samples than TCR-stimulated CB Treg cells.

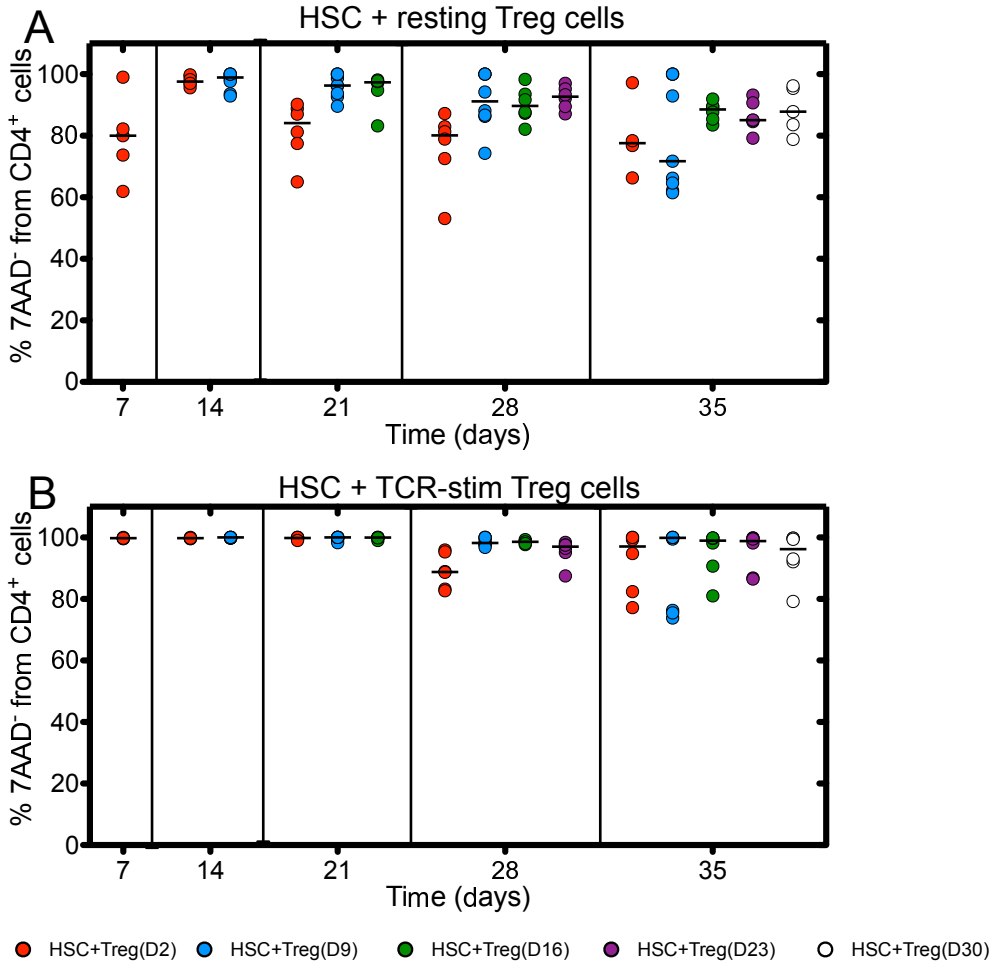
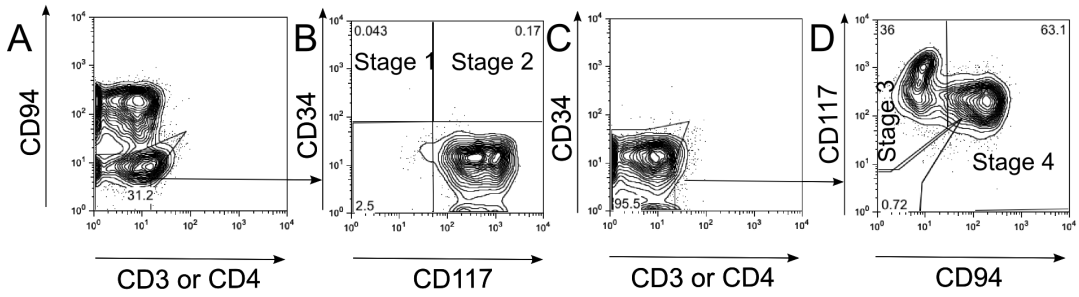


Figure 5.6: Viability of $CD4^+$ T cells in HSC cultures. Viability of $CD4^+$ T cells was assessed by flow cytometry using CD4, CD45 and 7-AAD. HSC were cultured in the presence or absence of allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B). The lines represent medians. n=5-6.

5.4 Phenotypic analysis of natural killer cell differentiation in the presence of regulatory T cells

5.4.1 NK cell differentiation stages

Next, it was determined whether TCR-stimulated Treg cells inhibited NK cell differentiation at a specific developmental time point. To evaluate this, the model by Freud and Caligiuri was used. The model identified four developmental stages of NK differentiation from $CD34^+$ HSC cells in human SLT based on the expression of CD3, CD34, CD117 and CD94 (Freud et al., 2006). Pro-NK cells (stage 1)($CD3^-CD34^+CD117^-CD94^-$) and pre-NK cells (stage 2)($CD3^-CD34^+CD117^+CD94^-$) were present during the first fourteen days of differentiation, whereas iNK cells (stage 3)($CD3^-CD34^-CD117^+CD94^-$) were detected after seven days of differentiation and $CD56^{bright}$ NK cells (stage 4)($CD3^-CD34^-CD117^{+/-}CD94^+$) after fourteen to 21 days of differentiation (**Figure 5.7** and **Figure 5.8).** **Figure 5.7** shows the gating strategy and definitions of each stage from a representative sample. In this study, CD4 was used instead of CD3 to detect T cells as TCR-stimulation of Treg cells with plate bound anti-CD3 may induce CD3 downregulation making the detection of this marker technically difficult.



Stage 1: CD3(-)CD34(+)CD117(-)CD94(-)
Stage 2: CD3(-)CD34(+)CD117(+)CD94(-)
Stage 3: CD3(-)CD34(-)CD117(+)CD94(-)
Stage 4: CD3(-)CD34(-)CD117(+/-)CD94(+)

Figure 5.7: Representative analysis of the stages of NK cell differentiation and maturation by flow cytometry. Flow cytometric analysis of the four stages of NK cell differentiation based on the expression of surface markers CD3, CD34, CD117 and CD94 at day 35, gated on $CD45^+$ cells. Stages 1 and 2 were gated as $CD3^-CD94^-CD34^+CD117^-$ and $CD3^-CD94^-CD34^+CD117^+$, respectively (A-B). Stages 3 and 4 were gated as $CD3^-CD34^-CD117^+CD94^-$ and $CD3^-CD34^-CD117^+CD94^+$, respectively (C-D).

To analyse the impact of CB Treg cells on each NK cell differentiation stage, the total cell numbers of the four NK cell stages in the different HSC cultures with or without resting or TCR-stimulated CB Treg cells were analysed. When resting CB Treg cells were added at key time point during HSC cultures, no difference was detected in the total cell numbers of any of the NK cell stages analysed (**Figure 5.8B.1, C.1, D.1, E.1** and **Figure 5.9A**). In contrast, TCR-stimulated CB Treg cells exerted different degrees of suppression on NK cell differentiation depending on the time point at which they were added to HSC cultures. When TCR-stimulated CB Treg cells were added at day 2 and 30 of HSC cultures, an effect on NK cell differentiation was not observed at any of the time points studied (**Figure 5.8B.2)**. However, when TCR-stimulated CB Treg cells were added at day 9, there was a slight decrease in the number of pro-NK cells at day 14 ($p=0.03$) (**Figure 5.8C.2),** but this effect was lost during subsequent days of HSC cultures. In contrast, iNK cell numbers were reduced at day 21 ($p=0.03$) (**Figure 5.8D.2)**, at day 28 ($p=0.01$) (**Figure 5.8E.2)** and at day 35 ($p=0.0079$) **(Figure 5.9B).** Likewise, total numbers of $CD56^{bright}$ NK cells were also reduced at days 28 ($p=0.07$) (**Figure 5.8E.2)** and 35 ($p=0.0079$) (**Figure 5.9B**).

When TCR-stimulated CB Treg cells were added at day 16, only significant reduction in the number of iNK cells (stage 3) was only noted at day 28 of culture ($p=0.008$) (**Figure 5.8E.2)**. A reduction in the number of $CD56^{bright}$ NK cells was observed at day 35 of culture ($p=0.0159$) (**Figure 5.9B**). In addition, when TCR-stimulated CB Treg cells were added at day 23, a reduction in the number of $CD56^{bright}$ NK cells at day 35 was observed ($p=0.0079$) (**Figure 5.9B**).

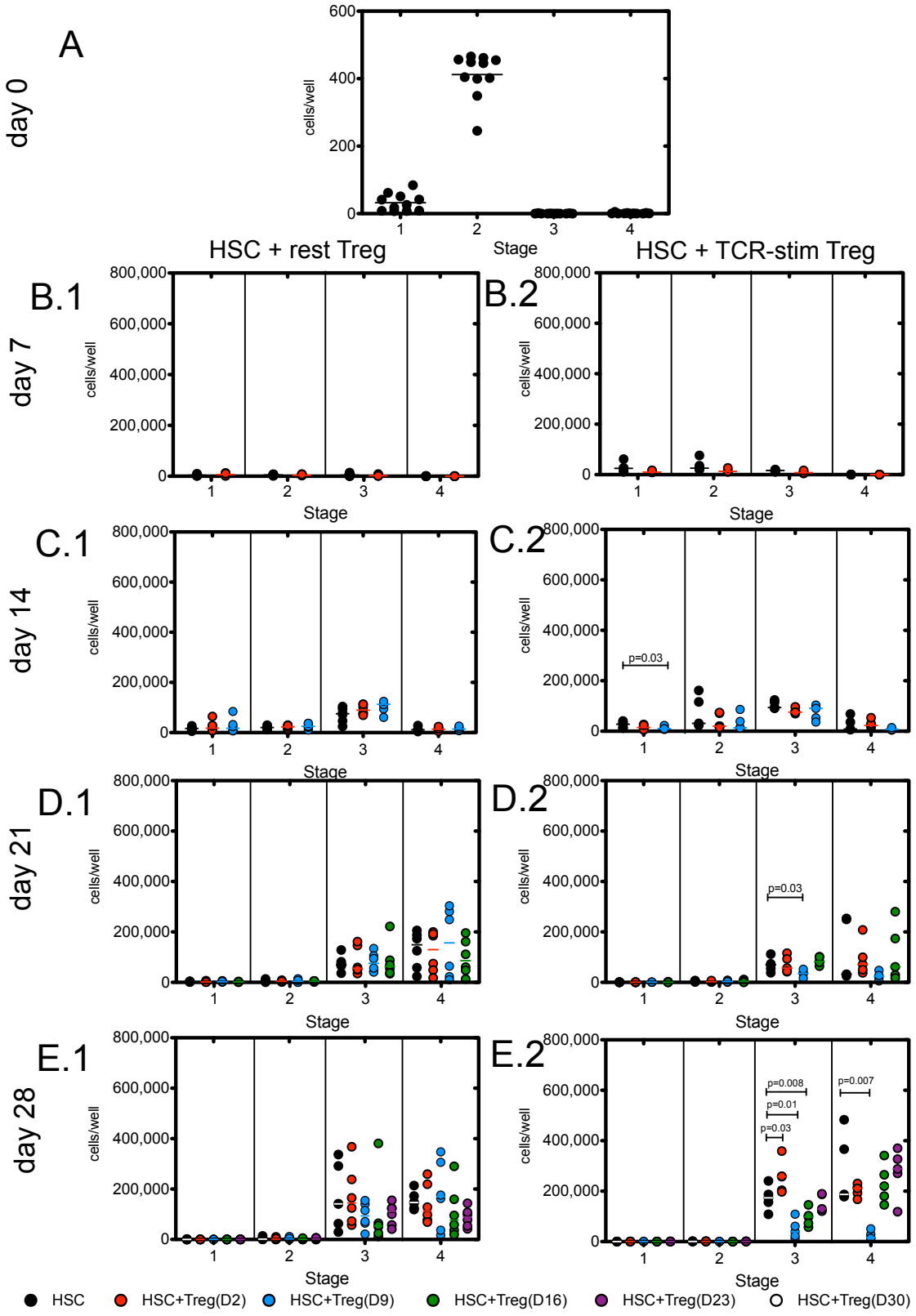


Figure 5.8: Assessment of the number of cells in each NK cell differentiation stage at days 0, 7, 14, 21 and 28 of HSC cultures. HSC were cultured in the presence or absence of allogeneic resting (left panels) or TCR-stimulated CB Treg cells (right panels) added at day 2, 9, 16, 23 and 30 of HSC culture. Flow cytometric analysis of Stages 1-4 based on CD4, CD34, CD117 and CD94 expression at day 0 (A), 7 (B.1-2), 14 (C.1-2), 21 (D.1-2), and 28 (E.1-2). Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell numbers per well. n=5-8.

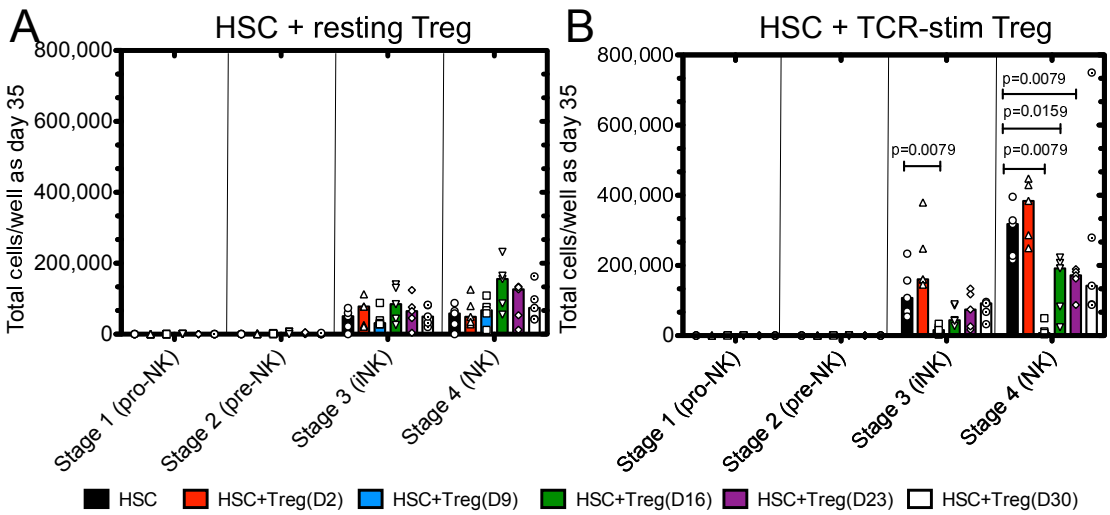


Figure 5.9: Assessment of the number of cells in each NK cell differentiation stage at day 35 of HSC cultures. HSC were cultured in the presence or absence of allogeneic resting (A) or TCR-stimulated (B) CB Treg cells added at day 2, 9, 16, 23 and 30 of HSC culture at a ratio of 1:4 (Treg cells: HSC). Flow cytometric analysis of Stages 1-4 based on CD4, CD34, CD117 and CD94 expression at day 35 of NK cell differentiation. The lines represent medians. Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell numbers per well. n=5-8.

Figure 5.10 and **Figure 5.11** illustrate total numbers of iNK cells and CD56^{bright} NK cells throughout HSC cultures with or without TCR-stimulated CB Treg cells. TCR-stimulated CB Treg cells, when added at day 9, 16 and 23 of HSC cultures, decreased iNK cell numbers, with the strongest effect observed when added at day 9 (**Figure 5.10; red line**). The addition of TCR-stimulated CB Treg cells at this particular time point also led to a reduction in iNK cell numbers in the following days of HSC culture. Cell numbers were then constant for the rest of the HSC differentiation after day 9.

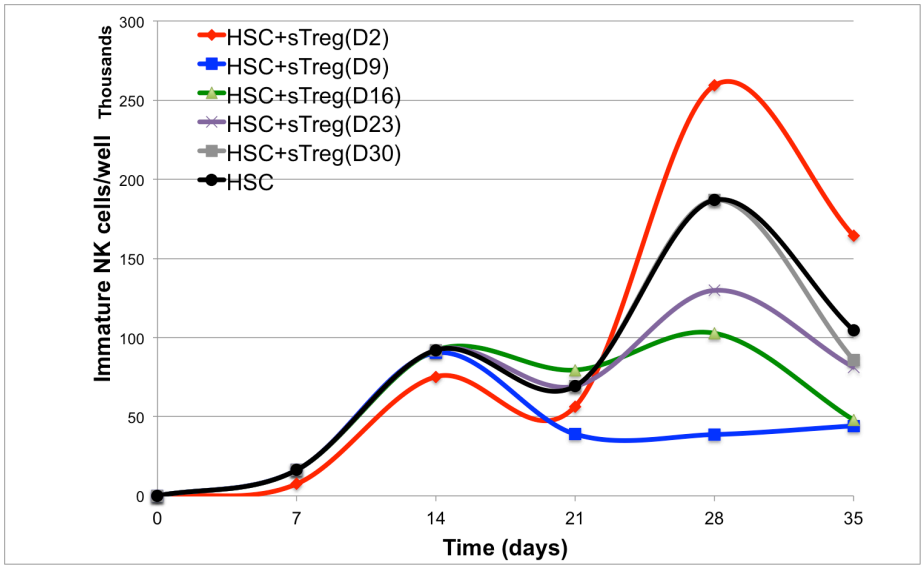


Figure 5.10: Total numbers of iNK cells in HSC cultures in the presence or absence of TCR-stimulated CB Treg cells. HSC were cultured in the presence or absence of allogeneic TCR-stimulated CB Treg cells at different time points at a ratio of 1:4 (Treg cells:HSC), except for day 2 where the ratio was 1:1. iNK cells were gated as $CD4^{-}CD34^{-}CD117^{+}CD94^{-}$ cells. Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell numbers per well. The values represent medians. n=5-8.

Likewise, total numbers of $CD56^{bright}$ NK cells were also reduced when TCR-stimulated CB Treg cells were added at day 9, 16 and 23 of HSC cultures, with the strongest effect observed when TCR-stimulated CB Treg cells were added at day 9 (**Figure 5.11**).

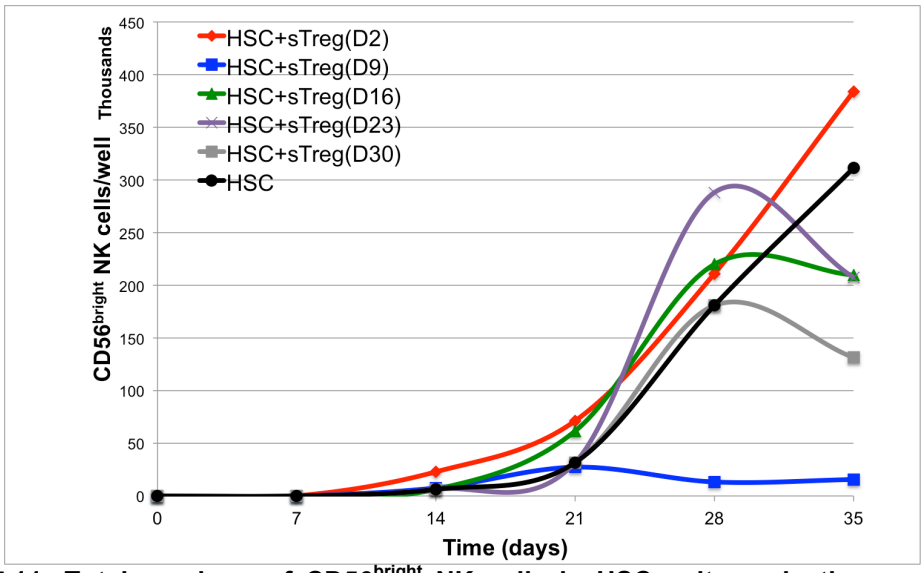


Figure 5.11: Total numbers of $CD56^{bright}$ NK cells in HSC cultures in the presence or absence of TCR-stimulated CB Treg cells. HSC were cultured in the presence or absence of allogeneic TCR-stimulated CB Treg cells at different time points at a ratio of 1:4 (Treg cells: HSC), except for day 2 where the ratio was 1:1. $CD56^{bright}$ NK cells were gated as $CD3^{-}CD34^{-}CD117^{+/-}CD94^{+}$ cells. Total $CD56^{bright}$ cell numbers were calculated from NK cell frequencies determined by flow cytometry and total cell numbers per well. The values represent medians. n=5-8.

To determine why TCR-stimulated CB Treg cells had the strongest impact when added at day 9 of HSC culture, the frequency of expression of each of the four stages of NK cell differentiation was measured. Between days 7 and 14, low frequencies of iNK cells and CD56^{bright} NK cells were detected. It is therefore likely that the subsets of cells present at the time when TCR-stimulated CB Treg cells were added were the only cells detected at the end of HSC cultures. Collectively, these results suggest that TCR-stimulated CB Treg cells have a negative impact on NK cell differentiation, with the strongest effect observed on HSC commitment to the NK cell lineage.

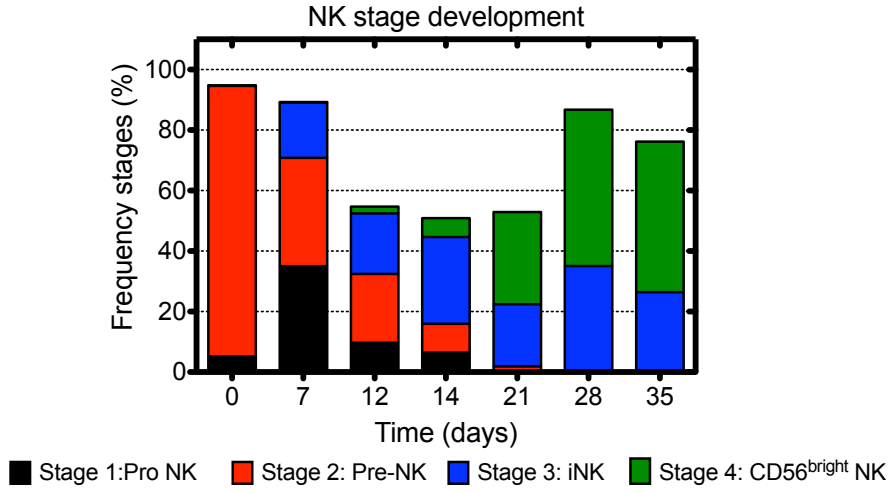


Figure 5.12: Frequency of cells in each NK cell differentiation stage in HSC cultures. HSC were differentiated for 35 days and frequencies of Stage 1-4 NK cells were assessed based on CD4, CD34, CD117 and CD94 expression at days 0, 7, 14, 21, 28 and 35. The values represent medians. n=11

5.4.2 Expression of the natural killer cell maturation markers CD94 and CD16

Based on the findings of Cooper and colleagues showing that PB CD56^{bright} NK cells are CD94^{+}CD16$^{+/-}$ and PB CD56^{dim} NK cells are CD94$^{+/-}$CD16^{+}, and that CD56^{dim} NK cells derive from CD56^{bright} NK cells (Cooper et al., 2001a), Freud and Caligiuri reported a fifth stage of NK cell differentiation based on the expression of CD94 and CD16 by CD56^{+} NK cells (Freud and Caligiuri, 2006). Thus, it was investigated whether TCR-stimulated CB Treg cells could also impair these intermediate stages of NK cell differentiation.

To evaluate this, allogeneic resting or TCR-stimulated CB Treg cells were added at different time points of NK cell differentiation. Total cell counts for the three different intermediate stages defined as $CD56^{+}CD94^{-}CD16^{-}$, $CD56^{+}CD94^{+}CD16^{-}$ and $CD56^{+}CD94^{+}CD16^{+}$ were measured at each time point. The latter representing the most mature NK cells. **Figure 5.13** shows the gating strategy used to identify these three populations.

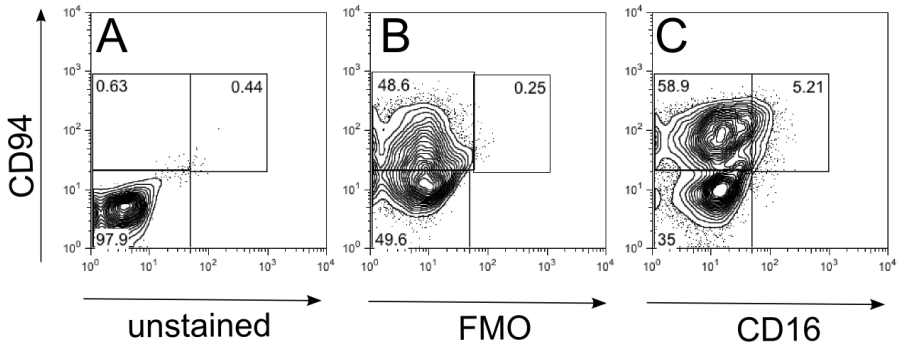


Figure 5.13: Expression of CD94 and CD16 on $CD56^{+}$ NK cells at day 35 of HSC cultures. Expression of CD94 and CD16 on HSC-derived $CD56^{+}$ NK cells at day 35 of differentiation (HSC alone). (A) Unstained cells. (B) Fluorescence minus one control for CD16. (C) Identification of three defined intermediate stages of $CD56^{+}$ NK cells based on the expression of CD94 and CD16 markers. Data is a representative sample of nine independent experiments (HSC).

It was observed that all three intermediate stages of NK cell differentiation were affected by the addition of TCR-stimulated CB Treg cells (**Figure 5.14B**) whereas no effect was observed when resting CB Treg cells were added at any indicated time point during HSC cultures (**Figure 5.14A**) as compared to control cultures. Importantly, an inhibitory effect on all intermediate stages was noted when TCR-stimulated CB Treg cells were added at day 9 of HSC cultures (**Figure 5.14B**). $CD56^{+}CD94^{-}CD16^{-}$ cell numbers were reduced by 75% ($p=0.01$), whereas $CD56^{+}CD94^{+}CD16^{-}$ cells ($p=0.0008$) and $CD56^{+}CD94^{+}CD16^{+}$ cells ($p=0.004$) were reduced by 90% and 95% respectively.

A reduction in the number of cells in these intermediate stages of NK cell differentiation was also observed when TCR-stimulated CB Treg cells were added at day 16 with a decrease in $CD56^{+}CD94^{-}CD16^{-}$ cell numbers of 75% ($p=0.01$) and in $CD56^{+}CD94^{+}CD16^{-}$ cell numbers of 50% ($p=0.0008$), whereas

when TCR-stimulated CB Treg cells were added at day 23 a decrease in $CD56^+CD94^+CD16^-$ cell numbers of 40% was observed (p=0.003).

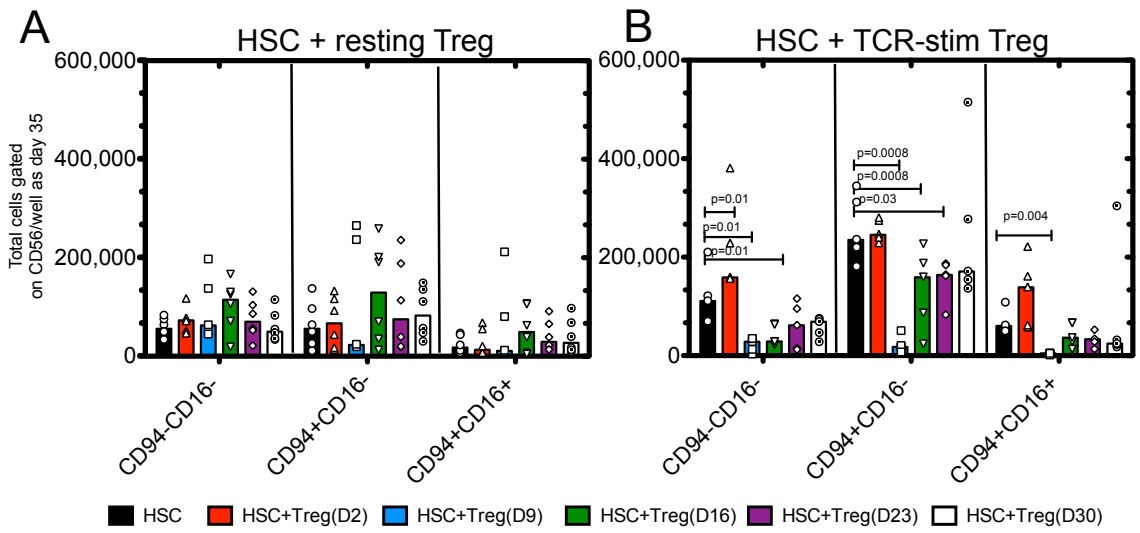


Figure 5.14: Total cell numbers of NK cells in the intermediate stages of NK cell maturation from HSC cultures. HSC were cultured in the presence or absence of allogeneic resting (A) or TCR-stimulated CB Treg cells (B) added at day 2, 9, 16, 23, and 30 of differentiation at a ratio of 1:4 (Treg cells:HSC). Flow cytometric analysis of three NK cell populations based on CD94 and CD16 expression gated on $CD56^+$ cells at day 35 of NK cell differentiation. Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell numbers per well. n=4-6.

5.4.3 Expression of myeloid markers

NK cell differentiation can be impaired *in vitro* by the addition of TCR-stimulated CB Treg cells at specific time points. A recent study reported that TGF-β can skew HSC differentiation towards cells of the myeloid lineage rather than the lymphoid lineage (Challen et al., 2010). Since TGF-β is one of the mechanisms by which Treg cells can inhibit NK cell effector functions (Ghiringhelli et al., 2005, Smyth et al., 2006, Zhou et al., 2010), it was determined whether CB Treg cells skewed HSC differentiation towards cells of the myeloid lineage rather than NK cells in this model.

To evaluate this, the expression of CD33 was analysed. CD33 is a marker expressed by myeloid progenitor cells and is reported to be a specific marker of the myeloid lineage (Freeman et al., 1995). Neither resting nor TCR-stimulated CB Treg cells induced an increase in the frequency of $CD33^+$ cells in the HSC cultures at any of the time points analysed (**Figure 5.15A-B**). However, CD33 MFIs were reduced when resting Treg cells were added at day 9 of culture

(p=0.03) (**Figure 5.15C-D**). These results suggest that Treg cells, regardless of their state of activation, do not favour HSC differentiation towards cells of the myeloid lineage in this system.

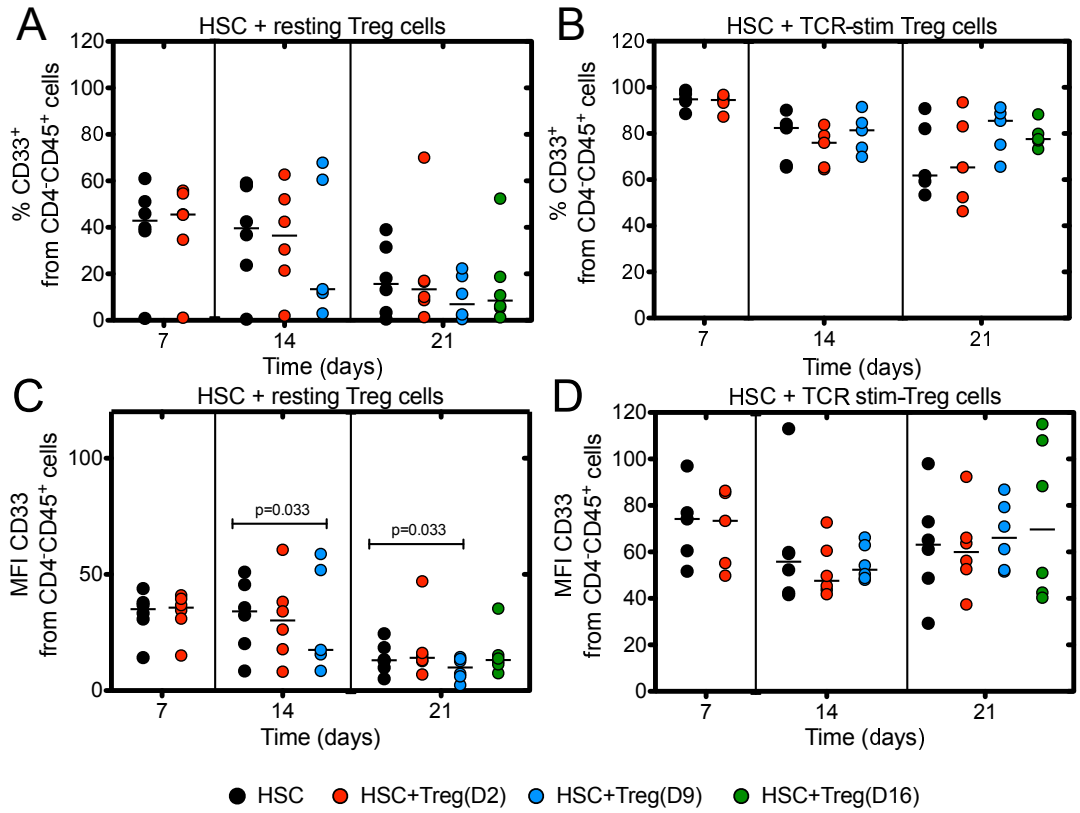


Figure 5.15: Analysis of CD33 expression in HSC cultures. Frequency of $CD33^+$ cells gated from $CD4^-CD45^+$ cells in HSC cultures in the presence or absence of allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B) added at days 2, 9 and 16 of HSC cultures. MFI of CD33 expression (gated on $CD4^-CD45^+$ cells) in the presence or absence of resting CB Treg cells (C) or TCR-stimulated CB Treg cells (D) added at day 2, 9 and 16 of HSC cultures. The values are represented as medians. n=5-8.

5.5 Phenotypic analysis of differentiated NK cells in the presence of regulatory T cells

5.5.1 Expression of activating and inhibitory receptors

The integration of signals provided by activating or inhibitory receptors upon the detection of infected cells or tumours determines NK cell function (Pegram et al., 2011). It has been shown that upregulation of NKG2D on NK cells by Tcon cells can be abrogated by the addition of Treg cells (Bergmann et al., 2011) and

that Treg cells can directly downregulate NKG2D on NK cells (Ghiringhelli et al., 2005). However, it is unknown whether Treg cells can impact on the repertoire of activating and inhibitory receptors expressed by NK cells during differentiation. To evaluate this, the expression of activating receptors such as CD16, DNAM-1, NKG2D, NKp30, NKp46 and 2B4 on NK cells was evaluated at day 35 of HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. These receptors were analysed because they are important for the killing capacity of NK cells (Lanier et al., 1988, Bottino et al., 2000, Sivori et al., 2000, Ferlazzo et al., 2002, Raulet, 2003, Byrd et al., 2007, Gilfillan et al., 2008).

Addition of resting CB Treg cells had no effect on the expression of NKp30, NKG2D, 2B4, or NKp46 by differentiated NK cells when added at any time point during HSC culture (**Figure 5.16A**). However, a significant decrease was observed in the frequency of CD16-expressing NK cells when resting CB Treg cells were added at day 9 of HSC cultures (4% to 2.5%; p=0.044). Moreover, DNAM-1 expression was reduced in frequency on differentiated NK cells when co-cultured with CB resting Treg cells added at day 23 (90% to 70%; p=0.007) and day 30 of HSC culture (90% to 70%; p=0.01). Surprisingly, no difference was observed in the expression of any of the activating receptors studied on the differentiated NK cells when TCR-stimulated CB Treg cells were added to HSC cultures (**Figure 5.16B),** except for DNAM-1, which was upregulated when co-cultured with TCR-stimulated Treg cells added at day 2 of HSC culture.

