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FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

IN VITRO DETERMINATION AND FUNCTIONAL MATURATION OF NATURAL KILLER CELLS FROM UMBILICAL CORD BLOOD PROGENITOR CELLS

MARIA JOÃO NASCIMENTO DE PINHO

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Em cumprimento do disposto no referido Decreto-Lei declara que participou ativamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a ativa colaboração dos outros autores.

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PREFACE

"Uma vez escolhido o caminho, jamais olhemos para trás"

Paulo Coelho - Escritor brasileiro

A presente tese integra-se numa linha de investigação sobre o contributo, de determinadas células do sistema imune, na imunoterapia aplicada a tumores – as células "Natural Killer".

Uma colaboração inicial entre o Departamento de Genética e a Universidade de Duesseldorf, levada a cabo pelo Professor Doutor Mário Sousa, levou a que me fosse proposto o desafio de participar nesse alargado estudo de melhor conhecer e caraterizar as células Natural Killer. Deste modo poderíamos tirar o máximo partido de todas as suas potencialidades para serem aplicadas como terapia em casos de tratamento de doenças, nomeadamente leucemias.

Foi algo que na altura achei irrecusável, uma vez que estaria a contribuir para o conhecimento mais aprofundado das células Natural Killer e no seu contributo para terapias celulares na luta contra tumores. Ao mesmo tempo foi um desafio, pois saía completamente do meu domínio científico.

No decurso desta colaboração, estagiei no *Institute for Transplantation Diagnostics and Cell Therapeutics da Universidade Heinrich Heine de Düsseldorf* onde aprendi diversas técnicas de seleção e diferenciação de células estaminais. O estudo implicava a utilização de células estaminais hematopoiéticas isoladas do Sangue do Cordão Umbilical (SCU). As amostras de SCU eram fáceis de obter uma vez que o Instituto se localizava a paredes meias com o *Jose Carreras-Cord Blood Bank* de Duesseldorf. No decorrer desta colaboração, imediatamente o Professor Doutor Alberto Barros se tornou meu mentor e o Professor Michael Punzel meu co-orientador.

Após esta "aterragem" dentro de uma área que me era totalmente desconhecida, comecei a pensar que todos tínhamos tido a má opção de ter aceite esta proposta. Além disso, após o regresso da Alemanha, verifiquei que nada no meu Departamento estava adaptado às experiências que necessitava de fazer. E o co-orientador a 1600 Km de distância…

PREFÁCIO

PREFACE

Procuramos e fizemos colaborações com diversas instituições para que pudéssemos desenvolver as nossas experiências. Seguiram-se períodos de angústia uma vez que, não só, estava eu aqui sozinha a tentar diferenciar células, mas também o meu co-orientador saíra do Hospital em Duesseldorf para ir trabalhar para uma outra instituição. Fiquei sem saber como dominar o leme… Com isto quero dizer que, na verdade, muitas vezes foi difícil conciliar o trabalho com o seguimento dos estudos mas para mim foi muito atrativo dar continuidade à minha vida académica. Para além disso, comecei a afeiçoar-me às minhas células! E aos poderes que elas poderiam trazer no benefício do tratamento de doenças!

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- My co-Supervisor, Professor Michael Punzel for leading my way with his vast knowledge of science. Thank you for sharing it with me and for your friendship. I really felt satisfied when you keep saying that what I'm doing is fantastic. I really miss your words!

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O meu co-Orientador "não oficial", Professor Doutor Mário Sousa por ter sido o impulsionador deste projeto, por estar permanentemente interessado no meu trabalho, pela persistência na busca de resultados e, principalmente, por ter sido o motor da minha motivação pela ciência… Obrigada por ter sempre a porta aberta (e a qualquer hora) para me receber! Obrigada por tudo, Professor!

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PREFACE

🝰 Os meus colegas de laboratório Vânia e Joel pela constante ajuda prestada principalmente no último e apertado troço deste trajeto. Às vezes, temos amigos que não sabíamos que tínhamos… e vocês foram, sem dúvida, um grande apoio e fontes de motivação. Obrigada!

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Por tudo isto, a presente tese de doutoramento não é apenas o texto que se segue…

ABSTRACT

Natural Killer (NK) cells are a subset of blood lymphocytes that play an important role in the innate immune response against virally infected and transformed cells – independent of the major histocompatibility complex (MHC). Autologous NK cells are suppressed by the recognition of inhibitory "self"-MHC class-I molecules. Cells that are susceptible to NK-cell mediated killing lack inhibitory ligands or have surface molecules that engage activating receptors. Based on these findings, NK cell-based adoptive immunotherapy has been introduced in the clinical setting – however due to the limited amounts of NK-cells and obstacles in NK-cell activation status – there are currently only few clinical studies performed using NK-cells for tumour-immunotherapy.

In the course of this work, we attempted to generate, expand and differentiate NK cells under different conditions, starting with hematopoietic stem and progenitor cells from human umbilical cord blood (UCB) - in order to recapitulate NK cell ontogeny and activation.

In addition, we investigated molecular mechanisms such as candidate transcription factors involved in the expression of NK cell specific genes.

We found that NK cells can be successfully generated and differentiated from UCB-progenitors and identified several known as well as only recently described transcription factors involved in activating NK-cells and eliciting certain effector functions.

Thus, we characterized the development of human NK cells in vitro. For the first time, we described the involvement of certain transcription factors which are implicated in the determination of human NK cell fate and certain activation mechanisms. This could add important knowledge to better understand the molecular and cellular base of NK-cell development and clinical activation to facilitate future clinical applications.

RESUMO

As células *Natural Killer* (NK) pertencem a um subgrupo de linfócitos do sangue periférico que apresentam uma função muito importante na resposta imune inata contra células anómalas ou infetadas por vírus, independentemente do complexo de histocompatibilidade major (MHC). As células NK autólogas são inibidas através do reconhecimento de moléculas inibitórias do MHC.

As células suscetíveis à destruição mediada pelas células NK não apresentam ligandos inibitórios ou então possuem moléculas à superfície que se ligam a recetores activadores das células NK. Foi com base nestes pressupostos que a imunoterapia adotiva baseada em células NK foi introduzida na prática clínica. No entanto, devido à quantidade limitada de células NK e aos obstáculos em conseguir células NK ativas, atualmente existem ainda poucos estudos clínicos com base na aplicação células NK para a imunoterapia de tumores.

No decorrer deste trabalho procurou-se gerar, expandir e diferenciar células NK em diferentes condições, usando células estaminais e progenitoras hematopoiéticas do sangue do cordão umbilical (SCU), como população inicial, de forma a recapitular a ontogenia e ativação das células NK.

Além disso, foram investigados os mecanismos moleculares, nomeadamente os fatores de transcrição envolvidos na expressão de genes específicos das células NK.

Descobrimos que as células NK podem ser geradas e diferenciadas com sucesso a partir de células progenitoras do SCU e identificamos alguns fatores de transcrição (quer já conhecidos quer descritos recentemente) envolvidos na ativação das células NK e que permitem determinadas funções efetoras.

Deste modo, caracterizamos o desenvolvimento in vitro das células NK humanas. Pela primeira vez, descrevemos o envolvimento de determinados fatores de transcrição que estão implicados quer na determinação da identidade das células NK quer em determinados mecanismos de ativação. Isto pode acrescentar conhecimentos importantes para melhor conhecer a base celular e molecular do desenvolvimento das células NK facilitando futuras aplicações clínicas.

Acronyms and Abbreviations:

ACT	allogenic cell transfer
ALL	acute lymphoblastic leukaemia
AML	acute myelogenous leukaemia
APC	antigen-presenting cell
ADCC	antibody dependent cellular cytotoxicity
A-NK	Il-2 activated NK
A-NK12	transgenic modified NK cells with IL-12
BM	bone marrow
BRM	biological response modifier
CLP	common lymphoid precursor
CMV	cytomegalovirus
CTLD	C-type lectin like domain
DD	death domain
DED	death effector domain
DC	dendritic cell
DR	death receptor
DAMP	damage-associated molecular patterns
DAP	DNAX-activation protein
EBV	epstein-barr virus
Ets	E-twenty six
FDA	Food and Drug Administration
Flt3	fms-like tyrosine kinase 3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GvHD	graft *versus* host disease
GvL	graft *versus* leukaemia
GvT	graft *versus* tumour
GMP	good manufacturing practice
HCMV	human cytomegalovirus
HCT	hematopoietic cell transfer
HIV	human immunodeficiency virus
HMGB1	high-mobility group protein B1
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
HLA	human leukocyte antigen
IC	immunocytokines
Id	inhibitor of DNA binding protein
iDC	immature dendritic cell

Ig	immunoglobulin
IFN	interferon
IL	interleukin
iNK	immature NK cells
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KARAP	killer-cell activating receptor-associated protein
KIR	killer cell Immunoglobulin-like receptor
LAK	lymphokine-activated killer
LGL	large granular lymphocyte
LRC	leukocyte Ig-like receptor complex
mDC	mature dendritic cell
MHC	major histocompatibility complex
MIC	stress-inducible MHC class I-related chain
mNK	mature NK cell
MM	multiple myeloma
NCR	natural cytotoxicity receptor
NK	natural killer cell
PB	peripheral blood
PAMP	pathogen-associated molecular pattern
PI3	Phosphatidilinositol 3
PRR	pattern recognition receptors
pNK	precursor NK cell
RCC	renal cell carcinoma
SCT	stem cell transfer
SHP	protein tyrosine phosphatase
SLT	second lymphoid tissue
SCF	stem cell factor
TCD	T cell depleted
TGF	transforming growth factor
TRAIL	TNF-related apoptosis-inducing ligand
TF	Transcription Factor
TLR	Toll-like receptors
TNF	tumour necrosis factor
UCB	umbilical cord blood
ULBP	UL16-binding protein
VDUP-1	Vitamin D(3) upregulated protein 1

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INTRODUCTION

1. NATURAL KILLER CELLS MORPHOLOGY

Natural Killer (NK) cells are Bone Marrow (BM) derived cytolytic lymphocytes, which comprise approximately 10% of all Peripheral Blood (PB) lymphocytes. Morphologically, NK cells are large granular lymphocytes (LGL) that express CD56 cell surface molecules and, unlike T and B cells, do not express receptors that require somatic gene rearrangements to generate receptor diversity and specificity (Ag receptors), as well CD3 (*Lanier et al., 1986*) (Fig. 1). In fact, unlike T-cells, they can kill targets without prior sensitization and exhibit spontaneous cytotoxicity activity towards cells that do not express class I molecules of the Major Histocompatibility Complex (MHC) (*Borrego et al., 2002*).

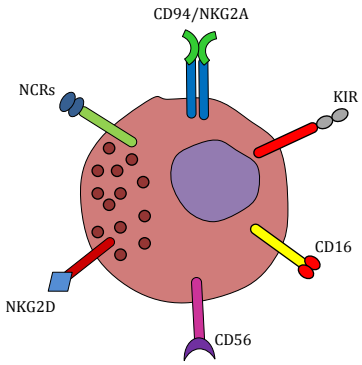


Figure 1 – NK cells are morphologically Large Granular Lymphocytes (LGL) characterized phenotipically by diverse receptors on their surface.

Although representing one of the first lines of immune defence, NK cells exhibit many features normally associated with adaptive immunity. While NK cells do not fully conform to the definition of adaptive immunity, they also differ from members of the innate immune system. For instance, NK cells do not mediate phagocytosis and lack bactericidal enzymatic systems. Rather, NK cells are characterized by several important effector functions, including their capacity to spontaneously lyse susceptible targets.

