EFFECTS OF MYCOPHENOLATE MOFETIL AND CYCLOSPORIN A ON CORD BLOOD AND PERIPHERAL BLOOD NATURAL KILLER CELLS

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Abbreviations

7-AAD	7-Aminoactinomycin D
μM	Micromolar
ADCC	Antibody-dependent cellular cytotoxicity
ADP	Adenosine diphosphate
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATP5B	Beta subunit of the mitochondrial ATP synthase
ATG	Anti T-lymphocyte globulin
BM	Bone marrow
BMT	Bone marrow transplantation
Ca^{2+}	Calcium
CB	Cord blood
CBMC	Cord blood mononuclear cell
CBT	Cord blood transplantation
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxy fluorescein succinimidyl ester
CsA	Cyclosporine A
Ct	Threshold cycle
dADP	Deoxyadenosine diphosphate
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
dGDP	deoxyguanosine diphosphate
dGTP	deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EDTA	Ethylenediamine tetraacetic acid
e.g.	*exempli gratia* (the same as “for example”)
FACS	Fluorescent-activated cell scanner
FasL	Fas Ligand
FBS	Foetal bovine serum

Fc	Fragment crystallisable
G-CSF	Granulocyte-colony-stimulating factor
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GvH	Graft versus host
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
h	Hours
HLA	Human leucocyte antigen
HPC	Haematopoietic progenitor cell
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
i.e.	*Id est* (the same as “that is”)
IFN	Interferon
IL	Interleukin
IMP	Inosine monophosphate
IMPDH	Inosine-5’-monophosphate dehydrogenase
KIR	Killer immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor subfamily G member 1
LAK	Lymphokine-activated killer
LAMP	Lysosomal-associated membrane glycoprotein
LFA	Lymphocyte function-associated antigen
LN	Lymph node
MET	Methotrexate
MHC	Major Histocompatibility Complex
mHSC	Mobilised haematopoietic stem cell
mL	Millilitre
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
mPB	Mobilised peripheral blood
mRNA	Messenger ribonucleic acid
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T cells
ng	Nanogram
NK	Natural killer

NKp	NK cell related protein
NKPs	NK cell precursors
NKR	NK-cell receptor
OD	Optical density
PB	Peripheral Blood
PBL	Peripheral blood lymphocytes
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-streptomycin
PILR	Paired Ig-like receptor
PMA	Phorbol 12-myristate 13-acetate
PRPP	Phosphoribosyl pyrophosphate
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SC	Stem cell
SD	Standard deviation
TCR	T cell receptor
TNC	Total nucleated cell
TNF	Tumour necrosis factor
TOP 1	Topoisomerase 1
TRM	Treatment-related mortality
UBC	Ubiquitin C
UK	United Kingdom
VCAM	Vascular cell adhesion protein

Summary

Haematopoietic stem cell transplantation (HSCT) is used to treat haematological malignancies and bone marrow (BM) failures. For many years BM was the only source of haematopoietic stem cells (SCs) for transplantation, but now SCs from mobilised peripheral blood (mPB) and cord blood (CB) are increasingly used. Cord blood transplantation (CBT) presents several advantages compared to bone marrow transplantation (BMT) including a greater tolerance to histocompatibility leukocyte antigen (HLA) disparity, a lower risk of graft versus host disease (GvHD) and a preserved graft versus leukaemia (GvL) effect. However, GvHD remains a major cause of morbidity and mortality after HSCT.

Cyclosporine A (CsA) and methotrexate (MET) were the standard immunosuppressive drugs used as prophylaxis treatment for GvHD in the United Kingdom (UK), but over the past twenty years new immunosuppressive drugs were developed in order to improve the efficacy and decrease the side effects of CsA and MET, such as mycophenolate mofetil (MMF), anti-T lymphocyte globulin (ATG), Campath and Rapamycin (RAPA).

CsA and MMF have been successfully used alone or in combination to prevent GvHD. The effects of those two immunosuppressive drugs have been studied on T cells, but little is known about their effects on natural killer (NK) cells.

NK cells provide the beneficial GvL effect without causing GvHD, contributing to reduced relapse rates. Moreover, NK cells reconstitute early after CBT providing GvL and the first line of defence against infectious organisms. Several studies have shown that NK cells are beneficial for clinical outcome.

To determine the effects of MPA, CsA and the combination of the drugs on peripheral blood (PB) and CB NK cells an approach based on flow cytometry and quantitative real time PCR was used.

MPA and MPA+CsA induced cell death of CB NK cells, but not of PB NK cells. The immunosuppressants did not have a significant effect on the expression of the activation markers CD69 and NKp44 by NK cells, but significantly reduced CB NK cell proliferation. MPA reduced perforin production by PB NK cells, while CsA and MPA+CsA drastically decreased PB and CB NK cell degranulation.

This study showed that all drugs tested had significant effects on NK cells that could jeopardise their beneficial effects after HSCT. We demonstrated that CB NK cells were more sensitive to the drugs than PB NK cells. However, this difference of sensibility could not be explained by significant differences in the expression of the drug targets, IMPDH1, IMPDH2 and NFATc between PB and CB NK cells. These data suggest that the immunosuppressive drug dosage could be adjusted for CBT or maybe alternatives to MMF

and CsA could be used in order to prevent GvHD, without jeopardising NK cell functionality.

Resumo

O transplante de células estaminais hematopoiéticas é utlizado para tratar doenças hematológicas malignas e falhas da medula óssea. Durante muitos anos a medula óssea foi a única fonte de células estaminais hematopoiéticas utilizadas em transplantes, mas agora células estaminais mobilizadas do sangue periférico e células estaminais do cordão umbilical são cada vez mais utilizadas. Algumas das vantagens do transplante do cordão umbilical em comparação com o transplante de medula óssea incluem uma maior tolerância a incompatibilidades do antígeno leucocitário humano, uma menor incidência da doença do transplante contra o hospedeiro e obtenção do efeito imunológico do enxerto contra leucemia. No entanto, a doença do transplante contra o hospedeiro continua sendo a maior causa de morbilidade e mortalidade após o transplante de células estaminais hematopoiéticas.

Cisclosporina A e metotrexato eram os fármacos imunossupressores padrão utilizados no tratamento profilático da doença do enxerto contra o hospedeiro no Reino Unido, mas ao longo dos últimos vinte anos novos fármacos imunossupressores têm sido desenvolvidos com o objectivo de melhorar a eficácia e minimizar os efeitos secundários desses fármacos. Alguns exemplos dos fármacos de nova geração desenvolvidos são o micofenolato de mofetila, globulina anti-linfócito T, campath e rapamicina.

Ciclosporina A e micofenolato de mofetila têm sido eficazmente utilizados individualmente ou em combinação para prevenir a doença do enxerto contra o hospedeiro. Os efeitos desses dois fármacos imunossupressores têm sido estudados em linfócitos T mas sabe-se muito pouco sobre os seus efeitos nas células *natural killer* (NK).

As células NK exercem o efeito imunológico benéfico do enxerto contra leucemia sem provocar a doença do enxerto contra o hospedeiro, contribuindo assim para a redução da reincidência da doença. Para além do mais, as células NK reconstituem num curto período de tempo após o transplante do cordão umbilical, proporcionando não só a primeira linha de defesa contra agentes infecciosos, mas também o efeito imunológico do enxerto contra leucemia. Vários estudos têm demostrado que as células NK têm um efeito benéfico no resultado clínico dos pacientes.

Para determinar os efeitos do ácido micofenólico, ciclosporina A e da combinação dos fármacos nas células NK do sangue periférico e do cordão umbilical, foi utilizada uma metodologia baseada em citometria de fluxo e reações quantitativa de polimerase em cadeia detectada em tempo real.

O micofenolato de mofetil e a sua combinação com ciclosporina A induziu a morte celular das células NK do cordão umbilical, mas não das células NK do sangue periférico. Os imunossupressores não tiveram um efeito significativo na expressão dos marcadores de ativação CD69 e NKp44 pelas células NK, mas reduziram significativamente a proliferação das células NK do cordão umbilical. O micofenolato de mofetila também

reduziu a produção de perforina pelas células NK do sangue periférico, enquanto que a ciclosporina A e a combinação dos fármacos reduziu drasticamente a degranulação das células NK de ambas as fontes.

Este estudo mostrou que todos os fármacos testados apresentam efeitos significativos nas células NK que podem prejudicar o seu efeito benéfico após o transplante de células estaminais hematopoiéticas. Demonstramos ainda que as células NK do cordão umbilical são mais susceptíveis aos imunossupressores do que as células NK do sangue periférico. No entanto, esta diferença de susceptibilidade não pode ser explicada com base em diferenças significativas dos alvos moleculares dos fármacos, como *IMPDH1*, *IMPDH2* e *NFATc*, entre células NK do sangue periférico e do cordão umbilical. Estes resultados sugerem que a dosagem dos fármacos imunossupressores pode ser ajustada para o transplante do cordão umbilical ou então alternativas ao micofenolato de mofetila e ciclosporina A devem ser pensadas de forma a prevenir a doença do enxerto contra o hospedeiro sem prejudicar a funcionalidade das células NK.

Introduction

1. Human Natural Killer Cells

Natural killer (NK) cells are large, granular, bone marrow (BM)-derived cells that constitute approximately 10-15% of human peripheral blood lymphocytes (PBL), however, this proportion can vary with age [1]. They are an essential element of the innate immune system, mediating early defence through cellular cytotoxicity against infectious organisms or transformed cells without previous sensitisation and through production of a wide variety of chemokines and cytokines that influence other cellular compartments of the immune system [1, 2].

NK cell receptors are germline-encoded, but unlike B and T cells, they do not undergo somatic recombination [1]. It is the balance of signals from activating and inhibitory receptors that determines the outcome of NK cell function [3]. Every healthy cell expresses major histocompatibility complex (MHC) class I molecules that are recognised by specific inhibitory NK cell receptors. This interaction allows NK cells to recognise self from non-self preventing auto-immunity [3, 4]. Infections and tumour transformation lead to a loss of MHC class I expression, triggering NK cell activation, as proposed by the "missing-self" hypothesis [5].

NK cells use several mechanisms to kill their cellular targets, including calcium (Ca^{2+}) dependent exocytosis of perforin and granzyme cytotoxic proteins, Fas/FasL mediated apoptosis, membrane bound or secreted cytokines such as tumor necrosis factor-α (TNF-α) [6] and antibody dependent cellular cytotoxicity (ADCC) [7].

Besides target recognition, NK cell effector functions can be triggered by cytokine stimulation. Infected or activated dendritic cells (DCs) and macrophages produce cytokines such as interferon-α/β (IFN-α/β), interleukin (IL)-12, IL-15 and IL-18 that stimulate NK cells to produce other cytokines including IFN-γ and TNF-α. In addition, several chemokines are also secreted [8, 9]. Regulatory pathways that control NK cell cytokine production are particularly relevant during early phases of inflammatory response [8].

1.1. NK Cell Subsets

Two distinct NK cell subsets in humans can be characterised according to their relative expression of CD56 and to major functional differences: cytolytic activity, cytokine production and homing capabilities [10, 11].

The majority (90%) of human NK cells are $CD56^{dim}$, meaning they have low surface density expression of CD56. This population is primarily $CD16^{+}$, killer cell immunoglobulin-like receptor $(KIR)^{+}$ and display low cytokine production, but potent cytotoxicity. $CD56^{dim}$ cells predominate in peripheral blood (PB) and inflamed tissues. In contrast, a minority of human NK cells (10%) are $CD56^{bright}$, have low or absent CD16 and KIR expression, have low cytolytic activity, but release high levels of cytokines. They predominate in lymph nodes (LN) (Figure 1) [10, 11].

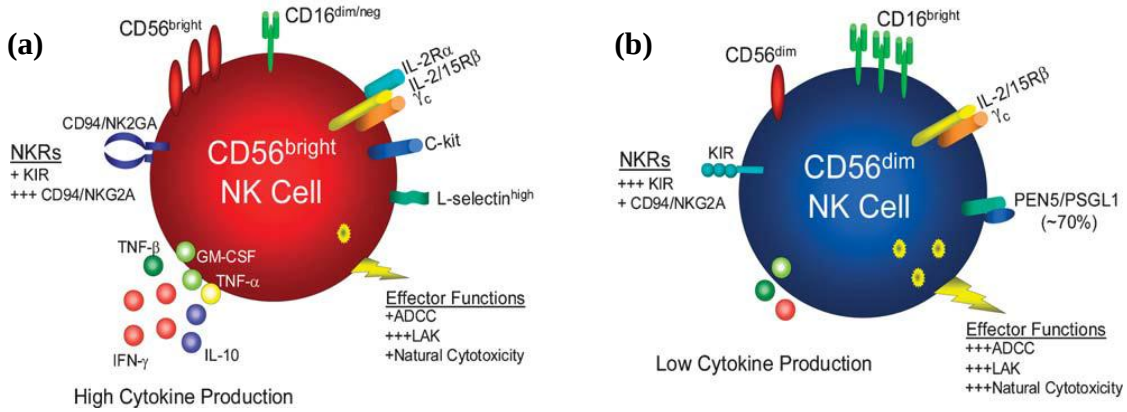

Figure 1. Schematic of human NK cell subsets exhibiting differential receptor profiles and functions. (a) $CD56^{bright}$ NK cells produce high levels of cytokines and exhibit high lymphokine-activated killer (LAK) activity but low natural cytotoxicity and antibody-dependent cellular activity. (b) By contrast, $CD56^{dim}$ NK cells produce low levels of cytokines but are potent mediators of ADCC, LAK and natural cytotoxicity. $CD56^{dim}$ have a more granular morphology and express high-level of KIRs when compared with $CD56^{bright}$ cells. However, $CD56^{bright}$ NK cells display a higher expression of CD94/NKG2A and L-selectin. From Cooper et al., 2001 [11].