MFI levels for all receptors were also studied on differentiated NK cells in control HSC cultures and HSC cultures with resting CB Treg cells (**Figure 5.17A**) or TCR-stimulated CB Treg cells (**Figure 5.17B**). Notably, NKp30 MFI on differentiated NK cells was reduced from 150 a.u. to 100 a.u when co-cultured with resting Treg cells added at day 9, 16, 23 and 30 ($p<0.005$, respectively). DNAM-1 MFI was also reduced in NK cells when co-cultured with resting Treg cells added at day 23 (p=0.005) and 30 (p=0.01). However, increased MFI was detected in NKG2D-expressing NK cells when co-cultured with resting Treg cells added at day 30 (p=0.002). When TCR-stimulated Treg cells were added at day 2 of HSC cultures, differentiated NK cells showed an

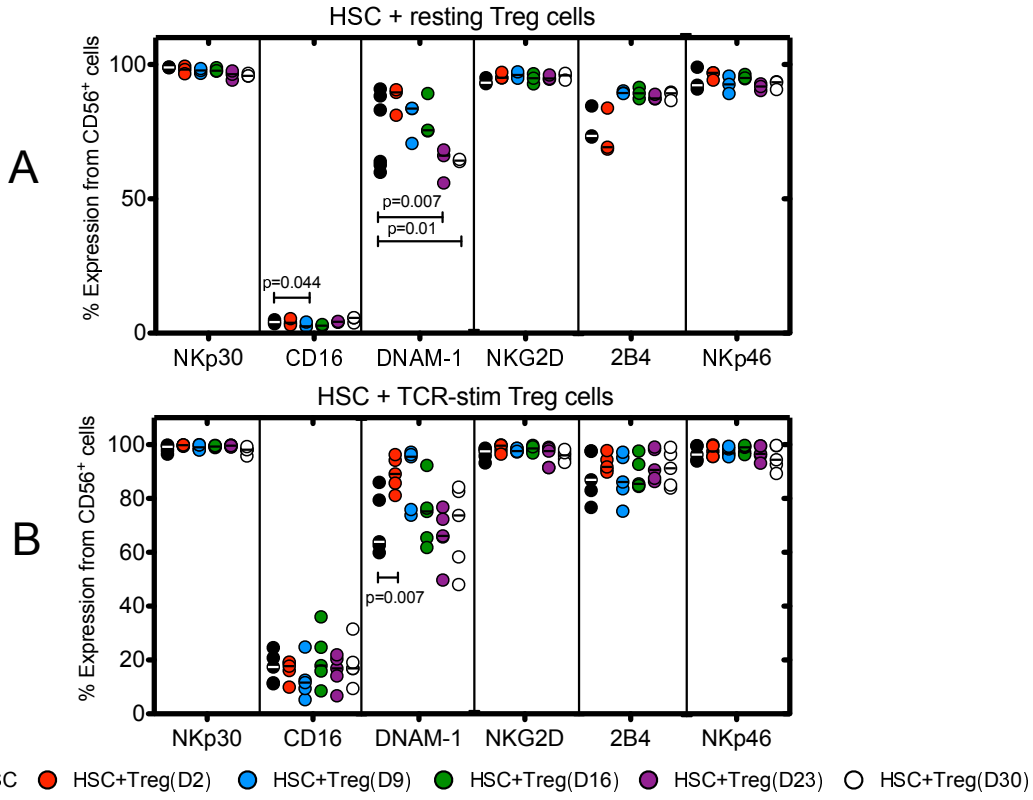


Figure 5.16: Expression of NK cell activating receptors on NK cells at day 35 of HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. Flow cytometric analysis of NKp30, CD16, DNAM-1, NKG2D, 2B4 and NKp46 expression on NK cells at day 35 of culture. (A) Controls (HSC) and Treg cell/HSC co-cultures with allogeneic resting CB Treg cells. (B) Controls and Treg cell/HSC co-cultures with allogeneic TCR-stimulated CB Treg cells. CB Treg cells were added at a ratio of 1:4 (Treg cells:HSC) at different time points of HSC cultures except for day 2 where the ratio was 1:1. The lines are represented as medians. n=3-5.

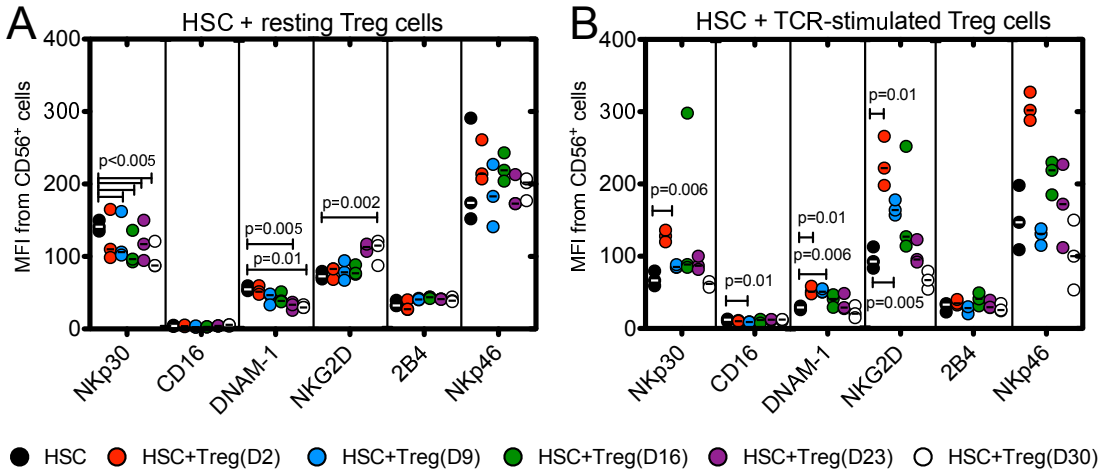


Figure 5.17: MFI of NK cell activating receptors on differentiated NK cells in the presence or absence of resting or TCR-stimulated CB Treg cells. Flow cytometric analysis of NKp30, CD16, DNAM-1, NKG2D, 2B4, and NKp46 MFI for NK cells at day 35 of NK cell differentiation. (A) Controls (HSC) and Treg cell/HSC co-cultures with allogeneic resting CB Treg cells. (B) Controls and Treg cell/HSC co-cultures with allogeneic TCR-stimulated CB Treg cells. CB Treg cells were added at a ratio of 1:4 (Treg cells:HSC) at different time points, except for day 2 where the ratio was 1:1. The lines are represented as medians. n=3.

increased in MFIs in NKp30 (p=0.006), DNAM-1 (p=0.01) and NKG2D (p=0.01). Likewise, CD16 (p=0.01), DNAM-1 (p=0.006) and NKG2D (p=0.005) were increased when TCR-stimulated Treg cells were added at day 9.

5.5.2 Expression of homing and chemokine receptors

NK cell differentiation can take place in the BM, thymus, liver, SLT and spleen (Huntington et al., 2007), all of which are sites of potential interaction with Treg cells. When differentiated, NK cells can migrate to SLT and inflamed peripheral tissues by upregulating expression of α4β7, CCR7, CXCR1 and CXCR3 (Erle et al., 1994, Morohashi et al., 1995, Uksila et al., 1997, Campbell et al., 2001) and downregulating CXCR4, a homing receptor for the BM (Bernardini et al., 2008). As TGF-β has been shown to upregulate CXCR4 and CXCR3 expression on resting NK cells (Castriconi et al., 2013), it is plausible that CB Treg cells can also modulate the expression of homing and chemokine receptors on differentiated NK cells from HSC cultures, TGF-β being key in Treg cell-mediated suppression of mature PB NK cells (Ghiringhelli et al., 2005).

In order to explore this, expression of α4β7, CCR7, LFA-1 and CXCR4 was analysed by flow cytometry on NK cells in HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. Expression of all homing receptors studied was not affected by the presence of resting CB Treg cells, except for CXCR4, for which a reduction in expression was observed at day 23 of culture (p=0.023; 5% reduction in total $CXCR4^+$ NK cells) (**Figure 5.18A).**

TCR-stimulated CB Treg cells had an impact on the expression of integrin β7, LFA-1, CXCR4 and CCR7 on NK cells, particularly when TCR-stimulated CB Treg cells were added at day 9 of HSC cultures (**Figure 5.18B**), with increases in expression of integrin β7 and LFA-1 by 10% (p=0.055) and 26% (p=0.045) respectively, and a decrease in CXCR4 expression of 12% (p=0.01). Moreover, when TCR-stimulated CB Treg cells were added at later time points of HSC culture, an impact on CCR7 and LFA-1 expression was detected. CCR7-expressing NK cells were reduced by ~15% at days 16 (p=0.02) and days 30

(p=0.007) whereas LFA-1 was increased by 10% at days 16, 23 and 30 (p=0.03, respectively).

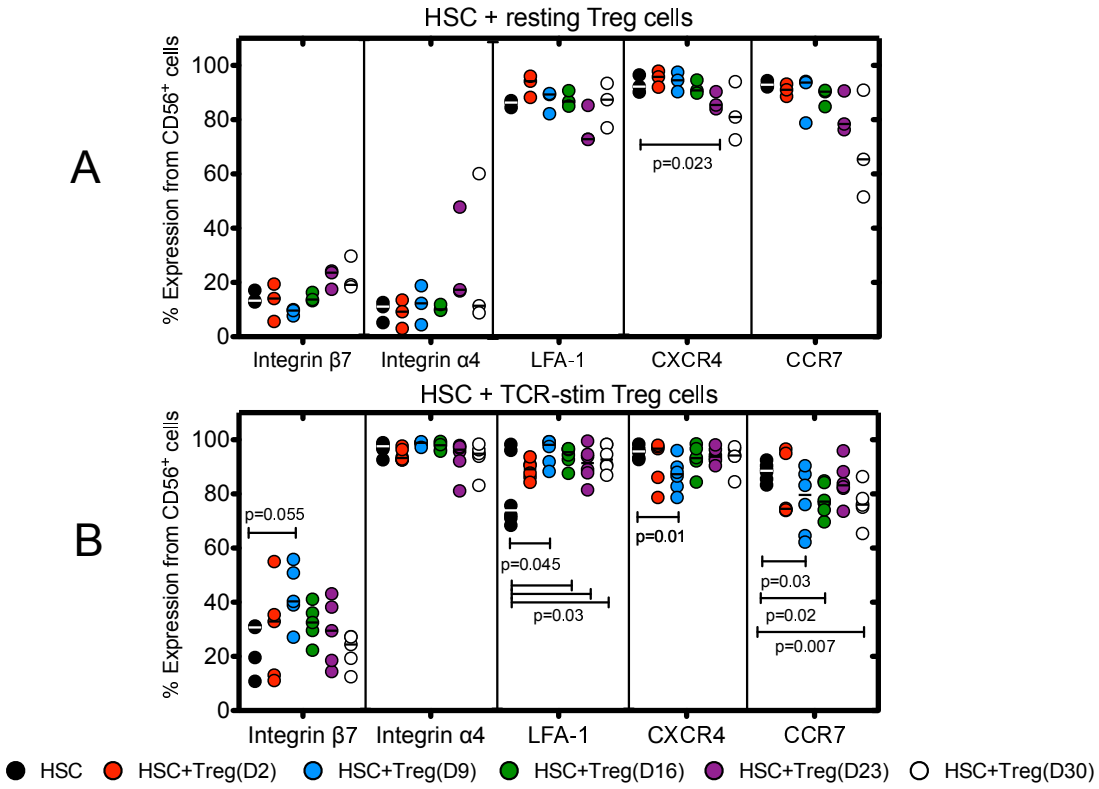


Figure 5.18: Expression of homing and chemokine receptors at day 35 of HSC cultures in the presence or absence of resting or TCR-stimulated CB Treg cells. Flow cytometric analysis of integrins α4 and β7, LFA-1, CXCR4, and CCR7 expression by differentiated NK cells at day 35 of culture. (A) Controls (HSC) and co-cultures with allogeneic resting CB Treg cells. (B) Controls and co-cultures with allogeneic TCR-stimulated CB Treg cells. CB Treg cells were added at a ratio of 1:4 (Treg cells:HSC) at different time points, except when added at day 2 where the ratio was 1:1. The lines are represented as medians. n=5-8.

Regarding MFI levels of the receptors previously studied, no difference was observed between HSC cultures regardless of whether resting or TCR-stimulated CB Treg cells were added or not (**Figure 5.19A-B**). Collectively, these results suggest that CB Treg cells can modulate the trafficking repertoire of differentiated NK cells.

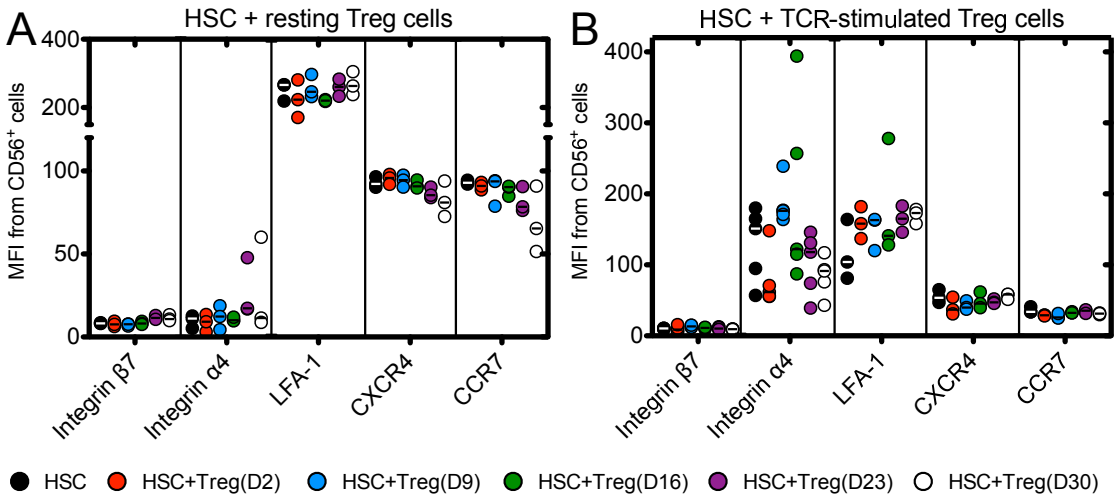


Figure 5.19: MFI of homing receptors on NK cells at day 35 of culture in the presence or absence of resting or TCR-stimulated CB Treg cells. Integrins α4 and β7, LFA-1, CXCR4, and CCR7 MFI on differentiated NK cells at day 35 of culture. (A) Controls (HSC) and co-cultures with allogeneic resting CB Treg cells. (B) Controls and co-cultures with allogeneic TCR-stimulated CB Treg cells. Treg cells were added at a ratio of 1:4 (Treg cells:HSC) at different time points, except for day 2 where the ratio was 1:1. The lines are represented as medians. n=5-8.

5.6 Effect of regulatory T cells on natural killer cell function

Next, the functional capacities of those NK cells that acquire a NK cell phenotype were analysed in the presence of resting or TCR-stimulated CB Treg cells added at key time points of HSC cultures.

5.6.1 Interferon-γ secretion

It has been reported that PB Treg cells can reduce IFN-γ production by NK cells in the presence of IL-12 but not IL-2 or IL-15 (Ghiringhelli et al., 2005). As IFN-γ plays a key role in NK cell function, the ability of resting or TCR-stimulated CB Treg cells to impair IFN-γ production by the differentiated NK cells was determined. To assess this, IFN-γ secretion by the differentiated NK cells was measured in response to different stimuli: K562 cells at a 1:1 ratio (K562 cells:NK cells) and PMA/ION as a positive control. Here, it was found that CB Treg cells, regardless of the time of addition to the cultures and state of stimulation, did not impair IFN-γ secretion by differentiated NK cells (**Figure 5.20A-B**). Under non-stimulated conditions and with K562 stimulation,

differentiated NK cells secreted between 0 and 450 pg/ml of IFN-γ respectively in all conditions tested. Moreover, when differentiated NK cells were stimulated with PMA/ION, IFN-γ levels ranged between 2 500 and 10 000 pg/ml in all conditions tested (**Figure 5.20A-B**). Hence, these results suggest that CB Treg cells do not impact on cytokine production by differentiated NK cells.

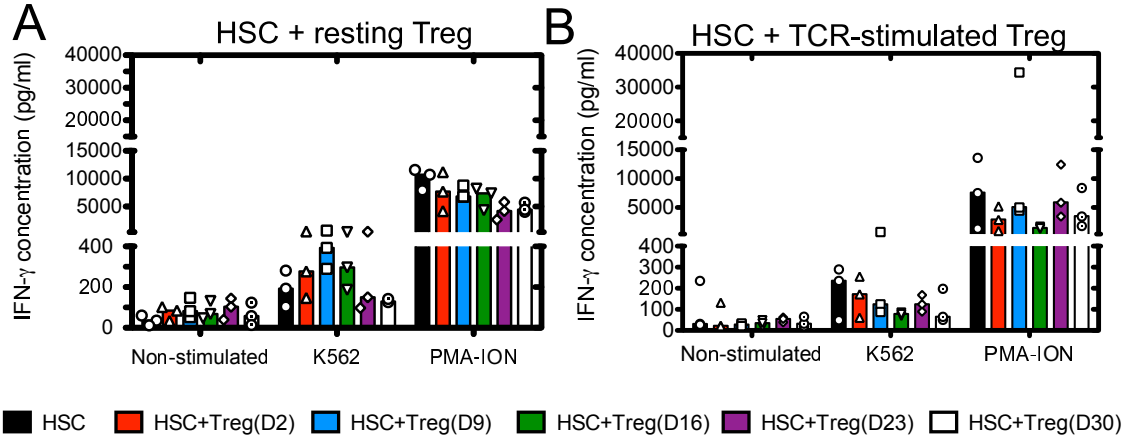


Figure 5.20: IFN-γ secretion by differentiated NK cells at day 35 of HSC cultures. HSC were cultured with allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B) added at days 2, 9, 16, 23 or 30 of HSC cultures. NK cells from controls (HSC) and Treg cell/HSC co-cultures were stimulated or not with K562 cells at a ratio of 1:1 or PMA/ION for 2 h. Supernatants were collected and analysed by ELISA. Results are represented as means of duplicate wells. The values shown in the graphs represent medians. n=3-6.

5.6.2 Natural killer cell cytotoxicity

The results presented in Chapter 4 showed that autologous and allogeneic TCR-stimulated CB Treg cells could inhibit the killing capacity of activated CB NK cells in the absence of cytokines. Therefore, it is plausible that TCR-stimulated CB Treg cells could also affect the killing capacity of differentiated NK cells. To evaluate this, allogeneic resting or TCR-stimulated CB Treg cells were added at key time points during HSC cultures and the impact of CB Treg cells on differentiated NK cells was measured by the lysis of K562 using chromium release assays.

These results showed that CB Treg cells, regardless of their activation state and time of addition during HSC cultures, did not impair the killing capacity of differentiated NK cells (**Figure 5.21**). Overall, it was demonstrated that the

functions of the cells that acquire a mature NK cell phenotype are not affected by the presence of CB Treg cells.

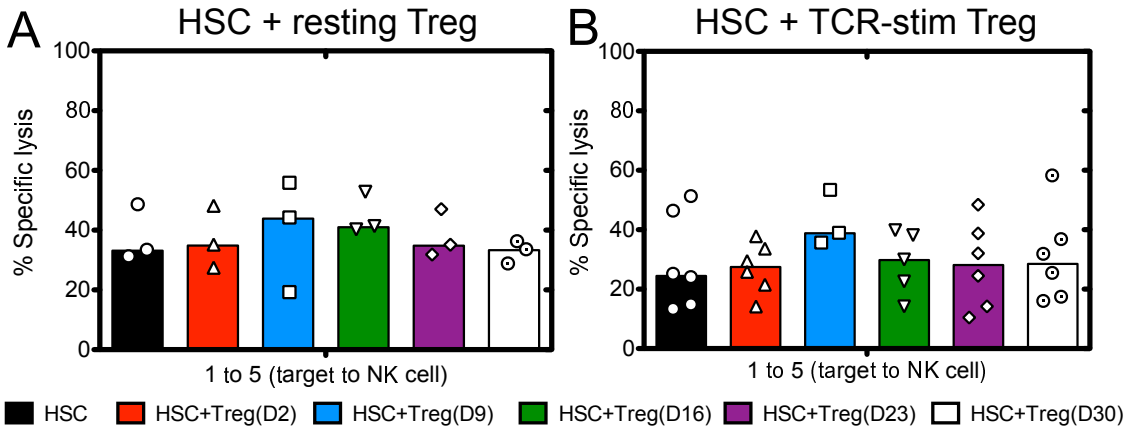


Figure 5.21: Cytolytic activity of differentiated NK cells at day 35 of HSC cultures. HSC were cultured with allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B) added at days 2, 9, 16, 23 or 30 of HSC cultures. Cytotoxicity of NK cells alone or in co-culture was measured at day 35 of NK cell differentiation against K562 targets at a E:T ratio of 5:1. Results of the chromium release assay are represented as means of triplicate wells. Percentage of specific lysis was determined by the following equation: % lysis= [(experimental release - spontaneous release)/(maximum release - spontaneous release)]*100. The values represent medians. n=3-6.

5.6.3 Degranulation assay

The expression of CD107a (LAMP-1) by NK cells correlates with levels of degranulation (Alter et al., 2004). In Chapter 4, a trend towards reduction in expression of CD107a on activated NK cells co-cultured with allogeneic TCR-stimulated CB Treg cells in the absence of cytokines was observed. Therefore, it was next investigated whether the expression of CD107a on differentiated NK cells could also be affected by the presence of CB Treg cells. To study this, allogeneic resting or TCR-stimulated CB Treg cells were added to HSC cultures at key time points during HSC cultures and CD107a expression on differentiated NK cells was analysed when unstimulated or after stimulation with K562 cells or PMA/ION.

The degranulation of differentiated NK cells was similar to the results observed with chromium release assays; however statistical significance was detected in some conditions. When non-stimulated, less than 10% of differentiated NK cells degranulated (**Figure 5.22**). The results were similar for differentiated NK cells

from HSC cultures with or without resting or TCR-stimulated CB Treg cells. When differentiated NK cells were stimulated with K562 cells, 20-40% of NK cells were CD107a-positive for all conditions tested (**Figure 5.22A-B**). An increased of 5% of CD107a expression was particularly observed when resting and TCR-stimulated Treg cells were added at day 9 (p=0.02 and p=0.01, respectively) and day 16 (p=0.01 and p=0.02, respectively) of HSC cultures. After PMA/ION stimulation, ~60% of NK cells from HSC cultures with resting CB Treg cells degranulated (**Figure 5.22A**), whereas 20-50% of NK cells from HSC cultures with TCR-stimulated CB Treg cells degranulated (**Figure 5.22B**). Upregulation of CD107a was detected when in HSC were cultured with TCR-stimulated Treg cells added at days 2 (p=0.003), 9 (p=0.008) and 16 (p=0.01). Collectively, these results suggest that NK cells that differentiated in the cultures where CB Treg cells were present were functional and even showed slightly enhanced cytotoxicity. However, statistical significance was not observed in chromium release assays.

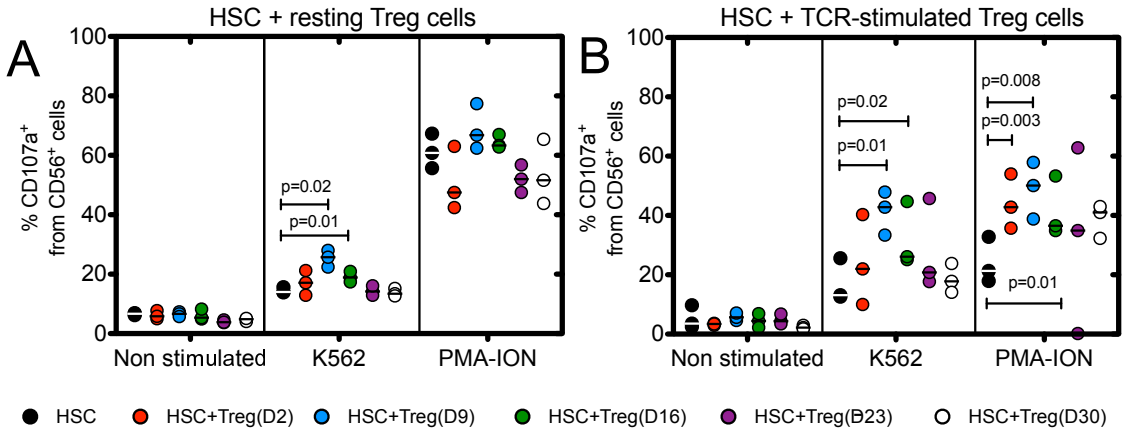


Figure 5.22: CD107a expression on differentiated NK cells in the presence or absence of resting or TCR-stimulated CB Treg cells. HSC were cultured with allogeneic resting CB Treg cells or TCR-stimulated CB Treg cells added at days 2, 9, 16, 23 or 30 of HSC culture. At day 35 of HSC cultures, differentiated NK cells were incubated or not with K562 cells (1:1 ratio) or PMA/ION for 2 h. (A-B) Percentage of $CD56^+$ NK cells expressing CD107a under different stimuli. (C-D) MFI of CD107a expressing $CD56^+$ NK cells. The lines represent medians. n=3.

5.7 Gene expression analysis of natural killer cell differentiation in the presence of regulatory T cells

Marcoe and colleagues demonstrated the impact of TGF-β on NK cell differentiation in mice by inhibiting TFs such as Gata-3 and T-bet, that are crucial for NK cell maturation (Marcoe et al., 2012). Therefore, it is possible that CB Treg cells also induce the downregulation of these TFs in NK cell differentiation. For this, mRNA expression of genes involved in NK cell differentiation (E4bp4, Id2, Pu.1 and Tox), NK cell maturation (Helios, Irf-2, T-bet, Bcl11b, Eomes and Gata-3), and NK cell function (IFN-γ) (Martin-Fontecha et al., 2011, Luevano et al., 2012c, Male et al., 2012) were analysed.

At day 9 of HSC cultures, resting or TCR-stimulated Treg cells were added to the cultures at a ratio of 1:4 (Treg cell:HSC) and co-cultured until day 12 or day 35. Then, HSC were separated from Treg cells by cell sorting and then analysed by real time PCR. This protocol was used in the following three sections. Since no effect was observed on NK cell differentiation when HSC were co-cultured with resting Treg cells, they were used as a negative control for this study.

5.7.1 Transcription factors involved in natural killer cell differentiation

mRNA expression of E4bp4, Id.2, and Pu.1 were similar in HSC co-cultured with resting or TCR-stimulated CB Treg cells until day 12 or day 35 of culture (**Figure 5.23A-B**). However, at day 12, TCR-stimulated CB Treg cells induced higher mRNA expression of Tox by HSC than resting CB Treg cells ($p=0.02$), yet, this effect was no longer observed at day 35 of culture (**Figure 5.23B**), suggesting that the effect mediated by Treg cells occurs within the first three days of Treg cell/HSC co-cultures.

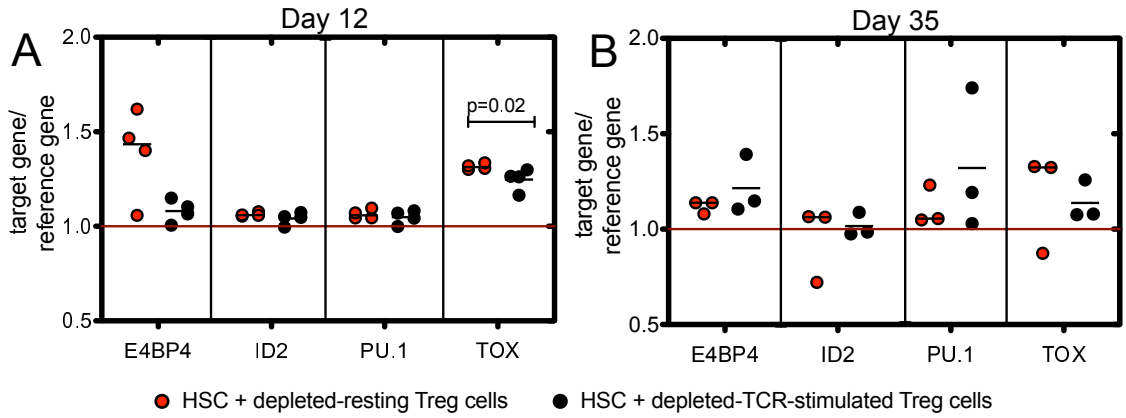


Figure 5.23: Expression of TFs involved in NK cell differentiation. Allogeneic resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures. HSC and Treg cells were separated by cell sorting at day 12 or day 35 of culture. Total RNA from Treg cell-depleted HSC was extracted and analysed by real time PCR. (A) mRNA expression levels of E4bp4, Id2, Pu.1 and Tox at day 12 of culture. (B) mRNA expression levels of E4bp4, Id2, Pu.1 and Tox at day 35 of culture. The lines represent medians. n=3-4.

5.7.2 Transcription factors involved in natural killer cell maturation

TCR-stimulated CB Treg cells increased mRNA expression levels of Helios (p=0.02), T-bet (p=0.02), Bcl11b (p=0.02), and Gata-3 (p=0.02) in HSC at day 12 of co-culture (**Figure 5.24A,C)** but not of Irf-2 or Eomes. The effect of TCR-stimulated CB Treg cells on the expression levels of Helios, Irf-2 and T-bet in HSC was not observed at day 35 of co-cultures (**Figure 5.24B)**. Bcl11b, Eomes, and Gata-3 mRNA expression levels in HSC could not be tested at day 35 of cultures due to the low amounts of total RNA obtained from these cultures.

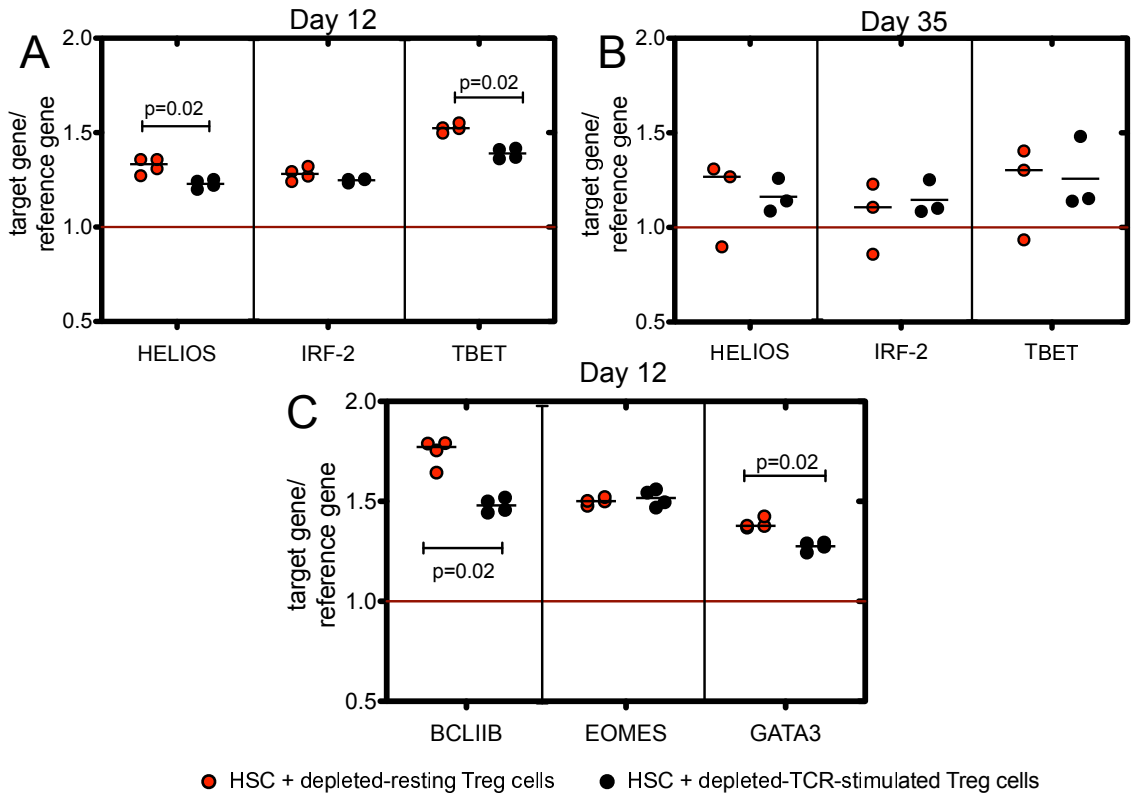


Figure 5.24: Expression of TFs involved in NK cell maturation. Allogeneic resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures. HSC and Treg cells were separated by cell sorting at day 12 or day 35 of culture. Total RNA from Treg cell-depleted HSC was extracted and analysed by real time PCR. (A, C) mRNA expression levels of Helios, Irf-2, Bcl11b, Eomes and Gata-3 at day 12 of culture. (B) mRNA expression levels of Helios, Irf-2 and T-bet at day 35 of culture. Bcl11b, Eomes and Gata-3 could not be tested at day 35 of culture due to lack of total RNA on the samples. The lines represent medians. n=3-4.

5.7.3 Natural killer cell function: analysis of IFN-γ mRNA expression

Even though IFN-γ production does not occur before NK cells differentiate into $CD56^{bright}$ cells (Freud et al., 2006), it is possible that IFN-γ gene transcription could be impaired at early stages in the presence of CB Treg cells. Interestingly, HSC co-cultured with TCR-simulated CB Treg cells until day 12 showed higher IFN-γ mRNA levels than HSC co-cultured with resting CB Treg cells (p=0.02) (**Figure 5.25**). IFN-γ expression could not be tested at day 35 (day 9-35 co-cultures) in HSC because of the low amount of total RNA obtained from these cultures.

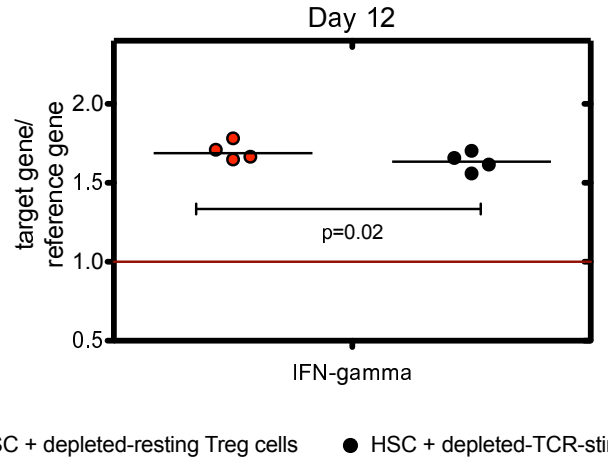


Figure 5.25: IFN-γ mRNA levels in HSC co-cultured with Treg cells. Allogeneic resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures. HSC and Treg cells were separated by cell sorting at day 12 of culture. Total RNA from HSC was extracted and analysed by real time PCR. The lines represent medians. n=4.

Altogether, no downregulation of any of the studied TFs involved in NK cell differentiation and maturation was detected for HSC cultured with TCR-stimulated CB Treg cells. These results provide evidence to suggest that HSC are not negatively affected at the molecular level when cultured with TCR-stimulated CB Treg cells.

5.8 Analysis of regulatory T cells on haematopoietic stem cell cultures

Several studies have suggested the ability of Treg cells to “reprogram” into pro-inflammatory cells, allowing cells to adapt to different conditions (Gao et al., 2012). For instance, Yang and colleagues demonstrated that a subset of tTreg cells could lose Foxp3 expression in the presence of IL-6 and TCR-stimulation and acquire a Th17 phenotype (Yang et al., 2008). Hence, it is unknown if the conditions used in this study for NK cell differentiation could favour Treg cells to change their phenotype. To investigate this, the expression of Foxp3 and LAP in CB Treg cells was analysed by flow cytometry and the mRNA expression levels of TFs that have been described as master regulators of $CD4^+$ T cell subsets such as T-bet (Th1 cells), Gata-3 (Th2 cells), Rorc (Th17 cells), and Foxp3 (Treg cells) were assessed by real time PCR.