Natural Killing involves exocytosis of perforin-and granzyme-containing cytoplasmic granules via a metabolically active process [reviewed in (*Smyth et al., 2005*)]. NK cells are also equipped with a variety of destructive arms and can also eliminate target cells via FasL- (*Oshimi et al., 1996*) and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated pathways, sometimes in a developmentally related fashion (*Zamai et al., 1998*).

An equally important function of NK cells involves their capacity to promptly produce cytokines and chemokines (*Cooper et al., 2001a; Robertson, 2002*) which serve to shape adaptive immune responses.

Together, these functional activities place NK cells in a position to eliminate susceptible targets through multiple mechanisms and to recruit and amplify the inflammatory response.

Human NK cells comprise two subsets that are phenotipically and functionally distinct *(Cooper et al., 2001a*). These NK subsets are identified based on the cell surface density of the "NK cell marker" CD56 together with CD16 (Fig. 2). The majority (≈90%) of human NK cells express low levels (dim) of CD56 and high levels (bright) of CD16, whereas a minority (≈10%) is $CD56^{bright}$ and $CD16^{dim}$ or $CD16^{-}$ (*Cooper et al., 2001a*). The immunoregulatory $CD56^{bright}CD16^{-}$ NK subset lacks perforin and appears prepared for cytokine secretion. They are abundant in lymph nodes and tonsils, both of which are areas of immune cell production and maturation (*Ferlazzo and Munz, 2004*).

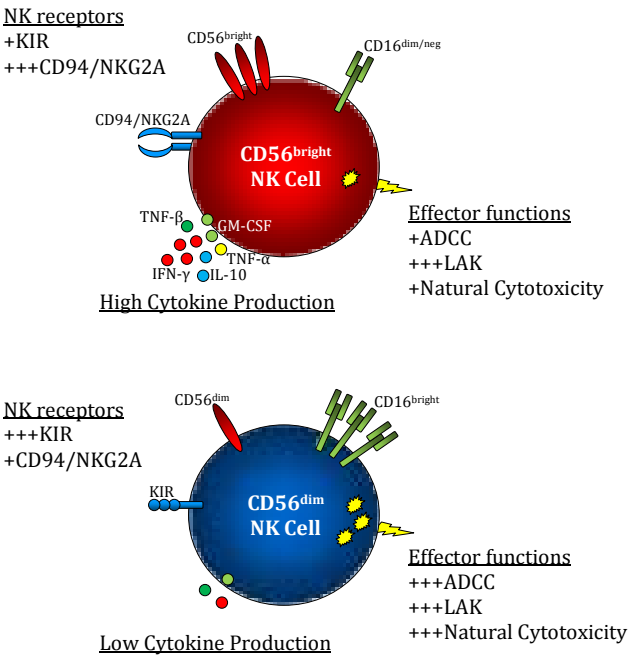


Figure 2 – $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets exhibit differential receptor profiles and innate immune functions.

The more cytotoxic (i.e. high perforin-expressing) $CD56^{low}CD16^{high}$ subset functions as effectors of natural and antibody-dependent target cell lysis (*Cooper et al., 2001b*) and exhibits tumouricidal properties. They circulate in the blood and spleen and may be recruited into inflamed tissues (*Penack et al., 2005*). Although resting $CD56^{dim}$ NK cells are more cytotoxic against NK-sensitive targets than $CD56^{bright}$ cells, IL-2 or IL-12 activated $CD56^{bright}$ NK cells exhibit similar or enhanced cytotoxicity against NK targets compared to $CD56^{dim}$ cells (*Caligiuri et al., 1990*).

In addition, $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets show differences in their NK receptor repertoires. Resting $CD56^{bright}$ NK cells are large agranular cells and express high levels of the C-type lectin CD94/NKG2 family with only very small fractions expressing killer immunoglobulin-like receptors (KIRs).

Resting $CD56^{dim}$ NK cells, however, express CD16, KIRs and C-type lectin NK receptors at high surface density along with an abundance of cytoplasmic granules (*Caligiuri et al., 1990; Jacobs et al., 2001*).

2. NATURAL KILLER CELLS DEVELOPMENT

The development of NK cells from hematopoietic stem cells (HSCs) is guided by environmental cues and intrinsic responsiveness of precursor cells to external signals. As hematopoietic progenitors progress in differentiation towards NK lineage two concomitant processes occur:

(1) acquisition of NK specific gene expression pattern, and

(2) gradual loss of the ability to express genes characteristic for other lineages.

NK cells are derived from $CD34^+$ progenitors, and the BM is considered the main site of NK cell generation in adults, providing cytokines, growth factors and stromal cells necessary for NK cell development (*Colucci et al., 2003*).

Interactions of hematopoietic progenitors with the environment provide growth factors and morphogenic signals that affect lineage fate and guide functional maturation via the triggering of inhibitory and/or activating receptors. The progression from multipotent hematopoietic precursors to mature NK (mNK) cells can be described on the basis of stages of NK cell development.

The most commonly accepted model of NK cell development represents NK cell lineage decision as a linear scheme. Once committed to the NK cell lineage, precursor NK (pNK) cells acquire phenotypic and functional qualities that characterize peripheral mNK cells. The dominant population of human peripheral blood NK cells – the $CD56^{dim}CD16^+$ subset – corresponds to the final stage of maturation, whereas $CD56^{bright}$ NK cells are considered to be immature and recently differentiated intermediates (*Ferlazzo et al., 2004b; Chan et al., 2007*). However, an opposing view is that $CD56^{bright}$ and $CD56^{dim}CD16^+$ NK cells represent two distinct terminal differentiation states, in support of a branching model of NK cell development. The ability of one cell type to transition into another (*Loza and Perussia, 2004; Chan et al., 2007*) supports the notion that they correspond to distinct states of activation.

Human NK cell development can be divided into phases, which differ in cytokine responsiveness of progenitor cells.

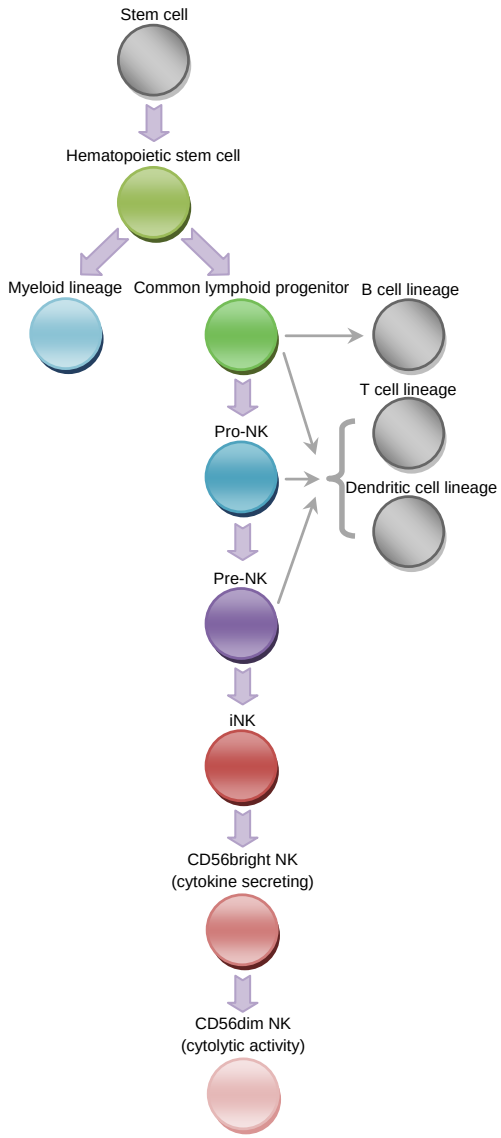


Figure 3 – NK development progresses through a series of stages starting from a common lymphoid progenitor (CLP), which gives rise to the pro-NK, pre-NK, iNK, $CD56^{bright}$ NK and $CD56^{dim}$ NK cell types. Specific stages shown by grey arrows may divert away from the NK cell lineage to become B, T or dendritic cells (DCs).

Development of precursor NK cells (pNK)

NK cells begin their differentiation pathway in the common lymphoid progenitor (CLP) (Fig. 3). After that, five stages of developing human NK cells can be identified within second lymphoid tissue (SLT): pro-NK cells, pre-NK cells, iNK cells, $CD56^{bright}$ NK and $CD56^{dim}$ NK cells (*Freud et al., 2006*). Transcription factors (TFs) involved in the initial NK cell expansion and development include the E-twenty six (Ets) family members PU.1 and Ets-1, Ikaros (a zinc-finger family member), Vitamin D3 upregulated protein-1 (VDUP-1) and the inhibitors of DNA binding (Id) proteins: Id2 and Id3 (*Vosshenrich et al., 2005b*) (Fig. 4). Development of pNK also involves interactions between HSC and stromal cells in vivo because highly purified HSC generate few NK cells in liquid culture (even with high levels of IL-15), whereas NK cell development is greatly enhanced when HSC are cultured on relevant stromal cell lines (*Miller et al., 1994; Shibuya et al., 1995; Mrozek et al., 1996; Yu et al., 1998*).

The first step to becoming a NK cell involves making a commitment to becoming a Natural Killer. This point of no return means that other developmental options within the hematopoietic system are no longer available.

The molecular signals delivered by stromal cells that induce NK commitment are unknown but must include those that control CD122 (IL-15Rβ) expression. Hematopoietic precursors having this profile are defined as pNK and result from a sequential loss of pluripotency as HSC differentiate to more committed hematopoietic precursors. Although, early studies of human NK cell differentiation from hematopoietic precursors used IL-2 (*Miller et al., 1992*), this cytokine is not abundant in the BM microenvironment.

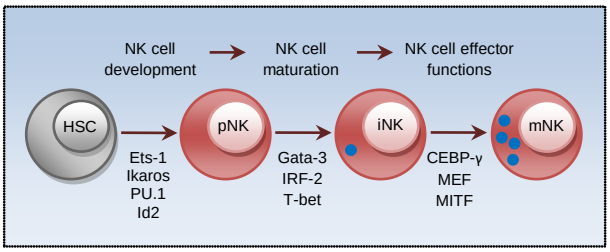


Figure 4 – Transcription Factors (TFs) that condition NK cell development (Ets-1, Id2, Ikaros, PU.1), maturation (Gata-3, IRF-2, T-bet) and functional differentiation of mNK cells (CEPB-γ, MEF, MITF).

As IL-15 is expressed by BM stroma, it was a possible candidate for NK progenitor development and expansion (*Mrozek et al., 1996; Puzanov et al., 1996*). The receptor for IL-15 shares β and γ subunits with IL-2 receptor, explaining the redundancy between IL-2 and IL-15 in vitro.

Similarly, the deficiency of the IL-15 receptor β-subunit (CD122, shared with IL-2R) also results in a profound decrease in NK cells (*Gilmour et al., 2001*).

In line with this, CD122 (IL-15Rβ) has been used to isolate pNKs (*Rosmaraki et al., 2001*).