1.2. NK Cell Development

As all cells of the haematopoietic system human NK cells derive from $CD34^{+}$ haematopoietic stem cells (HSCs). It is believed that the BM microenvironment is needed for NK cell full maturation [12]. BM ablation studies in mice provided the first evidence of its importance for NK cell development *in vivo* [13, 14].

Mature NK cells do not express CD34 and are $CD3^{-}CD56^{+}$ [15] although additional antigens are required to distinguish between functionally mature NK cells and immature intermediates.

Human NK cell development can be divided into different stages. Briefly, NK cell progenitors in the BM interact with stromal growth factors and generate an intermediate NK cell precursor ($CD34^{+}$IL-2/IL-15R$\beta^{+}CD56^{-}$). Becoming responsive to IL-15, this precursor is able to develop into a functional $CD56^{+}$ NK cell [12].

Freud et al. identified and characterised 4 distinct stages of NK cell differentiation, using $CD34^{+}$ haematopoietic progenitor cells (HPCs) from secondary lymphoid tissues (SLT), as follows: stage 1 CD34(+)CD117(-)CD94(-); stage 2, CD34(+)CD117(+)-CD94(-); stage 3, CD34(-)CD117(+)CD94(-); and stage 4, CD34(-)CD117(+/-)CD94(+). CD56 expression progressively increases as NK cell differentiation progresses from one stage to another to reach a level similar to PB $CD56^{bright}$ NK cells at stage 4 of differentiation. They also showed that the commitment to NK cell lineage occurs at stage 3, prior to NK cell functional maturity, which occurs from stage 4 [16].

When generated *in vitro*, NK cells were $CD56^{bright}$ not $CD56^{dim}$. This finding can be explained by the requirement of other soluble factors or cell-cell interactions that are not present *in vitro*. Parrish-Novak et al. showed that IL-21 in combination with IL-15 is involved in the development of $CD56^{+}CD16^{+}$ NK cells from BM HPCs *in vitro* [17]. However, it is not clear whether these cells are phenotypically and functionally similar to PB $CD56^{dim}$ NK cells. Several studies support the hypothesis that $CD56^{dim}$ population might represent the terminally differentiated stage of NK cell development [16, 18, 19].

1.3. NK Cell Recognition and NK Cell Receptors

Similarly to T cells, human NK cell recognition occurs through cell surface receptors. While T cells possess T cell receptors (TCRs), NK cells display an array of molecules; e.g. immunoglobulin-like and C-type lectin receptors.

Normal and healthy cells expressing MHC class I molecules will be specifically recognised by inhibitory NK cell receptors that will prevent NK cell activation while the interaction with transformed or non-self cells that have lost or exhibit altered MHC class I molecules expression will result in NK cell activation. Even though this is the main mechanism of NK cell recognition, MHC class I molecules are not always required for protection from NK cell lysis, and inhibition

by MHC class I molecules may not always be sufficient to prevent NK cell mediated cytotoxicity. For example class I-deficient non-haematopoietic tissues, such as skin grafts, cannot be rejected by NK cells, whereas NK cells are still able to kill some virus-infected cells that maintain the expression of MHC class I molecules [20, 21].

Three major superfamilies of NK cell receptors (NKRs) have been described in humans: the KIR superfamily, the C-type lectin superfamily and the natural cytotoxicity receptor (NCRs) family.

KIRs specifically recognise classical human leucocyte antigens (HLA-A, -B and –C), but also non-classical molecules such as HLA-G. They appear to play a role in the induction of NK cell tolerance to self, allowing the development of NK cells that are responsive against target cells, but tolerant to self MHC positive cells in a process named “licensing” [22].

C-type lectin receptors are selectively expressed by NK cells and cytotoxic T lymphocytes. These receptors react to the level of non-classical MHC class I molecules expressed on the surface of potential target cells. CD69 is a C-type lectin receptor, which appears to be the earliest molecule to be expressed on the surface of T and NK cells during activation [23]. Another example of C-type lectin receptor is the CD94/NKG2 heterodimer [24, 25]. NKG2D is the best-characterised NK cell activating receptor and is also a C-type lectin surface receptor distantly related to the NKG2 family, but expressed as a homodimer [26].

NK cell related protein (NKp) 46, NKp44 and NKp30 are part of the NCR family, which play an important role in the recognition of tumour cells. NKp46 and NKp30 are constitutively expressed by all PB NK cells and are not expressed by other immune cells [27, 28]. NKp44 is not expressed by resting NK cells, but is upregulated after activation such as IL-2 stimulation and may be important for the cytotoxicity of these cells [29]. NKp44 is also expressed by a proportion of $\gamma\delta$ T cells and NK22 cells [30]. NCR do not recognise MHC molecules, but viral hemagglutinins, heparin sulfate proteoglycan and nuclear factors released from tumour cells [31-33].

CD16 is a receptor expressed on NK cells that does not fit into any of the categories described above. This receptor is a member of the Fc receptor family, which recognises IgG. In contrast to receptors previously described that mediate natural cytotoxicity, CD16 mediates ADCC. The engagement of CD16 with its

ligand is capable of triggering by itself cytotoxicity and cytokine release of resting NK cells [34].

Other receptors have been implicated in the process of triggering NK cell-mediated cytotoxicity including natural killer receptor 2B4 (CD244) [35], CD2 (T11) [36], CD40 ligand [37], DNAX accessory molecule-1 (DNAM-1, also known as CD226) [38], killer cell lectin-like receptor subfamily G member 1 (KLRG1) [39], lymphocyte function-associated antigen-1 (LFA-1, also known as CD11a) [40], NKp80 [41] and paired Ig-like receptor (PILR) [42]. Most of these receptors require synergies with other receptors to activate cytotoxicity and cytokine production by resting NK cells. For activated NK cells, coactivation of specific pairs of receptors enhances NK cell cytotoxicity and cytokine production [34].

2. CB NK Cells

Phenotypic and functional similarities and differences between PB and cord blood (CB) NK cells have been reported [43]. Some investigators have reported CB NK cells as mature [44], while others have reported them as immature [45, 46].

NK cells are more abundant in CB than in PB, constituting up to 33% of CB lymphocytes [47]. The two major populations of NK cells, $CD56^{dim}$ and $CD56^{bright}$ exist in similar proportions in CB and PB [44, 48], however, Luevano et al. reported a slightly higher percentage of $CD56^{bright}$ cells in CB [46].

To assess the functionality of NK cells, the expression of activating and inhibitory receptors by CB and PB NK cells have been studied, the data from three different studies are summarised in Table 1.

Table 1. Comparative expression of inhibitory and activating receptors on CB and PB NK cells. ↑ higher expression on CB, → similar expression on CB, ↓ lower expression on CB, compared to PB, --- not studied.

	Inhibitory Receptors			**Activating Receptors**	
	CD158a	CD158b	NKG2A	NKG2D	NKp46
Luevano et al. 2012 [46]	$\downarrow^{dim}\downarrow^{bright}$	$\downarrow^{dim}\rightarrow^{bright}$	$\uparrow^{dim}\uparrow^{bright}$	$\rightarrow^{dim}\rightarrow^{bright}$	$\rightarrow^{dim}\downarrow^{bright}$
Wang et al. 2007 [49]	→	↓	↑	→	→
Dalle et al. 2005 [44]	→	→	---	---	---

The acquisition of lytic activity is associated with NK cell maturity. Some studies suggested that the expression of cytotoxic molecules such as FasL,

perforin and granzyme B do not differ between CB and PB NK cells, while other studies showed a lower percentage of CB NK cells expressing these molecules and others even showed an increase in the perforin and granzyme B producing-CB NK cells (Table 2). Despite controversies in the expression of cytotoxicity-related molecules by CB NK cells (Table 2), it seems consensual that resting CB NK cells are less cytotoxic than resting PB NK cells against K562 cells [44, 46, 48]. A lower expression of adhesion molecules and a higher expression of inhibitory receptors might account for the reduced CB NK cell cytotoxicity [46, 48, 49]. Nevertheless, CB NK cell cytotoxicity is enhanced after IL-2, IL-12 or IL-15 stimulation [44-46].

Table 2. Comparative expression of cytotoxicity-related molecules in CB and PB NK cells. ↑ higher expression on CB, → similar expression on CB, ↓ lower expression on CB, compared to PB.

	Fas-L	TRAIL	Perforin	Granzyme B
Luevano et al. 2012 [46]	↓^{dim}→^{bright}	→^{dim}→^{bright}	↓^{dim}→^{bright}	↓^{dim}→^{bright}
Wang et al. 2007 [49]	→	→	→	↓
Dalle et al. 2005 [44]	→	→	↑	↑

Several studies have compared IFN-γ production by CB and PB NK cells stimulated with phorbol 12-myristate 13-acetate (PMA) and an ionophore. Wang et al. and Dalle et al. showed no differences in the expression of IFN-γ by NK cells from CB and PB [44, 49], but Luevano et al. studied both NK cell subsets, showing a lower percentage of $CD56^{dim}$ subset expressing IFN-γ and a trend for the $CD56^{bright}$ subset [46].

In addition to the characterisation of the phenotypic and functional properties, the questions of the development and homing properties of CB NK cells compared to PB NK cells were recently addressed by Luevano et al. [46]. This study showed that even though the majority of CB NK cells are in the stage 4 according to the developmental stages defined by Freud et al. [16], their maturation seems to be incomplete. Moreover, CB NK cells seem to have a great potential to migrate to the BM, but not to LN as observed for PB NK cells [46].

3. Haematopoietic Stem Cell Transplantation

Haematopoietic stem cell transplantation (HSCT) is a common treatment of malignant (e.g. leukaemia and lymphoma) and non-malignant (e.g. thalassemia

and inborn errors of metabolism) disorders [50]. The number of HSCT performed worldwide has been increasing and the overall survival has improved due to advances in techniques, such as non-myeloablative conditioning, cord blood transplantation (CBT) and donor lymphocyte infusion allowing a higher number of people who are in need of HSCT to benefit from this treatment [51, 52].

3.1. Type of Donor and Source of Stem Cells

75% of patients in need of HSCT do not have an HLA-matched sibling, thus the recruitment of alternative donors such as matched unrelated volunteers and partially mismatched donors or the use of unrelated CB are required. For many years, BM was the only source of HSCs. Today, other sources of HSCs are used including CB units, granulocyte-colony-stimulating factor (G-CSF)-mobilised PB (mPB) HSCs/HPCs [53].

PB does not naturally contain a high percentage of HSCs/HPCs, but they can be mobilised from BM with G-CSF and/or AMD3100 (Plerixafor). Thereby, it is possible to obtain a high percentage of HSCs/HPCs in a volume of blood up to 20 L, with fewer side-effects for the donor. Unfortunately, mPB also contains a high percentage of T cells, increasing the incidence of GvHD [54].

CB is increasingly used as a source of SCs for transplantation mainly when suitable donors are not found. CB is rich in HSCs, but limited in volume. Despite the slow immune reconstitution and the increased risk of infections, CBT presents several advantages including off-the-shelf availability, a greater tolerance to HLA disparity and a lower risk of GvHD [55, 56]. For children, CB is now often chosen over mPB and BM [57].

3.2. Preparative Regimens

Prior to HSCT, a preparative regimen is required in order to eradicate cancer cells and to ablate the immune system of the patient preventing graft rejection [58].

Myeloablative regimens such as total-body irradiation (TBI) are very effective in eliminating tumour cells, consequently reducing relapse rates. The effectiveness of these regimens is due to the independence of blood supply and local shielding of organs. Thus, TBI can reach sites that chemotherapy cannot, however, these regimens are associated with high toxicity [59]. To reduce

toxicity, fractionated dose of TBI combined with chemotherapeutic agent has been used [58].

Lately, an innovative approach combined reduced intensity conditioning (RIC) with alloreaction in the graft-versus-host (GvH) direction. The RIC is mainly immunosuppressive, while the GvH alloreactivity results in recognition and elimination of malignant cells. This is known as the graft-versus-leukaemia (GvL) effect. Older recipients and patients with co-morbidities are the ones who benefit the most of this preparative regimen, otherwise they would not be able to undergo HSCT [60].

Radiation-free regimens, including chemotherapeutic drugs as busulfan and cyclophosphamide are also available [61].