5.8.1 Regulatory T cell phenotype

Treg cells were identified at the end of HSC/Treg cell co-cultures by the expression of Foxp3 and the TGF-β precursor protein LAP (Khalil, 1999). Resting or TCR-stimulated CB Treg cells were added at key time points of HSC cultures. Foxp3 and LAP expression by $CD4^+$ T cells was analysed by flow cytometry at day 35 of HSC cultures.

When resting CB Treg cells were added at any key time point during HSC cultures, Foxp3 MFI levels ranged between 25 and 45 a.u. (**Figure 5.26A**). In contrast, TCR-stimulated CB Treg cells maintained higher MFI levels for Foxp3 of ~100 a.u. (**Figure 5.26B**). This can be clearly observed in the histogram plots shown in **Figure 5.26C**. LAP expression was not detected in CB Treg cells for any of the culture conditions tested (**Figure 5.26C**). This could be due to the fast downregulation of LAP on Treg cells upon TCR-stimulation, which results in the availability of TGF-β.

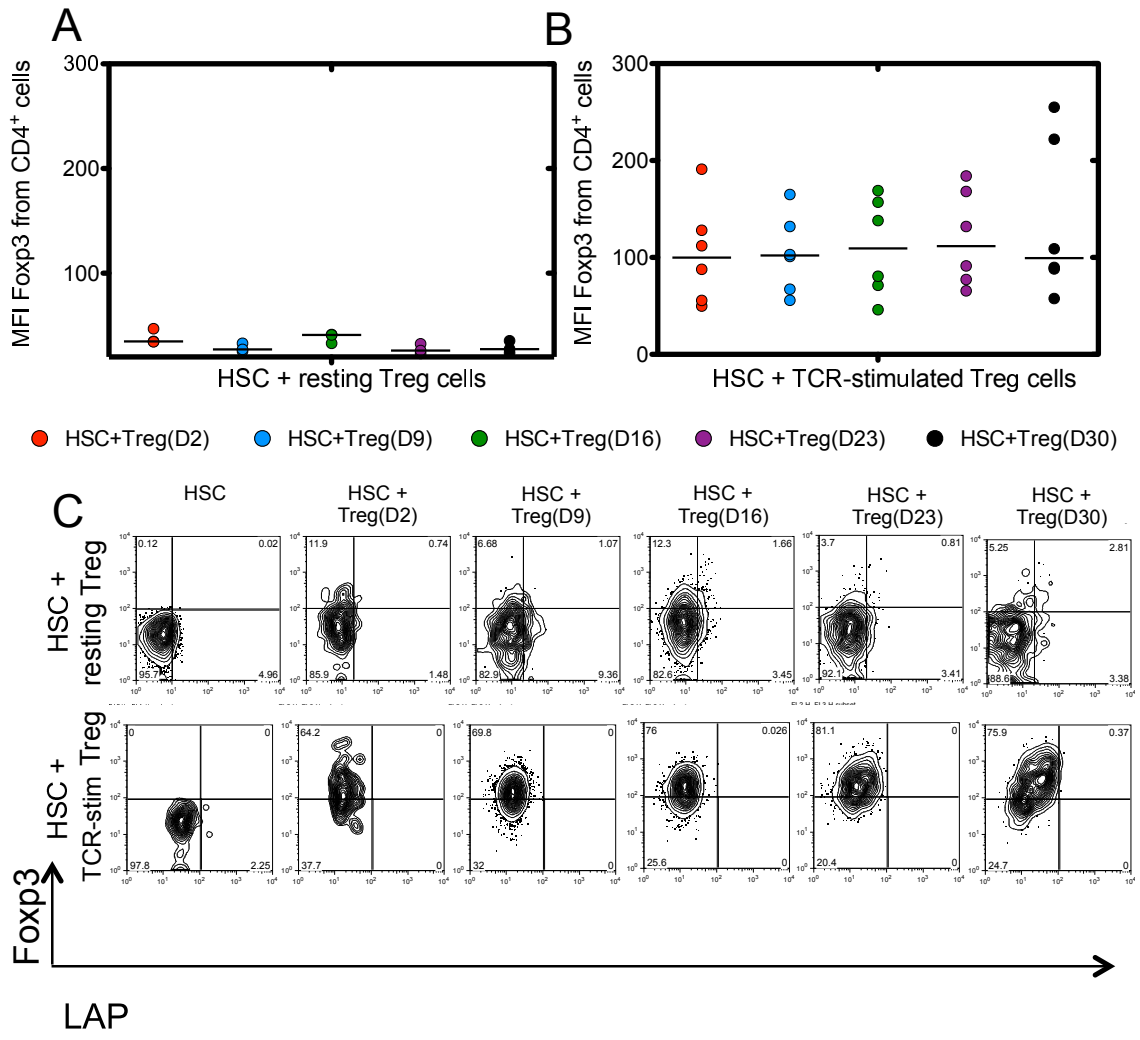


Figure 5.26: Phenotypic analysis of CB Treg cells in Treg cell/HSC co-cultures. Flow cytometric analysis of Foxp3 gated on $CD4^+$ cells. Resting CB Treg cells (A) or TCR-stimulated CB Treg cells (plate bound anti-CD3/soluble anti-CD28) (B) were added at key time points of HSC cultures. $CD56^+$ cells were used as negative controls for Foxp3 expression. (C) Representative flow cytometric analysis of Foxp3 and LAP expression on controls and in co-cultures (HSC+ resting or TCR-stimulated CB Treg cells). The lines represent medians. n=3-6.

5.8.2 Gene expression analysis of regulatory T cells in haematopoietic stem cell cultures

To evaluate whether CB Treg cells change their phenotype after being added to HSC cultures, the mRNA expression of Foxp3 and TFs that are characteristic of other $CD4^+$ T cell subsets, was determined. These included T-bet for Th1 cells, Gata-3 for Th2 cells and Rorc for Th17 cells. Resting or TCR-stimulated CB Treg cells were analysed before or after being added at day 9 of HSC cultures. To eliminate effects on Treg cells potentially induced by cytokines used in the HSC cultures (i.e. IL-3, IL-7, IL-15, Flt3 and SCF) or factors released by the

EL08.1D2 feeder layer cells, CB Treg cells were re-isolated from HSC by cell sorting three days later (D12 of cultures), before RNA extraction and analysis by real time PCR.

Interestingly, Foxp3 mRNA levels were significantly downregulated in resting CB Treg cells added for three days to HSC cultures when compared to resting CB Treg cells (p=0.028) before addition to HSC cultures. Likewise, Gata-3 (p=0.028) and Rorc (p=0.028) were also downregulated in resting CB Treg cells after being added to HSC cultures for three days (**Figure 5.27**). In contrast, T-bet and IFN-γ expression which was similar before and after being added to HSC cultures.

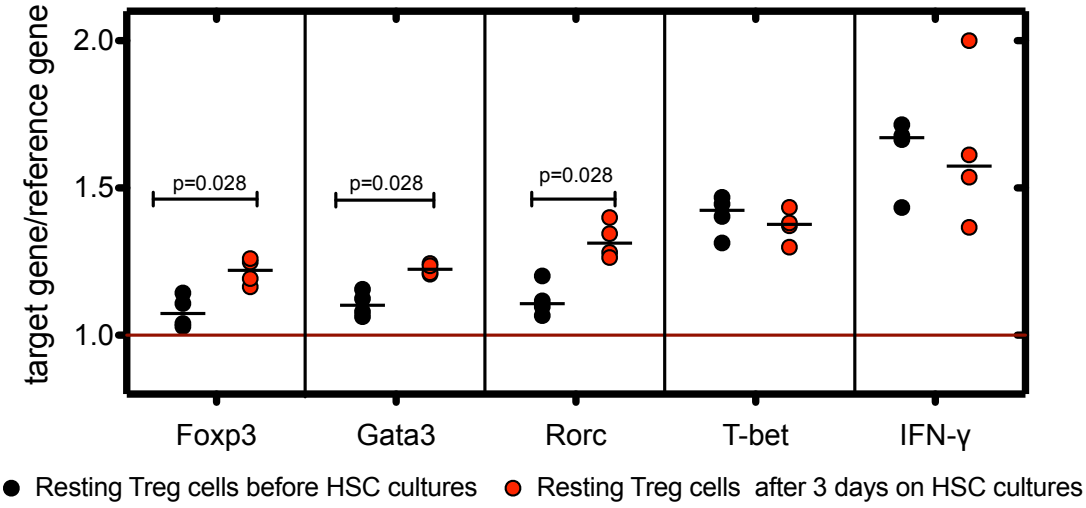


Figure 5.27: Gene expression of signature TFs of CD4 T helper subsets on resting CB Treg cells. HSC were cultured in the presence or absence of resting CB Treg cells at day 9 of differentiation and then $CD4^+$ cells were separated by cell sorting at day 12 for analysis by real time PCR. TFs Foxp3, Gata-3, Rorc, T-bet, and IFN-γ were assessed. Freshly isolated CB Treg cells (black) were used as controls. The lines represent medians. n=4.

As for TCR-stimulated CB Treg cells, only Foxp3 mRNA levels were downregulated three days after addition to HSC cultures (p=0.015). However, no difference was observed in the expression of Gata-3, Rorc, T-bet or IFN-γ between TCR-stimulated CB Treg cells before or after being added to HSC cultures (**Figure 5.28**). Therefore, CB Treg cells do not change their phenotype after three days of culture under HSC culture conditions.

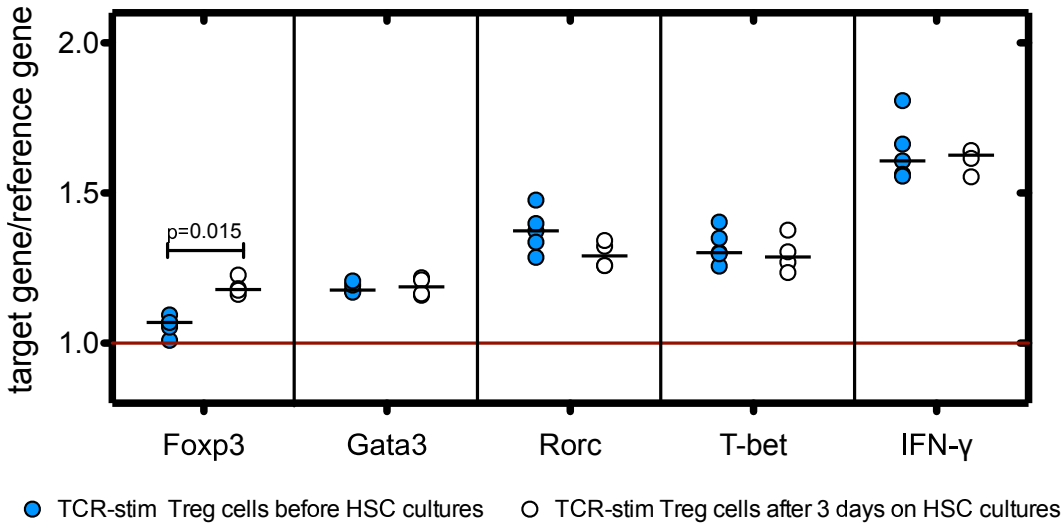


Figure 5.28: Gene expression of signature TFs of CD4 T helper subsets on TCR-stimulated CB Treg cells. HSC were cultured in the presence or absence of TCR-stimulated CB Treg cells at day 9 of differentiation and then $CD4^+$ cells were separated by cell sorting at day 12 for analysis by real time PCR. TFs Foxp3, Gata-3, Rorc, T-bet and IFN-γ were assessed. TCR-stimulated CB Treg cells before being added to HSC cultures (blue) were used as controls. The lines represent medians. n=4.

5.9 Discussion

Treg cell-based therapies to prevent GvHD should be carefully assessed since they could compromise NK cell-mediated control of infections and GvL as well as immune reconstitution. Currently, these results and the work of others have demonstrated that Treg cells can abrogate NK cell effector function under certain conditions (Trzonkowski et al., 2004, Ghiringhelli et al., 2005, Romagnani et al., 2005, Sun et al., 2010, Zhou et al., 2010, Bergmann et al., 2011). However, it remains unknown if Treg cells could also compromise NK cell differentiation.

In this chapter, the impact of CB Treg cells on NK cell differentiation was evaluated using a well-established *in vitro* model of NK cell differentiation from CB $CD34^+$ HSC (Grzywacz et al., 2006, Luevano, 2013) in which allogeneic resting or TCR-stimulated CB Treg cells were added at key time points during HSC cultures. The impact of CB Treg cells on NK cell differentiation was measured by the analysis of cell count, viability, frequency of NK cell stages, NK cell phenotype, NK cell functions and gene expression.

First, the potential inhibition of CB Treg cells on NK cell differentiation was determined by the analysis of NK cell production at the end of HSC cultures (day 35). Remarkably, 90% reduction in total NK cell numbers was observed at the end of HSC cultures in the presence of TCR-stimulated CB Treg cells, but not with resting CB Treg cells. The effect was particularly evident when TCR-stimulated CB Treg cells were added at day 9 of differentiation, time point at which HSC commitment to the NK cell lineage occurs. To date, no information is available on the effects of Treg cells on NK cell differentiation. However, various studies have focused on the effect of Treg cells on HSC and mature NK cells. Fujisaki and colleagues (Fujisaki et al., 2011) showed reduced HSC viability (70-90%) upon Treg cell depletion in a mouse model of HSCT, suggesting by this that Treg cells act as facilitators of allogeneic tolerance. Conversely, others have observed suppression of mature NK cell numbers in the presence of Treg cells. For instance in *Scurfy* mice, characterised by the

lack of Foxp3 expression that is necessary for the development of Treg cells, enhanced NK cell proliferation is observed (Brunkow et al., 2001). Also, depletion of Treg cells by anti-CD25 mAb injection in C57BL/6 mice (Ghiringhelli et al., 2005) or by diphtheria toxin in $Foxp3^{DTR}$ mice (Kim et al., 2007) increases NK cell numbers in the LN and spleen.

Furthermore, the analysis of defined NK cell stages of differentiation using the expression of CD34, CD117, CD94 and CD3 (Freud et al., 2006) highlights that stages 1 and 2 were not impaired in the presence of CB Treg cells, whereas stage 3 and 4 cells were severely compromised. In fact, it seems that NK cell differentiation is completely abrogated upon CB Treg cell addition (particularly at day 9), suggesting that Treg cells specifically suppress HSC commitment to the NK cell lineage. It may be possible that CB Treg cells induce cell cycle arrest, as observed in studies with PB Treg cells and mature NK cells (Romagnani et al., 2005); however, this still requires further investigation.

Recent work from Challen and colleagues has demonstrated the capacity of TGF-β1 to favour differentiation of HSC into myeloid rather than lymphoid lineage cells in transplanted mice (Challen et al., 2010). Since TGF-β is reported to be key for Treg cell-mediated suppression, it is plausible that Treg cells can also induce myeloid lineaged differentiation in this model. Expression of the myeloid cell marker CD33 was assessed during the first weeks of NK cell differentiation in the presence or absence of resting or TCR-stimulated CB Treg cells. No upregulation in expression of this marker were observed in the HSC co-cultured with resting or TCR-stimulated CB Treg cells, regardless of the time at which Treg cells were added. The discrepancy between this study and the work of Challen and colleagues may be due to differences in the source of HSC used, since Challen and colleagues used myeloid and lymphoid-biased HSC subsets based on the expression of the CD150 marker in mice.

These data provide evidence that CB Treg cells impose constraints on the commitment of HSC to the NK cell lineage, but what is the state of the few NK cells that still acquire a full NK cell phenotype at the end of these cultures? The phenotype of differentiated NK cells was assessed by analysing expression of

activating receptors (NKp30, CD16, DNAM-1, NKG2D, 2B4 and NKp460) and receptors associated with trafficking (integrins α4 and β7, L-selectin, LFA-1, CXCR4 and CCR7). Interestingly, TCR-stimulated Treg cells did not affect expression of any of the activating receptors analysed on those differentiated NK cells. These results were unexpected since NKG2D and NKp30 downregulation on NK cells by TGF-β has been previously described (Castriconi et al., 2003). However, when resting Treg cells where added at late time points of HSC cultures (D23 and D30), downregulation of CD16 and DNAM-1 was observed in differentiated NK cells. These results is in fact consistent to the results observed on freshly isolated CB NK cells when co-cultured with resting Treg cells (Chapter 4).

TCR-stimulated CB Treg cells did modulate the trafficking repertoire of differentiated NK cells. CXCR4 is a crucial chemokine receptor for NK cell development and homing to the BM (Beider et al., 2003, Noda et al., 2011). In this study, it was found that resting CB Treg cells added at day 23 of culture and TCR-stimulated CB Treg cells added at day 9 of culture induced the downregulation of CXCR4 on differentiated NK cells. These results suggest that Treg cells may induce NK cells to leave the BM. However, it was surprising to also observe CXCR4 downregulation induced by resting CB Treg cells. This can be explained by the findings of Ghiringhelli and colleagues who demonstrate that PB Treg cells can decrease NK cell effector functions through membrane-bound TGF-β on Treg cells without the need for TCR-stimulation (Ghiringhelli et al., 2005).

Downregulation of CCR7, accompanied with increased expression of integrin β7, was detected on the differentiated NK cells in cultures with TCR-stimulated CB Treg cells, suggesting that the ability of these cells to migrate to the LN is reduced in these conditions. Interestingly, Reeves and colleagues found similar results in symian immunodeficiency virus infected (SIV)-macaques in which infected NK cells showed reduced expression of CCR7 and increased expression of integrin α4β7, suggesting a preferential migration into the gut rather than the LN (Reeves et al., 2010). Furthermore, it seems as though loss of CCR7 expression by NK cells may correlate with enhanced functional

capacity. In HIV-1 patients, $CCR7^{-}CD56^{bright}$ NK cells display increased cytolytic potential, higher activation state and a more differentiated phenotype (Hong et al., 2012). However, the findings presented in this study indicate no enhancement in NK cell functions. Studies such as transwell migration assays are required to further demonstrate that Treg cells modulate the migratory capacity of differentiated NK cells in this system.

Remarkably, the few cells that acquire a NK cell phenotype were functional, as tested by cytotoxicity assays, degranulation assays and the analysis of IFN-γ production by ELISA. This seems to be in line with the existing literature, which describes an effect on NK cell functions exclusively in the absence of cytokines, proposed as a mechanism of NK cell tolerance (Gasteiger et al., 2013a, Kerdiles et al., 2013, Sitrin et al., 2013).

Given that CB Treg cells inhibit commitment of HSC to the NK cell lineage, one could expect TFs involved in NK cell differentiation to be differentially expressed by HSC in the presence of TCR-stimulated CB Treg cells. Studies in mice highlight the importance of E4BP4, Pu.1, Id2 and Tox for NK cell differentiation, as deficient-mice in these TFs have reduced NK cell numbers (Colucci, 2001, Boos et al., 2007, Gascoyne et al., 2009, Yun et al., 2011). In this study, no change in expression in HSC of any of the TFs analysed was observed in the presence of TCR-stimulated CB Treg cells, except for Tox, for which increased expression was observed. As for TFs involved in NK cell maturation, TCR-stimulated CB Treg cells added at day 9 of cultures induced enhanced expression of Helios, T-bet, Bcl11b, and Gata-3, TFs necessary for NK cell maturation. There are two possible explanations for these observations. Firstly, given that all previous studies that analysed TFs involved in NK cell differentiation and function were performed in mice, it is possible that the functions of these TFs may differ in humans. The second explanation is based on the fact that cell numbers were constant for the remainder of the culture period after addition of TCR-stimulated Treg cells at day 9 of cultures, which may suggest that the Treg cell-depleted HSC that were analysed were the only cells that managed to acquire a full NK cell phenotype.

Culture of Treg cells using HSC culture conditions consisiting of a feeder layer and cytokines such as IL-3, IL-15, IL-17, SCF and FLT-3L has never been described; therefore, it was assessed whether Treg cells could maintain their phenotype in these conditions, as plasticity is an evolutionary characteristic of T cells (Gao et al., 2012). Cellular and molecular analyses of CB Treg cells were performed at the end of HSC cultures and at the time point where the strongest effect was observed on HSC (day 9 and co-cultured for three days). The presence of Treg cells was confirmed by measuring expression of Foxp3 by intranuclear staining with no difference in expression levels of TFs associated with other $CD4^+$ T cells subsets including Gata-3, Rorc or T-bet. In fact, the viability and proliferation of TCR-stimulated CB Treg cells observed in HSC cultures (particularly at day 9 and to a lesser extent at the other time points) could be accounted for their affinity for IL-15 (particularly CB Treg cells) that induces Treg cell proliferation in the same manner as IL-2 (Lee et al., 2009a). Reduced cell numbers after cell sorting did not allow us to perform a molecular study at the end point of NK cell differentiation (day 35). This, accompanied with Foxp3 methylation studies would provide more evidence on the state of TCR-stimulated CB Treg cells in these particular conditions.

This study demonstrates for the first time the suppressive capacity of TCR-stimulated CB Treg cells on NK cell differentiation, particularly when HSC commitment to the NK cell lineage occurs. The similarity of results of studies on TGF-β on NK cell differentiation in mice with ours may suggest Treg cells as a source of TGF-β during ontogeny that negatively regulate NK cell differentiation, as presented in **Table 5.1**.

Table 5.1: Comparison of the results presented in this study (*time point where the strongest effect was observed; HSC+TCR-stimulated Treg cells (D9)) with existing studies on the impact of soluble TGF-β on NK cell ontogeny and functions.

Finding	This study*	Studies with the addition of TGF-β
HSC numbers and viability	Not impaired	Decreased proliferative capacity *in vivo* (Larsson et al., 2003, Larsson and Karlsson, 2005, Yamazaki et al., 2009), and decreased viability *in vivo* (Fujisaki et al., 2011) Suppressive effect with high concentrations of TGF-β and stimulatory effects with low concentrations of TGF-β (Kale, 2004, Kale and Vaidya, 2004)
NK cell numbers	Decreased by 90%	Decreased by 95-98% (Marcoe et al., 2012)
NK cell viability	Not impaired	N/D
NK cell stages	Blockage of Stage 3-4 of NK cell differentiation.	Decreased numbers of Stage F mature NK cells (Marcoe et al., 2012)
Expression of CD16 and CD94	Decreased	Blockage on maturation (Marcoe et al., 2012)
Presence of myeloid lineage cells	No upregulation is observed in the expression of CD33 myeloid marker	Myeloid-based HSC proliferate better than lymphoid-biased HSC *in vivo* (Challen et al., 2010)
NK cell phenotype	Activating receptors not impaired. Decrease in CXCR4 and CCR7-positive NK cells. Increase in integrin β7 and LFA-1-positive NK cells.	Upregulated expression of CXCR4 and CXCR3 on PB NK cells (Castriconi et al., 2013). Downregulated expression of NKG2D and NKp30 (Castriconi et al., 2003)
NK cell functions	Not impaired	Decreased IFN-γ production by mature NK cells in mice (Laouar et al., 2005). Decreased cytotoxicity by mature NK cells (Castriconi et al., 2003) Cytotoxicity not impaired (Ghiringhelli et al., 2005)
NK cell TFs involved in differentiation	Increased Tox mRNA expression in TCR-stimulated Treg cell-depleted HSC	N/D
NK cell TFs involved in maturation and function	Increased Helios, T-bet, Bcl11b, Gata-3 and IFN-γ mRNA expression in TCR-stimulated Treg cell-depleted HSC	Increased T-bet and Gata-3 in TGF-β resistant mNK cells (Marcoe et al., 2012)

6 Mechanism of suppression of regulatory T cells on natural killer cell differentiation

6.1 Introduction

The work presented in this thesis as well as data reported by other groups demonstrate the capacity of Treg cells to suppress NK functions (Trzonkowski et al., 2004, Ghiringhelli et al., 2005, Smyth et al., 2006, Sun et al., 2010, Zhou et al., 2010). The mechanisms by which Treg cells suppress NK cell effector functions have been studied in humans and mice and are summarised in **Table 1.2**. Ghiringhelli and colleagues showed that TGF-β neutralisation reverted Treg cell-mediated inhibition of NK cell effector functions in GIST patients and healthy individuals (Ghiringhelli et al., 2005). Moreover, Smyth and colleagues demonstrated that neutralisation of TGF-β but not IL-10 in mice could restore NK cell effector functions and revert Treg cell-mediated suppression (Smyth et al., 2006). Trzonkowski and colleagues confirmed that IL-10 does not play a role in Treg cell-mediated suppression of NK cells. This was observed in an *in vitro* model in which human Treg cells inhibit NK cell effector functions in the presence of APCs; yet neutralisation of IL-10 did not restore NK cell functions (Trzonkowski et al., 2004). Altogether, these reports suggest that TGF-β, but not IL-10, is the mediator by which Treg cells suppress NK cells.

It has also been reported that Treg cells require cell contact to exert suppressive functions (Ghiringhelli et al., 2005, Smyth et al., 2006, Zhou et al., 2010, Bergmann et al., 2011). Smyth and colleagues showed that cell contact between NK cells and Treg cells was necessary for suppression to occur in their *in vitro* culture system. Moreover, the authors investigated whether NK cell effector functions could be restored after depletion of Treg cells. Indeed, NK cells displayed similar levels of cytotoxicity against tumour cells after Treg cells were depleted, thus suggesting that Treg cells require contact with NK cells to mediate suppression.

Treg cells can also suppress NK cells by IL-2 deprivation. Sitrin and colleagues observed that Treg cells regulate NK cells via IL-2 in a mouse model of diabetes, since IL-2 neutralisation can revert this effect and IL-2 supplementation enabled NK cells to overcome Treg cell suppression (Sitrin et al., 2013). Gasteiger and colleagues confirmed these findings in a $Foxp3^{DTR}$ mouse model in which Treg cells can be depleted by treatment with diphtheria toxin (Gasteiger et al., 2013b). In this model, the authors observed that the inhibition of NK cell cytotoxicity caused by Treg cells could be reverted in the absence of Treg cells, but induced upon IL-2 neutralisation. These reports described an additional mechanism by which Treg cells suppress mature NK cells; however no information is available on how Treg cells can suppress NK cell differentiation.

In Chapter 5, it was demonstrated that TCR-stimulated CB Treg cells inhibit NK cell differentiation, particularly if added when HSC commitment to the NK cell lineage occurs. This chapter seeks to (i) determine the mechanism(s) by which CB Treg cells suppress NK cell differentiation, (ii) assess whether CB Treg cells require cell contact to be suppressive and (iii) determine whether CB Treg cells can also exert suppression by cytokine deprivation.

6.2 Measurement of TGF-β and IL-10 in haematopoietic stem cell cultures

6.2.1 TGF-β and IL-10 secretion in haematopoietic stem cell cultures

Most studies have identified TGF-β as the mediator responsible for NK cell suppression by Treg cells from human PB cells and in mice (Ghiringhelli et al., 2005, Smyth et al., 2006, Lundqvist et al., 2009, Zhou et al., 2010). Hence, TGF-β and also IL-10 were evaluated as candidates for inhibition of NK cell differentiation by CB Treg cells. For this, resting and TCR-stimulated Treg cells were added on day 9 of HSC culture as these co-cultures exhibited the strongest suppressive effect on NK cell differentiation.

TGF-β secretion was analysed every week for 35 days in HSC cultures with or without resting or TCR-stimulated CB Treg cells added on day 9 of culture. When TCR-stimulated CB Treg cells were added to HSC cultures, ~1 500 pg/ml of TGF-β were detected at all time points assessed. Importantly, for HSC (controls) and co-cultures where resting CB Treg cells were added on day 9 of culture, lower levels of TGF-β secretion (~800 pg/ml) were detected at all time points as compared to the co-cultures with TCR-stimulated CB Treg cells (**Figure 6.1**). This suggests that TCR-stimulated CB Treg cells secrete higher levels of TGF-β. However, no statistical significance was observed between cultures, which may be explained by the high background secretion of TGF-β by HSC and NK cells (Chen et al., 2009).

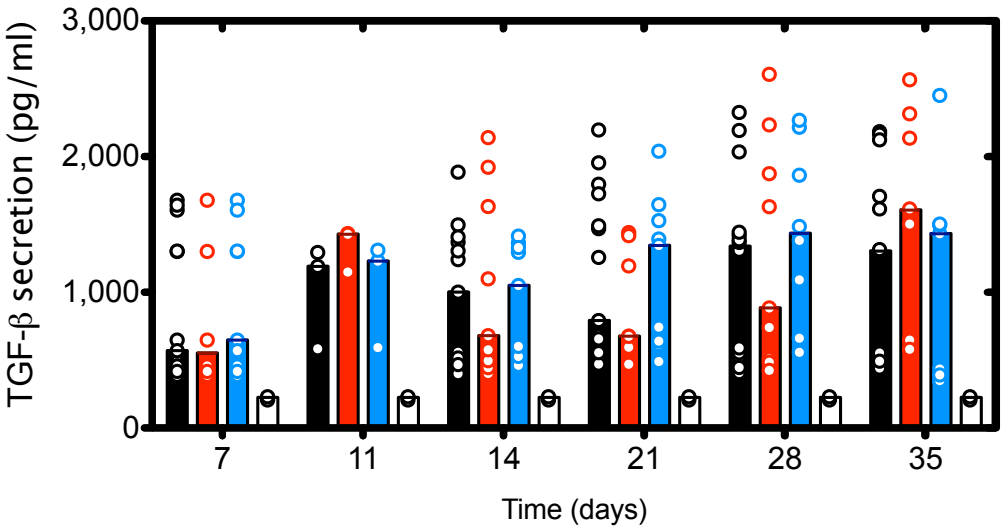


Figure 6.1: TGF-β secretion levels in HSC cultures with or without Treg cells. Allogeneic resting CB Treg cells (red) or TCR-stimulated CB Treg cells (blue) were added on day 9 of HSC cultures. HSC cultured alone were used as controls (black) and NK cell media was used as negative control (white). Supernatants were collected every week for 35 days and analysed by ELISA. The results are means of duplicate wells and the values shown in the graphs represent medians. n=3-15.

IL-10 secretion was also evaluated in the same cultures. Notably, when TCR-stimulated CB Treg cells were added at day 9 of HSC cultures, a peak in IL-10 secretion was clearly observed (**Figure 6.2**). In the co-cultures between HSC and TCR-stimulated CB Treg cells, ~80 pg/ml of IL-10 was detected at day 14 of culture ($p<0.0001$) followed by a gradual reduction over time. It is noteworthy that IL-10 amounts observed in this study are biologically relevant, as similar levels have been observed in the bladder (20-40 pg/ml) of healthy individuals (Duell et al., 2012) and for PB Treg cells stimulated with PMA/ION (~100 pg/ml) (Duhen et al., 2012).

When resting CB Treg cells were added to HSC cultures at day 9, IL-10 production was also detected with a peak of secretion at day 21 (~90 pg/ml, $p<0.0001$) that gradually decreased over the remaining weeks (**Figure 6.2**). IL-10 has only been reported to be secreted by TCR-stimulated and not resting Treg cells (Milward et al., 2013). Therefore, it is possible that the feeder layer cells used in this HSC culture system trigger IL-10 secretion by resting CB Treg cells. These results suggest that both TGF-β and IL-10 could be involved in the mechanism by which CB Treg cells inhibit NK cell differentiation.

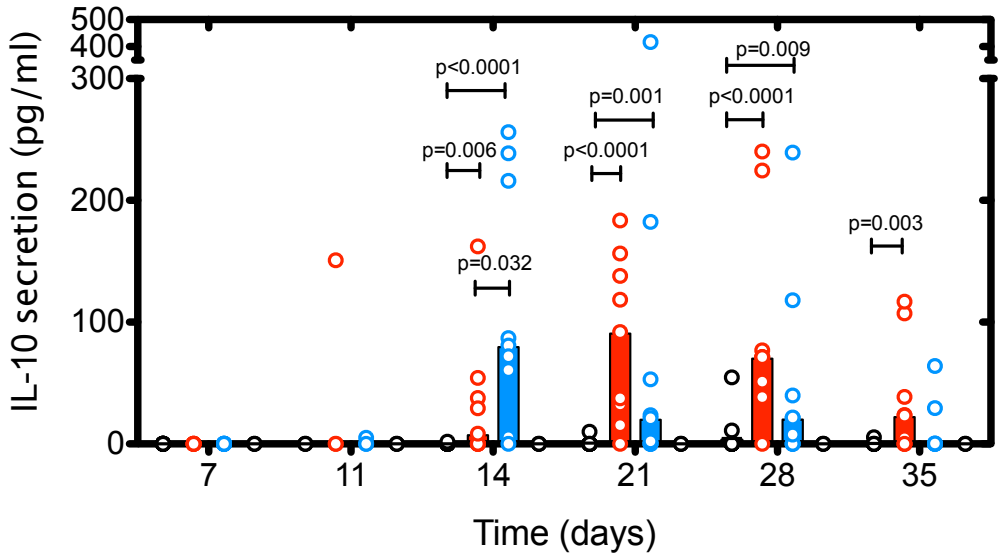


Figure 6.2: IL-10 secretion levels in HSC cultures with or without Treg cells. Allogeneic resting CB Treg cells (red) or TCR-stimulated CB Treg cells (blue) were added at day 9 of HSC cultures. HSC cultured alone were used as controls (black). NK cell media was used as negative control (white). Supernatants were collected every week for 35 days and analysed by ELISA. Results are represented as means of duplicate wells. The values in the graphs represent medians. n=3-15.

6.2.2 TGF-β and IL-10 gene expression by regulatory T cells added to haematopoietic stem cell cultures

The results presented in the previous section suggest that TGF-β and/or IL-10 may contribute to Treg cell mediated-inhibition of NK cell differentiation. If TGF-β and/or IL-10 are involved in inhibition of NK cell differentiation by Treg cells, one could expect to observe increased mRNA expression of these molecules. To assess this, resting or TCR-stimulated CB Treg cells were added to HSC cultures at the time point at which the strongest effect was observed (D9), re-isolated by cell sorting three days later (D12) and then RNA was extracted. TGF-β and IL-10 mRNA expression by Treg cells was then compared with resting or TCR-stimulated CB Treg cells before addition to HSC cultures.

For IL-10, similar levels of mRNA expression were observed in resting and TCR-stimulated Treg cells before and after culture with HSC (**Figure 6.3**). These results suggest that IL-10 may not be the molecule responsible for the suppression of CB Treg cells, or that IL-10 had already being translated and

secreted before being analysed. For TGF-β, higher levels of mRNA expression were detected in TCR-stimulated CB Treg cells after co-culture with HSC (p=0.031) when compared to TCR-stimulated CB Treg cells before culture (**Figure 6.3**).

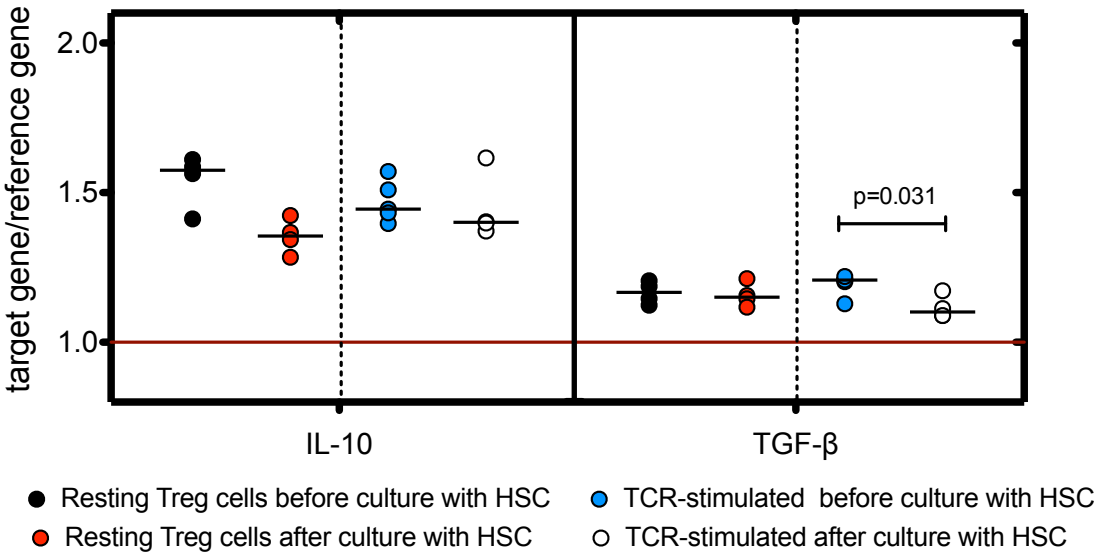


Figure 6.3: TGF-β and IL-10 gene expression in Treg cells after three days culture with HSC. HSC were cultured in the presence of allogeneic resting CB Treg cells (red) or TCR-stimulated CB Treg cells (white) at day 9 of HSC cultures and sorted at day 12. Freshly isolated CB Treg cells (black) or TCR-stimulated CB Treg cells for 24 h (blue) were used as controls. Total RNA was extracted from Treg cells, then TGF-β and IL-10 gene expression was assessed by real time PCR. The lines represent medians. n=3-4.