Cytokine signals play a general role in lymphoid commitment, including those delivered via fms-like tyrosine kinase receptor-3 (Flt3), CD117 (also known as c-kit), or γc-dependent receptors. These cytokines could influence NK commitment because pNK cells express these receptors (*Yu et al., 1998; Vosshenrich et al., 2005a*). Primitive, nonlineage specific growth factors, including stem cell factor (SCF), Flt3-ligand (Flt3-L) and IL-3, also influence NK cell development (*Williams et al., 1997*). These growth factors act upon the early hematopoietic precursors, inducing CD122 (IL-15Rβ) expression, thereby conferring IL-15 responsiveness (*Yu et al., 1998*). These findings suggest that at least one function of IL-15 is to provide survival signals to the developing NK cell (*Minagawa et al., 2002*).

Thus, NK cells are derived from $CD34^+$ HSCs and require cytokines present in the BM environment to mature. In fact, precursors of distinct lineages can be identified by the presence of specific growth factor receptors. The transition from pro-NK to pre-NK is marked by the gain of CD117 and by the ability to respond to IL-15 (*Freud et al., 2006*).

Maturation of NK cells

Pro-NK and pre-NK cells have the potential for non-NK lineage differentiation, whereas iNK cells are committed to the NK lineage. The latter are $CD34^-CD117^+CD94^-$ being CD34 (expressed on pro-NK and pre-NK cells) and CD94 (expressed on $CD56^{bright}$ NK cells) mutually exclusive antigens, implying that an intermediate cell type, which no longer expresses CD34 but does not yet express CD94, must exist (*Freud*

et al., 2006). NK cell differentiation is also controlled by TFs like Gata-3, T-bet and interferon regulatory factor (IRF)-2 (*Vosshenrich et al., 2005b*) (Fig. 4).

CD56 expression gradually accumulates at the population level as cells progress from the pre-NK stage to the iNK stage of maturation. Moreover CD56 expression is uniformly high within the $CD56^{bright}$ NK cell population (*Freud et al., 2006*). Although CD56 is typically considered a marker of mNK cells, the final stage of human NK cell maturation is marked by a decrease in CD56 and CD94 expression and an increase in CD16 (FcRγIII receptor) and KIR characterizing the $CD56^{dim}$ NK cell subset (Fig. 3) (*Caligiuri, 2008*).

During NK maturation, soluble factors [IL-12, IL-18, Interferon (IFN)-α/β] stimulate cytokine and chemokine production by NK cells as well as their lytic capacity.

Acquisition of effector functions

NK cells are restrained from auto-aggression by inhibitory receptors that are specific for MHC class I. During NK cell development, pNK acquire CD94/NKG2A. The linking of inhibitory receptor expression with effector mechanisms appears to be a form of tolerance during NK development and so, CD94/NKG2A expression is coordinated with attainment of functionality (activating receptor expression, cytotoxicity and IFN-γ production) (*Grzywacz et al., 2006*). The ligand for CD94/NKG2A is human leukocyte antigen (HLA)-E (*Braud et al., 1998; Lee et al., 1998b*) and the conserved sequence of this molecule assures that CD94/NKG2A will find its ligand on all healthy cells and tissues (*Kaiser et al., 2005*). Similarly, CD94 and NKG2A both have strikingly conserved sequences in the human population, compared with other NK receptors (*Shum et al., 2002*).

In contrast, different KIR receptors recognize only a selected set of HLA-C, B, or A molecules as their ligands (*Parham, 2005*). Thus, expression of a given KIR does not guarantee an effective inhibitory interaction with self-MHC. In this sense, it appears biologically justified that NK cells rely on the CD94/NKG2A as the first safety mechanism. In fact, all the $CD56^{bright}$ NK cells express high levels of CD94/NKG2A, in contrast to $CD56^{dim}CD16^{+}$ NK cells, where the majority of cells express at least one self-specific inhibitory receptor, but frequently it is a KIR and not CD94/NKG2A (Fig. 2). If $CD56^{dim}$ NK cells are derived from $CD56^{bright}$ NK cells, then this developmental transition would be associated with acquisition

of KIR and the loss of CD94/NKG2A. Alternatively, the existence of such CD94/NKG2A^{-}KIR^{+} CD56^{dim} NK cells might support the existence of another developmental pathway. Since licensing comes about through inhibitory interactions with self MHC (*Kim et al., 2005*), these "not licensed" NK cells have weak, if any, cytotoxicity despite expression of perforin and granzyme B.

The regulation of the development of human NK cells in CD56^{bright} or CD56^{dim} subsets has been partially clarified with the functional characterization of a novel cytokine, IL-21 (*Parrish-Novak et al., 2000*). In synergy with Flt3-L and IL-15, IL-21 promotes expansion and differentiation of NK cells from BM progenitors in vitro. While the combination of the three cytokines supports the development of CD56^{dim}CD16^{bright} highly lytic NK cells, in the absence of IL-21, the generation of CD56^{bright} NK cells lacking CD16 and KIRs is favoured (*Sivori et al., 2003*).

3. NATURAL KILLER CELL INTERACTION WITH OTHER CELLS

3.1 Natural Killer cell receptors and signalling

NK cell functions are regulated by at least 3 families of receptors, two of which recognize classical MHC class I molecules on the surface of target cells. These three families of receptors are structurally distinguished either as belonging to the Immunoglobulin (Ig) superfamily, such as the KIRs (Fig. 5) and Natural Cytotoxicity-Triggering Receptors (NCRs) (Fig. 8) or as members of the C-Type Lectin like Domain (CTLD) superfamily, such as CD94/NKG2s and NKG2D (Fig. 9). The human CTLD family of receptors includes NKG2A, -B, -C, -E and –F which form heterodimers with CD94 and recognize HLA-E, whereas KIR molecules recognize specific HLA-A, -B and -C allotype subsets as well as HLA-G ligands (*Uhrberg et al., 1997*).

In addition, NK cells also express other receptors such as FcγRIII receptor (CD16) (Fig. 10) and Toll-like receptors (TLRs) (Fig. 11). The FcγRIII receptor (CD16) that belongs to the Ig superfamily, known to be involved in antibody-dependent cellular cytotoxicity (ADCC), is the best-characterized membrane receptor responsible for triggering of lysis by NK cells. TLRs recognize structurally conserved molecules derived from pathogen-infected cells, also activating NK cell responses.

Killer cell Immunoglobulin-like Receptors (KIRs)

This family of receptors belongs to the Ig superfamily within the leukocyte Ig-like receptor complex (LRC) on chromosome 19q13.4, that probably has evolved by gene duplication (*Lanier, 2005*). Among the NK cell receptors, the KIRs comprise the most versatile and polymorphic family of receptors. The human KIR gene family contains 15 genes and 2 pseudogenes and the number of KIR genes in the genome of any given individual varies within the population. A database serving as a central depository for KIR sequences can be accessed at http://www.ebi.ac.uk:80/ipd/kir/index.html.

The KIRs are type I transmembrane glycoproteins that can be divided into two subfamilies based on the number of Ig-like domains in the extracellular portion of the protein (Fig. 5), being indicated by a 2D for two domain KIRs (designated KIR2D) or 3D for three domain KIRs (designated KIR3D) (*Marsh et al., 2003*).

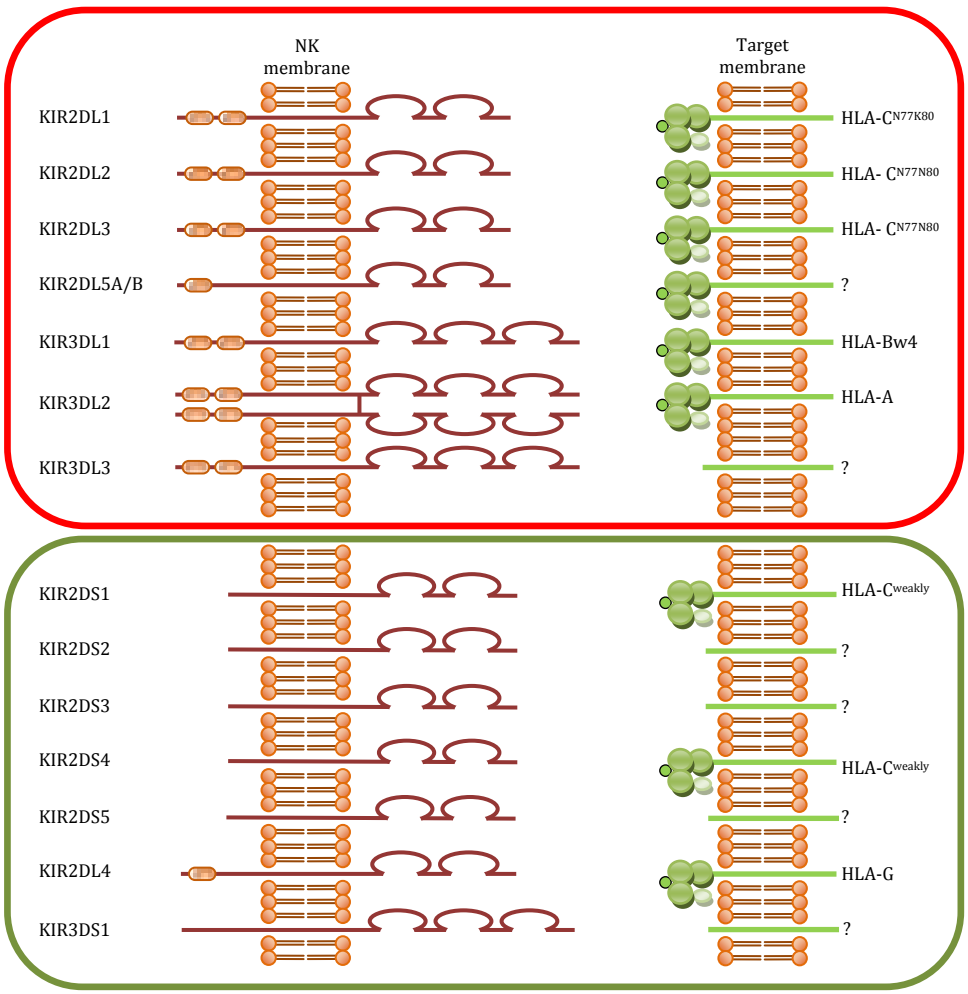


Figure 5 – Inhibitory (outlined in red) and activating (outlined in green) KIR receptors and their ligands.

These domains are designated D0, D1, and D2, with the D0 domain being the most N-terminal in KIR3D proteins, followed by the D1 and D2 domains. A short stalk region separates the Ig-like domains from the transmembrane segment, and the cytoplasmic domains are variable in length; some receptors possess long (L) cytoplasmic domains with one or two immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences, and other receptors have short (S) cytoplasmic domains without ITIM (*Marsh et al., 2003*) (Fig. 5). KIRs with short cytoplasmic domains (KIR2DS and KIR3DS) have a Lys residue, centrally located within their transmembrane region to allow for association with the DAP12 adapter protein. The extracellular Ig-like domains that are involved in classical MHC class I ligand binding, as well as

transmembrane and cytoplasmic regions, define the type of signal evoked on the NK cell (*Vilches and Parham, 2002*).

KIRs control the response of human NK cells by delivering inhibitory or activating signals upon binding to their MHC class I ligands expressed by target cells.