3.3. GvL versus GvHD

T cells contribute to a rapid engraftment and successful development of immune tolerance and immune reconstitution. Moreover, they are GvL effectors. Unfortunately, alloreactive T cells also cause GvHD. Hence, an immunosuppressive regimen is given after HSCT to suppress the immune system of the patient, in particular T cells, to prevent GvHD [62].

GvHD is a major cause of morbidity and mortality after HSCT and the global immunosuppression needed to prevent or treat it underlie the major reasons for transplant failures: infection and neoplastic relapse [63].

In turn, NK cells play a central role after HSCT providing GvL effect without causing GvHD [64]. Several studies have shown an association between circulating NK cells and a reduced risk of relapse, infections and mortality after HSCT [65, 66].

4. Immunosuppressive Drugs

Immunosuppressive drugs have been used to prevent the activation/proliferation of T cells post-transplantation, essential for the immune response that leads to undesirable GvHD.

In the United Kingdom, the standard prophylaxis treatment against GvHD includes cyclosporine A (CsA) and methotrexate (MET). However, a new generation of immunosuppressive drugs has been developed over the past two

decades to improve the efficacy of CsA and MET with minimal side effects. These drugs include mycophenolate mofetil (MMF), anti-T lymphocyte globulin (ATG), Campath, tacrolimus and Rapamycin (RAPA) [62, 67-70].

Different immunosuppressive agents have diverse mechanisms of action, such as blocking cell cycle progression (e.g. RAPA and MMF), blocking cytokine production through calcineurin inhibition (e.g. CsA and tacrolimus) or killing the cells that express its cellular target (e.g. Campath and ATG).

4.1. Mycophenolate Mofetil

MMF (CellCept®) is an orally bioavailable prodrug of mycophenolic acid (MPA), a fermentation product of several species of *Penicillium* [71-73]. MMF is the 2-morpholinoethyl ester of MPA [74]. It is quickly hydrolysed by esterases both *in vivo*, and in human peripheral blood mononuclear cells (PBMCs) cultures *in vitro*.

MMF has a bioavailability of around 94% and its metabolisation is primary hepatic [74, 75]. The half-life of the drug is of approximately 17 hours and its metabolites are excreted in the urine and in the bile [74].

In addition to its well-established transplant uses, MMF is also used for other diseases such as for the treatment of autoimmune diseases [76-79].

4.1.1. Mechanism of Action

MPA is a non-competitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme that catalyses the conversion of inosine monophosphate (IMP) into guanosine monophosphate (GMP) in the *de novo* pathway of purine biosynthesis (Figure 2). IMPDH inhibition decreases the guanine nucleotide pools and consequently blocks the *de novo* deoxyribonucleic acid (DNA) synthesis [72, 73].

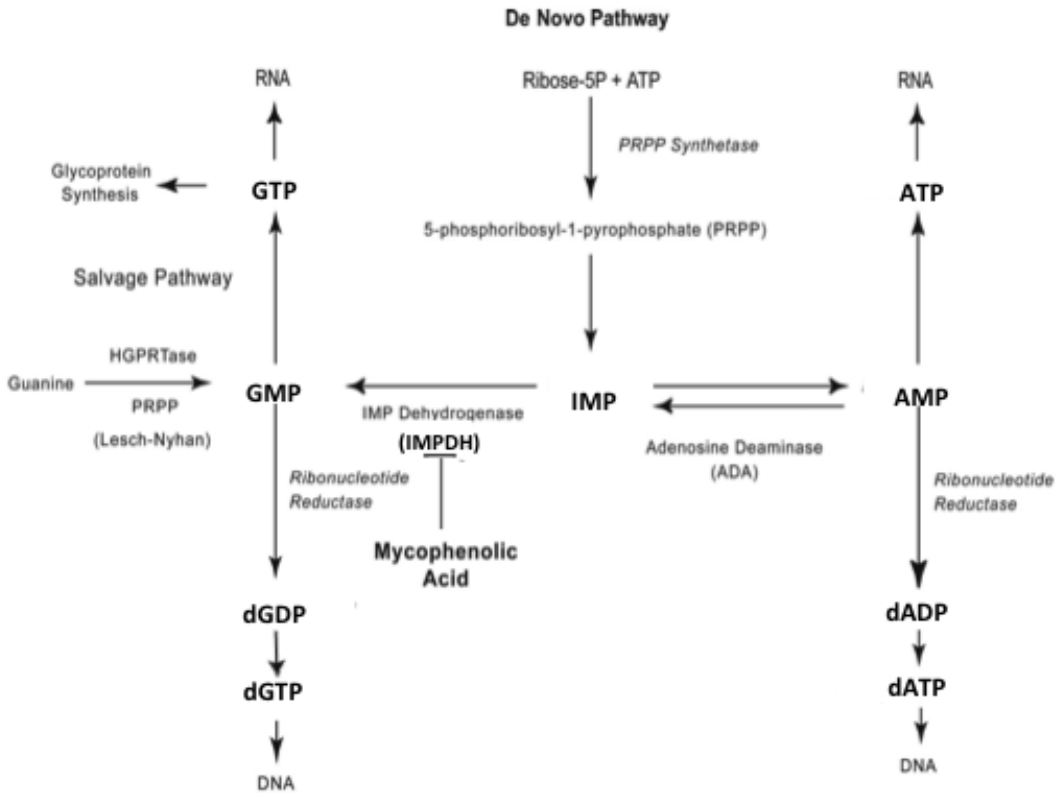

Figure 2. *De novo* and salvage pathways of purine biosynthesis. MPA inhibits IMPDH that catalyses the conversion of inosine monophosphate (IMP) into guanosine monophosphate (GMP). AMP, adenosine monophosphate; ATP, adenosine triphosphate; dADP, deoxyadenosine diphosphate; dATP, deoxyadenosine triphosphate; GTP, guanosine triphosphate; GMP, guanosine monophosphate; dGDP, deoxyguanosine diphosphate; dGTP, deoxyguanosine triphosphate; DNA, deoxyribonucleic acid; RNA ribonucleic acid. Adapted from Allison et al. 2005.

4.1.2. Effects on B and T Lymphocytes

There are two IMPDH isoforms: IMPDH1 and IMPDH2. While most of the cells express IMPDH1, IMPDH2 is almost exclusively expressed by B and T lymphocytes. Since MMF is 5 times more active on IMPDH2, it selectively affects the proliferation of activated lymphocytes, which is almost entirely dependent on the *de novo* pathway for purine synthesis [80].

Several papers have reported MMF/MPA as an inductor of apoptosis in T lymphocytes [81-83]. Human T lymphocytic and monocytic cell lines treated with MPA showed an increase in apoptotic cells [84]. Moreover, MMF supresses cytotoxic T lymphocyte generation [72].

Eugui et al. described MPA as an inhibitor of antibodies formation by activated B cells [71].

4.1.3. Effects on NK Cells

Recently, Ohata et al. described MPA as an inhibitor of PB NK cell proliferation and cytotoxic function. They suggested that the inclusion of MMF in the GvHD prophylaxis treatment may not be advantageous [85].

Eissens et al. demonstrated that MPA impairs human PB NK cell cytotoxicity due to differential effects on NK cell phenotype. The most important effects observed were the inhibition of the proliferation of the $CD56^{bright}CD16^{+/-}$ subset and the inhibition of IFN-γ production, suggesting that MPA might have a negative effect on NK cell mediated GvL responses *in vivo* after HSCT [86].

4.1.4. Effects on Dendritic Cells

DCs are the most important antigen presenting cells, especially during primary immune responses. Mehling et al. showed that MMF impairs the maturation and function of murine dendritic cells due to the downregulation of co-stimulatory molecules, CD40 and CD86, required for the maturation process. Similarly, studies on human monocyte-derived DCs showed that MPA suppresses their maturation and allostimulatory functions, highlighting a beneficial role for transplantation through tolerance induction [87].

4.1.5. Effects on Adhesion Molecules

An *in vitro* study conducted by Blaheta et al. showed that MMF suppresses in a dose-dependent manner the glycosylation and expression of several adhesion molecules such as vascular cell adhesion protein (VCAM)-1, E-selectin and P-selectin on T lymphocytes [88]. Furthermore, it was shown that MMF impairs T cell transendothelial migration [88]. *In vivo*, MMF decreased leucocytic infiltration into rat kidney allografts, reducing the incidence of acute rejection [89].

4.2. Cyclosporine A

The cyclic oligopeptide, CsA, was primarily described as derived from the fungus *Tolypocladium inflatum*, but several other microorganisms produce it. CsA presents a variety of biological activities, including immunosuppressive, anti-inflammatory, antifungal and antiparasitic properties [90].

Borel et al. were the first to show in 1976 that CsA delays the rejection of skin transplants and prevents/treats GvHD in mice [91].

Since approved for clinical use as immunosuppressant in 1983, CsA has been used in preventing allograft rejection and treating certain autoimmune diseases [90].

4.2.1. Mechanism of Action

Within the cell, CsA binds to cyclophilin and the complex CsA-cyclophilin inhibits calcineurin, a Ca^{2+}-dependent serine threonine phosphatase. This consequently inhibits the nuclear factor of activated T cells (NFAT) dephosphorylation, preventing its translocation into the nucleus. The repression of NFAT translocation into the nucleus prevents the transcription of early genes, such as IL-2, IL-4, IFN-γ, TNF, but also FasL and CD40L. Thus, CsA exerts its main immunosuppressive effect by inhibiting T cell activation [92].

4.2.2. Pharmacokinetic

CsA pharmacokinetic is highly variable in humans according to the type of organ transplanted, the co-administration with other drugs that can interact with CsA, the disease state and age of the patient. Its metabolisation occurs in the liver and the half-life of the drug is estimated as being between 6.4-8.7 hours [92].

4.2.3. Toxic Effects

In addition to its immunologic effects, CsA presents several side effects, partly due to calcineurin inhibition in non-lymphatic tissues. Among the most prominent side effects are nephrotoxicity and hypertension [92]. However, co-administration of CsA with other immunosuppressive drugs has shown a beneficial synergistic effect with a decrease of its toxic effects [90].

4.2.4. Effects on Lymphocytes and other Cell Types

In 1976, Borel and colleagues [91] thought that CsA was specific of lymphocytes, but over the years CsA was shown to have effects on a wide range

of cells. In Table 3 are summarised several studies reporting the effects of CsA on various cell types.

Table 3. Summary of studies reporting the effects of CsA on T cells, NK cells and other cell types. ↓inhibition; ↑induction; → no difference --- not applicable/not studied; AICD activation-induced cell death.

	T lymphocytes / cytotoxic T lymphocytes	NK cells	B cells / B cell line	Mast cells	Basophils
Degranulation	↓[93]	---	---	↓[94]	↓[94, 95]
Proliferation	↓[91]	↓[85, 96]; ↓(trend) [86]	---	---	---
Cytolytic function	↓[93]	↑[96]; ↓[97]; →[85, 86];	---	---	---
Cytokine production	↓IFN–γ and TNF [43]; ↑TGF-β [98]	↓IFN-γ [86]; ↑IFN-γ [96]	---	---	---
Apoptosis	↑[99]; ↓[100]	↑[96]	↓[100]	---	---
AICD	↓[101, 102]	---	↓[103]	---	---

5. Aim

MMF and CsA have been successfully used worldwide alone or in combination for GvHD prophylaxis [104-106]. Although the effects of these immunosuppressive drugs have been extensively studied in T cells, little is known about their effects on NK cells.

The aim of the present *in vitro* study was to gain a better understanding of the effects of MPA and/or CsA on PB and CB NK cell phenotype and function. This study intended to determine if CB NK cells are more sensitive to the immunosuppressants tested than PB NK cells. NK cells have been described as effectors of the GvL effect without causing GvHD. Thus, a fully functional NK cell population after HSCT is important to obtain a more favourable outcome. Hence we intended to identify the immunosuppressive regimen that preserves better NK cell functionality.

Materials and Methods

6. Collection of Blood Samples

PB samples from healthy volunteers were collected in sterile heparinised tubes. CB samples were obtained from the Anthony Nolan Cord Blood Bank, Nottingham, United Kingdom. Samples were collected, using routine banking procedures, into a CB donation bag containing a Citrate-Phosphate-Dextrose anticoagulant buffer. Only fresh CB units (up to 48h after collection) were used. All samples were collected under written informed consent.

7. Cell Isolation

7.1. Peripheral Blood Mononuclear Cell Isolation

The blood was diluted 1:1 in phosphate buffered saline (PBS) 1X (Lonza, Basel). Lympholyte-H® (Cederlane, Gateshead) density gradient was used to isolate PBMCs. Cells were washed and counted using trypan blue® (Sigma-Aldrich, Dorset) to assess cell viability. Thereafter, cells were either cultured in RPMI-1640 (Lonza) supplemented with 10% foetal bovine serum (FBS) (Lonza), 1% penicillin-streptomycin™ (Pen-Strep) (Lonza) and 0.1% β-mercaptoethanol (Gibco Invitrogen, Paisley) or resuspended in PBS 1X supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA (Gibco Invitrogen) for NK cell isolation.