6.3 TFG-β but not IL-10 recapitulates the effect of regulatory T cells on natural killer cell differentiation

It was demonstrated that resting and TCR-stimulated CB Treg cells secreted TGF-β and IL-10 when co-cultured with HSC and that TCR-stimulated Treg cells co-cultured with HSC for three days expressed higher levels of TGF-β mRNA. Hence, to confirm whether TGF-β and/or IL-10 are involved in TCR-stimulated CB Treg cell-mediated suppression of NK cell differentiation, blocking assays were performed using the TGF-β pathway inhibitor SB431542 (Inman et al., 2002, Rorby et al., 2012) and/or human IL-10 receptor α blocking mAb (Godfrey et al., 2005). However, toxicity caused by the addition of these reagents in this differentiation model did not allow continuation of this strategy.

Instead, it was assessed whether the effect of Treg cells on NK cell differentiation could be recapitulated by addition of recombinant human TGF-β and/ or IL-10 at different concentrations. Concentrations were chosen based on secretion levels observed in this study (~1 ng/ml TGF-β and ~0.1 ng/ml IL-10) and mentioned in the literature (Carson et al., 1995, Ghiringhelli et al., 2005, Rorby et al., 2012). Recombinant human TGF-β and/or IL-10 were added at day 9 of differentiation and then added weekly until day 35 of differentiation.

A reduction in the frequency of $CD56^+$ cells was observed when TGF-β was added at a minimum concentration of 2.5 ng/ml. (**Figure 6.4**). NK cell frequency gradually decreased when higher concentrations of TGF-β were used whereas no effect was noted when recombinant human IL-10 was added to the cultures. Interestingly, when TGF-β and IL-10 were both added to HSC cultures, an effect on NK cell frequency was also observed; however the reduction was not as striking as when TGF-β only was added to HSC cultures.

The expression of CD16 was also analysed, since it is important for NK cell maturation and cytotoxic functions (Cooper et al., 2001a). Notably, no reduction in expression of CD16 was observed in any of the conditions tested. These observations suggest that the addition of recombinant human TGF-β but not IL-10 at day 9 of HSC cultures emulates the effect of TCR-stimulated CB Treg cells on NK cell differentiation.

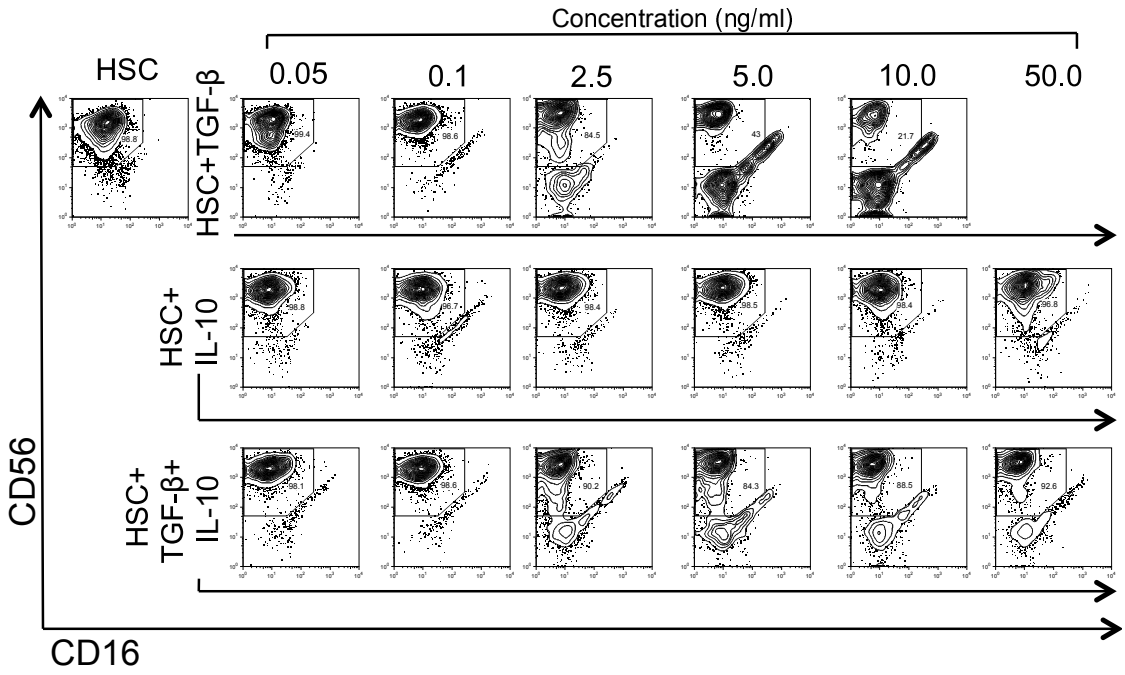


Figure 6.4: Effects of recombinant human TGF-β and/or IL-10 on HSC cultures. Recombinant human TGF-β and/or IL-10 were added weekly at different concentrations on days 9, 14, 21 and 28 of HSC cultures. Flow cytometric analysis of HSC was performed at day 35 of differentiation using CD56 and CD16 surface markers. Data is a representative example of seven independent experiments.

It was studied whether the addition of recombinant human TGF-β and/or IL-10 affected NK cell numbers and total cell numbers in this culture system. Total cell numbers were affected by the addition of recombinant human TGF-β at a minimum concentration of 2.5 ng/ml (64%; p=0.0006) (**Figure 6.5A, B, red bars**). Total cell numbers were further reduced when higher concentrations of TGF-β were used with reductions of 73% with 5.0 ng/ml TGF-β (p=0.0006) and 92% with 10.0 ng/ml (p=0.0006) observed. IL-10–treated HSC cultures were not as affected (**Figure 6.5A, B, blue bars**). Furthermore, the combination of both suppressive molecules showed an effect with 20 to 60% reduction in total cell numbers (**Figure 6.5B, white bars**).

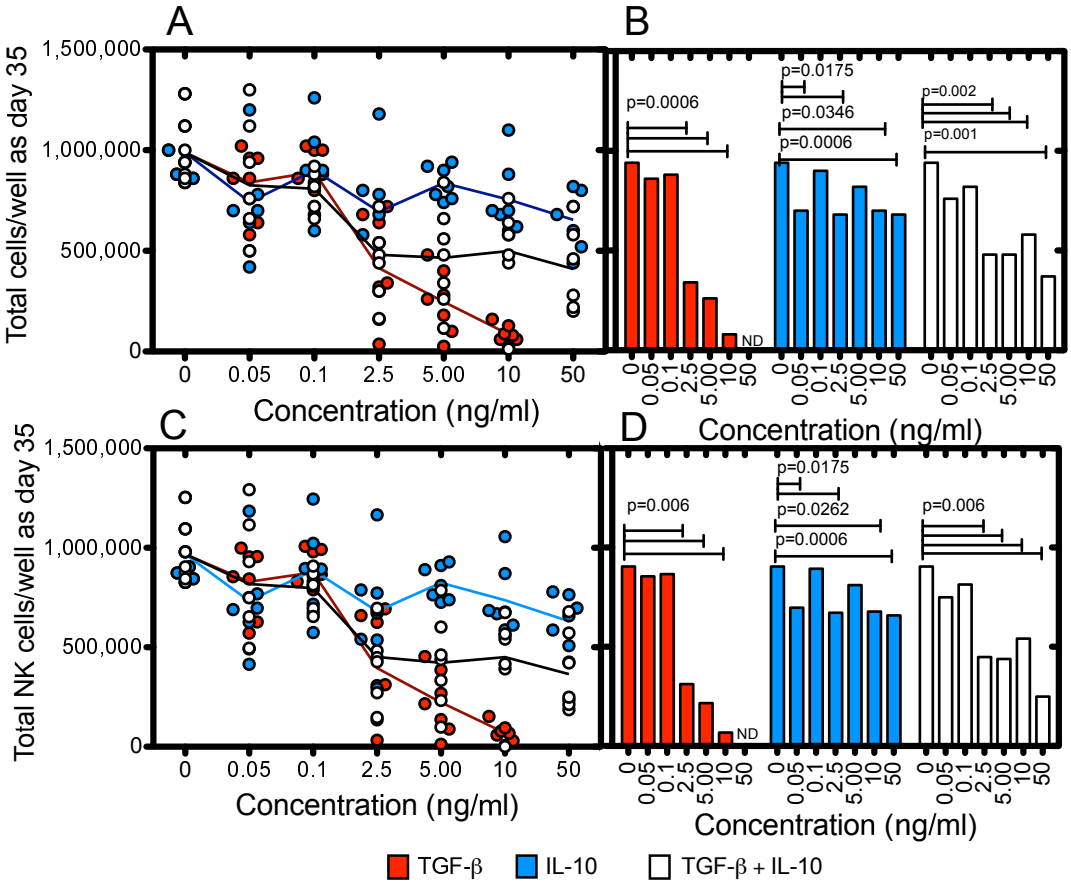


Figure 6.5: Total cell numbers and NK cell numbers in the presence of recombinant human TGF-β and/or IL-10. Recombinant human TGF-β and/or IL-10 were added weekly to HSC cultures at different concentrations. Total cell numbers (assessed using trypan blue) per well at day 35 of differentiation ± TGF-β and/or IL-10 at different concentrations (A). Summary plot and statistical analysis of panel A (B). Total NK cell numbers per well at day 35 of differentiation ± TGF-β and/or IL-10 at different concentrations (C). Summary plot and statistical analysis of panel C. NK cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell number per well (D). The lines represent medians. n=7.

A decrease in NK cell numbers was observed at day 35 of HSC cultures when the highest concentrations of recombinant human TGF-β were added. Reduction in NK cell numbers of 66% with 2.5 ng/ml (p=0.0006), 77% with 5.0 ng/ml (p=0.0006) and 96% with 10.0 ng/ml (p=0.0006) were observed (**Figure 6.5C, D, red bars**). Addition of IL-10 only caused a 20% decrease in NK cell numbers and this effect was non-dose dependent (**Figure 6.5C, D, blue bars**). Finally, the addition of both TGF-β and IL-10 caused ~50% reduction in NK cell numbers (starting from 2.5 ng/ml) (**Figure 6.5C, D, white bars**).

In order to ensure that this effect was not due to toxicity caused by the high concentrations of recombinant human TGF-β and/or IL-10 used, viability was

assessed in the cultures with the highest concentrations of these suppressive molecules. Flow cytometry analysis demonstrated no effect on viability for any of the treatments, with ~98% viable cells (7-AAD$^-$) in the lymphocyte gate for all cultures (**Figure 6.6**). These data suggest that recombinant human TGF-β is able to recapitulate the observed effects of TCR-stimulated CB Treg cells on NK cell differentiation when added at day 9 of HSC culture.

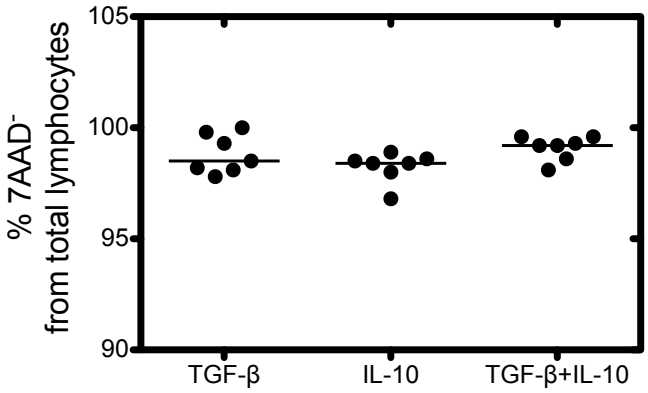


Figure 6.6: Viability of HSC cultures in the presence of recombinant human TGF-β and/or IL-10. Viability was measured in HSC cultures where the highest concentrations of recombinant human TGF-β (10 ng/ml) and/or IL-10 (50 ng/ml) were added weekly. CD45$^+$ viability was assessed by flow cytometry using CD45 and 7-AAD. The lines represent medians. n=7.

6.4 Regulatory T cell-mediated inhibition of natural cell differentiation is cell contact-dependent

Several studies focusing on the effects of Treg cells on NK cells demonstrated the importance of cell-to-cell contact for Treg cell-mediated suppression (Ghiringhelli et al., 2005, Smyth et al., 2006, Zhou et al., 2010, Bergmann et al., 2011). Hence, it is possible that CB Treg cells also require direct contact with HSC to inhibit their differentiation into NK cells. To evaluate this, HSC were cultured in the presence or absence of resting or TCR-stimulated CB Treg cells at key time points of HSC cultures, with Treg cells either being directly cultured together with HSC in the well or separated by a porous membrane of 0.4 µm (Transwell).

The suppressive effect of TCR-stimulated CB Treg cells added at day 9 of HSC cultures was lost when Treg cells and HSC where spatially separated by the

Transwell (p=0.037), showing similar NK cell numbers as controls (HSC) at the end of the differentiation (**Figure 6.7B)**. Furthermore, no difference was observed when resting CB Treg cells were cultured together or separated from HSC, regardless of the time point at which Treg cells were added to the cultures (**Figure 6.7A**). These results highlight the importance of cell-to-cell contact for TCR-stimulated CB Treg cells to suppress in this system and suggest that TGF-β may be surface bound rather than secreted by TCR-stimulated CB Treg cells.

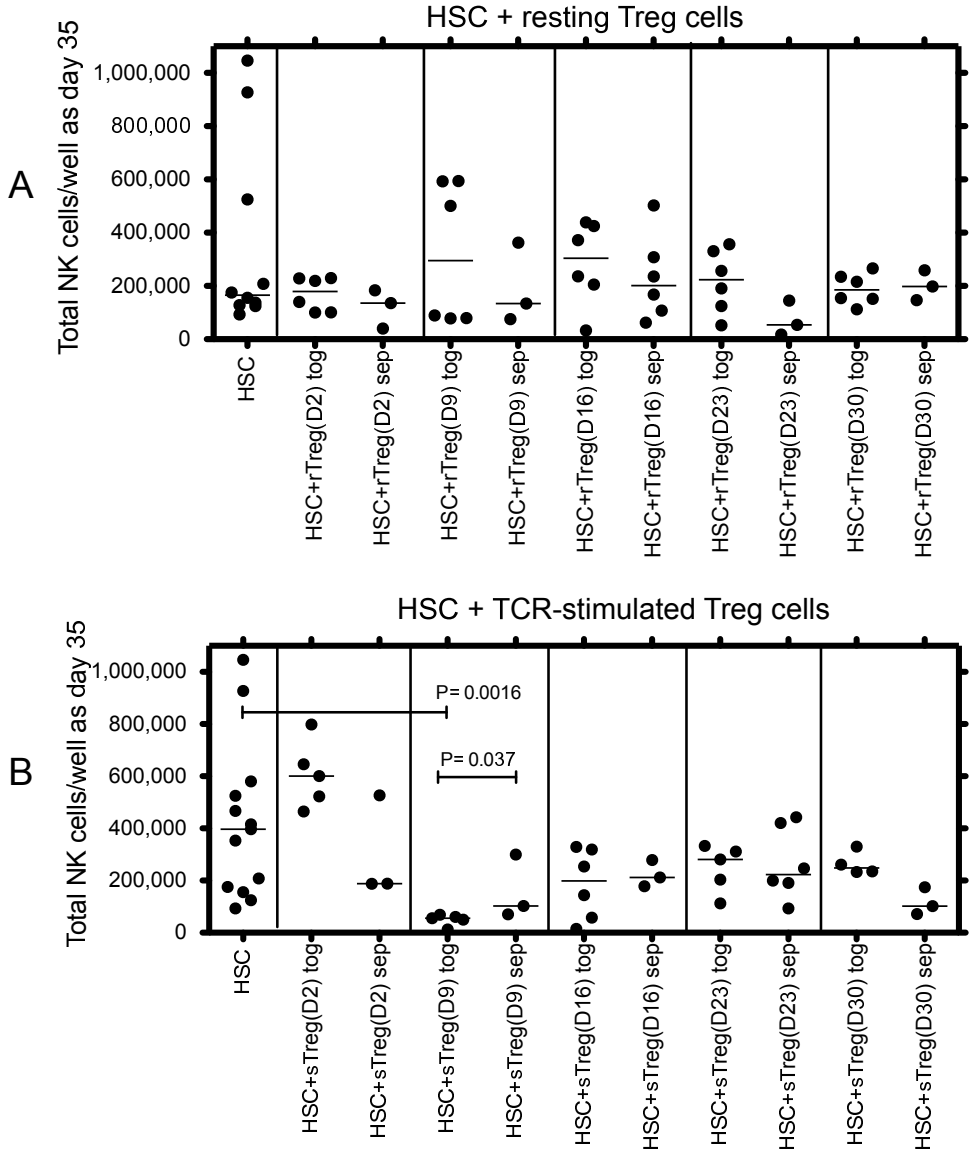


Figure 6.7: Treg cell-mediated suppression of NK cell differentiation is cell contact-dependent. HSC were cultured with allogeneic resting CB Treg cells (A) or TCR-stimulated CB Treg cells (B) added at key time points of NK cell differentiation. Treg cells were added directly to HSC cultures or were separated by a 0.4 µm porous membrane (Transwell) and analysed by flow cytometry at day 35 of culture. Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell number per well. The lines represent medians. n=3-13.

6.5 Natural killer cell differentiation can be "rescued" upon regulatory T cell depletion

Smyth and colleagues demonstrated the suppressive capacity of Treg cells against NK cells using a mouse tumour model (Smyth et al., 2006). They showed that Treg cells require contact to suppress NK cells as depletion of Treg cells with anti-CD25 mAb could revert NK cell functions *in vitro*. Hence, in this study it was investigated whether NK cell differentiation could be "rescued" by depletion of CB Treg cells after being co-cultured for a short period of time with Treg cells. In order to evaluate this, resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures and then depleted by cell sorting after a three day-co-culture. Sorted HSC were re-cultured on freshly irradiated feeder layer cells and cytokines were added. Total cell numbers of $CD4^+$ T cells, $CD56^+$ NK cells and intermediate stages of NK cell maturation were analysed in this study.

It was observed that Treg cell depletion "rescued" NK cell differentiation (**Figure 6.8**), with 89 to 97% of the cells being NK cells at day 35 of HSC cultures, with similar values to controls.

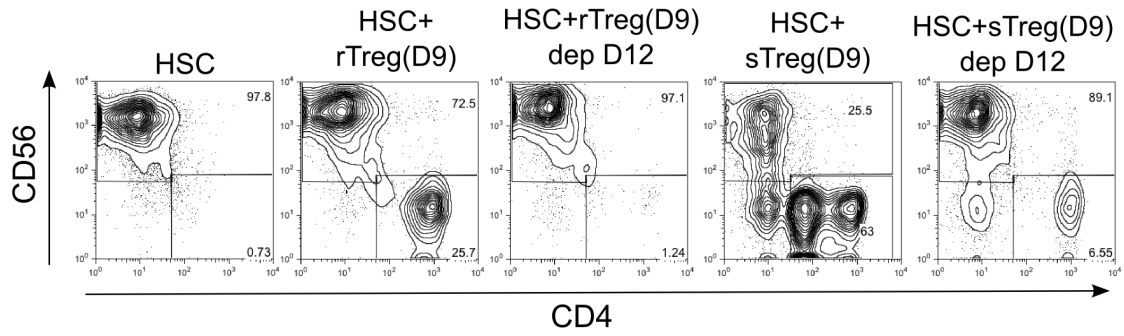


Figure 6.8: Flow cytometric analysis of Treg cell depleted-HSC at day 35 of NK cell differentiation. Resting or TCR-stimulated CB Treg cells were added on day 9 of HSC cultures and depleted on day 12 (after three days co-culture). NK cell and Treg cell populations were analysed by flow cytometry using the surface markers CD56 and CD4, respectively. Data is a representative of 3 or 4 independent experiments.

Total numbers of $CD45^+$ cells, NK cells ($CD56^+$) and Treg cells ($CD4^+$) at day 35 of HSC cultures are shown in **Figure 6.9A-B**. NK cell numbers from HSC cultures after Treg cell depletion were similar to controls, whereas reduced

numbers of NK cells were observed in co-cultures that contained TCR-stimulated CB Treg cells (p=0.05) (**Figure 6.9B**). Moreover, fold expansion rates were restored upon Treg cell depletion, showing equivalent numbers at the end of the 35-day culture period (**Figure 6.9C**).

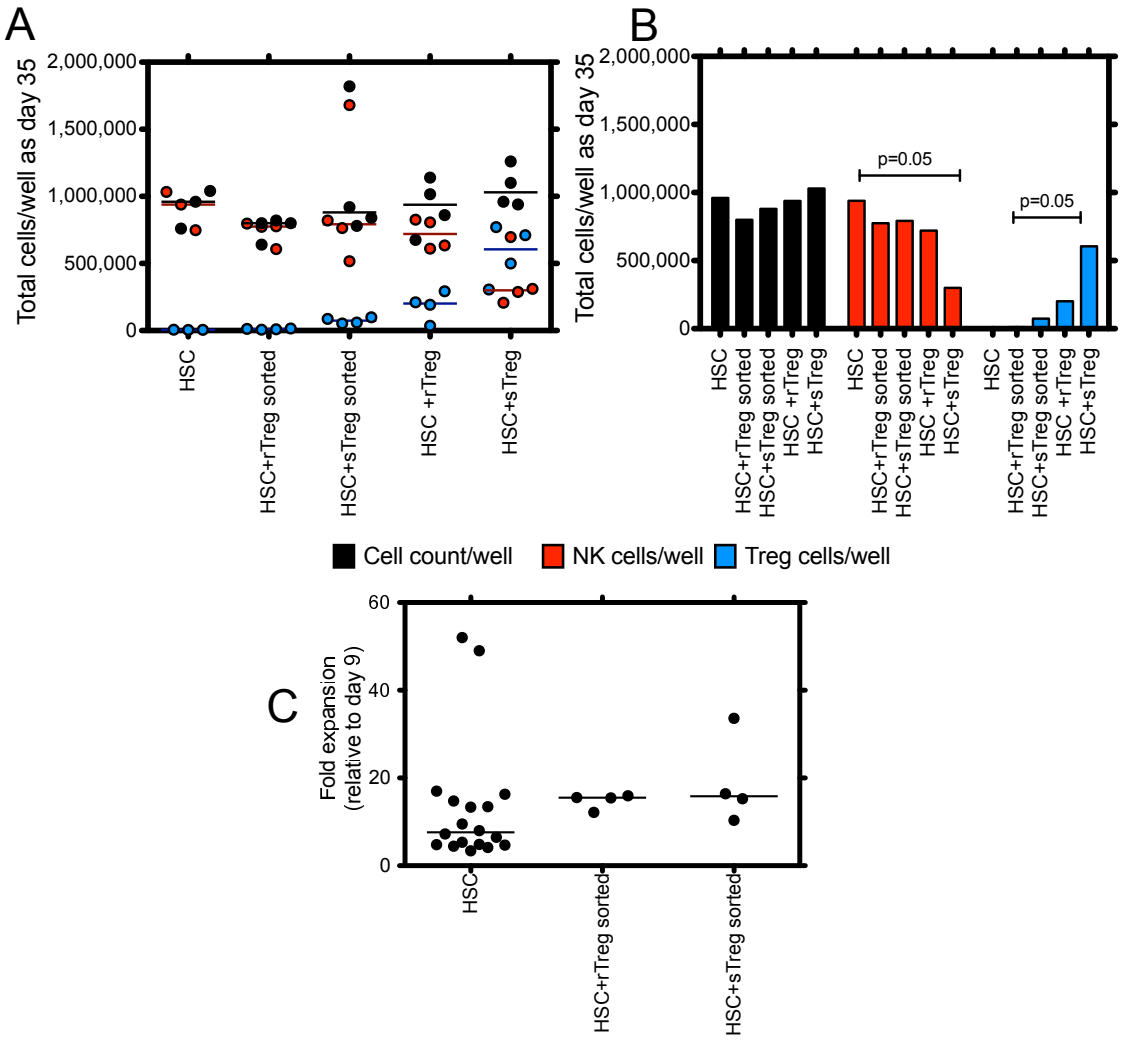


Figure 6.9: HSC differentiation into NK cells can be rescued after Treg cell depletion. Allogeneic resting or TCR-stimulated CB Treg cells were added at day 9 of HSC cultures and depleted at day 12 (after three days co-culture). Treg cell depleted-HSC were re-cultured with freshly irradiated feeder layer cells and cytokines. Total cells/ well (black), total NK cells/well (red) and total Treg cells/well (blue) at day 35 of NK differentiation (A). Summary plot and statistical analysis of graph shown in panel A (B). Fold expansion of controls (HSC) and resting or TCR-stimulated Treg cell depleted-HSC (C). Cell numbers were calculated from the NK cell and T cell frequencies determined by flow cytometry and total cell number per well. The lines represent medians. n=4-18.

Lastly, it was analysed whether NK cell maturation was completely restored on HSC cultures when Treg cells were depleted by assessing total cell numbers of three different intermediate stages of NK cell maturation that were defined as

$CD56^{+}CD94^{-}CD16^{-}$, $CD56^{+}CD94^{+}CD16^{-}$ and $CD56^{+}CD94^{+}CD16^{+}$ (see gating strategy in Section 5.4.2). Importantly, HSC cultures that were depleted of resting or TCR-stimulated CB Treg cells showed equivalent numbers of the three stages of NK cell maturation at the end of HSC cultures, as compared to HSC cultured alone (**Figure 6.10A**). Furthermore, MFI levels of CD94 and CD16 were not impaired in any of the conditions, but CD56 was slightly increased in both co-cultures (**Figure 6.10B**). These results suggest that HSC can be "rescued" after Treg cell depletion showing similar fold expansion rates, total cell numbers and NK cell maturation as controls. Moreover, these results highlight the fact that Treg cell persistence is required for TCR-stimulated CB Treg cell-mediated suppression of NK cell differentiation.

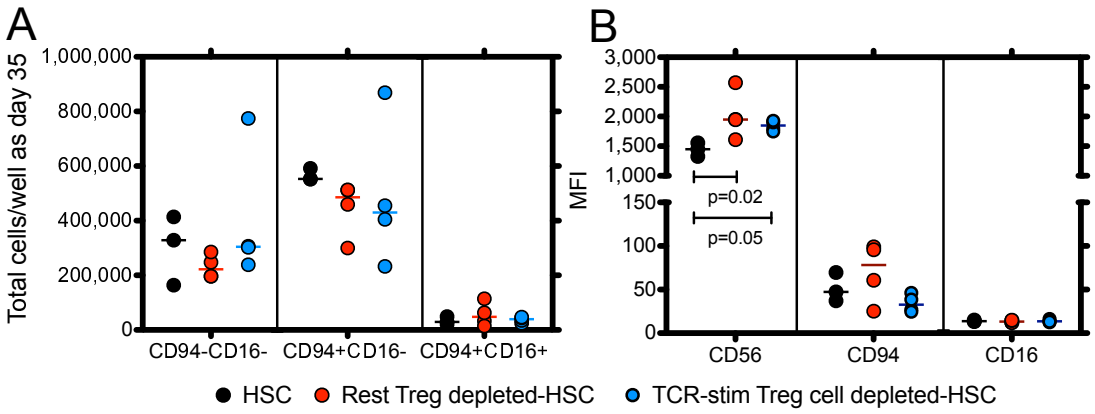


Figure 6.10: Intermediate stages of NK cell maturation are intact in Treg cell-depleted HSC cultures. Allogeneic resting or TCR-stimulated CB Treg cells were added or not on day 9 of HSC cultures and depleted on day 12 (after three days co-culture). Treg cell depleted-HSC were then re-cultured with freshly irradiated feeder layer cells and cytokines at a concentration of 50 000 cells/well. Frequency of expression of CD94 and CD16 on $CD56^{+}$ NK cells (A). MFI of CD56, CD94 and CD16 surface markers on NK cells (B). Cell numbers were calculated from the NK cell frequency determined by flow cytometry and total cell number per well. The values represent the medians. n=3-4

6.6 Regulatory T cell-mediated function is cytokine competition independent

It has recently been proposed that Treg cells can regulate NK cell functions via IL-2 (Gasteiger et al., 2013a, Gasteiger et al., 2013b, Sitrin et al., 2013), as has been previously reported for Treg cell-mediated suppression of Tcon cells

(Pandiyan et al., 2007). Therefore, it is plausible that CB Treg cells may inhibit NK cell differentiation by cytokine competition due to TCR-stimulated CB Treg cells having a higher affinity for the cytokines used in the HSC cultures. As a consequence, CB Treg cells would have a higher proliferative rate than resting CB Treg cells. To address this, resting or TCR-stimulated CB Treg cells were cultured using HSC culture conditions in the absence of HSC. Both resting and TCR-stimulated CB Treg cells exhibited a 30-fold expansion at day 35 day of culture (**Figure 6.11**). It seems that CB Treg cells, whether resting or TCR-stimulated, proliferate in the same way in response to the cytokines used in the *in vitro* system. Notably, it has been reported that IL-15, a crucial cytokine for NK cell differentiation, also induces Treg cell proliferation (Imamichi et al., 2008).

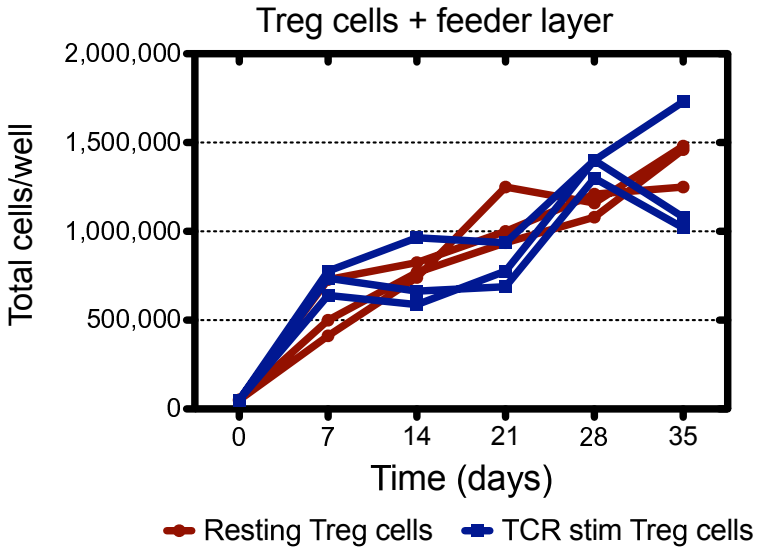


Figure 6.11: Resting and TCR-stimulated CB Treg cells show a similar proliferative profile under HSC culture conditions. Resting or TCR-stimulated CB Treg cells were cultured using HSC culture conditions in the absence of HSC. Cell numbers were calculated using trypan blue every week until day 35. The lines represent the medians. n=3

Furthermore, flow cytometric analysis clearly demonstrated that CB Treg cells do not persist in HSC cultures. It was noted that throughout the cultures, the proportion of $CD4^+$ cells gradually decreased under both conditions to reach only ~10-20% of the cultures by day 35 (**Figure 6.12A** and **Figure 6.12B**)

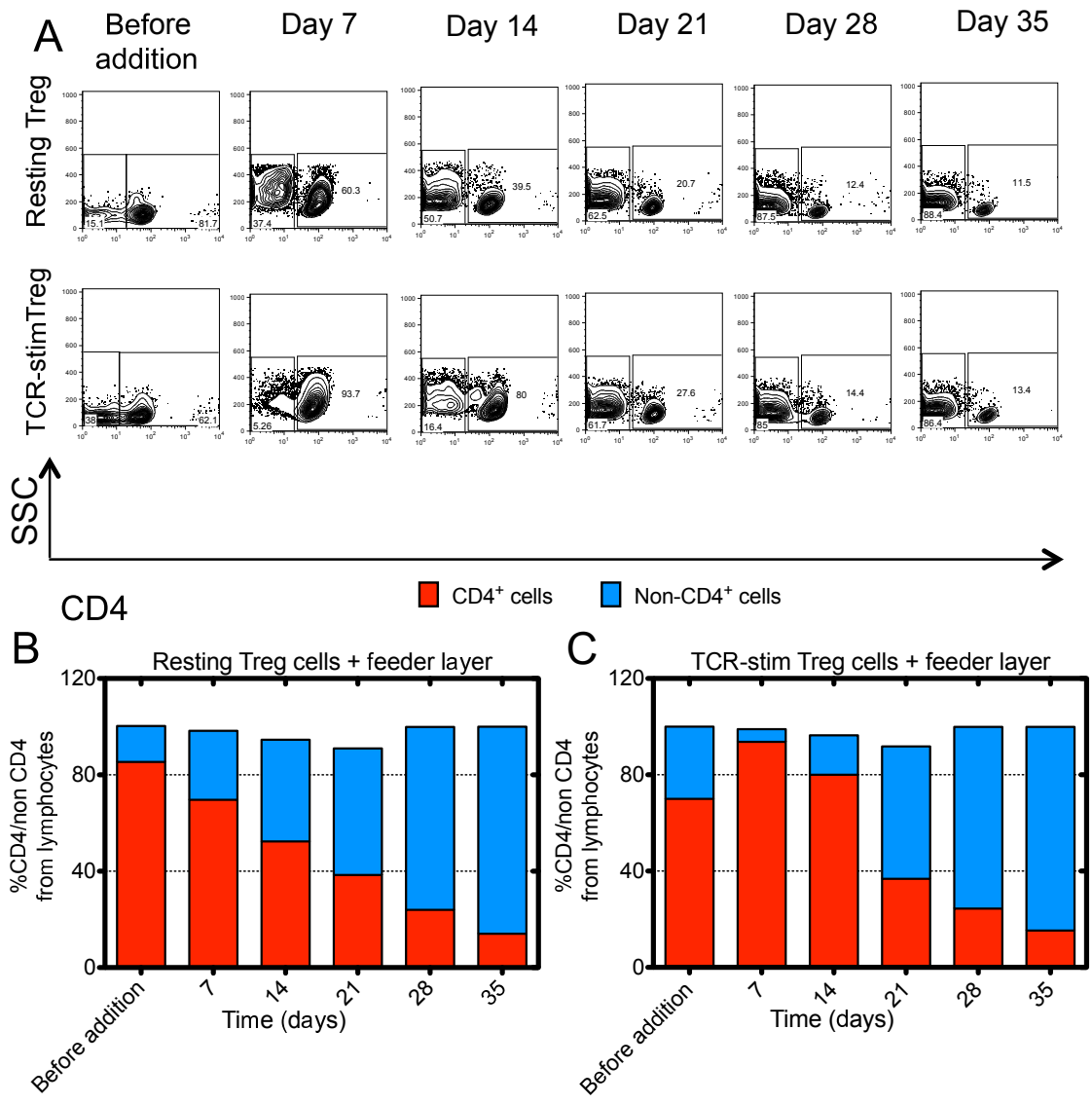


Figure 6.12: CD4 expression by Treg cells in HSC culture conditions. Resting or TCR-stimulated CB Treg cells were cultured using HSC culture conditions in the absence of HSC. Flow cytometric analysis of CD4 expression at days 0, 7, 14, 21, 28 and 35 (A). Data is a representative sample of 3 independent experiments per group. Proportion of CD4 and non-CD4 cells in resting CB Treg cell cultures (B). Proportion of CD4 and non-CD4 cells in TCR-stimulated Treg cell cultures (C). n=3. The values represent medians.