The *inhibitory* KIRs contain ITIM motives in their cytoplasmic domains, which are responsible for the delivery of the inhibitory signal upon receptor ligation (*Muta et al., 1994*). In fact, ITIMs are found in all inhibitory NK receptors, as well as in many other receptors expressed on hematopoietic cells (*Ravetch and Lanier, 2000*). KIRs recognize HLA-A, HLA-B, and HLA-C proteins (Fig. 5). While all inhibitory KIRs interact with MHC class I molecules, they manifest various allelic specificity. Two-domain KIRs (KIR2D) recognize HLA-C haplotypes while three-domain KIRs (KIR3D) recognize HLA-A/B haplotypes. The KIR3D receptors bind HLA-A3 and -A11 and some HLA-B proteins bearing the Bw4 motif (*Lanier, 2005*) (Fig. 5). The canonical ITIM motif [(I/V) xYxx(L/V)] contains a tyrosine residue that is critical for recruitment and docking by specific phosphatases (*Burshtyn et al., 1997*). Upon ligand binding, the tyrosine residue in the ITIM is phosphorylated by a Src homology region 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2 (Fig. 6). These phosphatases are recruited (through their SH2-domains) to ITIMS in the cytoplasmic domain of the receptors thereby blocking the progression of activation signals and preventing NK cell functions, i.e., cytotoxicity and cytokine production (*Lanier et al., 1998b*) (Fig. 6).

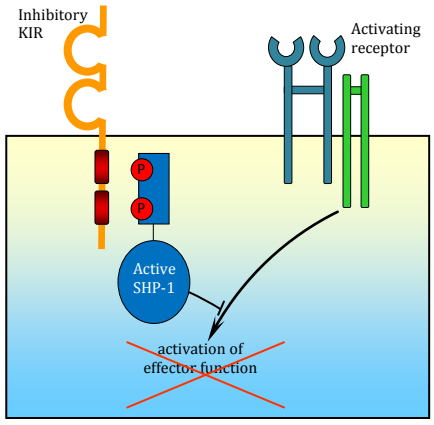


Figure 6 – Present view of the inhibition of cytotoxicity by KIRs. Engagement of KIRs by ligands induces ITIM phosphorylation and direct binding of SHP-1 or SHP-2 through SH2 domains, thereby transducing an inhibitory signal that intersects signals from activating receptors, thus abrogating effector functions.

The *activating* receptors in the human KIR family arose by gene duplication and conversion from inhibitory receptors (*Abi-Rached and Parham, 2005*) such that the extracellular domains of these activating receptors are highly homologous to their inhibitory counterparts. They share 95–98% sequence identity with the inhibitory forms but possess a truncated cytoplasmic domain and lack functional ITIMs (*Uhrberg et al., 1997*). Therefore, several of the activating KIR receptors have the ability to bind with low affinity to MHC class I ligands.

The charged acidic amino acid within the transmembrane regions of the activating KIR recruits adaptors with immunoreceptor tyrosine-based activating motifs (ITAMs). ITAMs comprise two copies of the motif Yxx(I/L) precisely spaced six or seven residues apart within the cytoplasmic domain of the activating receptors (*Lanier et al., 1998b; Moretta et al., 2000b*). In addition to KIR, an ITAM activation motif is found in the FcRγ and several other NK receptors with activating function. Activating KIRs and other NK activating receptors associate with a unique 12 kDa ITAM containing adaptor originally designated killer-cell activating receptor-associated protein (KARAP) or DNAX-activation protein (DAP)12, as shown in Fig. 7. DAP12 is a type I transmembrane protein expressed in NK cells that when phosphorylated leads to an association between DAP12 and ZAP70 and SYK protein tyrosine kinases (*McVicar and Burshtyn, 2001*). Unlike other adaptor molecules that signal through heterodimers, DAP12 signals are mediated by homodimerization. Activation of SYK/ZAP70 then ultimately leads to ERK phosphorylation, granule exocytosis and target cell lysis (Fig. 7).

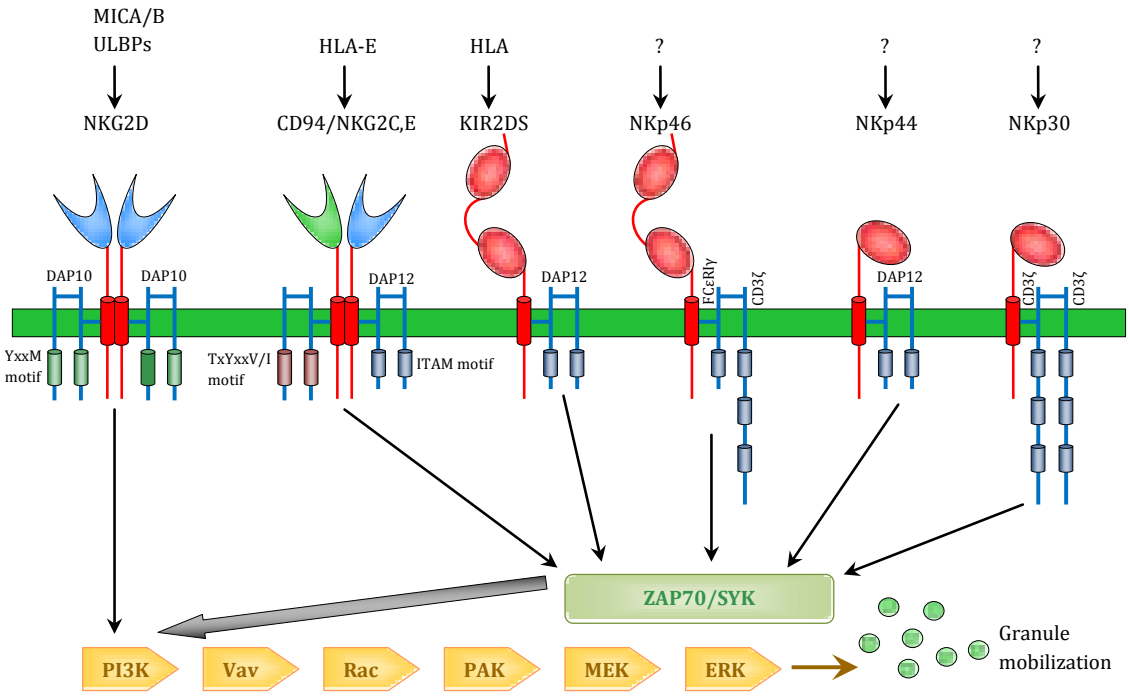


Figure 7 – Model of NK activating receptors. Activating NK receptors possess short cytoplasmic tails and an ITAM for adaptor protein binding. DAP12 homodimers associate with KIR activating receptors. Upon ligand engagement, the tyrosines in the ITAM of DAP12 becomes phosphorylated, and recruit SH2 domains of SYK/ZAP70 kinase. Upon NK receptor–ligand binding, adaptor proteins then associate with the intracellular domain of each receptor through charged interactions to activate PI3K or SYK/ZAP70.

Natural Cytotoxicity Receptors (NCRs)

NCRs, a first denomination given by Pende and colleagues (*Pende et al., 1999*), consist of two constitutively expressed receptors, NKp46 and NKp30, and an inducible receptor NKp44 (*Moretta et al., 2001b*). (Fig. 8). The NCRs include only activating variants and differ from KIRs in that they do not bind MHC ligands and, unlike KIRs and NKG2 receptors, are exclusively expressed on NK cells. Despite the important role played by the NCRs in recognition and killing of tumour cells (*Pessino et al., 1998; Vitale et al., 1998; Pende et al., 1999*), little is known about the nature and distribution of their ligands (Fig. 8). NCRs directly induce apoptosis after binding to ligands that indicate infection of a cell. The NCRs belong to the Ig superfamily and contain a charged amino acid in their transmembrane domain which associates with ITAM-bearing adaptor molecules (Fig. 7).

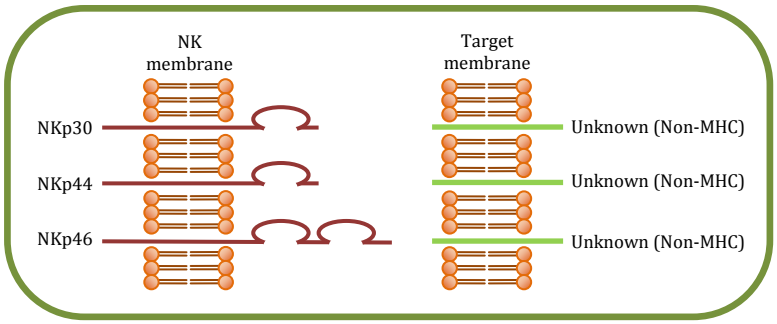


Figure 8 – Natural Cytotoxicity Receptors.

NKp46 was the first NCR to be identified (*Sivori et al., 1997; Pessino et al., 1998*) and is considered a major NK lysis receptor playing a dominant role in the activation of NK cells against various targets (*Sivori et al., 1997*). NKp46 is a 46 kDa glycoprotein that contains two C2 Ig-like extracellular domains. Crosslinking of NKp46 led to Ca^{2+} mobilization, cytotoxicity and cytokine release (*Sivori et al., 1997*). NKp46 binds and signals through CD3ζ/FcγR heterodimers that contain the activating ITAM motif (*Lanier, 2003*) as show in Fig. 7. Hemagglutinin molecules of different influenza strains were identified as the first specific NKp46 and NKp44 ligands (*Arnon et al., 2001; Mandelboim et al., 2001*).

NKp44 was the second NCR identified on human NK cells. It encodes a 44kDa surface glycoprotein and the activating signal of NKp44 is delivered via the association and phosphorylation of the DAP12 adaptor molecule (*Vitale et al., 1998; Cantoni et al., 1999*) (Fig. 7). NKp44 is not expressed on resting NK cells, but rather requires activation for its expression (*Vitale et al., 1998*). Similar to NKp46, very little is known

regarding the cellular ligands of NKp44 and the only ligand identified so far for this receptor is the influenza hemagglutinin protein (*Arnon et al., 2001*).

NKp30, the third NCR to be identified, functions in the killing of targets that are relatively resistant to NKp46/44-mediated killing, demonstrating that it probably recognizes ligands other than those recognized by NKp46/44 (*Pende et al., 1999; Moretta et al., 2000a*). NKp30 is a 30 kDa glycoprotein that contains one V-type Ig-like extracellular domain. NKp30 is selectively expressed on almost all human NK cells (*Pende et al., 1999; Manaster et al., 2008*). The transmembrane portion of NKp30 contains an arginine residue, which is probably involved in the association with CD3ζ chains for the transduction of the downstream activating signals (*Pende et al., 1999*) (Fig. 7).

CD94/NKG2 Receptors

CD94 and NKG2 are type II integral membrane glycoproteins that contain an extracellular C-type carbohydrate recognition domain and are encoded by genes clustered together in human chromosome 12p12.3-p13.2 (*Carretero et al., 1998*). The CD94 protein expresses on the cell surface and associates as a disulfide-linked heterodimer with NKG2A-C or NKG2E-F (Fig. 9) (*Lazetic et al., 1996; Brooks et al., 1997*).

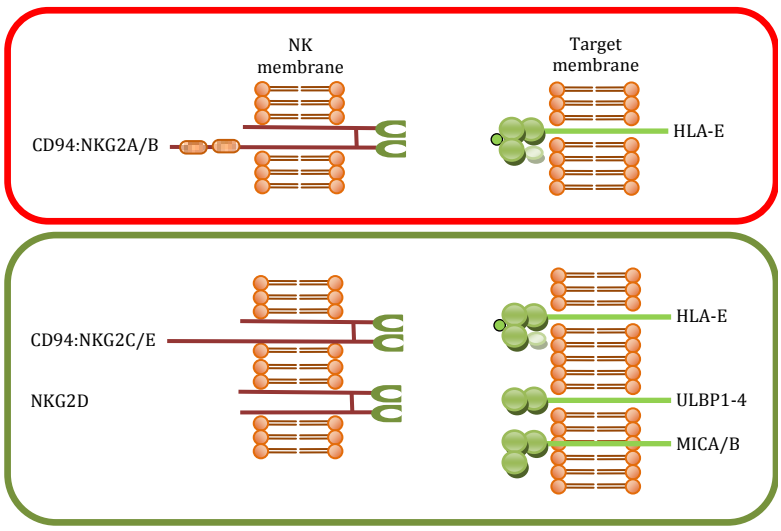


Figure 9 – C-Type Lectin like Domain (CTLD) superfamily of receptors, such as CD94/NKG2s and NKG2D. Activatory receptors are outlined in green and inhibitory in red.