7.2. Cord Blood Mononuclear Cell isolation

The blood was diluted 1:1 in RPMI-1640 supplemented with 10% FBS, 1% Pen-Strep, 33% trisodium citrate® (AnalaR, Poole) and 0.05 μM β-mercaptoethanol and allowed to equilibrate at room temperature for 45 minutes. Ficoll-Paque™ Plus (GE Healthcare, Chalfont St Giles) density gradient was used to isolate CB mononuclear cells (CBMCs). CBMCs were treated with lysing buffer 1X (BD Pharmingen, Oxford) at room temperature for 2 minutes to lyse red blood cells, washed and counted with Türk solution (methylene blue and 1% acetic acid (v/v)) to discriminate red blood cells and trypan blue to assess cell viability of white

blood cells. CBMCs were either cultured in RPMI-1640 supplemented with 10% FBS, 1% Pen-Strep and 50 µM β-mercaptoethanol or resuspended in PBS 1X supplemented with 0.5% BSA and 2 mM EDTA for NK cell isolation.

7.3. Natural Killer Cell isolation

CB samples were treated with the human granulocyte depletion cocktail (StemCell Technologies, Grenoble) before CBMCs isolation to deplete granulocytes that could block the column during the $CD3^-CD56^+$ enrichment step.

$CD3^-CD56^+$ populations were obtained by negative selection using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach) according to the manufacturer instructions; however, some modifications were applied to improve the quality of isolation. After labelling with the NK cell microbead cocktail, cells were washed with 25 mL of buffer rather than 1-2 mL of buffer per 10^7 cells, centrifuged at 300xg for 5 minutes and then resuspended in 5 mL of buffer instead of 500 µL (up to 10^8 cells). The magnetic separation was performed using LS columns (Miltenyi Biotec) up to $1x10^9$ total cells per column. The labelled cell suspension was applied to the column and washing steps were performed twice with 7 mL of PBS 1X supplemented with 0.5% BSA and 2 mM EDTA. The enriched NK cell fraction (negative fraction) was centrifuged; cells were resuspended in RPMI-1640 and counted with trypan blue.

8. Cell Culture

PBMCs and CBMCs were cultured in flat-bottom 96 well plates in RPMI-1640 supplemented with 10% FBS, 1% Pen-Strep, 50 µM β-mercaptoethanol without IL stimulation in order to test the effects of immunosuppressants on resting NK cells.

However, after 24 hours of culture, most CB NK cells were dead (Figure 3), while PB NK cells could withstand without stimulation for a longer time period.

For that reason the protocol had to be optimised to address the needs of CB NK cells and 1000 IU/mL of IL-2 was added to the cultures. Immunosuppressive drugs were added at the beginning of the culture.

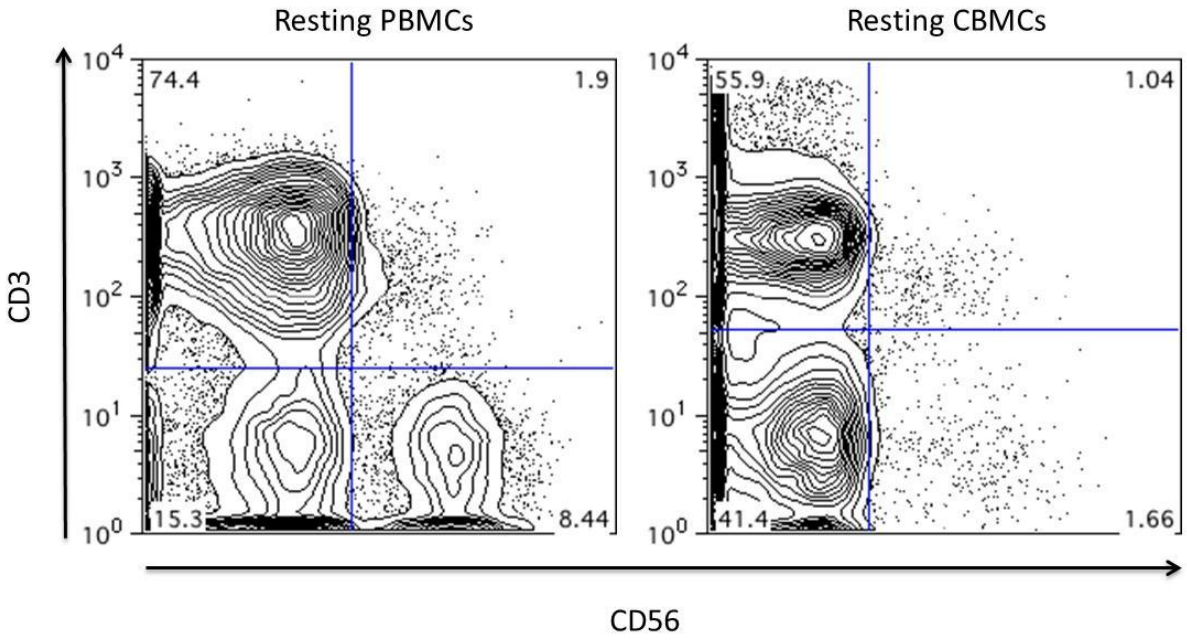

Figure 3. Comparison between resting PB and resting CB NK cell population at 24h after cell isolation. FACS plots from a representative donor showing staining for CD3 and CD56 of resting PBMCs compared to resting CBMCs in the absence of immunosuppressive drugs at 24h after cell isolation.

PB and CB isolated NK cells were cultured in round-bottom 96 well plates in RPMI-1640 supplemented with 10% FBS, 1% Pen-Strep, 50 μM β-mercaptoethanol and IL-2 at 1000 IU/mL for 5 days. Immunosuppressive drugs were added at day 5.

Cells were maintained in an incubator (Sanyo) at 37ºC, in a humidified atmosphere with 5% CO_2. The various concentrations of immunosuppressants used in different experiments are listed in Table 4.

Table 4. List of immunosuppressants and their corresponding concentration used in different experiments.

Immunosuppressants	Viability Activation	Granzyme B and perforin production	CD107a degranulation assay	Proliferation assay
MPA (μM)	2.5, 5, 7.5 and 10	5 and 10		
CsA (ng/mL)	5, 50, 500 and 5000	50 and 5000		
MPA (μM) + CsA (ng/mL)	2.5+5, 5+50, 7.5+500 and 10+5000	5+50 and 10+5000		

9. Phenotypic Analysis

The following anti-human monoclonal antibodies were supplied by BD Pharmingen: CD3-FITC (HIT3), CD3-PE (HIT3), CD3-PerCP (HIT3), CD16-FITC (NKp15), CD56-APC (B159), CD56-PE (B159), CD69-PerCP (L78), CD107a (H4A3), granzyme B-FITC (GB11) and perforin-PE (G9). 7-aminoactinomycin D (7-AAD), annexin-V-FITC, mouse IgG1 κ isotype control FITC (MOPC-21), mouse IgG2b κ isotype control PE (G9) were also supplied by BD Pharmingen. NKp44-APC (P44-8) was purchased from Biolegend (London).

For surface labelling, cells were incubated with antibodies in the dark at 4°C for 10 minutes, washed and resuspended in either cold PBS 1X with 2% FBS or Annexin-V binding buffer (BD Pharmingen) 1X.

Annexin-V and 7AAD were used to assess NK cell viability, while CD69 and NKp44 were the receptors chosen to study NK cell activation.

Perforin and granzyme B expression was assessed by intracellular staining after cell membrane permeabilisation with the Cytofix/Cytoperm™ kit from BD Pharmingen. Briefly, a 10% mouse serum blocking step preceded CD3, CD56 and CD16 surface labelling at 4°C for 10 minutes in the dark to avoid nonspecific binding. Cells were fixed and permeabilised with Cytofix/Cytoperm™ at 4°C for 20 minutes in the dark. After a second blocking step, cells were incubated either with the target or the isotype control antibodies in the dark at 4°C for 1 hour, washed twice with Perm/Wash™ buffer 1X and then resuspended in PBS 1X with 0.1% FBS.

Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences) and the data were analysed using FlowJo software (Tree Star, Oshland).

9.1. Gating Strategy

9.1.1. Effects of Immunosuppressants on NK Cell Viability

The lymphocyte population and the smaller population representing dying cells were gated based on forward and side scatter. Subsequent analyses were done on CD3-CD56+ cells. Cell death was determined by the expression of annexin-V and 7AAD (Figure 4).

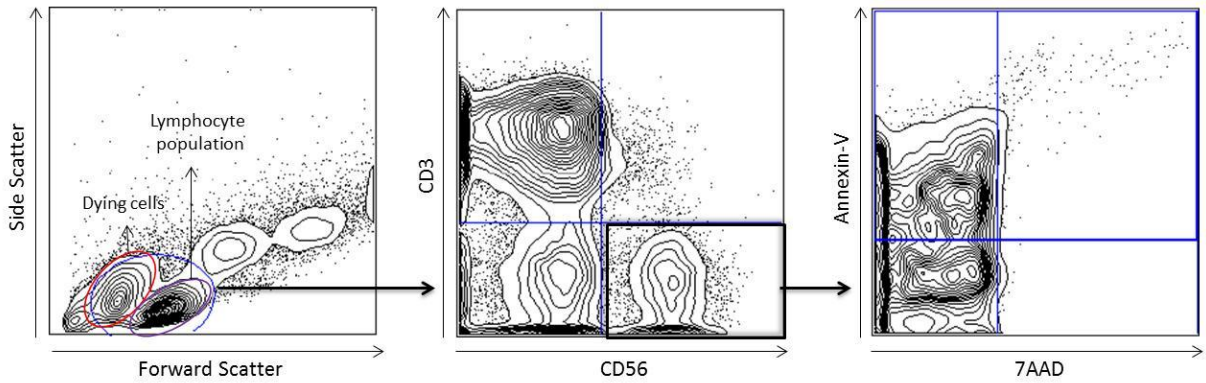

Figure 4. Gating strategy used for the viability study of NK cells. The example represents the analysis of one PBMCs sample. The same gating strategy was applied to CBMCs samples.

9.1.2. Effects of Immunosuppressants on NK Cell Activation

The lymphocyte population was gated based on forward and side scatter. CD69 and NKp44 mean fluorescence intensity (MFI) was determined on NK cells by gating on CD3-CD56+ cells (Figure 5).

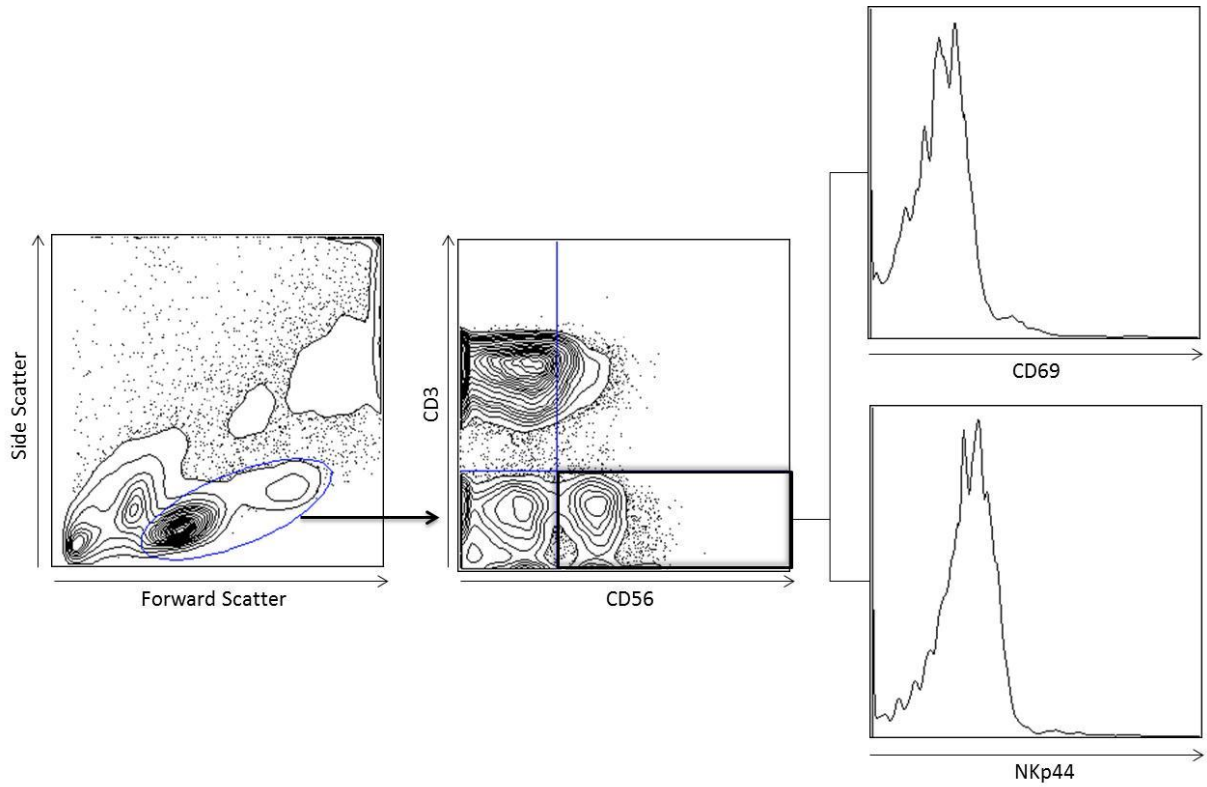

Figure 5. Gating strategy used to analyse the expression of activation markers on NK cells. The example represents the analysis of one PBMCs sample. The same gating strategy was applied to CBMCs samples.