In fact, it was found that the non-CD4$^+$ cells in these cultures, were CD56$^+$. While CD4$^+$ T cell numbers were decreasing, CD56$^+$ NK cell numbers increased, reaching 80-90% frequency of the total cultures (**Figure 6.13**). As only Treg cells were cultured in these experiments, one explanation for this could be the possible presence of HSC as contaminants in the Treg cell isolations that could differentiate into NK cells in the presence of these cytokines, but this requires further investigation. Collectively, these results

confirm that CB Treg cell-mediated suppression of NK cell differentiation is not due to cytokine competition.

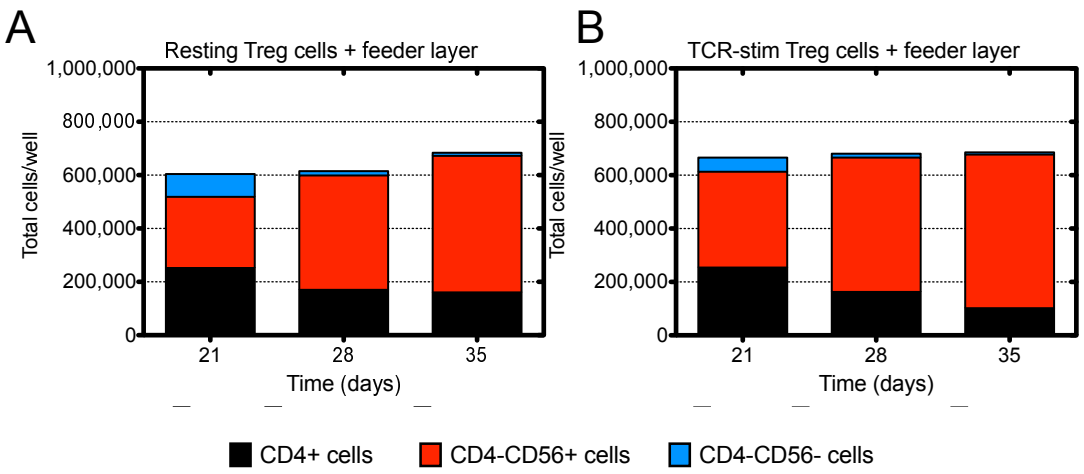


Figure 6.13: Analysis of total cell numbers in the final weeks of Treg culture using HSC culture conditions. Resting (A) or TCR-stimulated CB Treg cells (B) were cultured under HSC culture conditions in the absence of HSC. Flow cytometric analysis of CD4 and CD56 expression was performed at days 21, 28 and 35. Cell numbers were calculated from the T cell, NK cell and $CD4^{-}CD56^{-}$ frequencies determined by flow cytometry and total cell number per well. n=3

6.7 Discussion

In order to fully understand how Treg cell function can be manipulated for cell therapy, a thorough understanding of the mechanism(s) by which Treg cells suppress effector cells is required. To date, there is evidence that TGF-β mediates Treg cell suppression of NK cell function (Ghiringhelli et al., 2005, Smyth et al., 2006), however, the mechanism(s) by which Treg cells suppress NK cell differentiation were not known.

In this chapter the mechanisms by which CB Treg cells suppress NK cell differentiation were elucidated. First, high levels of TGF-β and IL-10 were detected in supernatants from co-cultures between HSC and TCR-stimulated CB Treg cells. However, delayed secretion of IL-10 by resting CB Treg cells was also observed. IL-10 production is reported to be exclusively detected upon strong TCR-stimulation (Milward et al., 2013). One possibility could be that the EL08.1D2 stromal feeder layer cells can stimulate Treg cells leading to IL-10 production; however, this requires further investigation.

In addition, it was demonstrated that TGF-β, but not IL-10, could recapitulate the effects of TCR-stimulated Treg cells on NK cell differentiation leading to a reduction in NK cell numbers (90%) in HSC cultures. This effect was observed with a minimum concentration of 2.5 ng/ml of TGF-β, which correlates with levels detected in the co-cultures (~2 ng/ml). A similar effect has also been observed in mice by Marcoe and colleagues who demonstrated that soluble TGF-β can also abrogate NK cell differentiation (95-98%) (Marcoe et al., 2012). Some authors have also suggested a dual effect of TGF-β on HSC with suppressive effects at high concentrations and stimulatory effects at low concentrations (Kale, 2004, Kale and Vaidya, 2004); yet this effect was not observed in this system.

Treg cells have previously been shown to suppress NK cell functions in a cell contact dependent manner (Ghiringhelli et al., 2005) and that depletion of Treg cells led NK cells to recover effector functions (Smyth et al., 2006). Similarly, here it was shown that cell contact between TCR-stimulated CB Treg cells and HSC was required for Treg cell-mediated inhibition of NK cell differentiation, since physical separation (using Transwells) of Treg cells from HSC or Treg cell depletion reverted this effect.

Treg cells can also suppress target cells by deprivation of available IL-2, thus causing reduced effector functions and control of NK cell activation (Gasteiger et al., 2013b, Sitrin et al., 2013). Since this study demonstrated that TCR-stimulated but not resting CB Treg cells inhibit NK cell differentiation, it was hypothesised that TCR-stimulated CB Treg cells would have more affinity for cytokines than resting CB Treg cells and as a consequence will have a higher proliferative rate, regardless of the presence of HSC. To assess this, CB Treg cells were cultured alone using HSC culture conditions. It was found that resting and TCR-stimulated CB Treg cell did not persist in these cultures with only 10% of the cells being $CD4^+$ T cells after 35 days in culture. Most of the cells were $CD56^+$, which could be explained by a small HSC population contaminating the isolated Treg cells. Only 500 HSC (~0.01% from total cells) are required to

achieve the expansion observed in this *in vitro* model, but whether this accounts for the expansion of $CD56^+$ cells observed still requires further assessment.

These results give a more thorough understanding of how CB Treg cells suppress NK cell differentiation, which will allow designing better Treg cell therapies.

7 Discussion

7.1 Scope of the study

Since the first HSCT performed in 1959, major progresses have been made in the selection of donors, conditioning regimens and HSC sources used. However, GvHD is still one of the major post-HSCT challenges, causing high morbidity and mortality rates. In view of their regulatory function, several groups have proposed Treg cells as an adoptive therapy to modulate or prevent GvHD (Trzonkowski et al., 2009, Brunstein et al., 2011b, Di Ianni et al., 2011, Edinger and Hoffmann, 2011). Preclinical and clinical studies have now demonstrated the safety of this therapy in transplanted patients but the potential impact of Treg cells on GvL and GvI is still controversial, requiring further investigation (Trenado, 2003, Maury et al., 2010, Brunstein et al., 2013). In view of the capacity of Treg cells to suppress NK cells, which are key effectors of the GvL effect (Ruggeri et al., 2002) and crucial for anti-viral responses (Quinnan et al., 1982), the effects of Treg cells on NK cell function and differentiation were studied. CB was selected as a model to analyse these potential effects as NK cell reconstitution occurs very early after transplant (Komanduri et al., 2007) and as NK cells constitute most of the lymphocytes in the circulation after CBT and are capable of killing leukaemia cells *ex-vivo* (Beziat et al., 2009), it is likely that NK cells mediate the GvL effect observed in the first months after CBT. Therefore, the use of CB Treg cells for adoptive therapy to prevent GvHD in CBT should be carefully reviewed, as there is potential for them to severely compromise NK cell functions.

7.2 Umbilical cord blood regulatory T cells exert suppressive capacity and can potentially interact with natural killer cells in diverse tissues

In order to study the effects of CB Treg cells on NK cell effector functions and differentiation, a thorough understanding of the phenotype and function of CB Treg cells was necessary. Notably, it was important to address whether CB Treg cells could migrate to potential sites where the interaction between Treg cells and NK cells may take place. In **Chapter 3**, it was demonstrated that CB Treg cells exhibit a naive phenotype and are capable of suppressing Tcon cells in the presence of adult APCs and polyclonal stimulation. In addition, a minimum cell ratio for CB Treg cell suppression of 1:4 (Treg cells to Tcon cells) was determined, and this was used for the suppression assays with NK cells. Consistent with published data, it was observed that CB Treg cells can secrete high levels of TGF-β, regardless of their TCR-stimulation state, and produce IL-10 upon TCR-stimulation (Milward et al., 2013). Finally, the homing properties of CB Treg cells were analysed and it was demonstrated that these cells exhibit high expression of surface markers associated with migration to the BM, LN and gut, but have low expression of surface markers associated with migration to inflammatory sites. These results were then compared with those published for the homing repertoire of CB NK cells (Luevano et al., 2012a) and it was found that both Treg cells and NK cells express markers associated with migration to the BM, LN (under certain conditions) and inflammatory sites, suggesting that this interaction may take place in the aforementioned tissues.

7.3 TCR-stimulated umbilical cord blood regulatory T cells can supress umbilical cord blood natural killer cells in the absence of IL-2

It has been reported in humans and mice that Treg cells can regulate NK cell functions in the absence of cytokines, whereas NK cells would bypass this suppression and exert their effector functions upon the presence of exogenous cytokines such as IL-2, IL-4 or IL-7 (Ghiringhelli et al., 2005, Gasteiger et al.,

2013a, Gasteiger et al., 2013b). In **Chapter 4** the effects of CB Treg cells on CB NK cell effector functions were studied under different conditions. In contrast to PB Treg cells, freshly isolated allogeneic resting CB Treg cells did not suppress cytotoxicity of resting PB NK cells in the absence of cytokines, which may suggest an immature state of Treg cells and the requirement of IL-2 stimulation for CB Treg cells to be functional (Godfrey et al., 2005). Conversely, a decrease in expression of the NK cell activating receptors NKG2D, CD16, NKp46 and DNAM-1 was observed on resting CB NK cells when co-cultured with resting CB Treg cells (allogeneic and autologous), but this effect was transient as statistical significance decreased over the period studied.

When IL-2 was added to the cultures neither resting nor TCR-stimulated CB Treg cells abrogated CB NK cell functions. As mentioned previously, this could be due to the presence of IL-2 conferring NK cells with the ability to override Treg cell-mediated suppression (Ghiringhelli et al., 2005) and/or the need for CB NK cell maturation for suppression by Treg cells, since CB NK cells also exhibit an immature phenotype (Luevano et al., 2012a). Therefore, activated NK cells were co-cultured with resting or TCR-stimulated Treg cells in the absence of cytokines. Interestingly, TCR-stimulated CB Treg cells could suppress cytotoxicity of activated NK cells in the absence of exogenous IL-2, whereas resting CB Treg cells did not exert any suppression. These findings are in agreement with published data, which highlight the suppressive capacity of Treg cells on NK cells in the absence of cytokines (Ghiringhelli et al., 2005). Another possibility is that mature NK cells are more susceptible to Treg cell-mediated suppression, which is in agreement with the results from Sungur and colleagues (Sungur et al., 2013), who observed a preferential expansion of licensed NK cells over unlicensed NK cells during infection with mouse CMV when Treg cells were depleted. Based on the assumption that the degree of licensing correlates with NK cell maturation, it may be possible that Treg cells could also selectively suppress specific NK cell populations. Hence, it would be interesting to analyse the effect of TCR-stimulated CB Treg cells on functions (i.e. cytotoxicity and cytokine secretion) of sorted CB $CD56^{bright}$ and $CD56^{dim}$ NK cells.

Treg cells had no effect on NK cell viability in any of the conditions tested. These observations differ to what has been shown by Grossman and colleagues, who showed lysis of autologous cells by human PB Treg cells via granzyme A (Grossman et al., 2004a). This suggests that CB Treg cells may exhibit a different pattern of expression of granzyme A as compared to PB Treg cells. Importantly, allogeneic and autologous Treg cells had similar effects on CB NK cell functions. Unfortunately, the mechanism of suppression by which TCR-stimulated Treg cells suppress NK cells could not be elucidated due to high variability within the samples.

Another key finding presented in **Chapter 4** was the ability of CB activated NK cells to lyse CB Treg cells in the presence of IL-2 after 72 h co-culture. This is consistent with several reports that demonstrate the ability of NK cells to control adaptive responses during infection in humans and mice (Brillard et al., 2007, Roy et al., 2008, Chin et al., 2010). Therefore, the expression of Fas (CD95) on CB Treg cells was evaluated. Fas expression was higher in TCR-stimulated CB Treg cells than in resting CB Treg cells, suggesting Fas as a potential receptor that promotes Treg cell lysis by activated CB NK cells. Further studies, including blocking of Fas on CB Treg cells in the presence of activated CB NK cells, would need to be performed to fully elucidate the mechanism by which NK cells lyse TCR-stimulated CB Treg cells.

These findings are in agreement with those of Gasteiger and colleagues, who suggested that Treg cells are unable to suppress NK cells in the presence of IL-2 (Gasteiger et al., 2013a, Gasteiger et al., 2013b). However, further experiments using activated CB NK cells and CB TCR-stimulated Treg cells in the presence of different cytokines would need to be performed in order to provide strong evidence that this concept applies to the *in vitro* system used in the present study. Experiments using activated NK cells co-cultured with resting or TCR-stimulated CB Treg cells with the addition of APCs and $CD4^+$ Tcon cells would provide more insight into this process of regulation. Also, the analysis of granzyme B expression could demonstrate why CB Treg cells are not able to lyse CB NK cells as compared to PB Treg cells. Furthermore, since a decrease in expression of activating receptors on NK cells was observed when resting CB

Treg cells were co-cultured with CB NK cells, it would be interesting to further analyse the expression of membrane-bound TGF-β in resting CB Treg cells, as this molecule has been reported to decrease NK cell effector functions in the absence of TCR-stimulation for PB Treg cells (Ghiringhelli et al., 2005). Additionally, one could perform TGF-β blocking assays to further elucidate whether CB Treg cells use this mechanism to decrease expression of the NK cell activating receptors NKG2D, CD16, NKp46 and DNAM-1. As for the ability of CB activated NK cells to lyse CB Treg cells, further studies are necessary to assess how NK cells cause Treg cell lysis, to determine whether Fas is the main cognate receptor that promotes Treg cell lysis, and whether other molecules such as perforin and granzyme also play a role in Treg cell lysis by NK cells.

Moreover, it will be key to validate these results *in vivo*. One strategy could be to evaluate the impact of CB Treg cells on GvT in a humanised $Rag^{-/-}\gamma^{-/-}$ model of multiple myeloma, recently optimised by Guichelaar and colleagues (Guichelaar et al., 2013). In this model, the authors co-infused multiple myeloma cells with PBMCs with or without autologous PB iTreg cells. Using this model they showed that iTreg cells suppressed GvT depending on the location of the tumour. Hence, instead of PBMCs it would be interesting to infuse CBMCs or NK cells with or without TCR-stimulated CB Treg cells, to evaluate if CB Treg cells exert the same effect *in vivo*. The advantage of this model is that since multiple myeloma cells reside within the BM, it is suitable for studying the interaction between CB NK cells and CB Treg cells.

7.4 TCR-stimulated umbilical cord blood regulatory T cells can suppress natural killer cell differentiation via TGF-β

In **Chapter 5,** the effects of resting or TCR-stimulated CB Treg cells on NK cell differentiation were evaluated using an *in vitro* model of NK cell differentiation (Grzywacz et al., 2006, Luevano, 2013). There was approximately 90% reduction in NK cell count when TCR-stimulated CB Treg cells were added at

the time point when HSC commit to the NK cell lineage, whereas no effect was observed when resting Treg cells were added at the same time point. NK cells that developed in those cultures had a normal phenotype and exhibited normal IFN-γ production and cytotoxicity, except for the expression of CD16, which was slightly reduced. Importantly, high levels of IL-10 and TGF-β were observed in those HSC cultures in which TCR-stimulated CB Treg cells were added.

As it was shown that TCR-stimulated CB Treg cells inhibit NK cell differentiation when HSC commit to the NK cell lineage, **Chapter 6** focused on elucidating the mechanisms by which CB Treg cells mediate this effect. Since no APCs were present in this system, TGF-β and IL-10 were selected as candidates of inhibition of NK cell differentiation. First, blocking assays were performed with the addition of the TGF-β inhibitor SB 431542, a human IL-10Rα blocking antibody, or the combination of both. However, toxicity caused by the addition of the IL-10Rα blocking antibody and the lack of inhibition of the TGF-β pathway in this system did not allow this approach to be pursued further. For this reason, the observed TCR-stimulated Treg cell-effect was recapitulated by the addition of soluble TGF-β, IL-10 or the combination of both to HSC cultures. Addition of TGF-β to HSC cultures induced a similar reduction in NK cell counts as when TCR-stimulated Treg cells were added to HSC cultures at day 9, whereas no effect was observed in the presence of soluble IL-10. To further confirm TGF-β as the main mechanism of suppression in this system, the phosphorylation of Smad2/3 in HSC was investigated after addition of Treg cells, as Smad2/3 are involved in the TGF-β signalling pathway. However, phosphorylation was not observed within this timeframe. This can be explained by the technical difficulty in obtaining both positive and cellular signals at the same time since phosphorylation is transient (30-60 min after stimulation) (Wang et al., 2009). Phosphorylation in HSC was observed 30 min after the addition of soluble TGF-β whereas no effect was observed with the addition of CB Treg cells to HSC cultures at day 9. This timeframe was selected assuming that CB Treg cells may take a few minutes to form the immune synapse with HSC; however, it is possible that this process may take longer. It was also demonstrated that the TCR-stimulated Treg-cell mediated effect observed in NK cell differentiation is cell contact dependent and cytokine competition independent. To date, the

effects of Treg cells on NK cell differentiation has not being studied, but there is strong evidence that TGF-β is involved in abrogating NK cell differentiation (Marcoe et al., 2012). Hence, this work suggests that Treg cells may impact on NK cell differentiation via TGF-β.

In summary, the conditions and the mechanism of suppression by which CB Treg cells can impair NK cell differentiation was demonstrated. It would be interesting to include APCs (indirect pathway of suppression by Treg cells) and/or $CD4^+$ Tcon cells in this system, as it has been observed that the latter can enhance NK cell maturation (Freud et al., 2006). Also, because of the use of other cell sources for HSCT, it would be interesting to perform this study with mobilised PB HSC. Moreover, the analysis of various transcription factors involved in NK cell differentiation was presented but it would be valuable to extend this study by microarray analysis. This technique would allow the screening of up to 30 000 genes at a time, thus providing a thorough understanding of the molecular mechanisms by which Treg cells suppress NK cell differentiation. Another interesting observation in this chapter was that at the time point where the strongest effect was observed, total cell numbers in the cultures were maintained constant until the end of the experiment, suggesting that potentially cell proliferation is blocked in this system. Therefore, it would be interesting to analyse whether the cell cycle of HSC is affected in the presence of Treg cells.

Finally, to validate these results, the translation to an animal model would be necessary. An animal model has been set up in collaboration with Dr Michael Blundell and Prof Adrian Thrasher at the Institute of Child Health in London, UK. The use of $Rag^{-/-}\gamma^{-/-}$ mice, which are characterised by the lack of T cells, B cells and NK cells, were selected to test the effect of Treg cells on NK cell differentiation based on an established humanised model of NK cell differentiation (Huntington et al., 2008). After irradiation, HSC were added at 1:1 ratio with resting or TCR-stimulated Treg cells and followed for 10 weeks. However, Treg cell persistence was not observed at the end of this experiment. Hence, further optimisation is required to obtain Treg cell persistence to enable assessment of the effects of Treg cells on NK cell differentiation *in vivo*.

7.5 Translation to the clinic

Formerly, studies in mice have reported the potential impact of transferred Treg cells on immune reconstitution. Nguyen and colleagues showed in a mouse model of GvHD using mismatched HSC and adoptive transfer of donor Tcon cells and donor Treg cells (1:1 ratio) at day 0, enhanced immune cell reconstitution including NK cell reconstitution and improved viral clearance in comparison to mice injected with Tcon cells and not Treg cells (Nguyen et al., 2008). However, whether adoptive Treg cells can compromise NK cell mediated-GvL is still debatable. While the killing capacity of A20 leukaemia cells (GvL effect) was observed in BM-transplanted Balb/c mice in the presence of recipient-alloantigen-specific Treg cells, Treg cells compromised the GvL effect when a different mouse strain and a different tumour cell line was used (Trenado, 2003). This discrepancy can be explained by the different characteristics of the tumour cell lines used (i.e. tumour localisation). Guichelaar and colleagues analysed tumours residing in different tissues in a humanised $Rag^{-/-}\gamma^{-/-}$ model whereby the potential of GvT suppression by Treg cells could be assessed (Guichelaar et al., 2013). PBMCs were used to induce T cell-mediated GvT and iTreg cells, infused or not, were used to determine if they have an impact of GvT. As expected, mice with PBMCs alone effectively induced GvT responses reducing tumour size. However, co-infusion of Treg cells inhibited GvT against tumours located outside the BM, but not those inside the BM. Notably, the authors identified that the secretion of IL-1β and IL-6 by BM stromal cells, neutralised the suppressive capacity of Treg cells by inducing differentiation into Th17 cells, suggesting for the first time in a humanised model that Treg cells can suppress T-cell mediated GvT only outside the BM, whereas a protective environment was maintained inside the BM.

In humans, the impact of CB Treg cells on GvL and viral clearance has not been fully addressed. Currently, we know that higher susceptibility to viral reactivation was observed in CB Treg cell-treated patients compared to historical controls (Brunstein et al., 2013). Also, the adoptive transfer of donor Treg cells to control GvHD caused by high doses of infused Tcon cell did not

impair NK cell reconstitution/maturation in haploidentical transplanted-patients (Di Ianni et al., 2011). This may suggest that under these particular conditions, Treg cells do not impair NK cell mediated GvL, possibly due to the elevated HSC numbers infused that may override a potential Treg cell-mediated effect. Hence, it would be interesting to study NK cell reconstitution in patients infused with normal HSC doses. Notably, a Treg cell mediated effect has also been observed in other clinical settings. In AML patients adoptively transferred with NK cells, Miller and colleagues observed that if >5% of Treg cells are present when NK cells are infused, elevated expansion of Treg cells are observed, directly correlating with decreased NK cell expansions which suggests possible Treg cell-mediated suppression of NK cells (Miller et al., unpublished data). In summary, all these studies provide evidence that Treg cells impair GvT or GvL, and even viral clearance. However, the underlying processes of suppression by which these effects occur are still not understood. Knowing that NK cells play a role in GvL, it is possible that Treg cells will directly suppress NK cells in addition to having an impact on NK cell differentiation.

This study demonstrated for the first time that TCR-stimulated CB Treg cells impair CB NK cell effector functions and NK cell differentiation *in vitro*. Particularly, it was shown that TCR-stimulated CB Treg cells suppress CB NK cells only in “steady state” conditions (i.e. absence of IL-2), as this effect is not observed in the presence of IL-2. In the context of HSCT, the occurrence of a cytokine storm in transplanted patients is characteristic of aGvHD (Cohen et al., 2000). There are two main processes in aGvHD that are considered to induce a cytokine storm. First, the conditioning regimen induces tissue damages and leads to activation of host APCs. Secondly, activated host APCs can activate donor T cells leading to clonal expansion and subsequent activation of cellular and inflammatory effectors which collectively produce high levels of cytokines such as IL-2, TNF-α, IFN-γ, IL-1 and IL-6 (Antin and Ferrara, 1992, Ferrara, 1993, Ferrara et al., 2009). Although reduced, GvHD still occurs in CB transplanted patients (Takahashi et al., 2004, MacMillan et al., 2009, Ponce et al., 2013). Hence, based on the assumptions that CB Treg cells can only exert suppression in homeostatic conditions and that CB NK cells would overcome this suppression in the presence of cytokines, it is then unlikely that CB Treg

cells would inhibit NK cell effector functions in transplanted patients with aGvHD.

Another important finding in this study was that TCR-stimulated CB Treg cells inhibit NK cell differentiation, particularly when HSC commitment to the NK cell lineage occurs. Notably, this inhibition was found regardless of the presence of cytokines, thus suggesting a potential clinical impact of a Treg cell therapy. As previously mentioned, CBT is characterised by early recovery of NK cells but a prolonged T cell reconstitution. Beziat and colleagues evaluated immune reconstitution in a cohort of 25 CB transplanted patients under RIC regimen and found that in the first 30 days post-transplantation, no T cells or B cells were detected, whereas high numbers of NK cells were observed. Importantly, these NK cells exhibited phenotypic features associated with maturity, which made them capable of killing leukemic blasts *in vitro* (Beziat et al., 2009), thus suggesting that at early stages after transplantation, NK cells may be effectors of GvL and viral clearance. In this study, it was demonstrated for the first time that CB Treg cells impair NK cell differentiation *in vitro*, with the strongest effect observed when HSC commit to NK cell lineage. If these results are confirmed *in vivo*, then it would be necessary to reassess the time frame of infusion of Treg cells in patients as it could severely impair NK cell-mediated GvL and viral clearance in the early stages (<30 days) after HSCT, thereby increasing the risk of relapse and infections. It is not known when HSC commit to NK cells *in vivo*, but it is likely that this process would occur within the first two weeks post-transplantation. Hence, it is proposed that if a Treg cell therapy is utilised to prevent or modulate GvHD, it would be prudent to consider infusing these cells before or after HSC commitment to NK cell lineage has occurred, thus avoiding a potential inhibition of NK cell differentiation by Treg cells.

In conclusion, this study identified the conditions by which CB Treg cells inhibit NK cell effector functions and NK cell differentiation, thus providing valuable information for the future design of Treg cell-based therapies for modulation or prevention of GvHD in HSCT.

8 Appendix

8.1 List of publications

S. Rani, B. Afzali, A. Kelly, L. Tewolde-Berhan, M. Hackett, A. S. Kanhere, **I. Pedroza-Pacheco**, H. Bowen, S. Jurcevic, R. G. Jenner, D. J. Cousins, J. A. Ragheb, P. Lavender and S. John. IL-2 regulates expression of C-MAF in human CD4 T Cells, The Journal of Immunology, 2011, 187(7):3721-9.

I. Pedroza-Pacheco, A. Madrigal, and A. Saudemont. Interaction between Natural killer cells and Regulatory T cells: Perspectives for immunotherapy, Cellular and Molecular Immunology, 2013, 10(3):222-9.

M. Luevano, A. Domogala, **I. Pedroza-Pacheco**, S. Derniame, M. Escobedo-Cousin, S. Querol, A. Madrigal, and A. Saudemont. Frozen UCB HSC generate higher number of functional NK cells *in vitro* than mobilized HSC or freshly isolated UCB HSC, Accepted with minor revision, PlosOne.

S. Derniame, J. Perrazo, M. Escobedo-Cousin, R. Alnabhan, M. Luevano, **I. Pedroza-Pacheco**, A. Madrigal, and A. Saudemont. Differential effects of mycophenolate mofetil and cord blood natural killer cells activated with interleukin-2, Under review, Cytotherapy.

8.2 List of presentations

Sept 2013: I. Pedroza-Pacheco, Cord blood regulatory T cells exert suppression on NK cell differentiation, Poster at the 14th Meeting of the Society for Natural Immunity, Heidelberg, Germany.

Apr 2013: I. Pedroza-Pacheco, Cord blood regulatory T cells exert suppression on NK cell differentiation, Poster at the 39th Annual Meeting of the European Group for Blood and Marrow Transplantation (EBMT), London, UK.

Feb 2013: I. Pedroza-Pacheco, Effect of Regulatory T cells on Natural killer cell differentiation, Talk at the 2nd NK cell meeting UK, Imperial College, London, UK.

Sept 2012: I. Pedroza-Pacheco, Effect of cord blood Regulatory T cells on Natural killer cell differentiation and function, Poster at the European Congress of Immunology (ECI), Glasgow, Scotland.

May 2012: I. Pedroza-Pacheco, Effect of cord blood Regulatory T cells on Natural killer cell differentiation and function, Poster at the 10th Cancer Immunotherapy (CIMT) Annual Meeting, Mainz, Germany.

Feb 2012: I. Pedroza-Pacheco, Regulatory T cells and Natural killer cell interaction. Oral presentation at the student conference, Cancer Institute, London, UK.

8.3 List of awards

2011: Award: Primer Design student sponsorship.

2011: Kitty Cookson travel grant: CIMT Annual Meeting, Mainz, Germany.

2011: UCL Graduate school travel grant: ECI conference, Glasgow, Scotland.

2010: Conacyt research fellowship: PhD fees and maintenance, UCL.

2010: Anthony Nolan research stipend: PhD maintenance, UCL.

9 References

Abbas, A. K. 2010. *Cellular and molecular immunology,* Philadelphia, Saunders Elsevier.

Abbas, A. K., Benoist, C., Bluestone, J. A., Campbell, D. J., Ghosh, S., Hori, S., Jiang, S., Kuchroo, V. K., Mathis, D., Roncarolo, M. G., Rudensky, A., Sakaguchi, S., Shevach, E. M., Vignali, D. A. & Ziegler, S. F. 2013. Regulatory T cells: recommendations to simplify the nomenclature. *Nature Immunology,* 14**,** 307-8.

Abbas, A. K., Murphy, K. M. & Sher, A. 1996. Functional diversity of helper T lymphocytes. *Nature,* 383**,** 787-93.

Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F. & Napolitani, G. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol,* 8**,** 639-46.

Afzali, B., Mitchell, P. J., Edozie, F. C., Povoleri, G. A., Dowson, S. E., Demandt, L., Walter, G., Canavan, J. B., Scotta, C., Menon, B., Chana, P. S., Khamri, W., Kordasti, S. Y., Heck, S., Grimbacher, B., Tree, T., Cope, A. P., Taams, L. S., Lechler, R. I., John, S. & Lombardi, G. 2013. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur J Immunol,* 43**,** 2043-54.

Agaugue, S., Marcenaro, E., Ferranti, B., Moretta, L. & Moretta, A. 2008. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. *Blood,* 112**,** 1776-83.

Alferink, J., Tafuri, A., Vestweber, D., Hallmann, R., Hammerling, G. J. & Arnold, B. 1998. Control of neonatal tolerance to tissue antigens by peripheral T cell trafficking. *Science,* 282**,** 1338-41.

Allan, D. S., Keeney, M., Howson-Jan, K., Popma, J., Weir, K., Bhatia, M., Sutherland, D. R. & Chin-Yee, I. H. 2002. Number of viable CD34(+) cells reinfused predicts engraftment in autologous hematopoietic stem cell transplantation. *Bone Marrow Transplantation,* 29**,** 967-72.

Allan, S. E., Crome, S. Q., Crellin, N. K., Passerini, L., Steiner, T. S., Bacchetta, R., Roncarolo, M. G. & Levings, M. K. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *International immunology,* 19**,** 345-54.

Alter, G., Malenfant, J. M. & Altfeld, M. 2004. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of immunological methods,* 294**,** 15-22.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C. & Mathis, D. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science,* 298**,** 1395-401.

Andrews, D. M., Estcourt, M. J., Andoniou, C. E., Wikstrom, M. E., Khong, A., Voigt, V., Fleming, P., Tabarias, H., Hill, G. R., van der Most, R. G., Scalzo, A. A., Smyth, M. J. & Degli-Esposti, M. A. 2010. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. *The Journal of experimental medicine,* 207**,** 1333-43.

Annacker, O., Pimenta-Araujo, R., Burlen-Defranoux, O., Barbosa, T. C., Cumano, A. & Bandeira, A. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *Journal of immunology,* 166**,** 3008-18.

Annes, J. P. 2003. Making sense of latent TGFbeta activation. *Journal of Cell Science,* 116**,** 217-224.

Antin, J. H. & Ferrara, J. L. 1992. Cytokine dysregulation and acute graft-versus-host disease. *Blood,* 80**,** 2964-8.

Apperley, J. & Masszi, T. 2012. Graft-versus-host disease. *Haematopoietic Stem Cell Transplantation, ESH-EBMT Handbook.* 6th ed.

Apperley, J. F., Mauro, F. R., Goldman, J. M., Gregory, W., Arthur, C. K., Hows, J., Arcese, W., Papa, G., Mandelli, F., Wardle, D. & et al. 1988. Bone marrow transplantation for chronic myeloid leukaemia in first chronic phase: importance of a graft-versus-leukaemia effect. *Br J Haematol,* 69**,** 239-45.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, II, Umesaki, Y., Itoh, K. & Honda, K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science,* 331**,** 337-41.

Baecher-Allan, C., Brown, J. A., Freeman, G. J. & Hafler, D. A. 2001. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol,* 167**,** 1245-53.

Balaji, K. N., Schaschke, N., Machleidt, W., Catalfamo, M. & Henkart, P. A. 2002. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *The Journal of experimental medicine,* 196**,** 493-503.

Ballen, K. K., Gluckman, E. & Broxmeyer, H. E. 2013. Umbilical cord blood transplantation: the first 25 years and beyond. *Blood,* 122**,** 491-8.

Barao, I., Hanash, A. M., Hallett, W., Welniak, L. A., Sun, K., Redelman, D., Blazar, B. R., Levy, R. B. & Murphy, W. J. 2006. Suppression of natural killer cell-mediated bone marrow cell rejection by CD4+CD25+ regulatory T cells. *Proc Natl Acad Sci U S A,* 103**,** 5460-5.

Barber, D. F., Faure, M. & Long, E. O. 2004. LFA-1 contributes an early signal for NK cell cytotoxicity. *Journal of immunology,* 173**,** 3653-9.

Barker, J. N., Weisdorf, D. J., DeFor, T. E., Blazar, B. R., McGlave, P. B., Miller, J. S., Verfaillie, C. M. & Wagner, J. E. 2005. Transplantation of 2 partially HLA-matched umbilical cord blood units to enhance engraftment in adults with hematologic malignancy. *Blood,* 105**,** 1343-7.

Batista, F. D. & Harwood, N. E. 2009. The who, how and where of antigen presentation to B cells. *Nature reviews. Immunology,* 9**,** 15-27.

Batista, F. D., Iber, D. & Neuberger, M. S. 2001. B cells acquire antigen from target cells after synapse formation. *Nature,* 411**,** 489-94.

Baume, D. M., Robertson, M. J., Levine, H., Manley, T. J., Schow, P. W. & Ritz, J. 1992. Differential responses to interleukin 2 define functionally distinct subsets of human natural killer cells. *European Journal of Immunology,* 22**,** 1-6.

Bautista, J. L., Lio, C. W., Lathrop, S. K., Forbush, K., Liang, Y., Luo, J., Rudensky, A. Y. & Hsieh, C. S. 2009. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol,* 10**,** 610-7.

Becknell, B. & Caligiuri, M. A. 2005. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Advances in immunology,* 86**,** 209-39.

Beider, K., Nagler, A., Wald, O., Franitza, S., Dagan-Berger, M., Wald, H., Giladi, H., Brocke, S., Hanna, J., Mandelboim, O., Darash-Yahana, M., Galun, E. & Peled, A. 2003. Involvement of CXCR4 and IL-2 in the homing and retention of human NK and NK T cells to the bone marrow and spleen of NOD/SCID mice. *Blood,* 102**,** 1951-8.

Bellone, G., Aste-Amezaga, M., Trinchieri, G. & Rodeck, U. 1995. Regulation of NK cell functions by TGF-beta 1. *J Immunol,* 155**,** 1066-73.

Berahovich, R. D., Lai, N. L., Wei, Z., Lanier, L. L. & Schall, T. J. 2006. Evidence for NK cell subsets based on chemokine receptor expression. *Journal of immunology,* 177**,** 7833-40.