Association of CD94 with the NKG2A (which contains an ITIM in its cytoplasmic tail) constitutes another MHC class I-specific inhibitory receptor (Fig. 9) (*Carretero et al., 1997; Le Drean et al., 1998; Palmieri et al., 1999*).

Conversely, CD94/NKG2C,E heterodimers serve as activating receptors (Fig. 9) and require association with the DAP12 adapter protein for stable expression on the cell surface and for signalling (*Braud et al., 1998; Lanier et al., 1998a*) (Fig. 7). The Lys residue in the transmembrane region of NKG2C associates with the Asp residue in the transmembrane segment of DAP12 and is required to generate a stable receptor complex (*Lanier et al., 1998a*) (Fig. 7). Expression of CD94/NKG2 receptors appears earlier in NK cell ontogeny than KIR in humans (*Mingari et al., 1997; Miller and McCullar, 2001*). Human CD94/NKG2A and CD94/NKG2C bind HLA-E (*Lanier, 2005*). An intriguing feature of HLA-E is that it assembles at the endoplasmic reticulum with peptides derived from the leader peptides of HLA-A, B, C and G (*Braud et al., 1997; Lee et al., 1998a; O'Callaghan et al., 1998*). So, expression of HLA-E on the cell surface is dependent upon availability of leader peptides provided by HLA-A, -B, -C, -G respectively, reflecting in this way the overall biogenesis of MHC class I proteins in the cells. Engagement of HLA-E in the absence of the peptide leader sequence fails to provide protection from direct cytotoxicity. NKG2A, B, E and F are inhibitory receptors that bind as a heterodimer with CD94 to the nonclassical MHC class Ib molecule HLA-E (*Braud et al., 1997*). Nonclassical HLA-E, like the classical human MHC class 1a molecules, possesses the ability to present antigen in conjunction with the heavy chain and β2-microglobulin invariant chain.

NKG2D receptor and ligands

NKG2D is a type II transmembrane-anchored glycoprotein that is expressed on all NK cells (*Lopez-Larrea et al., 2008*) as a disulfide-linked homodimer and, unlike other members of CTLD superfamily, doesn't form dimers with CD94 (*Wu et al., 1999*) (Fig. 9). This receptor is a member of the C-type lectin-activating receptor family that is evolutionarily conserved, encoded by a single gene located on the human chromosome 12p12-p13 demonstrating essentially no polymorphism (*Shum et al., 2002*). The NKG2D receptor recognizes cell surface glycoproteins structurally related to MHC class I, but most of these ligands are not encoded by genes in the MHC complex (*Raulet, 2003*) (Fig. 9). NKG2D ligands belong to two

relatively distant families related to MHC class I molecules. One family is composed of MHC class I chain-related gene (MIC)A/B molecules and the other by UL16-binding proteins (ULBP)1-5 (*Leong et al., 1998; Cosman et al., 2001; Sutherland et al., 2001; Sutherland et al., 2002*). MICA/B genes are encoded in the MHC region and they share structural and sequence similarity with MHC class I genes (28–35%). Like MHC class I proteins, MICA/B have α1-α2-α3 extracellular domains and transmembrane tails. However, they do not associate with β2-microglobulin or peptides. Crystal structures of NKG2D in complex with its ligands show that MICA interacts with the NKG2D dimer through the α1-α2 domains (*Bauer et al., 1998; Radaev et al., 2001*). ULBPs are also distant members of the MHC class I family, but they lack the α-3 domain (Fig. 9). ULBP1 and ULBP2 were discovered for their capacity to specifically interact with the human cytomegalovirus (HCMV) UL16 protein (*Cosman et al., 2001*).

In general, NKG2D serves as the primary activating receptor in activated NK cells, where NKG2D engagement alone triggers cytotoxicity, even in the presence of NK inhibitory receptors and their respective MHC class I ligands (*Bauer et al., 1999*). NKG2D couples to the transmembrane adaptor DAP10 for intracellular signalling, which can deliver a full cytotoxic response. NKG2D is uniquely dependent on the transmembrane adaptor molecule DAP10 (*Wu et al., 1999*). The human DAP10 gene codes for a 93 amino acid type I transmembrane protein with a Tyr-X-X-Met (YXXM) motif (*Wu et al., 1999*), as shown in Fig. 7. The YXXM motif is a conical docking site for the SH2 domain of the phosphatidylinositol 3 (PI3)-kinase p85 subunit, where a synthetic tyrosine-phosphorylated peptide derived from the DAP10 cytoplasmic domain containing this YXXM motif binds the p85 subunit *(Wu et al., 1999*). Thus, instead of activating the traditional ITAM-associated signalling events such as SYK and ZAP70, DAP10-associated recruitment and activation of PI3-kinase bypasses this step and directly activates downstream effector proteins during the cytotoxic response (*Upshaw et al., 2006*) (Fig. 7). PI3-kinase phosphorylation and recruitment of Grb2 are essential events in NKG2D signalling (*Billadeau et al., 2003; Upshaw et al., 2006*).

NKG2D homodimers assemble with two dimers of DAP10 proteins, thereby forming a hexameric structure (*Garrity et al., 2005*) with four potential YXXM binding sites. This serves to reduce the threshold of activation and to initiate multiple signalling events simultaneously.

FcγRIII (CD16)

FcγRIII (CD16), the low-affinity Fc receptor for IgG responsible for ADCC, was the first activating NK receptor identified (*Lanier et al., 1983; Perussia et al., 1983*) and it is, indeed, the best characterized (*Ravetch and Bolland, 2001*). In fact, CD16 was recently reported to be the most potent activating receptor on freshly isolated human NK cells, able to elicit strong cytotoxicity and cytokine production (*Bryceson et al., 2006*). FcγRIII is a type I transmembrane receptor containing two extracellular Ig-like domains (Fig. 10). Human CD16 associates with FcεRIγ (*Hibbs et al., 1989*) and/or CD3ζ (*Lanier et al., 1989*) that contains the ITAM motif for signal transduction (*Vivier et al., 1991*) (Fig. 7).

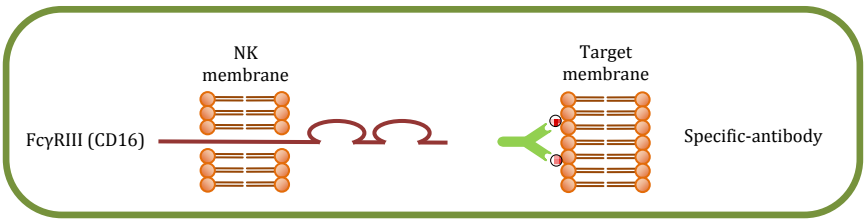


Figure 10 – FcγRIII (CD16) receptor and its ligand.

Thus, in addition to direct lysis of target cells, NK cells also possess the ability to mediate activation-dependent cell cytotoxicity via expression of FcRγIII (CD16) (*Leibson, 1997*). Many of the same signalling molecules participate in both direct NK lysis and ADCC due most likely to the conserved CD3ζ and FcεRIγ usage.

An additional role for CD16 on human NK cells was described as a lysis receptor that mediates the direct killing of some virus-infected and tumour cells, independent of antibody ligation (*Mandelboim et al., 1999*).

This allows NK cells to target cells against which a humoral response has been mobilized and to lyse cells through ADCC.

Toll-like receptors (TLRs)

An alternative mode of NK-cell activation has recently been identified thanks to the discovery that human NK cells can express TLRs (*Sivori et al., 2004*). TLRs are pattern recognition receptors (PRRs), which

trigger innate immune responses, providing both immediate protection against various pathogens and instructing the adaptive immune system through the induction of DC recruitment and maturation.

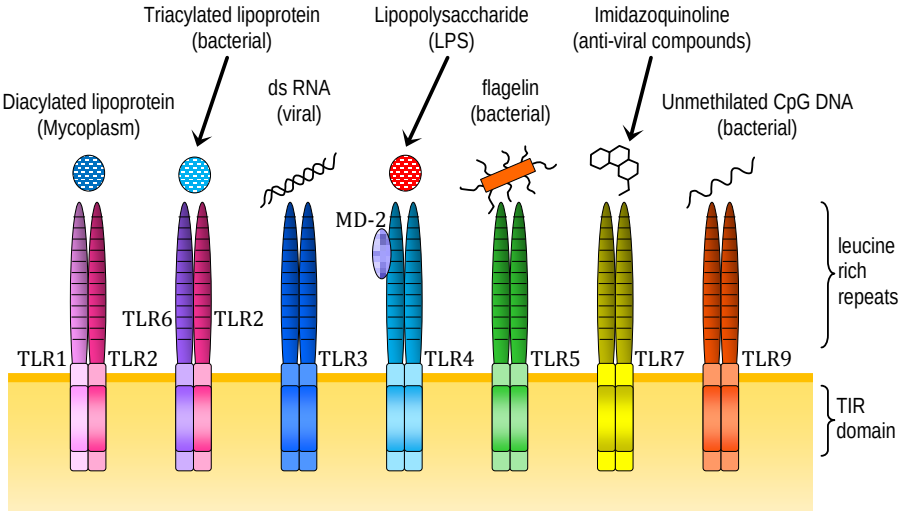


Figure 11 – Ligands recognized by TLR family. Each receptor consists in two identical TLR molecules (called homodimers), or two different TLRs (known as heterodimers).

Ten different TLRs have been described in humans, and most of their specific ligands have been identified (*Takeda and Akira, 2005*) (Fig. 11). The best known ligands of TLRs are the pathogen-associated molecular patterns (PAMP) molecules. These include lipopolysaccharide (LPS), recognized by TLR4; bacterial lipoproteins and lipoteichoic acids, recognized by TLR2; flagellin, recognized by TLR5; unmethylated CpG typical of bacterial and viral DNA, recognized by TLR9; double-stranded RNA (dsRNA) recognized by TLR3 and single-stranded RNA recognized by TLR7.

Recently, some damage-associated molecular pattern (DAMP) molecules have also been found to bind and activate TLRs (*Rubartelli and Lotze, 2007*).

Human NK cells, independent of their status of activation, express functional TLR2 (*Becker et al., 2003*), TLR3 (*Pisegna et al., 2004*) and TLR9 (*Sivori et al., 2004*) that enable their response to both viral and bacterial products leading, for example to the release of IFN-γ and TNF-α and to kill targets more efficiently (*Sivori et al., 2004; Marcenaro et al., 2008*) (Fig. 12).