10. Proliferation Assay

Freshly isolated NK cells were resuspended in PBS at 10^6 cells/mL and labelled with 2 μM carboxyfluorescein succinimidyl ester (CFSE) using the Cell Trace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen, Carlsbad) for 10 minutes in the dark at 37°C. Cells were washed twice with cold RPMI supplemented with 10% FBS and then cultured in RPMI supplemented with 10% FBS, 1% Pen-Strep, 50 μM β-mercaptoethanol and IL-2 at 1000 IU/mL in the presence or absence of immunosuppressive drugs for 5 days. Fresh media with 1000 IU/mL of IL-2 was added every 2 days and the cells were split when needed. Cells were analysed by flow cytometry at day 2 and day 5.

11. CD107a Degranulation Assay

Immunosuppressive drugs were added to IL-2 activated NK cells at day 5 of culture. At day 7 of culture NK cells were resuspended in 200 μL of fresh RPMI-1640 supplemented with 10% FBS, 1% Pen-Strep, 50 μM β-mercaptoethanol. The cells were stimulated with 100 ng/mL of PMA and 1 μg/mL of ionomycin for 2 hours at 37°C. 10% blocking mouse serum was added for 10 minutes at room temperature, the cells were then stained with CD56, CD3, CD16 and either CD107a or the corresponding isotype control antibodies, in PBS supplemented with 2% FBS and 2 mM EDTA for 45 minutes at 4°C. The cells were then washed, resuspended in PBS containing 2% FBS and 2 mM EDTA, and analysed by flow cytometry.

12. RNA isolation

RNA was extracted using the RNeasy® Mini Kit (Qiagen, West Sussex) from both freshly isolated and IL-2 (1000 IU/mL for five days) activated CB or PB NK cells according to the manufacturer's instructions. RNA concentration and purity were determined by measuring the optical density at 260 nm (OD_{260}) and the OD_{260}/OD_{280} ratio using a NanoDrop® ND-1000 spectrophotometer (Labtech, East Sussex).

13. Reverse Transcription

cDNA was synthesised from 200 ng of RNA using random primers (Promega, Southampton) and the SuperScript® III reverse transcriptase (Invitrogen). The mix was incubated for 50 minutes at 42°C and then 15 minutes at 70°C to inactivate the enzyme.

14. Real Time PCR

Quantitative real time polymerase chain reaction (PCR) was performed using a 7500 System SDS instrument (Applied Biosystems, Paisley). The data were analysed using the 7500 System SDS Software version 1.4 (Applied Biosystems). SYBR Green (Primer Design, Southamptom) was used as a fluorogenic probe. The β subunit of the mitochondrial ATP synthase (ATP5B), ubiquitin C (UBC) and topoisomerase I (TOP I) (Primer Design) were chosen as internal controls. The sequences of the primers used are listed in Table 5. Samples and internal controls were run in triplicates at the same time in a single 96-well optical reaction plate (ABI PRISM, Applied Biosystems). The comparative threshold cycle (Ct) method was used to quantify mRNAs.

Table 5. Primer Sequences.

	Forward (3'-5')	**Reverse (5'-3')**
IMPDH1	GCACACTGTGGGCGAT	GAGCCACCACCAGTTCA
IMPDH2	TCTTCAACTGCGGAGAC	CTGTAAGCGCCATTGCT
NFATc	GCCGCAGCACCCCTACCAGT	TTCTTCCTCCCGATGTCCGTCTCT

15. Statistical Analysis

An unpaired, 2-tailed Student t-test was performed using Excel® software (Microsoft Excel for MAC 2011, version 14.1.0, Berkshire) to analyse the statistical significance. Results are presented as mean ± standard deviation (SD). p values < 0.05 were considered statistically significant.

Results

1. Effects of Immunosuppressants on NK Cell Viability

PBMCs and CBMCs were cultured with 1000 IU/mL of IL-2 with increasing concentrations of MPA and/or CsA. Cells were stained with CD3, CD56, annexin-V and 7AAD to assess NK cell viability. None of the conditions tested decreased PB or CB NK cell viability (Figure 6), however, under these conditions there was a high percentage of NK cell death at 24 and 48 hours.

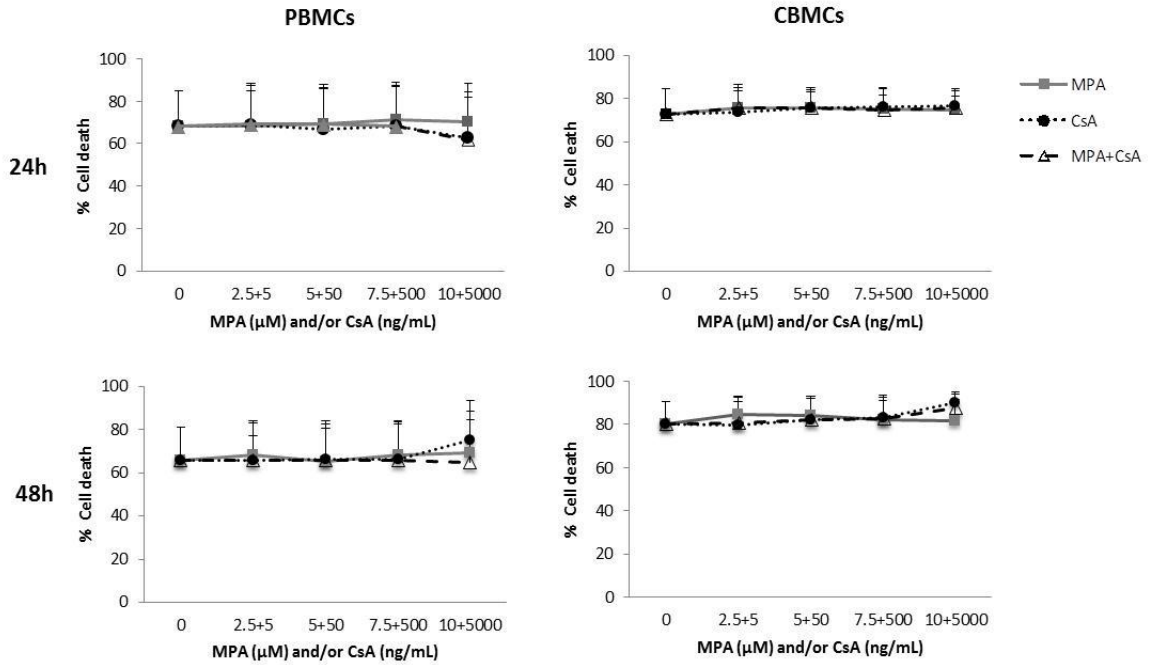

Figure 6. Influence of immunosuppressants on the viability of non-isolated NK cells. Percentage of NK cell death of IL-2 (1000 IU/mL) stimulated PBMCS (N=5) and CBMCs (N=5) at 24 and 48 hours after the addition of MPA (2.5, 5, 7.5 and 10 µM) and/or CsA (5, 50, 500, 5000 ng/mL).

As the previous *in vitro* culture conditions showed not to be ideal for NK cell survival, we decided to isolate and activate NK cells, prior to the drug addition in order to test the effects of the drugs on NK cell viability. PB and CB NK cells were isolated by negative selection and cultured with 1000 IU/mL of IL-2 for five days. At day 5 of culture, drugs were added at increasing concentrations. Cells were stained at day 6 and 7 of culture with CD56, annexin-V and 7AAD.

For up to day 6 of culture immunosuppressants did not affect PB or CB NK cell viability. Nevertheless, at day 7 of culture, MPA and MPA+CsA, not CsA,

increased the percentage of cell death of CB NK cells in a dose dependent manner compared to untreated cells. The immunosuppressants did not affect significantly PB NK cell viability, but MPA showed a trend towards an increase of cell death (Figure 7).

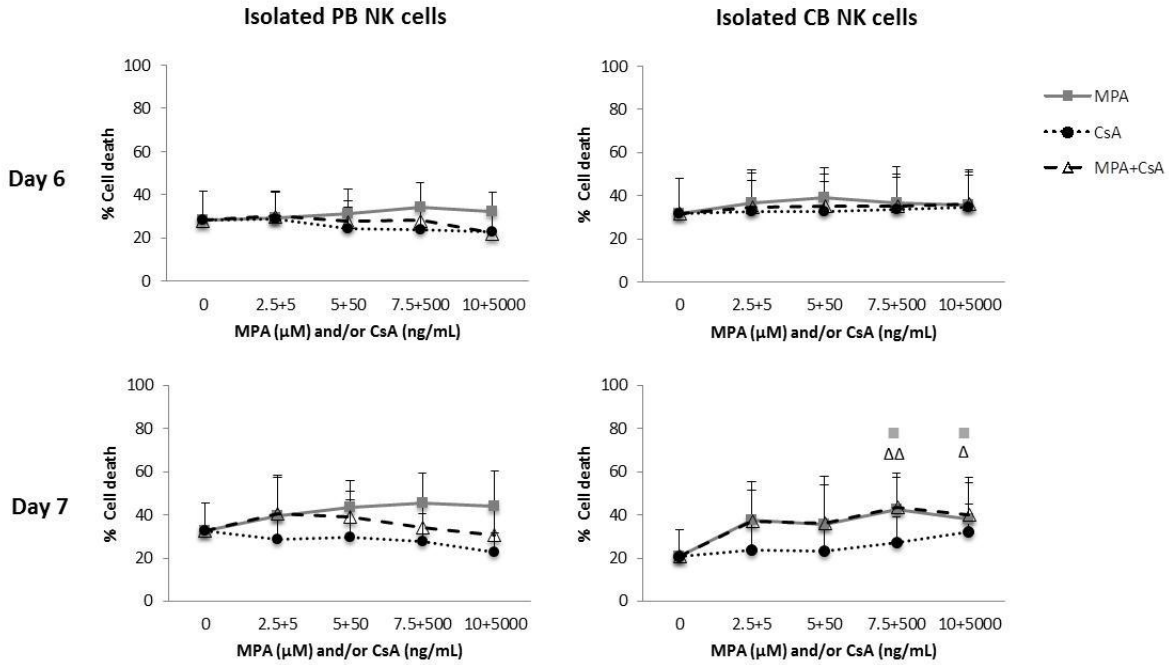

Figure 7. Influence of immunosuppressants on the viability of isolated NK cells. Percentage of cell death of isolated PB (N=5) and CB (N=6) NK cells activated with 1000 IU/mL of IL-2. MPA (2.5, 5, 7.5 and 10 µM) and/or CsA (5, 50, 500, 5000 ng/mL) were added at day 5 of culture and the cells were stained at day 6 and 7 of culture with annexin-V and 7AAD. ■$p<0.05$ for MPA-treated cells compared to untreated cells, $\Delta p<0.05$ and $\Delta\Delta p<0.01$ for MPA+CsA treated cells compared to untreated cells.

These data suggest that the drugs affected only activated NK cells. Besides CB NK cells were more sensitive to MPA and MPA+CsA than PB NK cells regarding cell viability.

2. Effects of Immunosuppressants on NK Cell Activation

CD69 is an early activation marker of NK cells. NKp44 is a natural cytotoxic receptor that is absent on freshly isolated lymphocytes, but is progressively expressed by all NK cells *in vitro* after activation such as culture with IL-2. This last receptor mediates tumour cell killing [29]. Therefore, we analysed the effects of the immunosuppressants on the expression of both activation markers, CD69 and NKp44.

No significant changes were observed in the expression of CD69 or NKp44 on non-isolated PB or CB NK cells treated with any of the immunosuppressants (Figure 8).

The drugs did not significantly affect the expression of either CD69 or NKp44 on isolated and activated NK cells, but some trends were observed. CsA showed a trend towards the downregulation of CD69 on PB but not on CB NK cells, while all the drugs showed a trend towards the downregulation of NKp44 expression on both PB and CB NK cells (Figure 9).

Thus, the immunosuppressants did not significantly affect the expression of CD69 and NKp44 when they were added to 5 days IL-2 activated PB or CB NK cells.