Bergmann, C., Wild, C. A., Narwan, M., Lotfi, R., Lang, S. & Brandau, S. 2011. Human tumor-induced and naturally occurring Treg cells differentially affect NK cells activated by either IL-2 or target cells. *European journal of immunology,* 41**,** 3564-73.

Beriou, G., Costantino, C. M., Ashley, C. W., Yang, L., Kuchroo, V. K., Baecher-Allan, C. & Hafler, D. A. 2009. IL-17-producing human peripheral regulatory T cells retain suppressive function. *Blood,* 113**,** 4240-9.

Bernardini, G., Sciume, G., Bosisio, D., Morrone, S., Sozzani, S. & Santoni, A. 2008. CCL3 and CXCL12 regulate trafficking of mouse bone marrow NK cell subsets. *Blood,* 111**,** 3626-34.

Betts, G., Jones, E., Junaid, S., El-Shanawany, T., Scurr, M., Mizen, P., Kumar, M., Jones, S., Rees, B., Williams, G., Gallimore, A. & Godkin, A. 2012. Suppression of tumour-specific CD4(+) T cells by regulatory T cells is associated with progression of human colorectal cancer. *Gut,* 61**,** 1163-71.

Beutler, B. 2004. Innate immunity: an overview. *Molecular immunology,* 40**,** 845-59.

Beziat, V., Nguyen, S., Lapusan, S., Hervier, B., Dhedin, N., Bories, D., Uzunov, M., Boudifa, A., Trebeden-Negre, H., Norol, F., Marjanovic, Z., Marie, J. P., Vernant, J. P., Debre, P., Rio, B. & Vieillard, V. 2009. Fully functional NK cells after unrelated cord blood transplantation. *Leukemia,* 23**,** 721-8.

Bielekova, B., Catalfamo, M., Reichert-Scrivner, S., Packer, A., Cerna, M., Waldmann, T. A., McFarland, H., Henkart, P. A. & Martin, R. 2006. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 5941-6.

Billadeau, D. D., Upshaw, J. L., Schoon, R. A., Dick, C. J. & Leibson, P. J. 2003. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol,* 4**,** 557-64.

Biron, C. A. 1997. Activation and function of natural killer cell responses during viral infections. *Current opinion in immunology,* 9**,** 24-34.

Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar-Mather, T. P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual review of immunology,* 17**,** 189-220.

Blazar, B. R., Murphy, W. J. & Abedi, M. 2012. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol,* 12**,** 443-58.

Bonadies, N., Pabst, T. & Mueller, B. U. 2010. Heterozygous deletion of the PU.1 locus in human AML. *Blood,* 115**,** 331-4.

Boos, M. D., Yokota, Y., Eberl, G. & Kee, B. L. 2007. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *The Journal of experimental medicine,* 204**,** 1119-30.

Borrego, F., Pena, J. & Solana, R. 1993. Regulation of CD69 expression on human natural killer cells: differential involvement of protein kinase C and protein tyrosine kinases. *Eur J Immunol,* 23**,** 1039-43.

Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Hopner, S., Centonze, D., Bernardi, G., Dell'Acqua, M. L., Rossini, P. M., Battistini, L., Rotzschke, O. & Falk, K. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood,* 110**,** 1225-32.

Bottino, C., Biassoni, R., Millo, R., Moretta, L. & Moretta, A. 2000. The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. *Human immunology,* 61**,** 1-6.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. & Forster, R. 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med,* 192**,** 1545-52.

Bresatz, S., Sadlon, T., Millard, D., Zola, H. & Barry, S. C. 2007. Isolation, propagation and characterization of cord blood derived CD4+ CD25+ regulatory T cells. *Journal of immunological methods,* 327**,** 53-62.

Brillard, E., Pallandre, J.-R., Chalmers, D., Ryffel, B., Radlovic, A., Seilles, E., Rohrlich, P. S., Pivot, X., Tiberghien, P., Saas, P. & Borg, C. 2007. Natural killer cells prevent CD28-mediated Foxp3 transcription in CD4+CD25– T lymphocytes. *Experimental Hematology,* 35**,** 416-425.

Brodin, P., Lakshmikanth, T., Johansson, S., Karre, K. & Hoglund, P. 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood,* 113**,** 2434-41.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F. & Ramsdell, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature genetics,* 27**,** 68-73.

Brunstein, C. G., Blazar, B. R., Miller, J. S., Cao, Q., Hippen, K. L., McKenna, D. H., Curtsinger, J., McGlave, P. B. & Wagner, J. E. 2013. Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation,* 19**,** 1271-3.

Brunstein, C. G., Fuchs, E. J., Carter, S. L., Karanes, C., Costa, L. J., Wu, J., Devine, S. M., Wingard, J. R., Aljitawi, O. S., Cutler, C. S., Jagasia, M. H., Ballen, K. K., Eapen, M. &

O'Donnell, P. V. 2011a. Alternative donor transplantation after reduced intensity conditioning: results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood,* 118**,** 282-8.

Brunstein, C. G., Miller, J. S., Cao, Q., McKenna, D. H., Hippen, K. L., Curtsinger, J., Defor, T., Levine, B. L., June, C. H., Rubinstein, P., McGlave, P. B., Blazar, B. R. & Wagner, J. E. 2010. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*.

Brunstein, C. G., Miller, J. S., Cao, Q., McKenna, D. H., Hippen, K. L., Curtsinger, J., Defor, T., Levine, B. L., June, C. H., Rubinstein, P., McGlave, P. B., Blazar, B. R. & Wagner, J. E. 2011b. Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood,* 117**,** 1061-70.

Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood,* 107**,** 159-66.

Byrd, A., Hoffmann, S. C., Jarahian, M., Momburg, F. & Watzl, C. 2007. Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44. *PLoS One,* 2**,** e1339.

Cai, L., Zhang, Z., Zhou, L., Wang, H., Fu, J., Zhang, S., Shi, M., Zhang, H., Yang, Y., Wu, H., Tien, P. & Wang, F. S. 2008. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. *Clinical immunology,* 129**,** 428-37.

Cai, Q., Dierich, A., Oulad-Abdelghani, M., Chan, S. & Kastner, P. 2009. Helios deficiency has minimal impact on T cell development and function. *Journal of immunology,* 183**,** 2303-11.

Caligiuri, M. A., Murray, C., Robertson, M. J., Wang, E., Cochran, K., Cameron, C., Schow, P., Ross, M. E., Klumpp, T. R., Soiffer, R. J. & et al. 1993. Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *The Journal of clinical investigation,* 91**,** 123-32.

Caligiuri, M. A., Zmuidzinas, A., Manley, T. J., Levine, H., Smith, K. A. & Ritz, J. 1990. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *The Journal of experimental medicine,* 171**,** 1509-26.

Cameron, M. J., Arreaza, G. A., Zucker, P., Chensue, S. W., Strieter, R. M., Chakrabarti, S. & Delovitch, T. L. 1997. IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *Journal of immunology,* 159**,** 4686-92.

Campbell, J. J., Qin, S., Unutmaz, D., Soler, D., Murphy, K. E., Hodge, M. R., Wu, L. & Butcher, E. C. 2001. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *Journal of immunology,* 166**,** 6477-82.

Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R. & Ley, T. J. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity,* 27**,** 635-46.

Carson, W. E., Lindemann, M. J., Baiocchi, R., Linett, M., Tan, J. C., Chou, C. C., Narula, S. & Caligiuri, M. A. 1995. The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood,* 85**,** 3577-85.

Castriconi, R., Cantoni, C., Della Chiesa, M., Vitale, M., Marcenaro, E., Conte, R., Biassoni, R., Bottino, C., Moretta, L. & Moretta, A. 2003. Transforming growth factor beta 1 inhibits

expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 4120-5.

Castriconi, R., Dondero, A., Bellora, F., Moretta, L., Castellano, A., Locatelli, F., Corrias, M. V., Moretta, A. & Bottino, C. 2013. Neuroblastoma-derived TGF-beta1 modulates the chemokine receptor repertoire of human resting NK cells. *Journal of immunology,* 190**,** 5321-8.

Cavaillon, J. M. 1994. Cytokines and macrophages. *Biomed Pharmacother,* 48**,** 445-53.

Cella, M., Fujikawa, K., Tassi, I., Kim, S., Latinis, K., Nishi, S., Yokoyama, W., Colonna, M. & Swat, W. 2004. Differential requirements for Vav proteins in DAP10- and ITAM-mediated NK cell cytotoxicity. *J Exp Med,* 200**,** 817-23.

Chalifour, A., Scarpellino, L., Back, J., Brodin, P., Devevre, E., Gros, F., Levy, F., Leclercq, G., Hoglund, P., Beermann, F. & Held, W. 2009. A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity,* 30**,** 337-47.

Challen, G. A., Boles, N. C., Chambers, S. M. & Goodell, M. A. 2010. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell stem cell,* 6**,** 265-78.

Chan, W. K., Kung Sutherland, M., Li, Y., Zalevsky, J., Schell, S. & Leung, W. 2012. Antibody-dependent cell-mediated cytotoxicity overcomes NK cell resistance in MLL-rearranged leukemia expressing inhibitory KIR ligands but not activating ligands. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 18**,** 6296-305.

Chang, C. C., Satwani, P., Oberfield, N., Vlad, G., Simpson, L. L. & Cairo, M. S. 2005. Increased induction of allogeneic-specific cord blood CD4+CD25+ regulatory T (Treg) cells: a comparative study of naive and antigenic-specific cord blood Treg cells. *Experimental Hematology,* 33**,** 1508-20.

Chen, G., Han, G., Wang, J., Wang, R., Xu, R., Shen, B., Qian, J. & Li, Y. 2009. Natural killer cells modulate overt autoimmunity to homeostasis in nonobese diabetic mice after anti-CD3 F(ab')2 antibody treatment through secreting transforming growth factor-beta. *The American Journal of Pathology,* 175**,** 1086-94.

Chen, M. L., Yan, B. S., Bando, Y., Kuchroo, V. K. & Weiner, H. L. 2008. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGFbeta-mediated function and enhanced suppression of experimental autoimmune encephalomyelitis. *Journal of immunology,* 180**,** 7327-37.

Chen, S., Kawashima, H., Lowe, J. B., Lanier, L. L. & Fukuda, M. 2005. Suppression of tumor formation in lymph nodes by L-selectin-mediated natural killer cell recruitment. *The Journal of experimental medicine,* 202**,** 1679-89.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. & Wahl, S. M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med,* 198**,** 1875-86.

Chia, J., Yeo, K. P., Whisstock, J. C., Dunstone, M. A., Trapani, J. A. & Voskoboinik, I. 2009. Temperature sensitivity of human perforin mutants unmasks subtotal loss of cytotoxicity, delayed FHL, and a predisposition to cancer. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 9809-14.

Chien, Y. H., Gascoigne, N. R., Kavaler, J., Lee, N. E. & Davis, M. M. 1984. Somatic recombination in a murine T-cell receptor gene. *Nature,* 309**,** 322-6.

Chin, A. I., Miyahira, A. K., Covarrubias, A., Teague, J., Guo, B., Dempsey, P. W. & Cheng, G. 2010. Toll-like receptor 3-mediated suppression of TRAMP prostate cancer shows the critical role of type I interferons in tumor immune surveillance. *Cancer research,* 70**,** 2595-603.

Chiossone, L., Vitale, C., Cottalasso, F., Moretti, S., Azzarone, B., Moretta, L. & Mingari, M. C. 2007. Molecular analysis of the methylprednisolone-mediated inhibition of NK-cell function: evidence for different susceptibility of IL-2- versus IL-15-activated NK cells. *Blood,* 109**,** 3767-75.

Choi, P. J. & Mitchison, T. J. 2013. Imaging burst kinetics and spatial coordination during serial killing by single natural killer cells. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 6488-93.

Choi, S. & Reddy, P. 2011. HDAC inhibition and graft versus host disease. *Mol Med,* 17**,** 404-16.

Civin, C. I., Strauss, L. C., Brovall, C., Fackler, M. J., Schwartz, J. F. & Shaper, J. H. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol,* 133**,** 157-65.

Cohen, S. B., Wang, X. N. & Dickinson, A. 2000. Can cord blood cells support the cytokine storm in GvHD? *Cytokine Growth Factor Rev,* 11**,** 185-97.

Colonna, M., Nakajima, H. & Cella, M. 1999. Inhibitory and activating receptors involved in immune surveillance by human NK and myeloid cells. *Journal of Leukocyte Biology,* 66**,** 718-22.

Colucci, F. 2001. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood,* 97**,** 2625-2632.

Condiotti, R., Zakai, Y. B., Barak, V. & Nagler, A. 2001. Ex vivo expansion of CD56+ cytotoxic cells from human umbilical cord blood. *Experimental Hematology,* 29**,** 104-13.

Cooper, M. A., Bush, J. E., Fehniger, T. A., VanDeusen, J. B., Waite, R. E., Liu, Y., Aguila, H. L. & Caligiuri, M. A. 2002. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood,* 100**,** 3633-8.

Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. 2001a. The biology of human natural killer-cell subsets. *Trends in immunology,* 22**,** 633-40.

Cooper, M. A., Fehniger, T. A., Ponnappan, A., Mehta, V., Wewers, M. D. & Caligiuri, M. A. 2001b. Interleukin-1beta costimulates interferon-gamma production by human natural killer cells. *European Journal of Immunology,* 31**,** 792-801.

Cooper, M. A., Fehniger, T. A., Turner, S. C., Chen, K. S., Ghaheri, B. A., Ghayur, T., Carson, W. E. & Caligiuri, M. A. 2001c. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood,* 97**,** 3146-51.

Copelan, E. A. 2006. Hematopoietic stem-cell transplantation. *N Engl J Med,* 354**,** 1813-26.

Cosmi, L., De Palma, R., Santarlasci, V., Maggi, L., Capone, M., Frosali, F., Rodolico, G., Querci, V., Abbate, G., Angeli, R., Berrino, L., Fambrini, M., Caproni, M., Tonelli, F., Lazzeri, E., Parronchi, P., Liotta, F., Maggi, E., Romagnani, S. & Annunziato, F. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. *J Exp Med,* 205**,** 1903-16.

Cosmi, L., Maggi, L., Santarlasci, V., Capone, M., Cardilicchia, E., Frosali, F., Querci, V., Angeli, R., Matucci, A., Fambrini, M., Liotta, F., Parronchi, P., Maggi, E., Romagnani, S. & Annunziato, F. 2010. Identification of a novel subset of human circulating memory

CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol,* 125**,** 222-30 e1-4.

Crome, S. Q., Lang, P. A., Lang, K. S. & Ohashi, P. S. 2013. Natural killer cells regulate diverse T cell responses. *Trends in immunology*.

Cupedo, T., Crellin, N. K., Papazian, N., Rombouts, E. J., Weijer, K., Grogan, J. L., Fibbe, W. E., Cornelissen, J. J. & Spits, H. 2009. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nature Immunology,* 10**,** 66-74.

Curotto de Lafaille, M. A., Kutchukhidze, N., Shen, S., Ding, Y., Yee, H. & Lafaille, J. J. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity,* 29**,** 114-26.

Curotto de Lafaille, M. A. & Lafaille, J. J. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity,* 30**,** 626-35.

D'Andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M. & Trinchieri, G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med,* 178**,** 1041-8.

Daniel, V., Sadeghi, M., Wang, H. & Opelz, G. 2013. CD4+ CD25+ Foxp3+ IFNgamma+ CD178+ human induced Treg (iTreg) contribute to suppression of alloresponses by apoptosis of responder cells. *Human immunology,* 74**,** 151-62.

Davies, J. K., Gribben, J. G., Brennan, L. L., Yuk, D., Nadler, L. M. & Guinan, E. C. 2008. Outcome of alloanergized haploidentical bone marrow transplantation after ex vivo costimulatory blockade: results of 2 phase 1 studies. *Blood,* 112**,** 2232-41.

Davis, D. M., Chiu, I., Fassett, M., Cohen, G. B., Mandelboim, O. & Strominger, J. L. 1999. The human natural killer cell immune synapse. *Proc Natl Acad Sci U S A,* 96**,** 15062-7.

Davis, S. J. & van der Merwe, P. A. 2001. The immunological synapse: required for T cell receptor signalling or directing T cell effector function? *Curr Biol,* 11**,** R289-91.

Davis, S. J. & van der Merwe, P. A. 2006. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol,* 7**,** 803-9.

De Maria, A., Bozzano, F., Cantoni, C. & Moretta, L. 2011. Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 728-32.

Delaney, C., Heimfeld, S., Brashem-Stein, C., Voorhies, H., Manger, R. L. & Bernstein, I. D. 2010. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nature medicine,* 16**,** 232-6.

Delves, P. J. & Roitt, I. M. 2000a. The immune system. First of two parts. *The New England journal of medicine,* 343**,** 37-49.

Delves, P. J. & Roitt, I. M. 2000b. The immune system. Second of two parts. *The New England journal of medicine,* 343**,** 108-17.

Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol,* 2**,** 1032-9.

Di Ianni, M., Falzetti, F., Carotti, A., Terenzi, A., Castellino, F., Bonifacio, E., Del Papa, B., Zei, T., Ostini, R. I., Cecchini, D., Aloisi, T., Perruccio, K., Ruggeri, L., Balucani, C., Pierini,

A., Sportoletti, P., Aristei, C., Falini, B., Reisner, Y., Velardi, A., Aversa, F. & Martelli, M. F. 2011. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood,* 117**,** 3921-8.

Di Santo, J. P. 2006. Natural killer cell developmental pathways: a question of balance. *Annual review of immunology,* 24**,** 257-86.

Di Stasi, A., Tey, S. K., Dotti, G., Fujita, Y., Kennedy-Nasser, A., Martinez, C., Straathof, K., Liu, E., Durett, A. G., Grilley, B., Liu, H., Cruz, C. R., Savoldo, B., Gee, A. P., Schindler, J., Krance, R. A., Heslop, H. E., Spencer, D. M., Rooney, C. M. & Brenner, M. K. 2011. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med,* 365**,** 1673-83.

Dieckmann, D., Plottner, H., Berchtold, S., Berger, T. & Schuler, G. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med,* 193**,** 1303-10.

Diehn, M., Alizadeh, A. A., Rando, O. J., Liu, C. L., Stankunas, K., Botstein, D., Crabtree, G. R. & Brown, P. O. 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc Natl Acad Sci U S A,* 99**,** 11796-801.

Ding, Y., Xu, J. & Bromberg, J. S. 2012. Regulatory T cell migration during an immune response. *Trends in immunology,* 33**,** 174-80.

Doubrovina, E. S., Doubrovin, M. M., Vider, E., Sisson, R. B., O'Reilly, R. J., Dupont, B. & Vyas, Y. M. 2003. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *Journal of immunology,* 171**,** 6891-9.

Doucey, M. A., Scarpellino, L., Zimmer, J., Guillaume, P., Luescher, I. F., Bron, C. & Held, W. 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nature Immunology,* 5**,** 328-36.

Duell, B. L., Carey, A. J., Tan, C. K., Cui, X., Webb, R. I., Totsika, M., Schembri, M. A., Derrington, P., Irving-Rodgers, H., Brooks, A. J., Cripps, A. W., Crowley, M. & Ulett, G. C. 2012. Innate transcriptional networks activated in bladder in response to uropathogenic Escherichia coli drive diverse biological pathways and rapid synthesis of IL-10 for defense against bacterial urinary tract infection. *Journal of immunology,* 188**,** 781-92.

Duffner, U., Lu, B., Hildebrandt, G. C., Teshima, T., Williams, D. L., Reddy, P., Ordemann, R., Clouthier, S. G., Lowler, K., Liu, C., Gerard, C., Cooke, K. R. & Ferrara, J. L. 2003. Role of CXCR3-induced donor T-cell migration in acute GVHD. *Experimental Hematology,* 31**,** 897-902.

Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D. J. 2012. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells. *Blood,* 119**,** 4430-40.

Eckmann, L. 2005. Defence molecules in intestinal innate immunity against bacterial infections. *Current opinion in gastroenterology,* 21**,** 147-51.

Edinger, M. & Hoffmann, P. 2011. Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol,* 23**,** 679-84.

Eikawa, S., Ohue, Y., Kitaoka, K., Aji, T., Uenaka, A., Oka, M. & Nakayama, E. 2010. Enrichment of Foxp3+ CD4 regulatory T cells in migrated T cells to IL-6- and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6. *Journal of immunology,* 185**,** 6734-40.

Erle, D. J., Briskin, M. J., Butcher, E. C., Garcia-Pardo, A., Lazarovits, A. I. & Tidswell, M. 1994. Expression and function of the MAdCAM-1 receptor, integrin alpha 4 beta 7, on human leukocytes. *Journal of immunology,* 153**,** 517-28.

Ermann, J., Hoffmann, P., Edinger, M., Dutt, S., Blankenberg, F. G., Higgins, J. P., Negrin, R. S., Fathman, C. G. & Strober, S. 2005. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. *Blood,* 105**,** 2220-6.

Escribano, L., Ocqueteau, M., Almeida, J., Orfao, A. & San Miguel, J. F. 1998. Expression of the c-kit (CD117) molecule in normal and malignant hematopoiesis. *Leuk Lymphoma,* 30**,** 459-66.

Evans, E. J., Esnouf, R. M., Manso-Sancho, R., Gilbert, R. J., James, J. R., Yu, C., Fennelly, J. A., Vowles, C., Hanke, T., Walse, B., Hunig, T., Sorensen, P., Stuart, D. I. & Davis, S. J. 2005. Crystal structure of a soluble CD28-Fab complex. *Nat Immunol,* 6**,** 271-9.

Fallarino, F., Grohmann, U., Hwang, K. W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M. L., Fioretti, M. C., Alegre, M. L. & Puccetti, P. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nature immunology,* 4**,** 1206-12.

Fehniger, T. A., Cai, S. F., Cao, X., Bredemeyer, A. J., Presti, R. M., French, A. R. & Ley, T. J. 2007. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. *Immunity,* 26**,** 798-811.

Fehniger, T. A., Cooper, M. A., Nuovo, G. J., Cella, M., Facchetti, F., Colonna, M. & Caligiuri, M. A. 2003. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood,* 101**,** 3052-7.

Ferlazzo, G., Pack, M., Thomas, D., Paludan, C., Schmid, D., Strowig, T., Bougras, G., Muller, W. A., Moretta, L. & Munz, C. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc Natl Acad Sci U S A,* 101**,** 16606-11.

Ferlazzo, G., Tsang, M. L., Moretta, L., Melioli, G., Steinman, R. M. & Munz, C. 2002. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *The Journal of experimental medicine,* 195**,** 343-51.

Fernandez, N. C., Treiner, E., Vance, R. E., Jamieson, A. M., Lemieux, S. & Raulet, D. H. 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood,* 105**,** 4416-23.

Ferrara, J. L. 1993. Cytokine dysregulation as a mechanism of graft versus host disease. *Curr Opin Immunol,* 5**,** 794-9.

Ferrara, J. L., Levine, J. E., Reddy, P. & Holler, E. 2009. Graft-versus-host disease. *Lancet,* 373**,** 1550-61.

Feuerer, M., Beckhove, P., Bai, L., Solomayer, E. F., Bastert, G., Diel, I. J., Pedain, C., Oberniedermayr, M., Schirrmacher, V. & Umansky, V. 2001. Therapy of human tumors in NOD/SCID mice with patient-derived reactivated memory T cells from bone marrow. *Nature medicine,* 7**,** 452-8.

Feuerer, M., Beckhove, P., Garbi, N., Mahnke, Y., Limmer, A., Hommel, M., Hammerling, G. J., Kyewski, B., Hamann, A., Umansky, V. & Schirrmacher, V. 2003. Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nature medicine,* 9**,** 1151-7.

Figueroa-Tentori, D., Querol, S., Dodi, I. A., Madrigal, A. & Duggleby, R. 2008. High purity and yield of natural Tregs from cord blood using a single step selection method. *J Immunol Methods,* 339**,** 228-35.

Filipovich, A. H., Weisdorf, D., Pavletic, S., Socie, G., Wingard, J. R., Lee, S. J., Martin, P., Chien, J., Przepiorka, D., Couriel, D., Cowen, E. W., Dinndorf, P., Farrell, A., Hartzman, R., Henslee-Downey, J., Jacobsohn, D., McDonald, G., Mittleman, B., Rizzo, J. D., Robinson, M., Schubert, M., Schultz, K., Shulman, H., Turner, M., Vogelsang, G. & Flowers, M. E. 2005. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant,* 11**,** 945-56.

Flannagan, R. S., Jaumouille, V. & Grinstein, S. 2012. The cell biology of phagocytosis. *Annual review of pathology,* 7**,** 61-98.

Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H. D., Bopp, T., Schmitt, E., Klein-Hessling, S., Serfling, E., Hamann, A. & Huehn, J. 2007. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol,* 5**,** e38.

Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature immunology,* 4**,** 330-6.

Frassoni, F., Varaldo, R., Gualandi, F., Bacigalupo, A., Sambuceti, G., Sacchi, N. & Podesta, M. 2010. The intra-bone marrow injection of cord blood cells extends the possibility of transplantation to the majority of patients with malignant hematopoietic diseases. *Best Pract Res Clin Haematol,* 23**,** 237-44.

Freeman, S. D., Kelm, S., Barber, E. K. & Crocker, P. R. 1995. Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. *Blood,* 85**,** 2005-12.

Freud, A. G. & Caligiuri, M. A. 2006. Human natural killer cell development. *Immunological reviews,* 214**,** 56-72.

Freud, A. G., Yokohama, A., Becknell, B., Lee, M. T., Mao, H. C., Ferketich, A. K. & Caligiuri, M. A. 2006. Evidence for discrete stages of human natural killer cell differentiation in vivo. *The Journal of experimental medicine,* 203**,** 1033-43.

Fujimaki, W., Takahashi, N., Ohnuma, K., Nagatsu, M., Kurosawa, H., Yoshida, S., Dang, N. H., Uchiyama, T. & Morimoto, C. 2008. Comparative study of regulatory T cell function of human CD25CD4 T cells from thymocytes, cord blood, and adult peripheral blood. *Clinical & developmental immunology,* 2008**,** 305859.

Fujisaki, J., Wu, J., Carlson, A. L., Silberstein, L., Putheti, P., Larocca, R., Gao, W., Saito, T. I., Lo Celso, C., Tsuyuzaki, H., Sato, T., Cote, D., Sykes, M., Strom, T. B., Scadden, D. T. & Lin, C. P. 2011. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature,* 474**,** 216-9.

Fukaura, H., Kent, S. C., Pietrusewicz, M. J., Khoury, S. J., Weiner, H. L. & Hafler, D. A. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *The Journal of clinical investigation,* 98**,** 70-7.

Fukuda, M. 1991. Lysosomal membrane glycoproteins. Structure, biosynthesis, and intracellular trafficking. *The Journal of biological chemistry,* 266**,** 21327-30.

Gallez-Hawkins, G. M., Franck, A. E., Li, X., Thao, L., Oki, A., Gendzekhadze, K., Dagis, A., Palmer, J., Nakamura, R., Forman, S. J., Senitzer, D. & Zaia, J. A. 2011. Expression of activating KIR2DS2 and KIR2DS4 genes after hematopoietic cell transplantation: relevance to cytomegalovirus infection. *Biol Blood Marrow Transplant,* 17**,** 1662-72.

Galy, A., Travis, M., Cen, D. & Chen, B. 1995. Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. *Immunity,* 3**,** 459-73.

Ganal, S. C., Sanos, S. L., Kallfass, C., Oberle, K., Johner, C., Kirschning, C., Lienenklaus, S., Weiss, S., Staeheli, P., Aichele, P. & Diefenbach, A. 2012. Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity,* 37**,** 171-86.

Gao, Y., Lin, F., Su, J., Gao, Z., Li, Y., Yang, J., Deng, Z., Liu, B., Tsun, A. & Li, B. 2012. Molecular mechanisms underlying the regulation and functional plasticity of FOXP3(+) regulatory T cells. *Genes and immunity,* 13**,** 1-13.

Gardner, J. M., Fletcher, A. L., Anderson, M. S. & Turley, S. J. 2009. AIRE in the thymus and beyond. *Curr Opin Immunol,* 21**,** 582-9.

Garin, M. I., Chu, C. C., Golshayan, D., Cernuda-Morollon, E., Wait, R. & Lechler, R. I. 2007. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood,* 109**,** 2058-65.

Garrod, K. R., Wei, S. H., Parker, I. & Cahalan, M. D. 2007. Natural killer cells actively patrol peripheral lymph nodes forming stable conjugates to eliminate MHC-mismatched targets. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 12081-6.

Gascoyne, D. M., Long, E., Veiga-Fernandes, H., de Boer, J., Williams, O., Seddon, B., Coles, M., Kioussis, D. & Brady, H. J. 2009. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nature immunology,* 10**,** 1118-24.

Gasteiger, G., Hemmers, S., Bos, P. D., Sun, J. C. & Rudensky, A. Y. 2013a. IL-2-dependent adaptive control of NK cell homeostasis. *The Journal of experimental medicine,* 210**,** 1179-87.

Gasteiger, G., Hemmers, S., Firth, M. A., Le Floc'h, A., Huse, M., Sun, J. C. & Rudensky, A. Y. 2013b. IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. *The Journal of experimental medicine,* 210**,** 1167-78.

Gavin, M. A., Torgerson, T. R., Houston, E., DeRoos, P., Ho, W. Y., Stray-Pedersen, A., Ocheltree, E. L., Greenberg, P. D., Ochs, H. D. & Rudensky, A. Y. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 6659-64.

Gershon, R. K. & Kondo, K. 1970. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology,* 18**,** 723-37.

Geuking, M. B., Cahenzli, J., Lawson, M. A., Ng, D. C., Slack, E., Hapfelmeier, S., McCoy, K. D. & Macpherson, A. J. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity,* 34**,** 794-806.

Ghiringhelli, F., Menard, C., Terme, M., Flament, C., Taieb, J., Chaput, N., Puig, P. E., Novault, S., Escudier, B., Vivier, E., Lecesne, A., Robert, C., Blay, J. Y., Bernard, J., Caillat-Zucman, S., Freitas, A., Tursz, T., Wagner-Ballon, O., Capron, C., Vainchencker, W., Martin, F. & Zitvogel, L. 2005. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *The Journal of experimental medicine,* 202**,** 1075-85.

Gilfillan, S., Chan, C. J., Cella, M., Haynes, N. M., Rapaport, A. S., Boles, K. S., Andrews, D. M., Smyth, M. J. & Colonna, M. 2008. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *The Journal of experimental medicine,* 205**,** 2965-73.

Gluckman, E. 2012. Choice of the donor according to HLA typing and stem cell source. *Haematopoietic Stem Cell Transplantation, ESH-EBMT Handbook.* 6 ed.

Gluckman, E., Broxmeyer, H. A., Auerbach, A. D., Friedman, H. S., Douglas, G. W., Devergie, A., Esperou, H., Thierry, D., Socie, G., Lehn, P. & et al. 1989. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N Engl J Med,* 321**,** 1174-8.

Gober, M. D., Fishelevich, R., Zhao, Y., Unutmaz, D. & Gaspari, A. A. 2008. Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis. *J Invest Dermatol,* 128**,** 1460-9.

Godfrey, W. R., Spoden, D. J., Ge, Y. G., Baker, S. R., Liu, B., Levine, B. L., June, C. H., Blazar, B. R. & Porter, S. B. 2005. Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood,* 105**,** 750-8.

Goldrath, A. W. & Bevan, M. J. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature,* 402**,** 255-62.

Gomez-Almaguer, D., Ruiz-Arguelles, G. J., del Carmen Tarin-Arzaga, L., Gonzalez-Llano, O., Gutierrez-Aguirre, H., Cantu-Rodriguez, O., Jaime-Perez, J., Carrasco-Yalan, A. & Giralt, S. 2008. Alemtuzumab for the treatment of steroid-refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant,* 14**,** 10-5.

Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. & Noelle, R. J. 2005. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *Journal of immunology,* 174**,** 1783-6.

Goulmy, E. 1996. Human minor histocompatibility antigens. *Curr Opin Immunol,* 8**,** 75-81.

Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J. H., Vossen, J., Gratwohl, A., Vogelsang, G. B., van Houwelingen, H. C. & van Rood, J. J. 1996. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med,* 334**,** 281-5.

Gracie, J. A., Robertson, S. E. & McInnes, I. B. 2003. Interleukin-18. *Journal of Leukocyte Biology,* 73**,** 213-24.

Grainger, J. R., Smith, K. A., Hewitson, J. P., McSorley, H. J., Harcus, Y., Filbey, K. J., Finney, C. A., Greenwood, E. J., Knox, D. P., Wilson, M. S., Belkaid, Y., Rudensky, A. Y. & Maizels, R. M. 2010. Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway. *J Exp Med,* 207**,** 2331-41.

Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. & Dustin, M. L. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science,* 285**,** 221-7.

Gratwohl, A. & Carreras, E. 2012. Principles of conditioning. *Haematopoietic Stem Cell Transplantation, ESH-EBMT Handbook.* 6 ed.

Gri, G., Piconese, S., Frossi, B., Manfroi, V., Merluzzi, S., Tripodo, C., Viola, A., Odom, S., Rivera, J., Colombo, M. P. & Pucillo, C. E. 2008. CD4+CD25+ regulatory T cells suppress mast cell degranulation and allergic responses through OX40-OX40L interaction. *Immunity,* 29**,** 771-81.

Griffith, L. M., Pavletic, S. Z., Lee, S. J., Martin, P. J., Schultz, K. R. & Vogelsang, G. B. 2008. Chronic Graft-versus-Host Disease--implementation of the National Institutes of Health Consensus Criteria for Clinical Trials. *Biol Blood Marrow Transplant,* 14**,** 379-84.

Grindebacke, H., Stenstad, H., Quiding-Jarbrink, M., Waldenstrom, J., Adlerberth, I., Wold, A. E. & Rudin, A. 2009. Dynamic development of homing receptor expression and memory

cell differentiation of infant CD4+CD25high regulatory T cells. *Journal of immunology,* 183**,** 4360-70.

Grossman, W. J., Revell, P. A., Lu, Z. H., Johnson, H., Bredemeyer, A. J. & Ley, T. J. 2003. The orphan granzymes of humans and mice. *Current opinion in immunology,* 15**,** 544-52.

Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P. & Ley, T. J. 2004a. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity,* 21**,** 589-601.

Grossman, W. J., Verbsky, J. W., Tollefsen, B. L., Kemper, C., Atkinson, J. P. & Ley, T. J. 2004b. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood,* 104**,** 2840-8.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M. G. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature,* 389**,** 737-42.

Grzywacz, B., Kataria, N., Sikora, M., Oostendorp, R. A., Dzierzak, E. A., Blazar, B. R., Miller, J. S. & Verneris, M. R. 2006. Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells. *Blood,* 108**,** 3824-33.

Guichelaar, T., Emmelot, M. E., Rozemuller, H., Martini, B., Groen, R. W., Storm, G., Lokhorst, H. M., Martens, A. C. & Mutis, T. 2013. Human regulatory T cells do not suppress the antitumor immunity in the bone marrow: a role for bone marrow stromal cells in neutralizing regulatory T cells. *Clin Cancer Res,* 19**,** 1467-75.

Haas, J., Hug, A., Viehover, A., Fritzsching, B., Falk, C. S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B., Storch-Hagenlocher, B., Krammer, P. H., Suri-Payer, E. & Wildemann, B. 2005. Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol,* 35**,** 3343-52.

Haddad, R., Guardiola, P., Izac, B., Thibault, C., Radich, J., Delezoide, A. L., Baillou, C., Lemoine, F. M., Gluckman, J. C., Pflumio, F. & Canque, B. 2004. Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood,* 104**,** 3918-26.