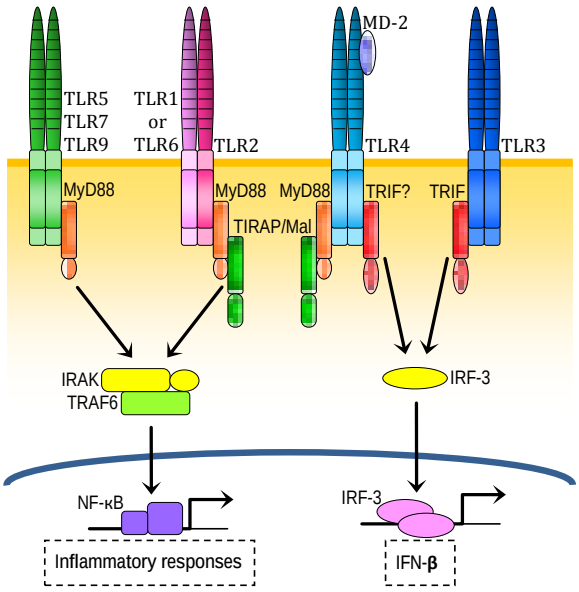


Figure 12 – IL-1R/TLR signaling pathways.

Recently, the high-mobility group protein B1 (HMGB1), a DAMP protein, has been proposed to enhance IFN-γ release from macrophage-stimulated NK cells.

Again, this is effective only when coupled with other pro-inflammatory cytokines, particularly with IL-2 in combination with IL-1 or IL-12 (*DeMarco et al., 2005*).

3.2 Natural Killer cell education

NK cells are educated by self-MHC class I at an immature stage of development in the BM. In this line, peripheral NK cells, which lack MHC class I molecules, are not educated as they respond poorly to receptor stimulation. However, upon cytokine stimulation, some human NK cells can acquire MHC class I receptors in vitro gaining responsiveness when the expressed KIR is specific for self-HLA (*Juelke et al., 2009*). Thus NK cells can functionally mature in an MHC class I dependent fashion but independent of their development in the BM. KIRs help NK cells discriminate between normal self and target cells by recognition of MHC class I molecules (*Colonna and Samaridis, 1995; Wagtmann et al., 1995*).

Binding of MHC class I molecules to KIR inhibits NK cell activation, and so, in the presence of a human MHC class I deficiency, NK cells are inactive (*Zimmer et al., 1998; Furukawa et al., 1999; Vitale et al., 2002*).

Therefore, MHC class I is important, not only for inactivation of mNK cells, but also in the progression towards functional maturity. NK cells apparently specialize in immune surveillance focused on monitoring cells for aberrant expression of MHC class I molecules. Genes of MHC, known as HLA in humans, are clustered on the short arm of chromosome 6.

The HLA system encodes structurally homologous cell surface glycoproteins characterized by a high degree of allelic polymorphism within human populations. The homologous HLA class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP) antigens are codominantly expressed and differ in their structure (Fig. 13), tissue distribution and characteristics in peptide presentation to T cells. An intact HLA class I trimer, composed of heavy chain, β2-microglobulin, and peptide, is required for KIR recognition. KIR can discriminate between different peptides presented by HLA-A, -B, or -C.

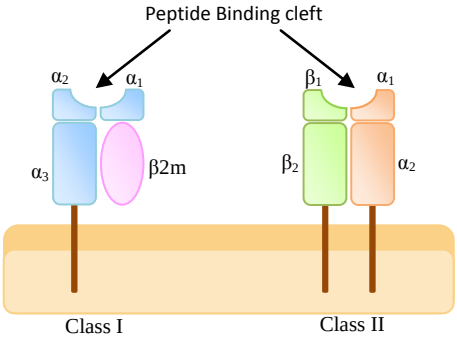


Figure 13 – Schematic representation showing the structure of human class I and class II MHC molecules. The HLA class I α-chain is non covalently associated with β2-microglobulin (β2m). The class II molecules are heterodimers composed of an α and a β-chain. The most distal domains of each of the two chains form the peptide binding site.

Although KIR recognition is clearly both peptide dependent and peptide selective, these receptors do not distinguish self from nonself peptides; thus, the biological relevance is not obvious. Binding of inhibitory KIR to their HLA class I ligands on potential target cells results in suppression of cytotoxicity and cytokine secretion by NK cells. Several classes of receptors are employed in the regulation of NK cell effector functions. Initial observations that NK cells could lyse MHC-negative targets (*Karre et al., 1986*) led to the formulation of the "missing-self" hypothesis (*Ljunggren and Karre, 1990*) that predicted the existence of NK cell negative regulatory receptors that would interact with MHC ligands and thereby spare target cell destruction by NK cells. According to the "missing-self" hypothesis, NK cells were proposed to provide immune surveillance for cells that had downregulated MHC class I, an event that frequently accompanies cellular transformation or infection with certain viruses. Until recently, a common misconception has been that NK cells attack any cell lacking MHC molecules. This notion is counterintuitive given documentation of the events involving cell-cell binding, Ca^{2+} mobilization, and synapse formation when NK cells encounter susceptible target cells that lack MHC class I.

A contemporary modification of the missing-self hypothesis might state, "NK cells patrol for abnormal cells that lack MHC class I or overexpress ligands for activating NK cell receptors". A corollary of the missing-self hypothesis is that failure of NK cells to respond to a potential target can be due either to

active inhibition mediated by the inhibitory receptors or alternatively, to the absence of sufficient activation signals to initiate a response (Fig. 14). An example of the latter situation may be represented by encounters between human erythrocytes and peripheral blood NK cells.

Although human red blood cells do not express MHC class I, NK cells do not attack them; therefore, erythrocytes may lack ligands capable of engaging the activating NK cell receptors. An alternative explanation for the inability of NK cells to harm normal tissues with low (e.g., neural tissues) or no (e.g., erythrocytes) MHC class I is the possibility that this target cell protection is mediated by inhibitory receptors recognizing non-MHC ligands.

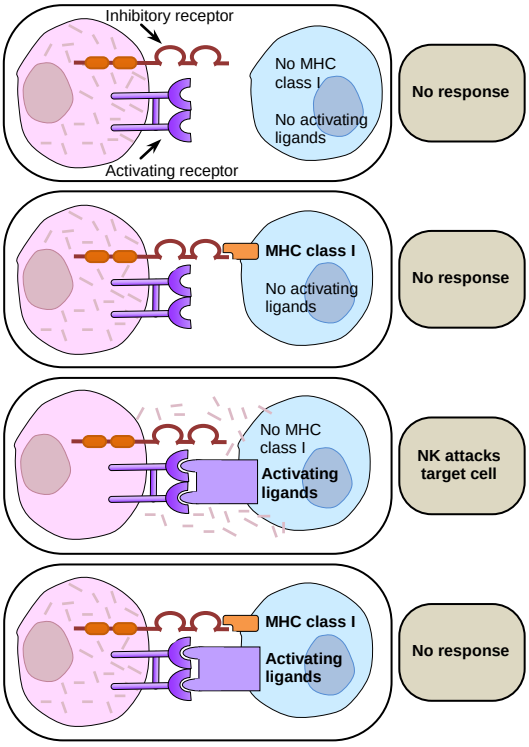


Figure 14 – Contemporary modification of the "missing-self" hypothesis showing the possible outcomes from encounters between NK cells/potential targets.

NK cell education requires signalling via the inhibitory receptors because mutations within the ITIM of inhibitory receptors render NK cells hyporesponsive even in the presence of the cognate MHC class I ligand of the mutant receptor (*Kim et al., 2005*).

Several distinct models have been proposed to explain how MHC class I recognition by inhibitory receptors improves the function of activating NK cell receptors and to explain the tolerance of NK cells lacking self-MHC class I (*Joncker and Raulet, 2008; Hoglund and Brodin, 2010; Orr and Lanier, 2010; Yokoyama et al., 2010a*).

Two main theories have been proposed for the role of KIR in the education of the NK cell.

The "licensing" (*Kim et al., 2005*) or "arming" (*Raulet and Vance, 2006*) models imply that activation receptors on iNK cells are by default nonresponsive (Fig. 15). Signals delivered by self-MHC-I receptors render activation pathways competent to respond to stimulation. Activation receptors remain functionally incompetent when NK cells cannot recognize MHC class I.

This hypothesis postulates that NK cells are initially unresponsive or "unlicensed" and that MHC class I engagement of inhibitory receptors during development licenses or "arms" these cells to become competent effector cells (*Kim et al., 2005*).

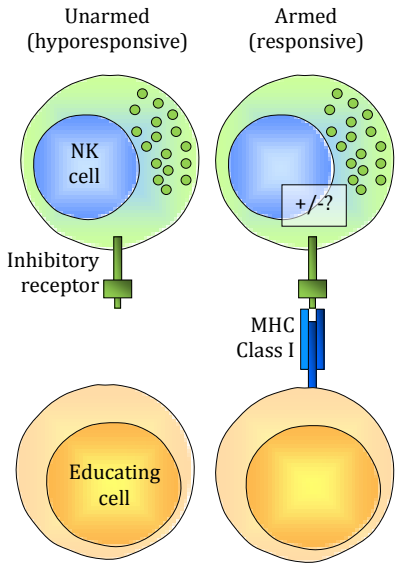


Figure 15 – In the arming model, signaling by an inhibitory receptor leads to NK cell functional competence; in the absence of arming, the NK cell remains hyporesponsive.

Thus, the arming/licensing model imply that MHC class I receptors transmit signals and/or perform other functions that render NK cell activation pathways responsive. This model attributes licensing directly to engagement of KIR (*Anfossi et al., 2006*). However, arguments against this theory note that licensing a NK cell requires an activation signal, whereas most KIR are inhibitory, so these receptors would have to switch from activator to inhibitor during NK cell development. Considering the complexity of signalling pathways and the likely alternative intracellular environment of a developing versus a mNK cell, this activator-inhibitor cell-signalling switch is plausible.

On the other hand, the "disarming" model (*Raulet and Vance, 2006*) implies that activation receptors on iNK cells become responsive by default (Fig. 16). If such NK cells acquire an inhibitory self-MHC class I receptor, NK cell activation signals are counter-balanced and this maintains the functionality of activation pathways. If NK cells fail to acquire an inhibitory receptor for self-MHC-I, persistent and unopposed activation by normal host cells induces NK cell hyporesponsiveness, render them anergic (*Gasser and Raulet, 2006*).

This model describes an additional unknown activating signal that, if left unopposed, would over stimulate the unlicensed NK cell leading to anergy, thus necessitating a balance of a second, inhibitory signal from KIR-MHC class I engagement (*Fernandez et al., 2005; Yokoyama and Kim, 2006*). According to this hypothesis, persistent stimulation of NK cells via one (or a few) activation receptor should be sufficient to induce hyporesponsiveness of most activation receptors and the inhibitory function of MHC class I receptors (which prevents persistent NK cell activation) is sufficient to ensure NK cell responsiveness.

Hyporesponsiveness could be induced in mNK cells and persistent stimulation by stress or viral ligands reversed the positive effect of NK cell education (*Coudert et al., 2008; Tripathy et al., 2008*).

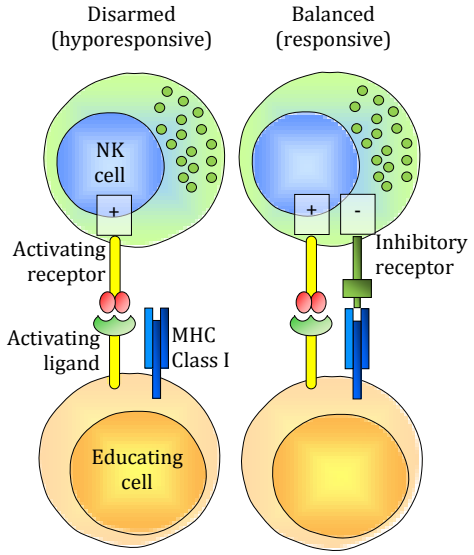


Figure 16 – In the disarming model, the NK cell is activated by default; however, in the absence of an inhibitory receptor for self-MHC class I molecules, the NK cell becomes anergic or hyporesponsive. The presence of an inhibitory receptor, in contrast, allows NK cell to be responsive.