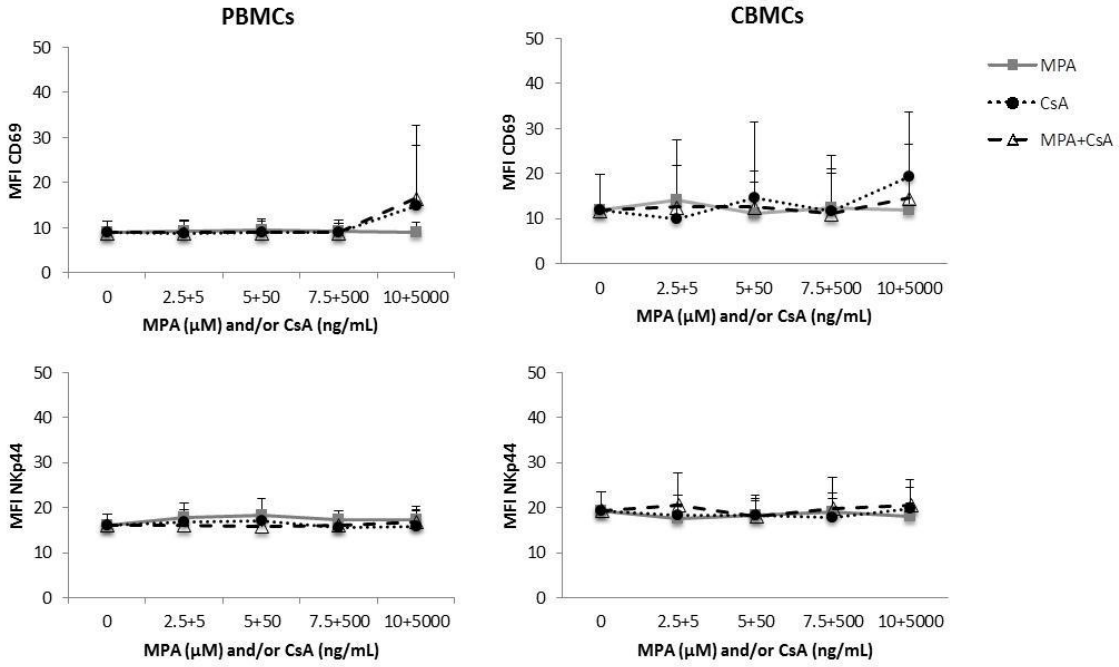

Figure 8. Influence of immunosuppressants on the expression of activation markers by non-isolated NK cells. MFI of CD69 and NKp44 activation markers on IL-2 (1000 IU/mL) stimulated PBMCS (N=5) and CBMCs (N=5) gated on NK cells at 24 and 48 hours after the addition of MPA (2.5, 5, 7.5 and 10 µM) and/or CsA (5, 50, 500, 5000 ng/mL).

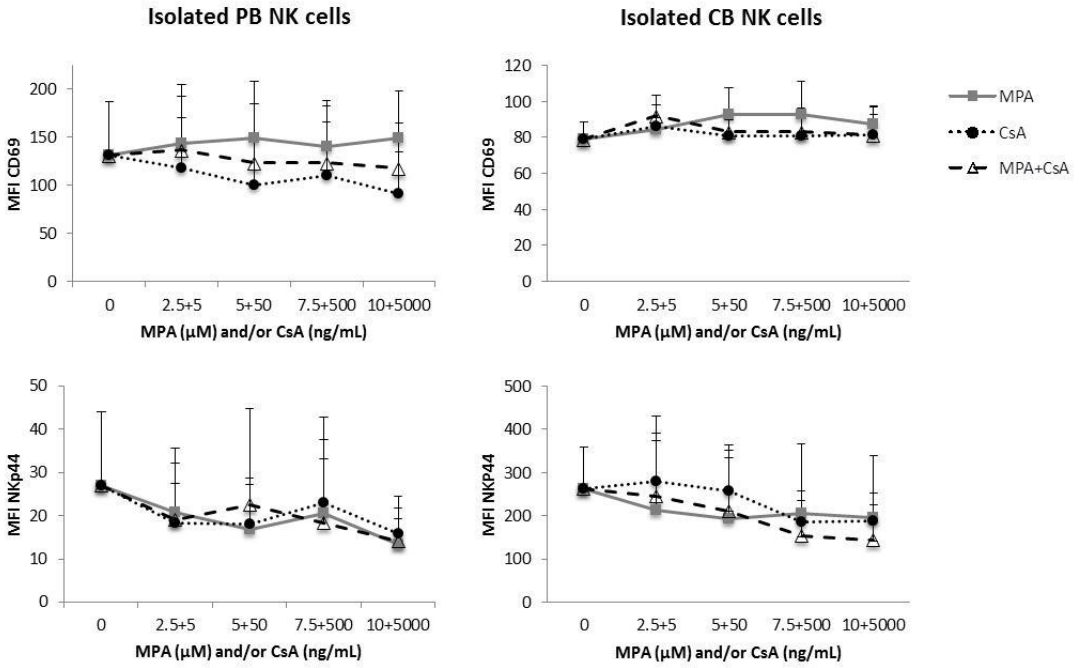

Figure 9. Influence of immunosuppressants on the expression of activation markers by isolated NK cells. MFI of CD69 and NKp44 activation markers on IL-2 (1000 IU/mL) activated PB (N=5) and CB (N=6) NK cells at day 7 of culture. MPA (2.5, 5, 7.5 and 10 µM) and/or CsA (5, 50, 500, 5000 ng/mL) were added at day 5 of culture.

3. Effects of Immunosuppressants on NK Cell Proliferation

To study the effects of MPA, CsA and MPA+CsA on NK cell proliferation, freshly isolated PB or CB NK cells were labelled with CFSE and cultured up to 5 days with 1000 IU/mL of IL-2 in the presence or absence of increasing concentration of MPA and/or CsA. The CFSE levels were determined by flow cytometry at day 2 and 5 of culture.

All NK cells proliferated at day 2 compared to day 0 when stimulated with 1000 IU/mL of IL-2. At day 2 the drugs did not affect the proliferation of PB or CB NK cells compared to untreated cells. At day 5 PB NK cells were divided into 2 groups as they showed differences in proliferation in the presence of the drugs. Two out of six samples tested (group 1) showed reduced proliferation in the presence of the drugs. MPA- and MPA+CsA-treated NK cells showed a similar inhibition of proliferation compared to untreated NK cells independently of the dose tested, while CsA decreased NK cell proliferation in a dose dependent manner. In group 2, none of the drugs inhibited NK cell proliferation (Figure 10).

In contrast, the immunosuppressive drugs inhibited NK cell proliferation of all CB NK cell samples. However, CsA ($p<0.05$) showed to be less potent than MPA ($p<0.001$) and MPA+CsA ($p<0.001$) (Figure 10).

Regarding proliferation, CB NK cells showed to be more sensitive to the immunosuppressants than PB NK cells.

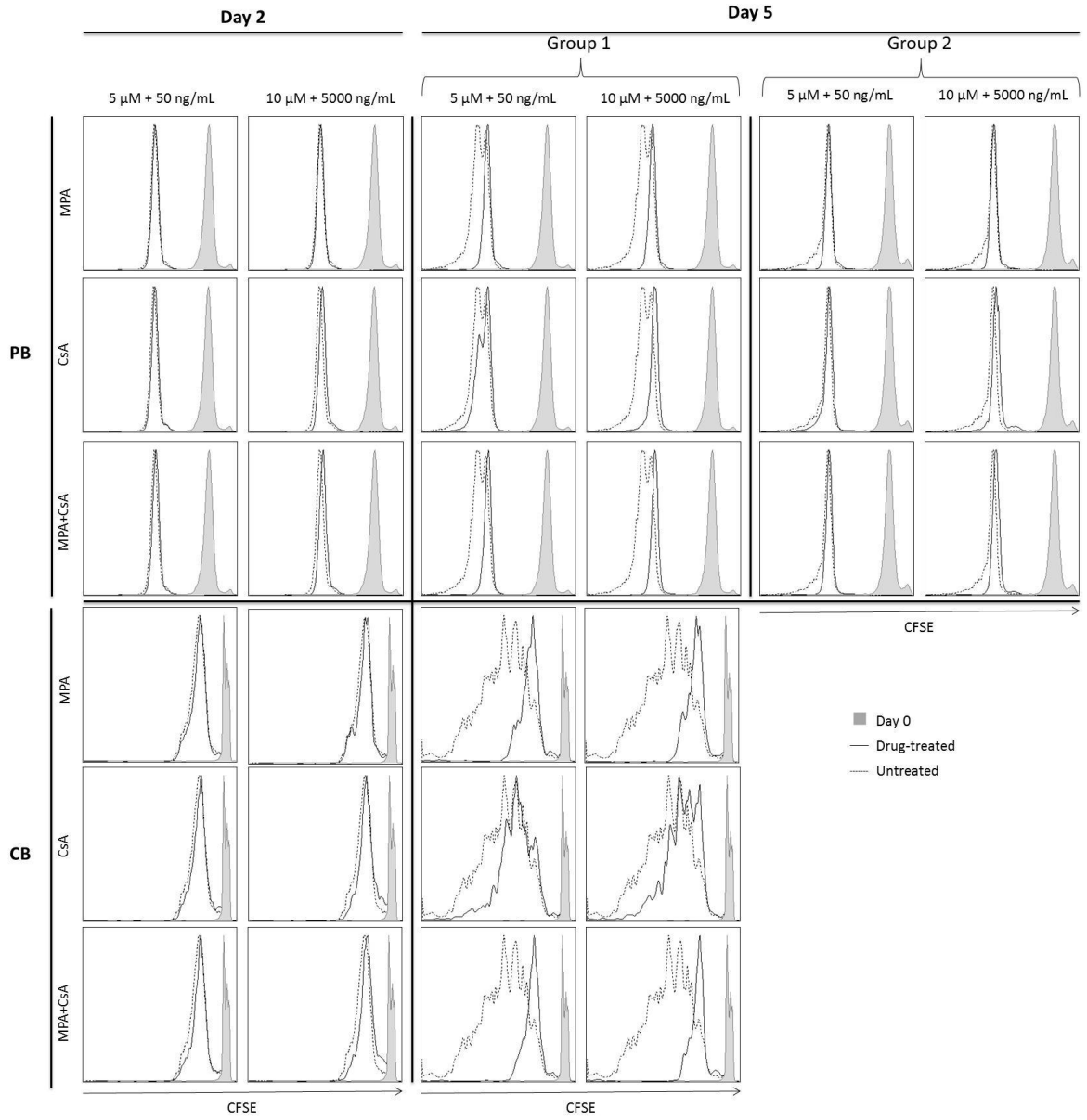

Figure 10. Influence of immunosuppressants on NK cell proliferation. CFSE division profile at day 2 and day 5 of IL-2 (1000 IU/mL) stimulated untreated (dashed line, open histograms) compared to drugs-treated (heavy line, open histograms) NK cells. The CFSE content at day 0 is represented by a heavy line, closed histograms. As at day 5 PB NK cells from different individuals showed distinct profiles, they were divided in group 1 (N=2), in which the immunosuppressive drugs inhibited NK cell proliferation and group 2 (N=4), in which the immunosuppressive drugs did not inhibit NK cell proliferation. Regarding CB NK cells, the figure shows a set of histograms from a representative sample. NK cells from 4 individual CB samples showed similar results.

4. Effects of Immunosuppressants on NK Cell Cytotoxicity

NK cells are important effectors of the innate immune system that mediate cytotoxicity against infectious organisms and tumour cells without prior sensitisation by exocytosis of perforin and granzyme B. Perforin creates pores in the cell membrane of the target cell while granzyme B, a serine protease, enters the cell via those pores and induces apoptosis.

To investigate the effects of immunosuppressants on NK cell killing machinery, NK cells were activated for 5 days with 1000 IU/mL of IL-2 and the drugs were added at day 5 of culture. At day 7 of culture granzyme B and perforin production was accessed by intracellular staining.

It is interesting to note that CB NK cells produced far less perforin than PB NK cells. Furthermore, the drugs affected differentially PB and CB NK cells.

The percentage of cells producing perforin was analysed and the perforin content/cell (MFI) was measured. MPA significantly decreased the percentage of perforin producing cells in a dose dependent manner, as well as the quantity of perforin produced by PB NK cells. Although CsA increased significantly the percentage of perforin producing cells, it did not significantly affect the global perforin production. Interestingly, although MPA+CsA significantly decreased the percentage of perforin producing PB NK cells, MPA+CsA only showed a trend to decrease the perforin production. In contrast, the combination of drugs seemed to have the opposite effect on CB NK cells, significantly increasing the percentage of perforin producing cells (Figure 11).

The percentage of granzyme B producing cells, as well as the quantity of granzyme B produced by these cells, was very similar between PB and CB NK cells.

The immunosuppressants did not show major effect on granzyme B production. Although CsA showed a trend towards the decrease of granzyme B production, only the highest concentration of the combination of drugs showed a significant decrease of granzyme B production by CB NK cells (Figure 12).