Haller, O. & Wigzell, H. 1977. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. *J Immunol,* 118**,** 1503-6.

Hallett, W. H., Ames, E., Alvarez, M., Barao, I., Taylor, P. A., Blazar, B. R. & Murphy, W. J. 2008. Combination therapy using IL-2 and anti-CD25 results in augmented natural killer cell-mediated antitumor responses. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation,* 14**,** 1088-99.

Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H. & Allison, J. P. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature,* 356**,** 607-9.

Haribhai, D., Williams, J. B., Jia, S., Nickerson, D., Schmitt, E. G., Edwards, B., Ziegelbauer, J., Yassai, M., Li, S. H., Relland, L. M., Wise, P. M., Chen, A., Zheng, Y. Q., Simpson, P. M., Gorski, J., Salzman, N. H., Hessner, M. J., Chatila, T. A. & Williams, C. B. 2011. A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity,* 35**,** 109-22.

Hayday, A. & Tigelaar, R. 2003. Immunoregulation in the tissues by gammadelta T cells. *Nature reviews. Immunology,* 3**,** 233-42.

Hegazy, A. N., Peine, M., Helmstetter, C., Panse, I., Frohlich, A., Bergthaler, A., Flatz, L., Pinschewer, D. D., Radbruch, A. & Lohning, M. 2010. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity,* 32**,** 116-28.

Helander, T. S. & Timonen, T. 1998. Adhesion in NK cell function. *Current topics in microbiology and immunology,* 230**,** 89-99.

Herberman, R. B., Nunn, M. E., Holden, H. T. & Lavrin, D. H. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *International Journal of Cancer,* 16**,** 230-239.

Herrera, O. B., Golshayan, D., Tibbott, R., Salcido Ochoa, F., James, M. J., Marelli-Berg, F. M. & Lechler, R. I. 2004. A novel pathway of alloantigen presentation by dendritic cells. *J Immunol,* 173**,** 4828-37.

Hertoghs, K. M., Moerland, P. D., van Stijn, A., Remmerswaal, E. B., Yong, S. L., van de Berg, P. J., van Ham, S. M., Baas, F., ten Berge, I. J. & van Lier, R. A. 2010. Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *The Journal of clinical investigation,* 120**,** 4077-90.

Hobo, W., Broen, K., van der Velden, W. J., Greupink-Draaisma, A., Adisty, N., Wouters, Y., Kester, M., Fredrix, H., Jansen, J. H., van der Reijden, B., Falkenburg, J. H., de Witte, T., Preijers, F., Schattenberg, T., Feuth, T., Blijlevens, N. M., Schaap, N. & Dolstra, H. 2013. Association of disparities in known minor histocompatibility antigens with relapse-free survival and graft-versus-host disease after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant,* 19**,** 274-82.

Hoffmann, P., Eder, R., Boeld, T. J., Doser, K., Piseshka, B., Andreesen, R. & Edinger, M. 2006. Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood,* 108**,** 4260-7.

Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. 2004. Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood,* 104**,** 895-903.

Hong, H. S., Ahmad, F., Eberhard, J. M., Bhatnagar, N., Bollmann, B. A., Keudel, P., Ballmaier, M., Zielinska-Skowronek, M., Schmidt, R. E. & Meyer-Olson, D. 2012. Loss of CCR7 expression on CD56(bright) NK cells is associated with a CD56(dim)CD16(+) NK cell-like phenotype and correlates with HIV viral load. *PLoS One,* 7**,** e44820.

Hori, S., Nomura, T. & Sakaguchi, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**,** 1057-61.

Houchins, J. P., Yabe, T., McSherry, C. & Bach, F. H. 1991. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *J Exp Med,* 173**,** 1017-20.

Hsu, P., Santner-Nanan, B., Dahlstrom, J. E., Fadia, M., Chandra, A., Peek, M. & Nanan, R. 2012. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. *The American Journal of Pathology,* 181**,** 2149-60.

Hua, J., Liang, S., Ma, X., Webb, T. J., Potter, J. P. & Li, Z. 2011. The interaction between regulatory T cells and NKT cells in the liver: a CD1d bridge links innate and adaptive immunity. *PLoS One,* 6**,** e27038.

Huan, J., Culbertson, N., Spencer, L., Bartholomew, R., Burrows, G. G., Chou, Y. K., Bourdette, D., Ziegler, S. F., Offner, H. & Vandenbark, A. A. 2005. Decreased FOXP3 levels in multiple sclerosis patients. *J Neurosci Res,* 81**,** 45-52.

Huang, C. T., Workman, C. J., Flies, D., Pan, X., Marson, A. L., Zhou, G., Hipkiss, E. L., Ravi, S., Kowalski, J., Levitsky, H. I., Powell, J. D., Pardoll, D. M., Drake, C. G. & Vignali, D. A. 2004. Role of LAG-3 in regulatory T cells. *Immunity,* 21**,** 503-13.

Huntington, N. D., Legrand, N., Alves, N. L., Jaron, B., Weijer, K., Plet, A., Corcuff, E., Mortier, E., Jacques, Y., Spits, H. & Di Santo, J. P. 2008. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *Journal of Experimental Medicine,* 206**,** 25-34.

Huntington, N. D., Legrand, N., Alves, N. L., Jaron, B., Weijer, K., Plet, A., Corcuff, E., Mortier, E., Jacques, Y., Spits, H. & Di Santo, J. P. 2009. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *The Journal of experimental medicine,* 206**,** 25-34.

Huntington, N. D., Nutt, S. L. & Carotta, S. 2013. Regulation of murine natural killer cell commitment. *Front Immunol,* 4**,** 14.

Huntington, N. D., Vosshenrich, C. A. & Di Santo, J. P. 2007. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nature reviews. Immunology,* 7**,** 703-14.

Hwang, E. S., Szabo, S. J., Schwartzberg, P. L. & Glimcher, L. H. 2005. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science,* 307**,** 430-3.

Hyka-Nouspikel, N., Lucian, L., Murphy, E., McClanahan, T. & Phillips, J. H. 2007. DAP10 deficiency breaks the immune tolerance against transplantable syngeneic melanoma. *Journal of immunology,* 179**,** 3763-71.

Ikawa, T., Kawamoto, H., Fujimoto, S. & Katsura, Y. 1999. Commitment of common T/Natural killer (NK) progenitors to unipotent T and NK progenitors in the murine fetal thymus revealed by a single progenitor assay. *J Exp Med,* 190**,** 1617-26.

Imamichi, H., Sereti, I. & Lane, H. C. 2008. IL-15 acts as a potent inducer of CD4(+)CD25(hi) cells expressing FOXP3. *European Journal of Immunology,* 38**,** 1621-30.

Inaba, K., Witmer, M. D. & Steinman, R. M. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J Exp Med,* 160**,** 858-76.

Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. & Hill, C. S. 2002. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular pharmacology,* 62**,** 65-74.

Jaatinen, T. & Laine, J. 2007. Isolation of hematopoietic stem cells from human cord blood. *Current protocols in stem cell biology,* Chapter 2**,** Unit 2A 2.

Jacobs, R., Stoll, M., Stratmann, G., Leo, R., Link, H. & Schmidt, R. E. 1992. CD16- CD56+ natural killer cells after bone marrow transplantation. *Blood,* 79**,** 3239-44.

Jacobson, C. A., Turki, A. T., McDonough, S. M., Stevenson, K. E., Kim, H. T., Kao, G., Herrera, M. I., Reynolds, C. G., Alyea, E. P., Ho, V. T., Koreth, J., Armand, P., Chen, Y. B., Ballen, K., Soiffer, R. J., Antin, J. H., Cutler, C. S. & Ritz, J. 2012. Immune reconstitution after double umbilical cord blood stem cell transplantation: comparison with unrelated peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant,* 18**,** 565-74.

Janeway, C. A., Jr. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today,* 13**,** 11-6.

Jenkins, M. K. & Schwartz, R. H. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med,* 165**,** 302-19.

Jevremovic, D., Billadeau, D. D., Schoon, R. A., Dick, C. J., Irvin, B. J., Zhang, W., Samelson, L. E., Abraham, R. T. & Leibson, P. J. 1999. Cutting edge: a role for the adaptor protein LAT in human NK cell-mediated cytotoxicity. *J Immunol,* 162**,** 2453-6.

Jinushi, M., Takehara, T., Tatsumi, T., Kanto, T., Groh, V., Spies, T., Kimura, R., Miyagi, T., Mochizuki, K., Sasaki, Y. & Hayashi, N. 2003. Expression and role of MICA and MICB in human hepatocellular carcinomas and their regulation by retinoic acid. *International journal of cancer. Journal international du cancer,* 104**,** 354-61.

Johansson, M. E., Ambort, D., Pelaseyed, T., Schutte, A., Gustafsson, J. K., Ermund, A., Subramani, D. B., Holmen-Larsson, J. M., Thomsson, K. A., Bergstrom, J. H., van der Post, S., Rodriguez-Pineiro, A. M., Sjovall, H., Backstrom, M. & Hansson, G. C. 2011. Composition and functional role of the mucus layers in the intestine. *Cellular and molecular life sciences : CMLS,* 68**,** 3635-41.

Joncker, N. T., Fernandez, N. C., Treiner, E., Vivier, E. & Raulet, D. H. 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *Journal of immunology,* 182**,** 4572-80.

Joncker, N. T. & Raulet, D. H. 2008. Regulation of NK cell responsiveness to achieve self-tolerance and maximal responses to diseased target cells. *Immunological reviews,* 224**,** 85-97.

Jonuleit, H. & Schmitt, E. 2003. The regulatory T cell family: distinct subsets and their interrelations. *Journal of immunology,* 171**,** 6323-7.

Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J. & Enk, A. H. 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med,* 193**,** 1285-94.

Josefowicz, S. Z., Lu, L. F. & Rudensky, A. Y. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annual review of immunology,* 30**,** 531-64.

Junghanss, C., Marr, K. A., Carter, R. A., Sandmaier, B. M., Maris, M. B., Maloney, D. G., Chauncey, T., McSweeney, P. A. & Storb, R. 2002. Incidence and outcome of bacterial and fungal infections following nonmyeloablative compared with myeloablative allogeneic hematopoietic stem cell transplantation: a matched control study. *Biol Blood Marrow Transplant,* 8**,** 512-20.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K. J., Podack, E. R., Zinkernagel, R. M. & Hengartner, H. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature,* 369**,** 31-7.

Kale, V. P. 2004. Differential activation of MAPK signaling pathways by TGF-beta1 forms the molecular mechanism behind its dose-dependent bidirectional effects on hematopoiesis. *Stem cells and development,* 13**,** 27-38.

Kale, V. P. & Vaidya, A. A. 2004. Molecular mechanisms behind the dose-dependent differential activation of MAPK pathways induced by transforming growth factor-beta1 in hematopoietic cells. *Stem cells and development,* 13**,** 536-47.

Kane, L. P., Lin, J. & Weiss, A. 2002. It's all Rel-ative: NF-kappaB and CD28 costimulation of T-cell activation. *Trends Immunol,* 23**,** 413-20.

Karre, K. 2008. Natural killer cell recognition of missing self. *Nature Immunology,* 9**,** 477-80.

Karre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature,* 319**,** 675-8.

Kebriaei, P. & Robinson, S. 2011a. Mesenchymal stem cell therapy in the treatment of acute and chronic graft versus host disease. *Front Oncol,* 1**,** 16.

Kebriaei, P. & Robinson, S. 2011b. Treatment of graft-versus-host-disease with mesenchymal stromal cells. *Cytotherapy,* 13**,** 262-8.

Kelly, D., King, T. & Aminov, R. 2007. Importance of microbial colonization of the gut in early life to the development of immunity. *Mutation research,* 622**,** 58-69.

Kerdiles, Y., Ugolini, S. & Vivier, E. 2013. T cell regulation of natural killer cells. *The Journal of experimental medicine,* 210**,** 1065-8.

Khalil, N. 1999. TGF-beta: from latent to active. *Microbes and infection / Institut Pasteur,* 1**,** 1255-63.

Khattri, R., Cox, T., Yasayko, S. A. & Ramsdell, F. 2003. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol,* 4**,** 337-42.

Kiessling, R., Klein, E., Pross, H. & Wigzell, H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European Journal of Immunology,* 5**,** 117-21.

Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature immunology,* 8**,** 191-7.

Kim, S., Poursine-Laurent, J., Truscott, S. M., Lybarger, L., Song, Y. J., Yang, L., French, A. R., Sunwoo, J. B., Lemieux, S., Hansen, T. H. & Yokoyama, W. M. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature,* 436**,** 709-13.

King, A., Burrows, T., Verma, S., Hiby, S. & Loke, Y. W. 1998. Human uterine lymphocytes. *Human reproduction update,* 4**,** 480-5.

Kishimoto, K., Yuan, X., Auchincloss, H., Jr., Sharpe, A. H., Mandelbrot, D. A. & Sayegh, M. H. 2004. Mechanism of action of donor-specific transfusion in inducing tolerance: role of donor MHC molecules, donor co-stimulatory molecules, and indirect antigen presentation. *J Am Soc Nephrol,* 15**,** 2423-8.

Komanduri, K. V., St John, L. S., de Lima, M., McMannis, J., Rosinski, S., McNiece, I., Bryan, S. G., Kaur, I., Martin, S., Wieder, E. D., Worth, L., Cooper, L. J., Petropoulos, D., Molldrem, J. J., Champlin, R. E. & Shpall, E. J. 2007. Delayed immune reconstitution after cord blood transplantation is characterized by impaired thymopoiesis and late memory T-cell skewing. *Blood,* 110**,** 4543-51.

Komatsu, N., Mariotti-Ferrandiz, M. E., Wang, Y., Malissen, B., Waldmann, H. & Hori, S. 2009. Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A,* 106**,** 1903-8.

Kondo, M., Weissman, I. L. & Akashi, K. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell,* 91**,** 661-72.

Kottke, T., Galivo, F., Wongthida, P., Diaz, R. M., Thompson, J., Jevremovic, D., Barber, G. N., Hall, G., Chester, J., Selby, P., Harrington, K., Melcher, A. & Vile, R. G. 2008. Treg depletion-enhanced IL-2 treatment facilitates therapy of established tumors using systemically delivered oncolytic virus. *Mol Ther,* 16**,** 1217-26.

Kotylo, P. K., Baenzinger, J. C., Yoder, M. C., Engle, W. A. & Bolinger, C. D. 1990. Rapid analysis of lymphocyte subsets in cord blood. *American journal of clinical pathology,* 93**,** 263-6.

Kreslavsky, T. & von Boehmer, H. 2010. gammadeltaTCR ligands and lineage commitment. *Semin Immunol,* 22**,** 214-21.

Krzewski, K. & Coligan, J. E. 2012. Human NK cell lytic granules and regulation of their exocytosis. *Frontiers in immunology,* 3**,** 335.

Krzewski, K., Gil-Krzewska, A., Nguyen, V., Peruzzi, G. & Coligan, J. E. 2013. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood,* 121**,** 4672-83.

Kukreja, A., Cost, G., Marker, J., Zhang, C., Sun, Z., Lin-Su, K., Ten, S., Sanz, M., Exley, M., Wilson, B., Porcelli, S. & Maclaren, N. 2002. Multiple immuno-regulatory defects in type-1 diabetes. *J Clin Invest,* 109**,** 131-40.

Kumar, V., Ben-Ezra, J., Bennett, M. & Sonnenfeld, G. 1979. Natural killer cells in mice treated with 89strontium: normal target-binding cell numbers but inability to kill even after interferon administration. *J Immunol,* 123**,** 1832-8.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F. & Kosaka, H. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med,* 184**,** 923-30.

Kurts, C., Miller, J. F., Subramaniam, R. M., Carbone, F. R. & Heath, W. R. 1998. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J Exp Med,* 188**,** 409-14.

Lanier, L. L. 2008. Evolutionary struggles between NK cells and viruses. *Nature reviews. Immunology,* 8**,** 259-68.

Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R. & Phillips, J. H. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol,* 136**,** 4480-6.

Lanier, L. L. & Phillips, J. H. 1996. Inhibitory MHC class I receptors on NK cells and T cells. *Immunol Today,* 17**,** 86-91.

Lanier, L. L., Ruitenberg, J. J. & Phillips, J. H. 1988. Functional and biochemical analysis of CD16 antigen on natural killer cells and granulocytes. *Journal of immunology,* 141**,** 3478-85.

Lanier, L. L., Serafini, A. T., Ruitenberg, J. J., Cwirla, S., Federspiel, N. A., Phillips, J. H., Allison, J. P. & Weiss, A. 1987. The gamma T-cell antigen receptor. *Journal of clinical immunology,* 7**,** 429-40.

Lanier, L. L., Testi, R., Bindl, J. & Phillips, J. H. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *The Journal of experimental medicine,* 169**,** 2233-8.

Laouar, Y., Sutterwala, F. S., Gorelik, L. & Flavell, R. A. 2005. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nature Immunology,* 6**,** 600-7.

Larsson, J., Blank, U., Helgadottir, H., Bjornsson, J. M., Ehinger, M., Goumans, M. J., Fan, X., Leveen, P. & Karlsson, S. 2003. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood,* 102**,** 3129-35.

Larsson, J. & Karlsson, S. 2005. The role of Smad signaling in hematopoiesis. *Oncogene,* 24**,** 5676-92.

Laughlin, M. J., Eapen, M., Rubinstein, P., Wagner, J. E., Zhang, M. J., Champlin, R. E., Stevens, C., Barker, J. N., Gale, R. P., Lazarus, H. M., Marks, D. I., van Rood, J. J., Scaradavou, A. & Horowitz, M. M. 2004. Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. *N Engl J Med,* 351**,** 2265-75.

Law, R. H., Lukoyanova, N., Voskoboinik, I., Caradoc-Davies, T. T., Baran, K., Dunstone, M. A., D'Angelo, M. E., Orlova, E. V., Coulibaly, F., Verschoor, S., Browne, K. A., Ciccone, A., Kuiper, M. J., Bird, P. I., Trapani, J. A., Saibil, H. R. & Whisstock, J. C. 2010. The structural basis for membrane binding and pore formation by lymphocyte perforin. *Nature,* 468**,** 447-51.

Ledbetter, J. A., Imboden, J. B., Schieven, G. L., Grosmaire, L. S., Rabinovitch, P. S., Lindsten, T., Thompson, C. B. & June, C. H. 1990. CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood,* 75**,** 1531-9.

Lee, C. C., Lin, S. J., Cheng, P. J. & Kuo, M. L. 2009a. The regulatory function of umbilical cord blood CD4(+) CD25(+) T cells stimulated with anti-CD3/anti-CD28 and exogenous interleukin (IL)-2 or IL-15. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology,* 20**,** 624-32.

Lee, S. H., Fragoso, M. F. & Biron, C. A. 2012. Cutting edge: a novel mechanism bridging innate and adaptive immunity: IL-12 induction of CD25 to form high-affinity IL-2 receptors on NK cells. *Journal of immunology,* 189**,** 2712-6.

Lee, S. H., Kim, K. S., Fodil-Cornu, N., Vidal, S. M. & Biron, C. A. 2009b. Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *The Journal of experimental medicine,* 206**,** 2235-51.

Leung, M. W., Shen, S. & Lafaille, J. J. 2009. TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. *J Exp Med,* 206**,** 2121-30.

Levings, M. K., Sangregorio, R., Sartirana, C., Moschin, A. L., Battaglia, M., Orban, P. C. & Roncarolo, M. G. 2002. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med,* 196**,** 1335-46.

Li, L., Godfrey, W. R., Porter, S. B., Ge, Y., June, C. H., Blazar, B. R. & Boussiotis, V. A. 2005. CD4+CD25+ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood,* 106**,** 3068-73.

Lim, H. W., Hillsamer, P., Banham, A. H. & Kim, C. H. 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *Journal of immunology,* 175**,** 4180-3.

Liu, G. Z., Gomes, A. C., Fang, L. B., Gao, X. G. & Hjelmstrom, P. 2008a. Decreased 4-1BB expression on CD4+CD25 high regulatory T cells in peripheral blood of patients with multiple sclerosis. *Clin Exp Immunol,* 154**,** 22-9.

Liu, M. F., Lin, L. H., Weng, C. T. & Weng, M. Y. 2008b. Decreased CD4+CD25+bright T cells in peripheral blood of patients with primary Sjogren's syndrome. *Lupus,* 17**,** 34-9.

Liu, M. F., Wang, C. R., Fung, L. L. & Wu, C. R. 2004. Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand J Immunol,* 59**,** 198-202.

Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., Fazekas de St Groth, B., Clayberger, C., Soper, D. M., Ziegler, S. F. &

Bluestone, J. A. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine,* 203**,** 1701-11.

Liu, Y., Zhang, P., Li, J., Kulkarni, A. B., Perruche, S. & Chen, W. 2008c. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol,* 9**,** 632-40.

Ljunggren, H. G. & Karre, K. 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today,* 11**,** 237-44.

Locksley, R. M., Killeen, N. & Lenardo, M. J. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell,* 104**,** 487-501.

Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol,* 17**,** 875-904.

Long, E. O. 2007. Ready for prime time: NK cell priming by dendritic cells. *Immunity,* 26**,** 385-7.

Lopez, J. A., Susanto, O., Jenkins, M. R., Lukoyanova, N., Sutton, V. R., Law, R. H., Johnston, A., Bird, C. H., Bird, P. I., Whisstock, J. C., Trapani, J. A., Saibil, H. R. & Voskoboinik, I. 2013. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood,* 121**,** 2659-68.

Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity,* 26**,** 503-17.

Luevano, M. 2013. *Generation of natural killer cells from umbilical cord blood stem cells, characterisation and application for immunotherapy.* Doctor of Philosophy, University College London.

Luevano, M., Daryouzeh, M., Alnabhan, R., Querol, S., Khakoo, S., Madrigal, A. & Saudemont, A. 2012a. The unique profile of cord blood natural killer cells balances incomplete maturation and effective killing function upon activation. *Human immunology,* 73**,** 248-57.

Luevano, M., Madrigal, A. & Saudemont, A. 2012b. Generation of natural killer cells from hematopoietic stem cells in vitro for immunotherapy. *Cellular & molecular immunology,* 9**,** 310-20.

Luevano, M., Madrigal, A. & Saudemont, A. 2012c. Transcription factors involved in the regulation of natural killer cell development and function: an update. *Frontiers in immunology,* 3**,** 319.

Lundqvist, A., Yokoyama, H., Smith, A., Berg, M. & Childs, R. 2009. Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. *Blood,* 113**,** 6120-7.

Lyons, A. B. & Parish, C. R. 1994. Determination of lymphocyte division by flow cytometry. *Journal of immunological methods,* 171**,** 131-7.

Mackall, C., Fry, T., Gress, R., Peggs, K., Storek, J., Toubert, A., Center for International, B., Marrow Transplant, R., National Marrow Donor, P., European, B., Marrow Transplant, G., American Society of, B., Marrow, T., Canadian, B., Marrow Transplant, G., Infectious Disease Society of, A., Society for Healthcare Epidemiology of, A., Association of Medical, M., Infectious Diseases, C., Centers for Disease, C. & Prevention 2009. Background to hematopoietic cell transplantation, including post transplant immune recovery. *Bone Marrow Transplant,* 44**,** 457-62.

Mackinnon, S., Hows, J. M. & Goldman, J. M. 1990a. Induction of a syngeneic graft-versus-leukemia effect following bone marrow transplantation for chronic myeloid leukemia. *Leukemia,* 4**,** 287-91.

Mackinnon, S., Hows, J. M. & Goldman, J. M. 1990b. Induction of in vitro graft-versus-leukemia activity following bone marrow transplantation for chronic myeloid leukemia. *Blood,* 76**,** 2037-45.

MacLennan, I. C. 1994. Germinal centers. *Annual review of immunology,* 12**,** 117-39.

MacMillan, M. L., Weisdorf, D. J., Brunstein, C. G., Cao, Q., DeFor, T. E., Verneris, M. R., Blazar, B. R. & Wagner, J. E. 2009. Acute graft-versus-host disease after unrelated donor umbilical cord blood transplantation: analysis of risk factors. *Blood,* 113**,** 2410-5.

Male, V., Nisoli, I., Gascoyne, D. M. & Brady, H. J. 2012. E4BP4: an unexpected player in the immune response. *Trends in immunology,* 33**,** 98-102.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. & Weaver, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature,* 441**,** 231-4.

Mantel, P. Y., Ouaked, N., Ruckert, B., Karagiannidis, C., Welz, R., Blaser, K. & Schmidt-Weber, C. B. 2006. Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol,* 176**,** 3593-602.

Marcoe, J. P., Lim, J. R., Schaubert, K. L., Fodil-Cornu, N., Matka, M., McCubbrey, A. L., Farr, A. R., Vidal, S. M. & Laouar, Y. 2012. TGF-beta is responsible for NK cell immaturity during ontogeny and increased susceptibility to infection during mouse infancy. *Nature immunology,* 13**,** 843-50.

Martin-Fontecha, A., Lord, G. M. & Brady, H. J. 2011. Transcriptional control of natural killer cell differentiation and function. *Cellular and molecular life sciences : CMLS,* 68**,** 3495-503.

Matzinger, P. & Bevan, M. J. 1977. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? *Cell Immunol,* 29**,** 1-5.

Mauri, C. & Ehrenstein, M. R. 2008. The 'short' history of regulatory B cells. *Trends in immunology,* 29**,** 34-40.

Maury, S., Lemoine, F. M., Hicheri, Y., Rosenzwajg, M., Badoual, C., Cherai, M., Beaumont, J. L., Azar, N., Dhedin, N., Sirvent, A., Buzyn, A., Rubio, M. T., Vigouroux, S., Montagne, O., Bories, D., Roudot-Thoraval, F., Vernant, J. P., Cordonnier, C., Klatzmann, D. & Cohen, J. L. 2010. CD4+CD25+ regulatory T cell depletion improves the graft-versus-tumor effect of donor lymphocytes after allogeneic hematopoietic stem cell transplantation. *Science translational medicine,* 2**,** 41ra52.

Mazzucchelli, R. & Durum, S. K. 2007. Interleukin-7 receptor expression: intelligent design. *Nature reviews. Immunology,* 7**,** 144-54.

McCullar, V., Oostendorp, R., Panoskaltsis-Mortari, A., Yun, G., Lutz, C. T., Wagner, J. E. & Miller, J. S. 2008. Mouse fetal and embryonic liver cells differentiate human umbilical cord blood progenitors into CD56-negative natural killer cell precursors in the absence of interleukin-15. *Experimental Hematology,* 36**,** 598-608.

Menning, A., Hopken, U. E., Siegmund, K., Lipp, M., Hamann, A. & Huehn, J. 2007. Distinctive role of CCR7 in migration and functional activity of naive- and effector/memory-like Treg subsets. *European Journal of Immunology,* 37**,** 1575-83.

Miller, J. S., Alley, K. A. & McGlave, P. 1994. Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. *Blood,* 83**,** 2594-601.

Miller, J. S. & McCullar, V. 2001. Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. *Blood,* 98**,** 705-13.

Milward, K., Issa, F., Hester, J., Figueroa-Tentori, D., Madrigal, A. & Wood, K. J. 2013. Multiple unit pooled umbilical cord blood is a viable source of therapeutic regulatory T cells. *Transplantation,* 95**,** 85-93.

Miyara, M. & Sakaguchi, S. 2011. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. *Immunol Cell Biol,* 89**,** 346-51.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., Mathian, A., Nakahata, T., Yamaguchi, T., Nomura, T., Ono, M., Amoura, Z., Gorochov, G. & Sakaguchi, S. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity,* 30**,** 899-911.

Moffett-King, A. 2002. Natural killer cells and pregnancy. *Nature reviews. Immunology,* 2**,** 656-63.

Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature,* 395**,** 82-6.

Monteiro, M., Almeida, C. F., Caridade, M., Ribot, J. C., Duarte, J., Agua-Doce, A., Wollenberg, I., Silva-Santos, B. & Graca, L. 2010. Identification of regulatory Foxp3+ invariant NKT cells induced by TGF-beta. *Journal of immunology,* 185**,** 2157-63.

Moo-Young, T. A., Larson, J. W., Belt, B. A., Tan, M. C., Hawkins, W. G., Eberlein, T. J., Goedegebuure, P. S. & Linehan, D. C. 2009. Tumor-derived TGF-beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer. *Journal of immunotherapy,* 32**,** 12-21.

Moretta, A., Bottino, C., Pende, D., Tripodi, G., Tambussi, G., Viale, O., Orengo, A., Barbaresi, M., Merli, A., Ciccone, E. & et al. 1990a. Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *The Journal of experimental medicine,* 172**,** 1589-98.

Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., Biassoni, R. & Moretta, L. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annual review of immunology,* 19**,** 197-223.

Moretta, A., Tambussi, G., Bottino, C., Tripodi, G., Merli, A., Ciccone, E., Pantaleo, G. & Moretta, L. 1990b. A novel surface antigen expressed by a subset of human CD3-CD16+ natural killer cells. Role in cell activation and regulation of cytolytic function. *The Journal of experimental medicine,* 171**,** 695-714.

Moretta, A., Vitale, M., Bottino, C., Orengo, A. M., Morelli, L., Augugliaro, R., Barbaresi, M., Ciccone, E. & Moretta, L. 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *The Journal of experimental medicine,* 178**,** 597-604.

Moretta, L., Bottino, C., Pende, D., Mingari, M. C., Biassoni, R. & Moretta, A. 2002. Human natural killer cells: their origin, receptors and function. *European Journal of Immunology,* 32**,** 1205-11.

Morohashi, H., Miyawaki, T., Nomura, H., Kuno, K., Murakami, S., Matsushima, K. & Mukaida, N. 1995. Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. *Journal of Leukocyte Biology,* 57**,** 180-7.

Moser, B. & Loetscher, P. 2001. Lymphocyte traffic control by chemokines. *Nature Immunology,* 2**,** 123-8.

Mosmann, T. R. & Coffman, R. L. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol,* 7**,** 145-73.

Mrozek, E., Anderson, P. & Caligiuri, M. A. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood,* 87**,** 2632-40.

Mucida, D., Kutchukhidze, N., Erazo, A., Russo, M., Lafaille, J. J. & Curotto de Lafaille, M. A. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest,* 115**,** 1923-33.

Muller, C. A., Autenrieth, I. B. & Peschel, A. 2005. Innate defenses of the intestinal epithelial barrier. *Cellular and molecular life sciences : CMLS,* 62**,** 1297-307.

Najafian, N., Chitnis, T., Salama, A. D., Zhu, B., Benou, C., Yuan, X., Clarkson, M. R., Sayegh, M. H. & Khoury, S. J. 2003. Regulatory functions of CD8+CD28- T cells in an autoimmune disease model. *J Clin Invest,* 112**,** 1037-48.

Nakamura, K., Kitani, A. & Strober, W. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *The Journal of experimental medicine,* 194**,** 629-44.

Ng, W. F., Duggan, P. J., Ponchel, F., Matarese, G., Lombardi, G., Edwards, A. D., Isaacs, J. D. & Lechler, R. I. 2001. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood,* 98**,** 2736-44.

Nguyen, V. H., Shashidhar, S., Chang, D. S., Ho, L., Kambham, N., Bachmann, M., Brown, J. M. & Negrin, R. S. 2008. The impact of regulatory T cells on T-cell immunity following hematopoietic cell transplantation. *Blood,* 111**,** 945-53.

Nguyen, V. H., Zeiser, R., Dasilva, D. L., Chang, D. S., Beilhack, A., Contag, C. H. & Negrin, R. S. 2007. In vivo dynamics of regulatory T-cell trafficking and survival predict effective strategies to control graft-versus-host disease following allogeneic transplantation. *Blood,* 109**,** 2649-56.

Nie, H., Zheng, Y., Li, R., Guo, T. B., He, D., Fang, L., Liu, X., Xiao, L., Chen, X., Wan, B., Chin, Y. E. & Zhang, J. Z. 2013. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. *Nature medicine,* 19**,** 322-8.

Niehues, T., Rocha, V., Filipovich, A. H., Chan, K. W., Porcher, R., Michel, G., Ortega, J. J., Wernet, P., Gobel, U., Gluckman, E. & Locatelli, F. 2001. Factors affecting lymphocyte subset reconstitution after either related or unrelated cord blood transplantation in children -- a Eurocord analysis. *Br J Haematol,* 114**,** 42-8.

Nielsen, N., Odum, N., Urso, B., Lanier, L. L. & Spee, P. 2012. Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. *PLoS One,* 7**,** e31959.

Ninin, E., Milpied, N., Moreau, P., Andre-Richet, B., Morineau, N., Mahe, B., Vigier, M., Imbert, B. M., Morin, O., Harousseau, J. L. & Richet, H. 2001. Longitudinal study of bacterial, viral, and fungal infections in adult recipients of bone marrow transplants. *Clin Infect Dis,* 33**,** 41-7.

Noda, M., Omatsu, Y., Sugiyama, T., Oishi, S., Fujii, N. & Nagasawa, T. 2011. CXCL12-CXCR4 chemokine signaling is essential for NK-cell development in adult mice. *Blood,* 117**,** 451-8.

Norcross, M. A. 1984. A synaptic basis for T-lymphocyte activation. *Ann Immunol (Paris),* 135D**,** 113-34.

North, J., Bakhsh, I., Marden, C., Pittman, H., Addison, E., Navarrete, C., Anderson, R. & Lowdell, M. W. 2007. Tumor-primed human natural killer cells lyse NK-resistant tumor targets: evidence of a two-stage process in resting NK cell activation. *J Immunol,* 178**,** 85-94.

Noval Rivas, M., Hazzan, M., Weatherly, K., Gaudray, F., Salmon, I. & Braun, M. Y. 2010. NK cell regulation of CD4 T cell-mediated graft-versus-host disease. *Journal of immunology,* 184**,** 6790-8.

Oestreich, K. J. & Weinmann, A. S. 2012. Master regulators or lineage-specifying? Changing views on CD4+ T cell transcription factors. *Nat Rev Immunol,* 12**,** 799-804.

Ohkura, N., Hamaguchi, M., Morikawa, H., Sugimura, K., Tanaka, A., Ito, Y., Osaki, M., Tanaka, Y., Yamashita, R., Nakano, N., Huehn, J., Fehling, H. J., Sparwasser, T., Nakai, K. & Sakaguchi, S. 2012. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. *Immunity,* 37**,** 785-99.

Oida, T., Xu, L., Weiner, H. L., Kitani, A. & Strober, W. 2006. TGF-beta-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. *Journal of immunology,* 177**,** 2331-9.

Olivares-Villagomez, D., Wang, Y. & Lafaille, J. J. 1998. Regulatory CD4(+) T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. *J Exp Med,* 188**,** 1883-94.

Oostendorp, R. A., Harvey, K. N., Kusadasi, N., de Bruijn, M. F., Saris, C., Ploemacher, R. E., Medvinsky, A. L. & Dzierzak, E. A. 2002. Stromal cell lines from mouse aorta-gonads-mesonephros subregions are potent supporters of hematopoietic stem cell activity. *Blood,* 99**,** 1183-9.

Orentas, R. J., Kohler, M. E. & Johnson, B. D. 2006. Suppression of anti-cancer immunity by regulatory T cells: back to the future. *Seminars in cancer biology,* 16**,** 137-49.

Ortega, C., Fernandez, A. S., Carrillo, J. M., Romero, P., Molina, I. J., Moreno, J. C. & Santamaria, M. 2009. IL-17-producing CD8+ T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines. *Journal of Leukocyte Biology,* 86**,** 435-43.

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. 2007. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature immunology,* 8**,** 1353-62.

Passweg, J. & Tyndall, A. 2007. Autologous stem cell transplantation in autoimmune diseases. *Semin Hematol,* 44**,** 278-85.

Peck, A. & Mellins, E. D. 2010. Precarious balance: Th17 cells in host defense. *Infect Immun,* 78**,** 32-8.