The expected functional properties were restored when these NK cells were cultured in the absence of the stimulating cells. These data show that mNK cells can adapt to excessive stimulation by reducing the functionality of activation receptors. Several studies demonstrated that NK cell education requires the educating MHC class I to be expressed on all cells or alternatively hyporesponsiveness is dominantly induced by lack of MHC class I, as would be predicted by the disarming hypothesis (*Johansson et al., 1997; Wu and Raulet, 1997*).

The SHP-1 and SHIP-1 phosphatases associated with inhibitory receptor signalling are dispensable for NK education, suggesting that inhibitory signals are not needed for NK cell education, supporting the licensing model (*Kim et al., 2005; Orr et al., 2010a*).

However, these findings are not incompatible with the disarming model because many inhibitory receptors also associate with SHP-2, so there might be signalling redundancy between the different phosphatases recruited by the inhibitory receptors.

Collectively, there is evidence that the establishment of functional competence of NK cell activation receptors and effector inhibition are two separable functions of MHC class I receptors (*Chalifour et al., 2009*), suggesting a model in which both functions of MHC class I receptors are essential to generate and maintain responsive NK cells.

Finally, recent data show that the response of NK cells to stimulation is graded rather than all-or-none. The response is related to how many inhibitory self-MHC class I receptors NK cells express, combined

with the affinity of these receptors for MHC class I (*Yawata et al., 2008; Brodin et al., 2009b; Joncker et al., 2009; Jonsson et al., 2010*).

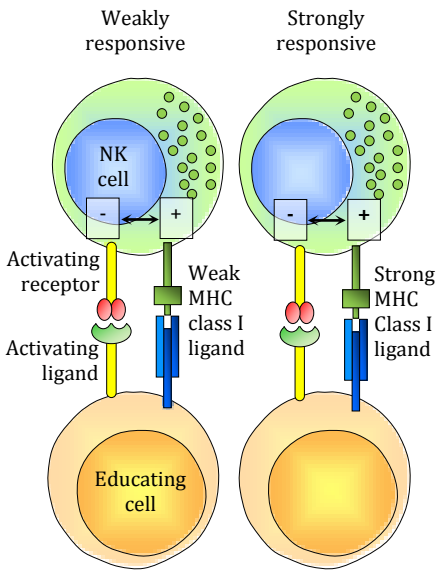


Figure 17 – In the rheostat model, NK cell education is dynamic. According to the strength of the inhibitory signal during NK cell education, the responding NK cell balances its activation threshold as a rheostat, which allows the maturation of NK cells to be optimally tuned by the inhibitory input.

This influences both the frequency of responding NK cells as well as the magnitude of the response of individual NK cells (*Brodin et al., 2009b*). This third "rheostat" model proposes that NK cell reactivity is tuned by the number of self-MHC class I inhibitory receptors that NK cell expresses and by the affinity of each receptor for self-MHC class I (*Raulet and Vance, 2006; Brodin et al., 2009a*) (Fig. 17).

NK cells that express two or more inhibitory receptors for self-MHC class I respond more frequently and possess stronger effector functions than NK cells with only one inhibitory receptor for self-MHC class I (*Brodin et al., 2009a; Joncker et al., 2009*).

The affinity of the interaction between the inhibitory receptor and its MHC class I ligand also influences the NK cell education process (*Jonsson et al., 2010*).

Thus, NK cell education is likely to be a quantitative process whereby NK cell responsive capacity is determined by the frequency and strength of engagement of inhibitory receptors with self MHC class I, either opposing chronic activating receptor stimulation (disarming) or by transmitting undefined activating signals themselves (licensing or arming).

3.3 Natural killer cell activity

NK cells were named plainly in 1975 for their "innate" and "cytotoxic" character (*Herberman et al., 1975; Kiessling et al., 1975*). Now, NK cells are heralded as the first wave of defence against a variety of pathogens in which they employ potent weaponry to kill infected and malignant cells. In addition, NK are

implicated in the elimination of autologous activated immune cells (T cells and macrophages) following an inflammatory response (*Spielman et al., 1998; Rabinovich et al., 2003; van Dommelen et al., 2006*).

NK cell mediate their activity through:

1) secreting proinflammatory cytokines (IFN-γ, TNF-α, etc.),

2) releasing cytoplasmic cytotoxic granules (granzyme and perforin) by exocytosis, and

3) the engagement of death receptors on target cells by their cognate ligands (e.g., FasL and TRAIL) on NK cells (*Janeway and Medzhitov, 2002*).

Cytokine production

NK cells represent an important source of immunoregulatory cytokines and chemokines. They bind other immune cells during the early phases of inflammatory responses and the resulting functional interactions shape both the innate immune response within inflamed peripheral tissues and the adaptive immune response found in SLT (*Biron et al., 1999; Vivier et al., 2008*). The effector or immunoregulatory functions of NK cells correlate with the levels of expression of the surface markers CD56 and CD16. In fact, $CD56^{bright}CD16^{dim/neg}$ NK cell subset, that represent the minor NK subset in blood (≈10%), is more dedicated to display immunomodulatory functions through secretion of cytokines (Cooper et al., 2001b; Jacobs et al., 2001). Upon target-cell binding, these NK cells produce cytokines such as IFN-γ, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (*Yokoyama et al., 2004*), defining a non-cytotoxic role for NK cells in host defence influencing the activity of other cells including macrophages, dendritic cells (DCs) and T cells (*Vivier et al., 2008*).

The most notably is the pro-inflammatory IFN-γ, which has pleiotropic effects on cells that modulate the adaptive immune response (*Schoenborn and Wilson, 2007*). IFN-γ is the major cytokine produced by $CD56^{bright}$ NK cells upon their detection of infected or cancerous cells (*Chawla-Sarkar et al., 2003*). It presents immune regulatory activity (*Maggi et al., 1992; Parronchi et al., 1992; Bradley et al., 1996*) as well as direct antiviral activity (*Cheney et al., 2002; Frese et al., 2002*). IFN-γ is a cytokine of much importance to proper innate and adaptive immune function. IFN-γ directly inhibits viral replication and activates the

innate and adaptive immune responses via multiple mechanisms. IFN-γ upregulates both MHC-I and MHC-II antigen presentation pathways, induces naïve $CD4^+$ T cells to differentiate into Th1 $CD4^+$ T cells and activates macrophages to increase phagocytosis, cytokine secretion and production of antimicrobials such as superoxide, nitric oxide (which inhibits viral replication) and hydrogen peroxide. IFN-γ also inhibits human immunodeficiency virus (HIV) replication during early infection (*Boehm et al., 1997; Shapshak et al., 2004*). The importance of IFN-γ to control infection is plainly demonstrated when pathogens considers a large amount of mechanisms to counteract the effects of IFN-γ. Inhibition of IFN-γ production via neutralization of IFN-γ stimulators is one such strategy.

Granule exocytosis

The regulation of perforin-dependent cytotoxicity implies the controlled release of effector granules containing perforin and granzymes stored in the NK cells cytoplasm (*Blott and Griffiths, 2002*). This release from NK cells is dependent on polarization of both microtubules and actin filaments in the cytoskeleton. Calcium signalling triggered by activating receptors initiates this cytoskeletal polarization, which positions the lytic granules so that they can be released at the effector cell-target cell interface (*Katz et al., 1982; Wulfing et al., 2003*).

The original granule exocytosis model describes the major pathway utilized by NK cells to carry out cytotoxic function being perforin the principle effector molecule able to kill cells via pore formation (*Henkart, 1985*). A second model called pore delivery model is consistent with a requirement for perforin in efficient granule-mediated killing (Fig. 18). It is characterized by the diffusion of granzymes through poly-perforin pores into the cytoplasm from within de novo vesicles created by the repair endocytosis process.

When the NK cells are sufficiently activated, they initiate an irreversible and unidirectional cytolitic response in which lytic granules are mobilized toward the NK Immune Synapse. Upon fusion of the granular vesicles with the plasma membrane, the lytic contents, perforin (*Dennert and Podack, 1983; Podack and Dennert, 1983*) and granzymes (*Jenne and Tschopp, 1988*), are released into the intercellular space.

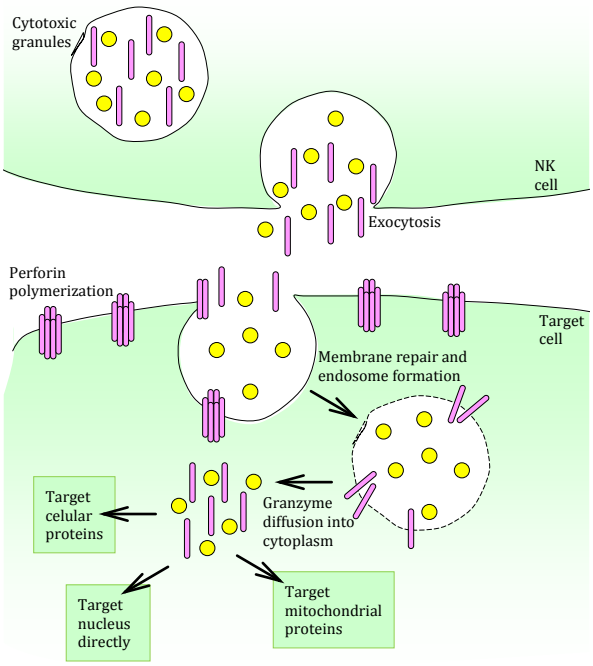


Figure 18 – Pore-delivery model of granzyme release.

Perforin monomers are exposed to extracellular levels of Ca^{2+} in the synaptic space, which elicits polymerization and insertion of the proteins into apposing target cell plasma membranes (*Young et al., 1986*). The resulting poly-perforin pores lead to a transient Ca^{2+} influx, triggering a membrane repair response in the target cell (*Podack, 1999; Keefe et al., 2005*). The repair process inadvertently leads to endocytosis of granzymes and other lytic granule components present within the synaptic space.

Granzymes are then delivered into the cytoplasm where they activate the apoptotic machinery by cleaving cellular substrates and leading to apoptosis. The death pathway initiated by these proteins was recently established (*Martinvalet et al., 2008*).