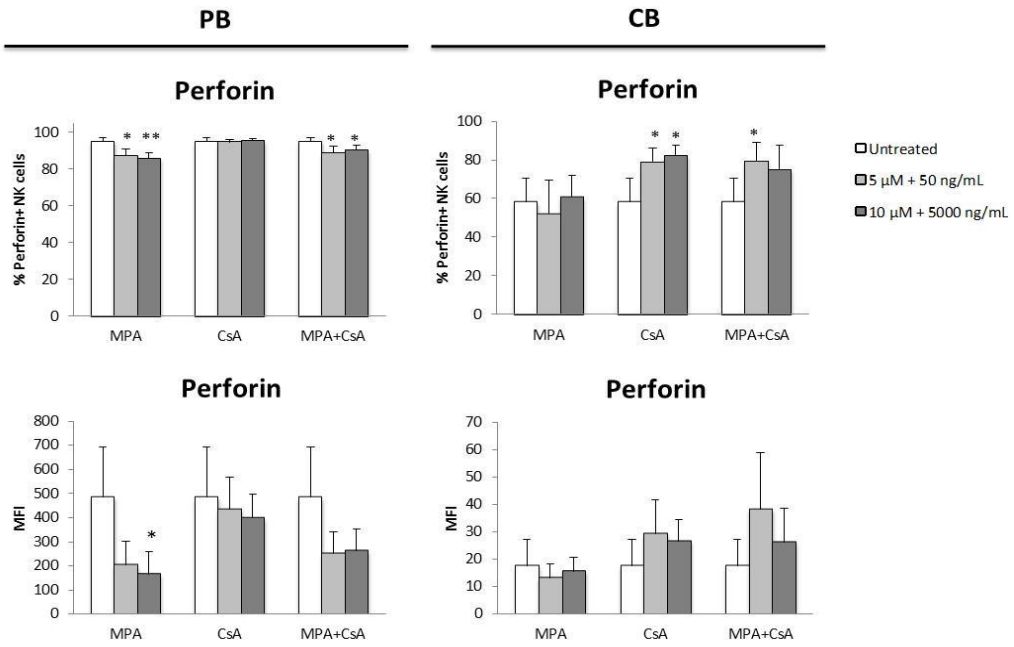

Figure 11. Influence of immunosuppressants on perforin expression by NK cells. Percentage of perforin-producing PB (N=4) and CB (N=4) NK cells (top graphs) and MFI of perforin produced by isolated PB (N=4) and CB (N=4) NK cells (bottom graphs) activated with 1000 IU/mL of IL-2 in the presence of MPA (5 and 10 µM), CsA (50 and 5000 ng/mL) and MPA+CsA (5 µM + 50 ng/mL and 10 µM + 5000 ng/mL). The drugs were added at day 5 of culture and the cells were stained at day 7 of culture. *p<0.05 and **p<0.01 compared to untreated cells.

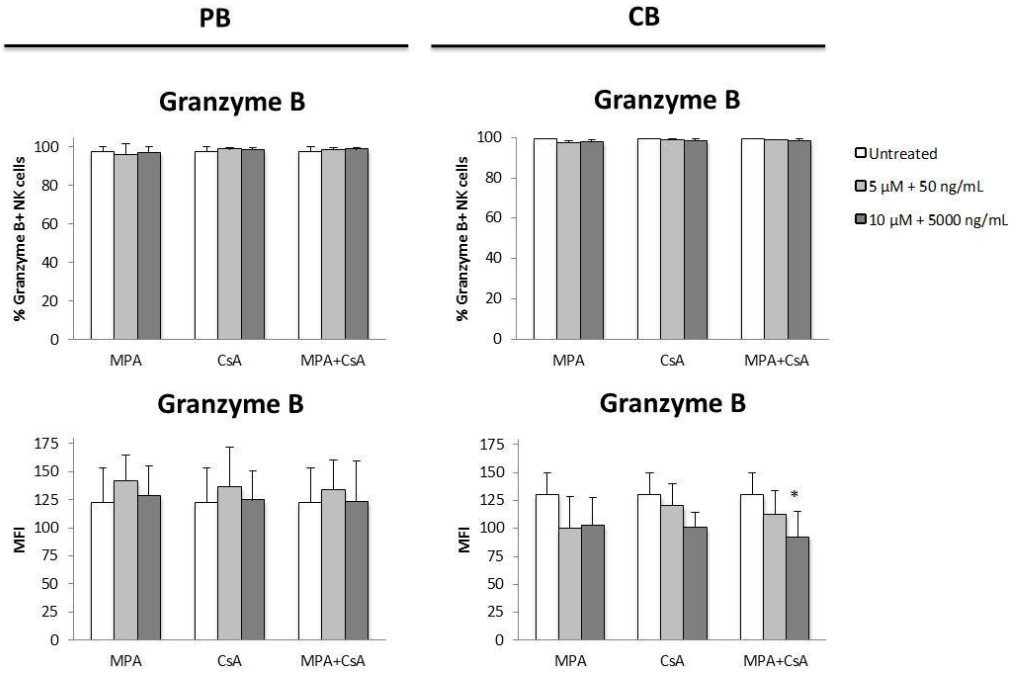

Figure 12. Influence of immunosuppressants on granzyme B expression by NK cells. Percentage of granzyme B-producing PB (N=4) and CB (N=4) NK cells (top graphs) and MFI of granzyme B produced by isolated PB (N=4) and CB (N=4) NK cells (bottom graphs) activated with 1000 IU/mL of IL-2 in the presence of MPA (5 and 10 µM), CsA (50 and 5000 ng/mL) and MPA+CsA (5 µM + 50 ng/mL and 10 µM + 5000 ng/mL). The drugs were added at day 5 of culture and the cells were stained at day 7 of culture. *p<0.05.

The previous data elucidated how the immunosuppressants affect the intracellular content of perforin and granzyme B. However, the cells are functional only if they are able to degranulate following stimulation.

Therefore, the cells were activated with 1000 IU/mL of IL-2 for five days and the drugs were added at day 5 of culture. At day 7 of culture cells were stained for CD107a after stimulation with 100 ng/mL of PMA and 1 µg/mL of ionomycin for 2 hours. CD107a, also known as lysosomal-associated membrane protein-1 (LAMP-1), is a marker of degranulation for cytotoxic lymphocytes such as CD8+ T cells and NK cells.

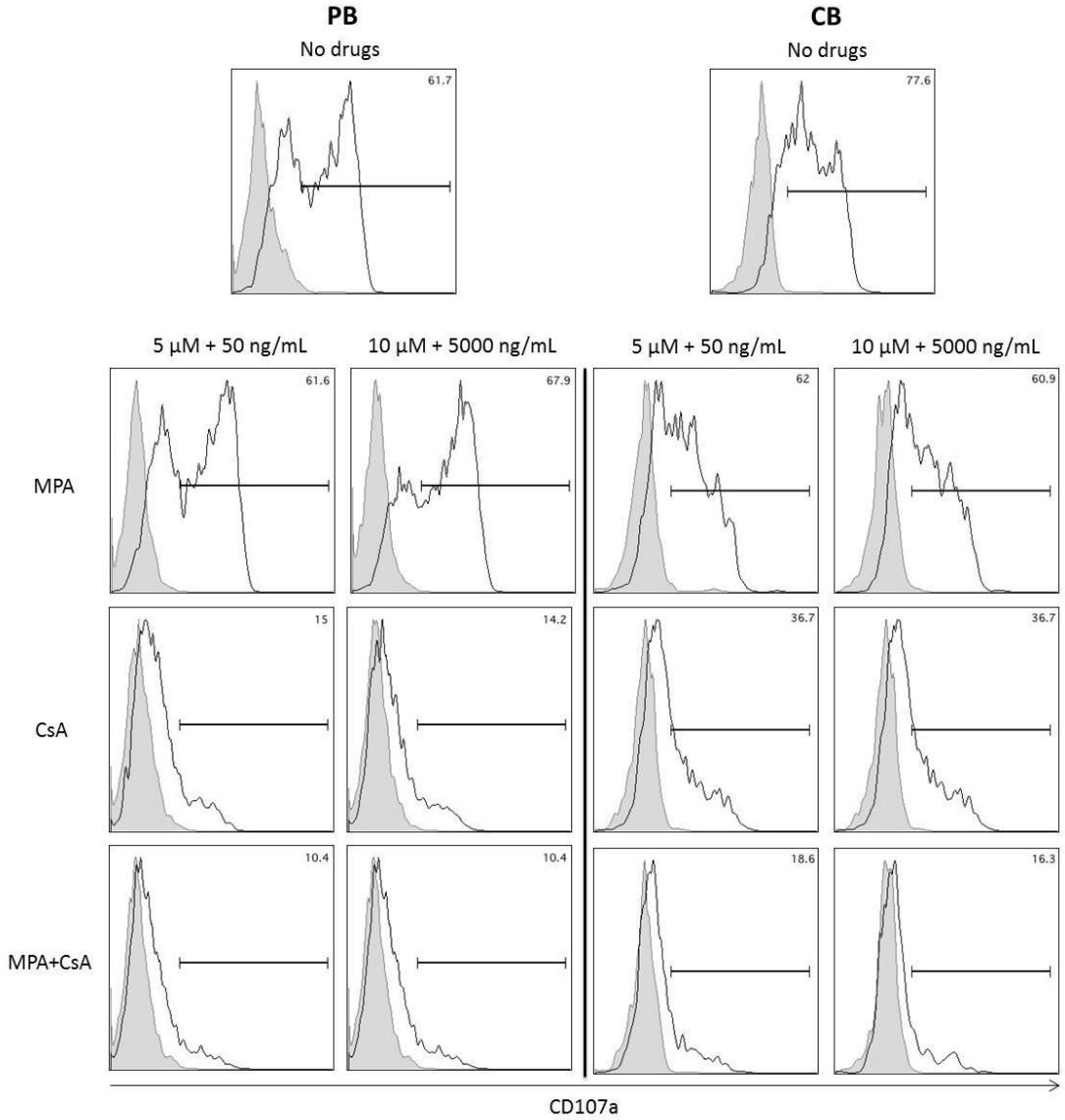

Figure 13. Influence of immunosuppressants on NK cell degranulation. Degranulation of PMA and ionomycin treated NK cells in the presence of immunosuppressive drugs. Flow cytometry histograms showing one representative example of CD107a surface expression on PB (N=3) and CB (N=4) untreated NK cells compared to MPA- (5 and 10 µM), CsA- (50 and 5000 ng/mL) and MPA+CsA- (5 µM+50 ng/mL and 10 µM+5000 ng/mL) treated NK cells at day 7 of culture after 2 hours of stimulation with 100 ng/mL of PMA and 1 µg/mL of ionomycin. Isotype controls are shown as closed histograms.

Figure 13 shows one representative example of PB and CB NK cell degranulation in the presence or absence of the drugs. In this experiment, although a great variability in the amount of degranulation was observed between samples, all the samples showed the same degranulation profile in the presence of the drugs. These preliminary data showed that CsA-treated PB and CB NK cells exhibited a drastic reduction of degranulation compared to untreated cells. MPA+CsA inhibited degranulation almost totally. Surprisingly, MPA slightly increased PB NK cell degranulation, but slightly decreased CB NK cell degranulation.

5. Relative Quantification of IMPDH1, IMPDH2 and NFATc Expression in PB and CB NK Cells by Quantitative Real Time PCR

To understand the difference of sensitivity to the immunosuppressants between PB and CB NK cells, a relative quantification of the drug targets; IMPDH1, IMPDH2 and NFATc expression by resting and activated PB and CB NK cells was performed by quantitative real time PCR.

No significant changes were observed in the expression of IMPDH1, IMPDH2 and NFATc between PB and CB resting or activated NK cells. However, activated CB NK cells showed a trend towards a reduced expression of IMPDH2 and NFATc compared to PB NK cells (Figure 14).

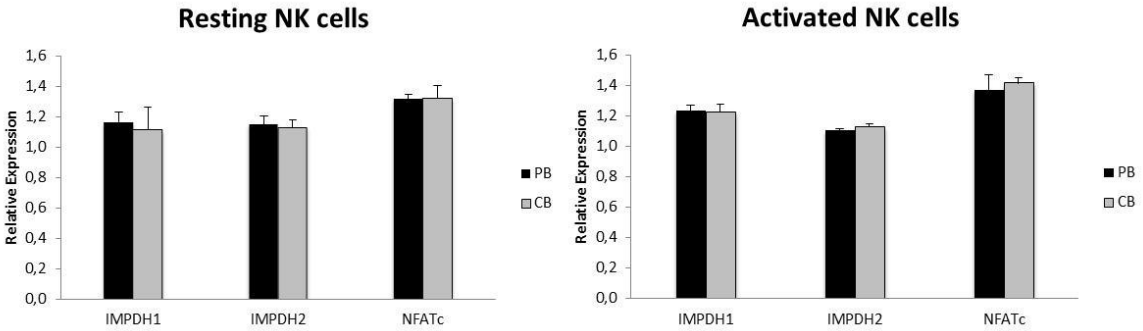

Figure 14. Relative expression of IMPDH1, IMPDH2 and NFATc mRNA levels by resting and activated NK cells. mRNA levels analysed by RT-PCR in freshly isolated PB (N=5) and CB (N=5) NK cells (left) and isolated PB (N=4) and CB (N=5) NK cells activated with IL-2 (1000 IU/mL) for 5 days (right).

Discussion

CB has been increasingly used as a source of SCs for transplantation as an alternative to BM and mPB due to a decreased GvHD incidence, off-the-shelf availability and a greater tolerance to HLA disparity. NK cells reconstitute early after CBT, providing GvL effect, without causing GvHD. These cells have already proven to be beneficial for clinical outcome [65, 66]. Therefore, it is important to study the effects of immunosuppressive drugs on NK cells in order to select a prophylaxis regimen with less impact on NK cell functionality. Although the effects of these drugs have been well studied on T cells, their effects on PB and CB NK cells have been scarcely studied. MPA and CsA have been successfully used worldwide, alone or in combination for GvHD prophylaxis. In the present study, we examined if CB NK cell sensitivity to MPA and/or CsA differs from their adult counterparts in terms of viability, activation, proliferation and cytotoxicity.