Pedroza-Pacheco, I., Madrigal, A. & Saudemont, A. 2013. Interaction between natural killer cells and regulatory T cells: perspectives for immunotherapy. *Cellular & molecular immunology,* 10**,** 222-9.

Pegram, H. J., Andrews, D. M., Smyth, M. J., Darcy, P. K. & Kershaw, M. H. 2011. Activating and inhibitory receptors of natural killer cells. *Immunology and cell biology,* 89**,** 216-24.

Pesenacker, A. M., Bending, D., Ursu, S., Wu, Q., Nistala, K. & Wedderburn, L. R. 2013. CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines. *Blood,* 121**,** 2647-58.

Petrie, E. J., Clements, C. S., Lin, J., Sullivan, L. C., Johnson, D., Huyton, T., Heroux, A., Hoare, H. L., Beddoe, T., Reid, H. H., Wilce, M. C., Brooks, A. G. & Rossjohn, J. 2008. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med,* 205**,** 725-35.

Pinho, M. J., Marques, C. J., Carvalho, F., Punzel, M., Sousa, M. & Barros, A. 2012. Genetic regulation on ex vivo differentiated natural killer cells from human umbilical cord blood CD34+ cells. *Journal of receptor and signal transduction research,* 32**,** 238-49.

Podack, E. R. & Dennert, G. 1983. Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature,* 302**,** 442-5.

Polansky, J. K., Schreiber, L., Thelemann, C., Ludwig, L., Kruger, M., Baumgrass, R., Cording, S., Floess, S., Hamann, A. & Huehn, J. 2010. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. *J Mol Med (Berl),* 88**,** 1029-40.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. & Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science,* 282**,** 2085-8.

Ponce, D. M., Gonzales, A., Lubin, M., Castro-Malaspina, H., Giralt, S., Goldberg, J. D., Hanash, A. M., Jakubowski, A., Jenq, R., Papadopoulos, E. B., Perales, M. A., van den Brink, M. R., Young, J. W., Boulad, F., O'Reilly, R. J., Prockop, S., Small, T. N., Scaradavou, A., Kernan, N. A., Stevens, C. E. & Barker, J. N. 2013. Graft-versus-host disease after double-unit cord blood transplantation has unique features and an association with engrafting unit-to-recipient HLA match. *Biol Blood Marrow Transplant,* 19**,** 904-11.

Potter, T. A., Grebe, K., Freiberg, B. & Kupfer, A. 2001. Formation of supramolecular activation clusters on fresh ex vivo CD8+ T cells after engagement of the T cell antigen receptor and CD8 by antigen-presenting cells. *Proc Natl Acad Sci U S A,* 98**,** 12624-9.

Quah, B. J., Warren, H. S. & Parish, C. R. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nature protocols,* 2**,** 2049-56.

Querol, S., Mufti, G. J., Marsh, S. G., Pagliuca, A., Little, A. M., Shaw, B. E., Jeffery, R., Garcia, J., Goldman, J. M. & Madrigal, J. A. 2009. Cord blood stem cells for hematopoietic stem cell transplantation in the UK: how big should the bank be? *Haematologica,* 94**,** 536-41.

Quinnan, G. V., Jr., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Moreschi, G., Santos, G. W., Saral, R. & Burns, W. H. 1982. Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N Engl J Med,* 307**,** 7-13.

Qureshi, O. S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E. M., Baker, J., Jeffery, L. E., Kaur, S., Briggs, Z., Hou, T. Z., Futter, C. E., Anderson, G., Walker, L. S. & Sansom, D. M. 2011. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science,* 332**,** 600-3.

Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature,* 381**,** 751-8.

Ralainirina, N., Poli, A., Michel, T., Poos, L., Andres, E., Hentges, F. & Zimmer, J. 2006. Control of NK cell functions by CD4+CD25+ regulatory T cells. *Journal of Leukocyte Biology,* 81**,** 144-153.

Ralainirina, N., Poli, A., Michel, T., Poos, L., Andres, E., Hentges, F. & Zimmer, J. 2007. Control of NK cell functions by CD4+CD25+ regulatory T cells. *J Leukoc Biol,* 81**,** 144-53.

Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nature reviews. Immunology,* 3**,** 781-90.

Redjimi, N., Raffin, C., Raimbaud, I., Pignon, P., Matsuzaki, J., Odunsi, K., Valmori, D. & Ayyoub, M. 2012. CXCR3+ T regulatory cells selectively accumulate in human ovarian carcinomas to limit type I immunity. *Cancer Res,* 72**,** 4351-60.

Reeves, R. K., Evans, T. I., Gillis, J. & Johnson, R. P. 2010. Simian immunodeficiency virus infection induces expansion of alpha4beta7+ and cytotoxic CD56+ NK cells. *Journal of virology,* 84**,** 8959-63.

Reibke, R., Garbi, N., Ganss, R., Hammerling, G. J., Arnold, B. & Oelert, T. 2006. CD8+ regulatory T cells generated by neonatal recognition of peripheral self-antigen. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 15142-7.

Reinhardt, R. L., Liang, H. E. & Locksley, R. M. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol,* 10**,** 385-93.

Rincon, M. & Flavell, R. A. 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *EMBO J,* 13**,** 4370-81.

Robertson, M. J. & Ritz, J. 1990. Biology and clinical relevance of human natural killer cells. *Blood,* 76**,** 2421-38.

Rocha, V., Labopin, M., Sanz, G., Arcese, W., Schwerdtfeger, R., Bosi, A., Jacobsen, N., Ruutu, T., de Lima, M., Finke, J., Frassoni, F., Gluckman, E., Acute Leukemia Working Party of European, B., Marrow Transplant, G. & Eurocord-Netcord, R. 2004. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med,* 351**,** 2276-85.

Rock, K. L., Benacerraf, B. & Abbas, A. K. 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J Exp Med,* 160**,** 1102-13.

Rodriguez, L., Azqueta, C., Azzalin, S., Garcia, J. & Querol, S. 2004. Washing of cord blood grafts after thawing: high cell recovery using an automated and closed system. *Vox sanguinis,* 87**,** 165-72.

Romagnani, C., Della Chiesa, M., Kohler, S., Moewes, B., Radbruch, A., Moretta, L., Moretta, A. & Thiel, A. 2005. Activation of human NK cells by plasmacytoid dendritic cells and its modulation by CD4+ T helper cells and CD4+ CD25hi T regulatory cells. *Eur J Immunol,* 35**,** 2452-8.

Romagnani, C., Juelke, K., Falco, M., Morandi, B., D'Agostino, A., Costa, R., Ratto, G., Forte, G., Carrega, P., Lui, G., Conte, R., Strowig, T., Moretta, A., Munz, C., Thiel, A., Moretta, L. & Ferlazzo, G. 2007. CD56brightCD16- killer Ig-like receptor- NK cells display longer

telomeres and acquire features of CD56dim NK cells upon activation. *Journal of immunology,* 178**,** 4947-55.

Roncador, G., Brown, P. J., Maestre, L., Hue, S., Martinez-Torrecuadrada, J. L., Ling, K. L., Pratap, S., Toms, C., Fox, B. C., Cerundolo, V., Powrie, F. & Banham, A. H. 2005. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol,* 35**,** 1681-91.

Ronchetti, S., Zollo, O., Bruscoli, S., Agostini, M., Bianchini, R., Nocentini, G., Ayroldi, E. & Riccardi, C. 2004. GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. *Eur J Immunol,* 34**,** 613-22.

Rook, A. H., Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Sporn, M. B., Burlington, D. B., Lane, H. C. & Fauci, A. S. 1986. Effects of transforming growth factor beta on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J Immunol,* 136**,** 3916-20.

Rorby, E., Nifelt Hagerstrom, M., Blank, U., Karlsson, G. & Karlsson, S. 2012. Human hematopoietic stem/progenitor cells overexpressing Smad4 exhibit impaired reconstitution potential in vivo. *Blood*.

Round, J. L. & Mazmanian, S. K. 2010. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci U S A,* 107**,** 12204-9.

Roy, S., Barnes, P. F., Garg, A., Wu, S., Cosman, D. & Vankayalapati, R. 2008. NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J Immunol,* 180**,** 1729-36.

Rudra, D., deRoos, P., Chaudhry, A., Niec, R. E., Arvey, A., Samstein, R. M., Leslie, C., Shaffer, S. A., Goodlett, D. R. & Rudensky, A. Y. 2012. Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nat Immunol,* 13**,** 1010-9.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W. D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., Martelli, M. F. & Velardi, A. 2002. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science,* 295**,** 2097-100.

Sabry, M., Tsirogianni, M., Bakhsh, I. A., North, J., Sivakumaran, J., Giannopoulos, K., Anderson, R., Mackinnon, S. & Lowdell, M. W. 2011. Leukemic priming of resting NK cells is killer Ig-like receptor independent but requires CD15-mediated CD2 ligation and natural cytotoxicity receptors. *J Immunol,* 187**,** 6227-34.

Sackstein, R. 2005. The lymphocyte homing receptors: gatekeepers of the multistep paradigm. *Current opinion in hematology,* 12**,** 444-50.

Sadler, T. 2010. *Langman's Medical embryology,* Philadelphia, Lippincot Williams & Wilkins.

Sakaguchi, S., Miyara, M., Costantino, C. M. & Hafler, D. A. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol,* 10**,** 490-500.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. 1995. 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. *J Immunol,* 155**,** 1151-64.

Sakaguchi, S., Toda, M., Asano, M., Itoh, M., Morse, S. S. & Sakaguchi, N. 1996. T cell-mediated maintenance of natural self-tolerance: its breakdown as a possible cause of various autoimmune diseases. *J Autoimmun,* 9**,** 211-20.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature,* 401**,** 708-12.

Sarris, M., Andersen, K. G., Randow, F., Mayr, L. & Betz, A. G. 2008. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity,* 28**,** 402-13.

Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A. & Saito, S. 2004. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Molecular human reproduction,* 10**,** 347-53.

Sauter, C., Abboud, M., Jia, X., Heller, G., Gonzales, A. M., Lubin, M., Hawke, R., Perales, M. A., van den Brink, M. R., Giralt, S., Papanicolaou, G., Scaradavou, A., Small, T. N. & Barker, J. N. 2011. Serious infection risk and immune recovery after double-unit cord blood transplantation without antithymocyte globulin. *Biol Blood Marrow Transplant,* 17**,** 1460-71.

Schaerli, P., Willimann, K., Lang, A. B., Lipp, M., Loetscher, P. & Moser, B. 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med,* 192**,** 1553-62.

Schmidt, R. E., MacDermott, R. P., Bartley, G., Bertovich, M., Amato, D. A., Austen, K. F., Schlossman, S. F., Stevens, R. L. & Ritz, J. 1985. Specific release of proteoglycans from human natural killer cells during target lysis. *Nature,* 318**,** 289-91.

Schub, N., Gunther, A., Schrauder, A., Claviez, A., Ehlert, C., Gramatzki, M. & Repp, R. 2011. Therapy of steroid-refractory acute GVHD with CD52 antibody alemtuzumab is effective. *Bone Marrow Transplant,* 46**,** 143-7.

Seaman, W. E., Blackman, M. A., Gindhart, T. D., Roubinian, J. R., Loeb, J. M. & Talal, N. 1978. beta-Estradiol reduces natural killer cells in mice. *J Immunol,* 121**,** 2193-8.

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S. I., Nanan, R., Kelleher, A. & Fazekas de St Groth, B. 2006a. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine,* 203**,** 1693-700.

Seddiki, N., Santner-Nanan, B., Tangye, S. G., Alexander, S. I., Solomon, M., Lee, S., Nanan, R. & Fazekas de Saint Groth, B. 2006b. Persistence of naive CD45RA+ regulatory T cells in adult life. *Blood,* 107**,** 2830-8.

Shalev, I., Liu, H., Koscik, C., Bartczak, A., Javadi, M., Wong, K. M., Maknojia, A., He, W., Liu, M. F., Diao, J., Winter, E., Manuel, J., McCarthy, D., Cattral, M., Gommerman, J., Clark, D. A., Phillips, M. J., Gorczynski, R. R., Zhang, L., Downey, G., Grant, D., Cybulsky, M. I. & Levy, G. 2008. Targeted deletion of fgl2 leads to impaired regulatory T cell activity and development of autoimmune glomerulonephritis. *J Immunol,* 180**,** 249-60.

Shaw, B. E. & Madrigal, A. 2012. Immunogenetics of allogeneic HSCT. *In:* Apperley, J., Carreras, E., Gluckman, E. & Masszi, T. (eds.) *Haematopoietic Stem Cell Transplantation, ESH-EBMT Handbook.* 6 ed.

Shilling, H. G., McQueen, K. L., Cheng, N. W., Shizuru, J. A., Negrin, R. S. & Parham, P. 2003. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood,* 101**,** 3730-40.

Shimizu, J., Yamazaki, S. & Sakaguchi, S. 1999. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *Journal of immunology,* 163**,** 5211-8.

Shin, H. J., Baker, J., Leveson-Gower, D. B., Smith, A. T., Sega, E. I. & Negrin, R. S. 2011. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood,* 118**,** 2342-50.

Shinkai, Y., Takio, K. & Okumura, K. 1988. Homology of perforin to the ninth component of complement (C9). *Nature,* 334**,** 525-7.

Simon, A. K., Jones, E., Richards, H., Wright, K., Betts, G., Godkin, A., Screaton, G. & Gallimore, A. 2007. Regulatory T cells inhibit Fas ligand-induced innate and adaptive tumour immunity. *Eur J Immunol,* 37**,** 758-67.

Sirianni, M. C., Businco, L., Seminara, R. & Aiuti, F. 1983. Severe combined immunodeficiencies, primary T-cell defects and DiGeorge syndrome in humans: characterization by monoclonal antibodies and natural killer cell activity. *Clin Immunol Immunopathol,* 28**,** 361-70.

Sitrin, J., Ring, A., Garcia, K. C., Benoist, C. & Mathis, D. 2013. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *The Journal of experimental medicine,* 210**,** 1153-65.

Sivori, S., Cantoni, C., Parolini, S., Marcenaro, E., Conte, R., Moretta, L. & Moretta, A. 2003. IL-21 induces both rapid maturation of human CD34+ cell precursors towards NK cells and acquisition of surface killer Ig-like receptors. *Eur J Immunol,* 33**,** 3439-47.

Sivori, S., Parolini, S., Falco, M., Marcenaro, E., Biassoni, R., Bottino, C., Moretta, L. & Moretta, A. 2000. 2B4 functions as a co-receptor in human NK cell activation. *European Journal of Immunology,* 30**,** 787-93.

Small, C. L., McCormick, S., Gill, N., Kugathasan, K., Santosuosso, M., Donaldson, N., Heinrichs, D. E., Ashkar, A. & Xing, Z. 2008. NK cells play a critical protective role in host defense against acute extracellular Staphylococcus aureus bacterial infection in the lung. *J Immunol,* 180**,** 5558-68.

Smith, P. A., Brunmark, A., Jackson, M. R. & Potter, T. A. 1997. Peptide-independent recognition by alloreactive cytotoxic T lymphocytes (CTL). *J Exp Med,* 185**,** 1023-33.

Smyth, L. A., Harker, N., Turnbull, W., El-Doueik, H., Klavinskis, L., Kioussis, D., Lombardi, G. & Lechler, R. 2008. The relative efficiency of acquisition of MHC:peptide complexes and cross-presentation depends on dendritic cell type. *J Immunol,* 181**,** 3212-20.

Smyth, M. J., Hayakawa, Y., Takeda, K. & Yagita, H. 2002. New aspects of natural-killer-cell surveillance and therapy of cancer. *Nature reviews. Cancer,* 2**,** 850-61.

Smyth, M. J., Teng, M. W., Swann, J., Kyparissoudis, K., Godfrey, D. I. & Hayakawa, Y. 2006. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol,* 176**,** 1582-7.

Sobecks, R. M., Askar, M., Thomas, D., Rybicki, L., Kalaycio, M., Dean, R., Avery, R., Mossad, S., Copelan, E. & Bolwell, B. J. 2011. Cytomegalovirus reactivation after matched sibling donor reduced-intensity conditioning allogeneic hematopoietic stem cell transplant correlates with donor killer immunoglobulin-like receptor genotype. *Exp Clin Transplant,* 9**,** 7-13.

Spanholtz, J., Tordoir, M., Eissens, D., Preijers, F., van der Meer, A., Joosten, I., Schaap, N., de Witte, T. M. & Dolstra, H. 2010. High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy. *PLoS One,* 5**,** e9221.

Stepp, S. E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P. A., Henter, J. I., Bennett, M., Fischer, A., de Saint Basile, G. & Kumar, V. 1999. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science,* 286**,** 1957-9.

Stinchcombe, J. C., Bossi, G., Booth, S. & Griffiths, G. M. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity,* 15**,** 751-61.

Stockis, J., Colau, D., Coulie, P. G. & Lucas, S. 2009. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. *European Journal of Immunology,* 39**,** 3315-22.

Street, S. E., Cretney, E. & Smyth, M. J. 2001. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood,* 97**,** 192-7.

Summers, C., Rankin, S. M., Condliffe, A. M., Singh, N., Peters, A. M. & Chilvers, E. R. 2010. Neutrophil kinetics in health and disease. *Trends Immunol,* 31**,** 318-24.

Sun, J. C. & Lanier, L. L. 2008. Cutting edge: viral infection breaks NK cell tolerance to "missing self". *J Immunol,* 181**,** 7453-7.

Sun, L., Wu, J. & Yi, S. 2012. Foxp3 is critical for human natural CD4+CD25+ regulatory T cells to suppress alloimmune response. *Transplant immunology,* 26**,** 71-80.

Sun, X., Wu, Y., Gao, W., Enjyoji, K., Csizmadia, E., Muller, C. E., Murakami, T. & Robson, S. C. 2010. CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice. *Gastroenterology,* 139**,** 1030-40.

Sungur, C. M., Tang-Feldman, Y. J., Ames, E., Alvarez, M., Chen, M., Longo, D. L., Pomeroy, C. & Murphy, W. J. 2013. Murine natural killer cell licensing and regulation by T regulatory cells in viral responses. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 7401-6.

Szanya, V., Ermann, J., Taylor, C., Holness, C. & Fathman, C. G. 2002. The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J Immunol,* 169**,** 2461-5.

Taams, L. S., Vukmanovic-Stejic, M., Smith, J., Dunne, P. J., Fletcher, J. M., Plunkett, F. J., Ebeling, S. B., Lombardi, G., Rustin, M. H., Bijlsma, J. W., Lafeber, F. P., Salmon, M. & Akbar, A. N. 2002. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. *Eur J Immunol,* 32**,** 1621-30.

Tai, X., Cowan, M., Feigenbaum, L. & Singer, A. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol,* 6**,** 152-62.

Takahashi, S., Iseki, T., Ooi, J., Tomonari, A., Takasugi, K., Shimohakamada, Y., Yamada, T., Uchimaru, K., Tojo, A., Shirafuji, N., Kodo, H., Tani, K., Takahashi, T., Yamaguchi, T. & Asano, S. 2004. Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. *Blood,* 104**,** 3813-20.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J. & Sakaguchi, S. 1998. 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,* 10**,** 1969-80.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. & Sakaguchi, S. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *The Journal of experimental medicine,* 192**,** 303-10.

Takeda, K., Cretney, E., Hayakawa, Y., Ota, T., Akiba, H., Ogasawara, K., Yagita, H., Kinoshita, K., Okumura, K. & Smyth, M. J. 2005. TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood,* 105**,** 2082-2089.

Tang, Q., Henriksen, K. J., Bi, M., Finger, E. B., Szot, G., Ye, J., Masteller, E. L., McDevitt, H., Bonyhadi, M. & Bluestone, J. A. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med,* 199**,** 1455-65.

Tarbell, K. V., Petit, L., Zuo, X., Toy, P., Luo, X., Mqadmi, A., Yang, H., Suthanthiran, M., Mojsov, S. & Steinman, R. M. 2007. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. *J Exp Med,* 204**,** 191-201.

Tarbell, K. V., Yamazaki, S., Olson, K., Toy, P. & Steinman, R. M. 2004. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med,* 199**,** 1467-77.

Taub, D. D., Sayers, T. J., Carter, C. R. & Ortaldo, J. R. 1995. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol,* 155**,** 3877-88.

ten Dijke, P. & Arthur, H. M. 2007. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol,* 8**,** 857-69.

Thomas, E. D., Lochte, H. L., Jr., Lu, W. C. & Ferrebee, J. W. 1957. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med,* 257**,** 491-6.

Thomas, S. M. & Brugge, J. S. 1997. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol,* 13**,** 513-609.

Thornton, A. M., Piccirillo, C. A. & Shevach, E. M. 2004a. Activation requirements for the induction of CD4+CD25+ T cell suppressor function. *Eur J Immunol,* 34**,** 366-76.

Thornton, A. M. & Shevach, E. M. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *The Journal of experimental medicine,* 188**,** 287-96.

Thornton, A. M. & Shevach, E. M. 2000. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *Journal of immunology,* 164**,** 183-90.

Thornton, C. A., Upham, J. W., Wikstrom, M. E., Holt, B. J., White, G. P., Sharp, M. J., Sly, P. D. & Holt, P. G. 2004b. Functional maturation of CD4+CD25+CTLA4+CD45RA+ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. *Journal of immunology,* 173**,** 3084-92.

Timonen, T., Patarroyo, M. & Gahmberg, C. G. 1988. CD11a-c/CD18 and GP84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. *Journal of immunology,* 141**,** 1041-6.

Tomblyn, M., Chiller, T., Einsele, H., Gress, R., Sepkowitz, K., Storek, J., Wingard, J. R., Young, J. A. & Boeckh, M. J. 2009. Guidelines for preventing infectious complications among hematopoietic cell transplant recipients: a global perspective. Preface. *Bone Marrow Transplant,* 44**,** 453-5.

Trenado, A. 2003. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *Journal of Clinical Investigation,* 112**,** 1688-1696.

Trinchieri, G. 1989. Biology of natural killer cells. *Advances in immunology,* 47**,** 187-376.

Trzonkowski, P., Bieniaszewska, M., Juscinska, J., Dobyszuk, A., Krzystyniak, A., Marek, N., Mysliwska, J. & Hellmann, A. 2009. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clinical immunology,* 133**,** 22-6.

Trzonkowski, P., Szmit, E., Myśliwska, J., Dobyszuk, A. & Myśliwski, A. 2004. CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. *Clinical Immunology,* 112**,** 258-267.

Uksila, J., Salmi, M., Butcher, E. C., Tarkkanen, J. & Jalkanen, S. 1997. Function of lymphocyte homing-associated adhesion molecules on human natural killer and lymphokine-activated killer cells. *Journal of immunology,* 158**,** 1610-7.

Vacca, P., Vitale, C., Montaldo, E., Conte, R., Cantoni, C., Fulcheri, E., Darretta, V., Moretta, L. & Mingari, M. C. 2011. CD34+ hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 2402-7.

Vaidya, S. V., Stepp, S. E., McNerney, M. E., Lee, J. K., Bennett, M., Lee, K. M., Stewart, C. L., Kumar, V. & Mathew, P. A. 2005. Targeted disruption of the 2B4 gene in mice reveals an in vivo role of 2B4 (CD244) in the rejection of B16 melanoma cells. *Journal of immunology,* 174**,** 800-7.

Valiante, N. M., Uhrberg, M., Shilling, H. G., Lienert-Weidenbach, K., Arnett, K. L., D'Andrea, A., Phillips, J. H., Lanier, L. L. & Parham, P. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity,* 7**,** 739-51.

Valmori, D., Merlo, A., Souleimanian, N. E., Hesdorffer, C. S. & Ayyoub, M. 2005. A peripheral circulating compartment of natural naive CD4 Tregs. *The Journal of clinical investigation,* 115**,** 1953-62.

van den Broek, M. F., Kagi, D., Zinkernagel, R. M. & Hengartner, H. 1995. Perforin dependence of natural killer cell-mediated tumor control in vivo. *European Journal of Immunology,* 25**,** 3514-6.

van Lier, R. A., Brouwer, M., Rebel, V. I., van Noesel, C. J. & Aarden, L. A. 1989. Immobilized anti-CD3 monoclonal antibodies induce accessory cell-independent lymphokine production, proliferation and helper activity in human T lymphocytes. *Immunology,* 68**,** 45-50.

Vely, F. & Vivier, E. 1997. Conservation of structural features reveals the existence of a large family of inhibitory cell surface receptors and noninhibitory/activatory counterparts. *J Immunol,* 159**,** 2075-7.

Vitale, C., Chiossone, L., Morreale, G., Lanino, E., Cottalasso, F., Moretti, S., Dini, G., Moretta, L. & Mingari, M. C. 2004. Analysis of the activating receptors and cytolytic function of human natural killer cells undergoing in vivo differentiation after allogeneic bone marrow transplantation. *Eur J Immunol,* 34**,** 455-60.

Vitale, M., Bottino, C., Sivori, S., Sanseverino, L., Castriconi, R., Marcenaro, E., Augugliaro, R., Moretta, L. & Moretta, A. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med,* 187**,** 2065-72.

Voogt, P. J., Goulmy, E., Fibbe, W. E., Veenhof, W. F., Brand, A. & Falkenburg, J. H. 1988. Minor histocompatibility antigen H-Y is expressed on human hematopoietic progenitor cells. *J Clin Invest,* 82**,** 906-12.

Vosshenrich, C. A., Garcia-Ojeda, M. E., Samson-Villeger, S. I., Pasqualetto, V., Enault, L., Richard-Le Goff, O., Corcuff, E., Guy-Grand, D., Rocha, B., Cumano, A., Rogge, L., Ezine, S. & Di Santo, J. P. 2006. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol,* 7**,** 1217-24.

Vosshenrich, C. A., Ranson, T., Samson, S. I., Corcuff, E., Colucci, F., Rosmaraki, E. E. & Di Santo, J. P. 2005. Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J Immunol,* 174**,** 1213-21.

Vyas, Y. M., Maniar, H. & Dupont, B. 2002. Cutting edge: differential segregation of the SRC homology 2-containing protein tyrosine phosphatase-1 within the early NK cell immune synapse distinguishes noncytolytic from cytolytic interactions. *J Immunol,* 168**,** 3150-4.

Waggoner, S. N., Cornberg, M., Selin, L. K. & Welsh, R. M. 2012. Natural killer cells act as rheostats modulating antiviral T cells. *Nature,* 481**,** 394-8.

Wagtmann, N., Rajagopalan, S., Winter, C. C., Peruzzi, M. & Long, E. O. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity,* 3**,** 801-9.

Walser, T. C., Ma, X., Kundu, N., Dorsey, R., Goloubeva, O. & Fulton, A. M. 2007. Immune-mediated modulation of breast cancer growth and metastasis by the chemokine Mig (CXCL9) in a murine model. *J Immunother,* 30**,** 490-8.

Wang, G., Matsuura, I., He, D. & Liu, F. 2009. Transforming growth factor-{beta}-inducible phosphorylation of Smad3. *J Biol Chem,* 284**,** 9663-73.

Weaver, C. T., Hatton, R. D., Mangan, P. R. & Harrington, L. E. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol,* 25**,** 821-52.

Weenink, S. M. & Gautam, A. M. 1997. Antigen presentation by MHC class II molecules. *Immunol Cell Biol,* 75**,** 69-81.

Weiden, P. L., Flournoy, N., Thomas, E. D., Prentice, R., Fefer, A., Buckner, C. D. & Storb, R. 1979. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. *N Engl J Med,* 300**,** 1068-73.

Welsh, R. M. & Waggoner, S. N. 2013. NK cells controlling virus-specific T cells: Rheostats for acute vs. persistent infections. *Virology,* 435**,** 37-45.

Welte, K., Foeken, L., Gluckman, E., Navarrete, C. & Cord Blood Working Group of the World Marrow Donor, A. 2010. International exchange of cord blood units: the registry aspects. *Bone Marrow Transplant,* 45**,** 825-31.

White, M., Lawless, M. W., O'Dwyer, M. J., Grealy, R., Connell, B. O., Stordeur, P., Kelleher, D., McManus, R. & Ryan, T. 2010. Transforming growth factor beta-1 and interleukin-17 gene transcription in peripheral blood mononuclear cells and the human response to infection. *Cytokine,* 50**,** 322-7.

Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricarelli, F. D., Byrne, G., McEuen, M., Proll, S., Appleby, M. & Brunkow, M. E. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet,* 27**,** 18-20.

Williams, N. S., Moore, T. A., Schatzle, J. D., Puzanov, I. J., Sivakumar, P. V., Zlotnik, A., Bennett, M. & Kumar, V. 1997. Generation of lytic natural killer 1.1+, Ly-49- cells from

multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates. *J Exp Med,* 186**,** 1609-14.

Wing, K., Ekmark, A., Karlsson, H., Rudin, A. & Suri-Payer, E. 2002. Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood. *Immunology,* 106**,** 190-9.

Wing, K., Larsson, P., Sandstrom, K., Lundin, S. B., Suri-Payer, E. & Rudin, A. 2005. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology,* 115**,** 516-25.

Wing, K., Lindgren, S., Kollberg, G., Lundgren, A., Harris, R. A., Rudin, A., Lundin, S. & Suri-Payer, E. 2003. CD4 T cell activation by myelin oligodendrocyte glycoprotein is suppressed by adult but not cord blood CD25+ T cells. *European Journal of Immunology,* 33**,** 579-87.

Wu, J. D. 2004. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *Journal of Clinical Investigation,* 114**,** 560-568.

Wu, J. D., Higgins, L. M., Steinle, A., Cosman, D., Haugk, K. & Plymate, S. R. 2004. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *J Clin Invest,* 114**,** 560-8.

Wysocki, C. A., Jiang, Q., Panoskaltsis-Mortari, A., Taylor, P. A., McKinnon, K. P., Su, L., Blazar, B. R. & Serody, J. S. 2005. Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. *Blood,* 106**,** 3300-7.

Yamaji, O., Nagaishi, T., Totsuka, T., Onizawa, M., Suzuki, M., Tsuge, N., Hasegawa, A., Okamoto, R., Tsuchiya, K., Nakamura, T., Arase, H., Kanai, T. & Watanabe, M. 2012. The development of colitogenic CD4(+) T cells is regulated by IL-7 in collaboration with NK cell function in a murine model of colitis. *Journal of immunology,* 188**,** 2524-36.

Yamazaki, S., Iwama, A., Takayanagi, S., Eto, K., Ema, H. & Nakauchi, H. 2009. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood,* 113**,** 1250-6.

Yamazaki, S., Iyoda, T., Tarbell, K., Olson, K., Velinzon, K., Inaba, K. & Steinman, R. M. 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med,* 198**,** 235-47.

Yamazaki, T., Yang, X. O., Chung, Y., Fukunaga, A., Nurieva, R., Pappu, B., Martin-Orozco, N., Kang, H. S., Ma, L., Panopoulos, A. D., Craig, S., Watowich, S. S., Jetten, A. M., Tian, Q. & Dong, C. 2008. CCR6 regulates the migration of inflammatory and regulatory T cells. *Journal of immunology,* 181**,** 8391-401.

Yang, X. O., Nurieva, R., Martinez, G. J., Kang, H. S., Chung, Y., Pappu, B. P., Shah, B., Chang, S. H., Schluns, K. S., Watowich, S. S., Feng, X. H., Jetten, A. M. & Dong, C. 2008. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity,* 29**,** 44-56.

Yoon, S. R., Lee, Y. S., Yang, S. H., Ahn, K. H., Lee, J. H., Lee, J. H., Kim, D. Y., Kang, Y. A., Jeon, M., Seol, M., Ryu, S. G., Chung, J. W., Choi, I. & Lee, K. H. 2010. Generation of donor natural killer cells from CD34(+) progenitor cells and subsequent infusion after HLA-mismatched allogeneic hematopoietic cell transplantation: a feasibility study. *Bone Marrow Transplant,* 45**,** 1038-46.

Yu, D., Rao, S., Tsai, L. M., Lee, S. K., He, Y., Sutcliffe, E. L., Srivastava, M., Linterman, M., Zheng, L., Simpson, N., Ellyard, J. I., Parish, I. A., Ma, C. S., Li, Q. J., Parish, C. R.,

Mackay, C. R. & Vinuesa, C. G. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity,* 31**,** 457-68.

Yu, J., Mao, H. C., Wei, M., Hughes, T., Zhang, J., Park, I. K., Liu, S., McClory, S., Marcucci, G., Trotta, R. & Caligiuri, M. A. 2010. CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. *Blood,* 115**,** 274-81.

Yu, J., Wei, M., Becknell, B., Trotta, R., Liu, S., Boyd, Z., Jaung, M. S., Blaser, B. W., Sun, J., Benson, D. M., Jr., Mao, H., Yokohama, A., Bhatt, D., Shen, L., Davuluri, R., Weinstein, M., Marcucci, G. & Caligiuri, M. A. 2006. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity,* 24**,** 575-90.

Yu, P., Gregg, R. K., Bell, J. J., Ellis, J. S., Divekar, R., Lee, H. H., Jain, R., Waldner, H., Hardaway, J. C., Collins, M., Kuchroo, V. K. & Zaghouani, H. 2005. Specific T regulatory cells display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. *J Immunol,* 174**,** 6772-80.

Yun, S., Lee, S. H., Yoon, S. R., Kim, M. S., Piao, Z. H., Myung, P. K., Kim, T. D., Jung, H. & Choi, I. 2011. TOX regulates the differentiation of human natural killer cells from hematopoietic stem cells in vitro. *Immunology letters,* 136**,** 29-36.

Zaia, J. A., Sun, J. Y., Gallez-Hawkins, G. M., Thao, L., Oki, A., Lacey, S. F., Dagis, A., Palmer, J., Diamond, D. J., Forman, S. J. & Senitzer, D. 2009. The effect of single and combined activating killer immunoglobulin-like receptor genotypes on cytomegalovirus infection and immunity after hematopoietic cell transplantation. *Biol Blood Marrow Transplant,* 15**,** 315-25.

Zamai, L., Del Zotto, G., Buccella, F., Galeotti, L., Canonico, B., Luchetti, F. & Papa, S. 2012. Cytotoxic functions and susceptibility to apoptosis of human CD56(bright) NK cells differentiated in vitro from CD34(+) hematopoietic progenitors. *Cytometry A,* 81**,** 294-302.

Zhang, N., Schroppel, B., Lal, G., Jakubzick, C., Mao, X., Chen, D., Yin, N., Jessberger, R., Ochando, J. C., Ding, Y. & Bromberg, J. S. 2009. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. *Immunity,* 30**,** 458-69.

Zhang, Z. X., Yang, L., Young, K. J., DuTemple, B. & Zhang, L. 2000. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nature medicine,* 6**,** 782-9.

Zhao, D. M., Thornton, A. M., DiPaolo, R. J. & Shevach, E. M. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood,* 107**,** 3925-32.

Zhou, H., Chen, L., You, Y., Zou, L. & Zou, P. 2010. Foxp3-transduced polyclonal regulatory T cells suppress NK cell functions in a TGF-β dependent manner. *Autoimmunity,* 43**,** 299-307.

Ziegler, S. F., Ramsdell, F. & Alderson, M. R. 1994. The activation antigen CD69. *Stem Cells,* 12**,** 456-65.

Zimmer, J., Andres, E. & Hentges, F. 2008. NK cells and Treg cells: a fascinating dance cheek to cheek. *European journal of immunology,* 38**,** 2942-5.

Zinkernagel, R. M. 1996. Response: immunology taught by darwin. *Science,* 272**,** 635a.

Zou, L., Barnett, B., Safah, H., Larussa, V. F., Evdemon-Hogan, M., Mottram, P., Wei, S., David, O., Curiel, T. J. & Zou, W. 2004. Bone marrow is a reservoir for CD4+CD25+

regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer research,* 64**,** 8451-5.