We first studied the effects of MPA and/or CsA on PB and CB NK cell viability. We showed that MPA significantly increase CB NK cell death. From our knowledge, we were the first to show that MPA induces cell death of CB NK cells in therapeutic and supratherapeutic doses at day 7 of culture. As MPA and the combination of the drugs increased cell death by 20%, this might impact on the post-transplant outcome, reducing the GvL effect and increasing the risk of infections. This data indicates that CB NK cells are more sensitive to MPA than their adult counterparts. In accordance with our results, a few papers have reported MPA as an inductor of apoptosis in lymphocytes [81-83]. Human T lymphocytic and monocytic cell lines treated with MPA also showed an increase in apoptosis [84]. On the other hand the role of CsA in inducing or inhibiting apoptosis is controversial [99, 102, 103, 107, 108]. This might be explained by experimental design differences and different cell type tested. In our experiments, CsA had no effects on PB or CB NK cell viability.

CsA is a calcineurin inhibitor that blocks NFAT translocation into the nucleus and consequently the transcription of several cytokine genes such as IL-2, IL-4 and IFN-γ, inhibiting cell activation. We investigated if the drugs affect NK cell activation by comparing the expression of CD69 and NKp44 by drug-treated NK cells with untreated NK cells. We found no significant difference in the expression of both activation markers, which is not in accordance with the results published

by others (Table 6). In addition to the different experimental design regarding the stimulus used for NK cell activation, drugs concentration and time of incubation, there is another major difference that must be underlined. We added the drugs to NK cells activated with IL-2 for 5 days, while in other studies the drugs were added to freshly isolated NK cells. Studies on T cell activation reported that if CsA was given within 1 hour after activation, it resulted in a total inhibition of T cell proliferation and function. On the other hand, if CsA was given more than 6 hours after activation there was little effect on T cell proliferation [109]. These data indicate that the time of drug addition is crucial, at least for CsA to prevent cell activation.

Table 6. Studies reporting the effects of MPA and CsA on the expression of CD69 and NKp44 by NK cells. ↓downregulation; →no difference. *p<0.05 and **p<0.01.

References	Source	Stimulus	Days	Drug Concentration	Activation marker expression
Wang et al. (2007)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	7	CsA 1000 ng/mL	↓NKp44 (p=0.06)
Lin et al. (2008)	PB/CB	IL-15 (10 ng/mL)	18 h	CsA 1000 ng/mL	PB: ↓CD69 (trend); CB: ↓ CD69*
Eissens et al. (2010)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	5	MPA 500 ng/mL ≈ 1.56 μM	↓NKp44**
				CsA 1000 ng/mL	→NKp44
				CsA 10-10000 ng/mL	↓CD69 (trend)
				MPA 10-10000 ng/mL ≈ 0.0312-31.2 μM	↓CD69 (trend)
Ohata et al. (2011)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	7	MPA 10 μg/mL ≈ 31.2 μM	↓NKp44*

Both Eissens et al. and Ohata et al. showed that MPA donwregulates NKp44 on PB NK cells while we only showed a trend towards the downregulation of NKp44 on these cells. Moreover, the results from Wang et al. and Eissens et al. are not in accordance about the effects of CsA on the expression of NKp44. Wang et al. showed a downregulation of NKp44, while Eissens et al. showed no significant differences in the expression of NKp44. We showed a trend towards the donwregulation of NKp44 by CsA on both PB and CB NK cells. MPA+CsA treated cells followed the same pattern that MPA or CsA treated cells. To our knowledge, the effects of MPA or CsA on NKp44 expression by CB NK cells were not studied by other groups. Nevertheless, CB and PB NK cells showed the same

pattern of NKp44 expression in the presence of drugs. However, it is interesting to note that CB NK cells expressed more NKp44 than PB NK cells.

None of the published studies showed a significant difference of CD69 expression when PB NK cells were treated by either MPA or CsA, but they showed a trend towards the downregulation of CD69, which is in accordance with our results. The only study on CB NK cells showed that CsA dowregulates CD69, but we found no significant difference compared with the untreated cells. This might be explained by experimental design differences in particular by the time of drug addition.

Comparing our results with what was published in the literature: we can conclude that there is a difference in the expression of activation markers if the immunosuppressive drugs are added to freshly isolated NK cells or if they are added to activated NK cells. Preliminary data obtained in our laboratory showed similar results to the literature when the drugs were added to freshly isolated NK cells. This might be relevant for immunotherapies of immunosuppressed patients using NK cell infusions, however further studies are needed.

In contrast with what was published by other groups (Table 7), we showed minor effects of the drugs on PB NK cell proliferation. Only two out of six samples treated with MPA and MPA+CsA showed a reduced proliferation at day 5 of culture. CsA also inhibited PB NK cell proliferation in these two samples in a dose dependent manner. MPA and MPA+CsA inhibited more potently CB NK cell proliferation than CsA.

Experimental design differences between our work and others might account for the contradictory results. In our study, we optimised the conditions for NK cell proliferation by providing close cell contact and adding fresh media plus IL-2 every two days, while in the other studies close cell contact was not provided and NK cells were cultured 5 to 9 days without refreshing the media or IL supply.

Table 7. Studies reporting the effects of MPA and CsA on NK cell proliferation by CFSE assay. ↓inhibition; →no difference. *p<0.05 and **p<0.01.

References	Source	Stimulus	Days	Drug Concentration	Proliferation
Wang et al. (2007)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	3, 5, 7 and 10	CsA 1000 ng/mL	→$CD56^{+}CD16^{-}$ ↓$CD56^{+}CD16^{+}$ ↓$CD56^{dim}CD16^{+}$ * ↓$CD56^{bright}CD16^{-}$ *
Eissens et al. (2010)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	5	MPA 100-10000 ng/mL ≈ 0.312-31.2 µM	↓***
				CsA 100-10000 ng/mL	↓(trend)
Ohata et al. (2011)	PB	IL-2 (100 IU/mL) + IL-15 (10 ng/mL)	1, 5 and 9	CsA 1000 ng/mL	↓
				MPA 1-100 µg/mL ≈ 0.312-31.2 µM	↓

Several studies reported controversial results regarding the effects of CsA on NK cell cytotoxicity. Eissens et al. and Ohata et al. recently reported that CsA did not impair IL-2 and IL-15 activated PB NK cell cytotoxicity against K562 cells [85, 86]. Ohata et al. also studied the effects of CsA on PB NK cell cytotoxicity against Daudi cells, showing a trend towards an increased cytotoxicity [85]. Moreover, Wang et al. reported that CsA improved cytolytic activity of IL-2 and IL-15 activated PB NK cells against K562 and Raji cells [96]. However, Lin et al. published that CsA did not affect K562 cytolysis by unstimulated CB and PB NK cells, but did significantly suppress K562 cytolysis by IL-15 activated CB and PB NK cells [97].

To our knowledge, there are few studies reporting the effects of MPA on NK cell cytotoxicity, but the existing data show that MPA impairs cytolytic activity of PB NK cells [85, 86].

Our study regarding the effects of MPA and/or CsA on NK cell cytotoxicity is based on the analysis of perforin and granzyme B intracellular production and in the capacity of NK cells to degranulate after activation. We showed that MPA significantly decreased perforin production by PB NK cells, but not by CB NK cells. These data could explain the impaired cytotoxicity of MPA-treated PB NK cells against K562 and Daudi cells published by other groups [85, 86]. CsA did not affect perforin levels in PB NK cells, which is in accordance with results published by Wang et al. [96]. In contrast, CsA significantly increased the percentage of

perforin producing CB NK cells and induced a trend towards the increase of global perforin production. Lin et al. reported that CsA did not affect intracellular perforin production of IL-15 activated CB NK cells [97].

Importantly, CsA drastically inhibited both PB and CB NK cell degranulation, shown by a significant decrease in CD107a expression, which might explain the impaired cytotoxicity of CsA-treated CB NK cells reported by Lin et al. [97]. However, this result is not in accordance with the observations made by Eissens et al., Ohata et al. and Wang et al. Despite the controversy in the results reported in these papers regarding the effects of CsA on NK cell cytotoxicity, none of them reported an impairment of PB NK cell cytotoxicity. On the other hand, several papers reported a blocking by CsA of Ca^{2+} dependent degranulation in a mast cell line, basophils and cytolytic T lymphocytes by CsA [93-95]. MPA+CsA showed a trend to reduce perforin production by PB NK cells, but showed a trend to increase perforin production by CB NK cells. Most importantly, MPA+CsA inhibited almost completely the degranulation of PB and CB NK cells suggesting an impairment of perforin and granzyme B release. It is interesting to note that despite the stimulation of CB NK cells with 1000 IU/mL of IL-2 perforin levels were much lower than in PB NK cells, although several papers have published that IL-2 enhances CBMCs cytotoxicity [110-112]. IL-2 activated CBMCs were shown to have a higher cytolytic activity than IL-2 activated PBMCs depending on the targets [110]. Moreover, Joshi et al. suggested that the increase of CBMCs cytotoxicity after IL-2 activation was mediated by FasL and by perforin/granzyme-mediated target cell lysis [110].

PB and CB NK cells expressed similar levels of granzyme B and, in general, the drugs did not seem to affect its expression. Only the highest concentration of MPA+CsA, a supratherapeutic dose, significantly decreased granzyme B production by CB NK cells.

We should bear in mind that perforin/granzyme B mediated lysis is not the only mechanism by which NK cells kill their targets. Other mechanisms, such as FasL mediated cytotoxicity and ADCC, are also used by NK cells against tumour cells and infectious organisms.

These data should be complemented with an *in vitro* cytotoxic assay against K562 cells to clarify what are the effects of the drugs on NK cell cytotoxicity against a haematopoietic malignant cell line and to confirm whether PB and CB NK cells are capable of comparable cytotoxicity.

Nevertheless, our data suggest that MPA, CsA and MPA+CsA could jeopardise NK cell cytotoxicity.

Regarding the effect of MPA on NK cell cytotoxicity, we would expect a higher sensitivity of PB NK cells compared to CB NK cells, as besides degranulation, MPA also decreased perforin production by PB NK cells, but not by CB NK cells.

In general, CB NK cells seemed to be more sensitive to the immunosuppressants than PB NK cells. Thus, we hypothesised that this difference could be explained by a lower expression of the known drug targets on CB NK cells compared to PB NK cells. However, the analysis of IMPDH1, IMPDH2 and NFATc expression by resting and activated PB and CB NK cells by quantitative real time PCR did not reveal significant difference of drug target expression between PB and CB NK cells. Therefore, we could not conclude on the mechanism underlying the higher sensitivity of CB NK cells. However, we should not exclude the hypothesis that the drugs could affect other molecular targets. To our knowledge, the quantification of drug targets in PB and CB NK cells has not been reported yet.

Conclusion

This work intended to compare the effects of MPA and/or CsA on PB and CB NK cells in order to provide a better understanding of the sensitivity to the drugs of NK cells from different sources.

All drugs tested showed significant effects on NK cells that could jeopardise their beneficial effect as prophylaxis after HSCT. Surprisingly, MPA was the only drug that seemed to preserve CB NK cell cytotoxicity via perforin/granzyme B secretion compared to untreated CB NK cells, however, CB NK cells seemed to be less cytotoxic than PB NK cells even after IL-2 stimulation. Another important effect observed was the increase of cell death caused by MPA and MPA+CsA on CB NK cells. We also showed that the drugs inhibited more efficiently CB NK cell proliferation than PB NK cell proliferation.

We demonstrated that CB NK cells are more sensitive to the drugs than PB NK cells since the same dosage showed a less severe effect or no effect at all on PB NK cells. These data suggest that, if confirmed by *in vivo* studies, the immunosuppressive drugs dosage could be adjusted for CBT or maybe alternatives to MMF and CsA could be considered in order to prevent GvHD and preserve NK cell functionality.

Future Perspectives

This study was part of a project aimed to study the effects of MMF and/or CsA on PB and CB immune cells, stem cell engraftment and immune reconstitution *in vitro*.

Regarding the *in vitro* effects of the immunosuppressive drugs on NK cells, it is crucial to test the cytotoxicity of the drug-treated NK cells against cancer cell lines. The most common cell line used to measure NK cell activity is K562, which is a human chronic myeloid leukemic cell line that does not express MHC class I molecules. Furthermore, it would be interesting to study the effects of the drugs on cytokine production, as CsA impairs cytokine production. Some cytokines produced by NK cells, such as IFN-γ and TNF were shown to play a role in the GvL effect mediated by NK cells [113, 114]. *In vivo* data are needed to support and complement the *in vitro* data. The most important *in vivo* experiment should answer whether the immunosuppressants decrease GvL effect or increase the risk of infections.

This project will provide a better understanding of the effects of the immunosuppressive drugs on PB and CB immune cells and SCs, and should help to identify the best immunosuppressive regimen as well as the dosage to control GvHD while preserving GvL effect and thus improve the outcome of patients after transplantation.

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