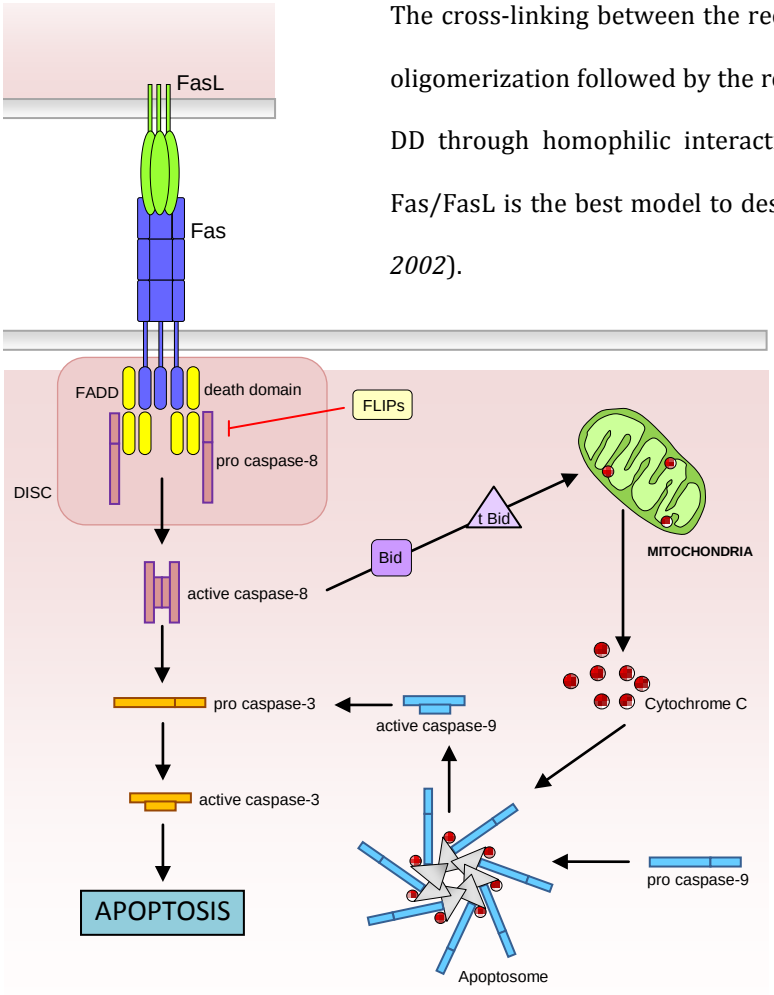
Death ligand interactions

Previous observations of rapid target cell death in the absence of extracellular Ca^{2+}, RNA or protein synthesis, granule exocytosis or perforin (*Trauth et al., 1989*) suggested the existence of alternative pathways of NK cell-mediated cytotoxicity. The extrinsic signalling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions leading to characteristic cytoplasmic and nuclear condensation and DNA fragmentation (*Itoh et al., 1991; Tartaglia et al., 1993*).

Several receptors, called Death Receptors (DR) of the TNF Receptors (TNFR) family are the most prominent players in the field of the extracellular signals leading to cell death (*Locksley et al., 2001*). These DRs have been identified as a subgroup with a predominant function in the induction of apoptosis. NK cells can express at least three death ligands: FasL, TNF-α and TRAIL (*Bhardwaj and Aggarwal, 2003*), all

of which induce apoptosis in their targets (*Montel et al., 1995; Osborne, 1996; Ashkenazi and Dixit, 1998; Kashii et al., 1999*).

Besides the diversity within members of TNFR superfamily, all of them contain cysteine-rich extracellular subdomains (*Naismith and Sprang, 1998*) and also contain a homologous cysteine-rich cytoplasmic domain, called death domain (DD) (*Ashkenazi and Dixit, 1998*) which plays a crucial role in transmitting the signal from the cell surface to intracellular signalling pathways (*Fulda and Debatin, 2004*).



The cross-linking between the receptor and the ligand induces receptor oligomerization followed by the recruitment of an adaptor protein to the DD through homophilic interaction. The signalling pathways through Fas/FasL is the best model to describe DRs signalling (Fig. 19) (*Wajant, 2002*).

The binding of Fas/FasL results in the recruitment of the adapter protein FADD (Fas-associated death domain) that interacts with DRs through a corresponding DD. These receptors can lead to a serial activations of a family of protease referred to caspases (*Singh et al., 1998*). FADD then associates with procaspase-8 via dimerization of the death effector domain (DED).

Figure 19 – NK cell-mediated apoptosis by FasL/Fas interaction.

At this point, a death-inducing signalling complex (DISC) is formed, resulting in the autocatalytic activation of procaspase-8 (*Kischkel et al., 1995*). This is then released into the cytosol to propagate the apoptotic signal. Once caspase-8 is activated, the execution phase of apoptosis is triggered.

DR-mediated apoptosis can be inhibited by family of proteins known as FLICE-inhibitor proteins (FLIPs), that competes with procaspase-8 recruitment to the DISC (*Guicciardi and Gores, 2009*), rendering FADD and caspase-8 ineffective (*Kataoka et al., 1998; Scaffidi et al., 1999*).

Upon initiator caspases activation, two distinct mechanisms can occur. In type I cells, large amounts of active initiator caspases are released from the DISC into the cytoplasm and cleave effector caspases triggering the proteolytic cascade. In type II, there are lower levels of DISC formation and thus lower level of active caspase-8 (*Scaffidi et al., 1998*). In this case, caspase-8 cleaves the proapoptotic Bcl-2 family protein Bid to generate truncated (t)Bid that will induce the t-Bid-mediated release of cytochrome C (Cyt C) from mitochondria. This leads to the formation of apoptosome, followed by activation of procaspase-9, which in turn cleaves downstream effector caspases. The type-II CD95 signalling induced apoptosis might be blocked by Bcl-2 family members (*Willis et al., 2003*), which inhibit mitochondrial alteration in type II, but not in type I cells (*Scaffidi et al., 1998; Fulda and Debatin, 2004*). Fas mediated apoptotic pathway in type I cells can also induce mitochondrial damage but only to amplify the signal, whereas in type II cells mitochondrial apoptotic signalling is essential to the execution of apoptosis (*Guicciardi and Gores, 2009*). After effector caspases activation (caspases-3, -6 and -7), also called execution caspases, specific cellular substrates are cleaved causing morphologic and biochemical changes characteristic of apoptosis, leading to the final cell disassembly (*Slee et al., 2001*). Caspase-3 is considered to be the most important of the executioner caspases and specifically activates the endonuclease CAD. In normal cells, CAD is coupled with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves ICAD releasing and activating CAD that cleaves DNA into oligonucleosomal fragments (*Sakahira et al., 1998*). Caspase-3 also cleaves cytoskeletal proteins inducing cytoskeleton disruption with disintegration into apoptotic bodies that are phagocytised avoiding an inflammatory response (*Slee et al., 2001*).

3.4 Interactions between Natural Killer cells and Dendritic Cells

DCs represent the most powerful antigen-presenting cells (APC) and are found in various tissues where they play a major role in antigen capture. Subsequent stimuli will induce further differentiation into

mature DCs (mDCs) and their migration into SLT. The potent APC activity of mDCs is due to the expression of costimulatory molecules and to the high levels of surface HLA molecules (*Banchereau et al., 2000*).

The first evidence of a crosstalk between NK cells and DCs demonstrated NK cell-dependent anti-tumour responses in mice bearing MHC class-I negative tumors in presence of DCs (*Fernandez et al., 1999*). In fact, the interaction between NK cells and DCs functions as an important regulator of the intensity of an innate immune responses. These two types of cells act during the initial phases of the innate response and influence the maturation of each other. NK cells also enhance ability of DCs to produce pro-inflamatory cytokines (*Degli-Esposti and Smyth, 2005; Walzer et al., 2005; Moretta et al., 2006b*).

Activation of NK cells by DCs

Interactions between NK cells and DCs are also important for the activation, expansion and maintenance of NK cells during microbial infections (*Andrews et al., 2003; Hochweller et al., 2008*) and they can occur through mechanisms involving direct contact or by way of soluble factors released by DCs.

SLT are important sites of NK cell activation, since a large amount of NK cells is located in uninflammed lymph nodes (*Ferlazzo et al., 2004b*). After an encounter with bacteria, DCs mature and migrate to SLT where they encounter $CD56^{bright}CD16^{-}$ NK cells. In course of DC-NK interplay, myeloid DCs by secreting NK-cells activating cytokines, promote the secretion of pro-inflammatory cytokines and cytotoxicity of NK cells (*Trinchieri, 2003; Alli and Khar, 2004; Walzer et al., 2005*).

Activation of NK cells by soluble factors – DCs produce IL-12 and IL-18 in response to a wide variety of pathogen-related agents (*Akira, 2000; Gerosa et al., 2002; Kikuchi et al., 2004*) and to endogenous signals from other cell types (*Hilkens et al., 1997; Snijders et al., 1998*). IL-18 acts as a costimulator with IL-12 enhancing the NK cell cytotoxic function (*Yu et al., 2001*) and, thus, the induction of IFN-γ by NK cells (*Trinchieri, 1998*). A membrane-bound form of DC-derived IL-15 also appears to be necessary to induce activation or at least proliferation of NK cells (*Ferlazzo et al., 2004a*) assuring also NK cell survival and apoptosis escape following interaction with mDCs (Fig. 20) (*Brilot et al., 2007*).

Activation of NK cells by cell contact - In addition to soluble factors, many studies indicate a role for cell-cell contact during DC-mediated NK cell activation showing that DCs can activate NK cells through the activating receptors NKp30, NKp46 and NKG2D (Fig. 20) (*Ferlazzo et al., 2002; Jinushi et al., 2003; Draghi et al., 2007*)

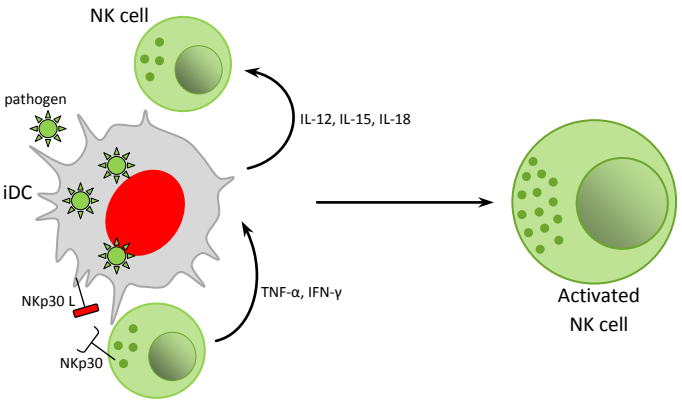


Figure 20 – Activation of Natural Killer cells by Dendrític Cells.

Enhanced IFN-γ production is also obtained when NK cells are stimulated by triggering of their surface receptors, supporting the hypothesis that surface receptor-ligand interactions are directly necessary for optimal NK cell activation (*Ortaldo et al., 2006*).

DC maturation by NK cells

Once activated, NK cells acquire the capability of killing myeloid immature DCs (iDCs) (DC "editing"). This effect is based on the capability of NK cells of discriminating between iDCs (that typically underexpress HLA-class I molecules) and mDCs that, after Ag uptake, up-regulate MHC-class I expression and are protected from NK cell killing (Fig. 21-a) (*Ferlazzo et al., 2001; Ferlazzo et al., 2003*). The editing process initiates with the engagement of the NKp30-activating receptor by its ligand expressed on DCs (*Ferlazzo et al., 2002*).

The activation status of both cell types seems to be critical. iDC are more susceptible to cytotoxicity than mDCs (*Della Chiesa et al., 2003*) and the decision also depends on the relative cell numbers involved: low NK cell to DC ratios result in DC activation, and high NK cell to DC ratios lead to DC killing (Fig. 21-b) (*Carbone et al., 1999; Piccioli et al., 2002*).

Moreover, resting NK cells are less likely to kill DCs and more likely to activate them, while IL-2 activated NK cells are more likely to induce DCs lysis (Fig. 21-c) (*Piccioli et al., 2002; Mailliard et al., 2003*). It seems that there is a check of the quality of DCs undergoing maturation ("editing" process) (*Moretta, 2002;*

Moretta et al., 2005), as only DCs undergoing this NK-mediated quality control would become fully mature and capable of inducing priming of protective and cytotoxic Th1 responses.

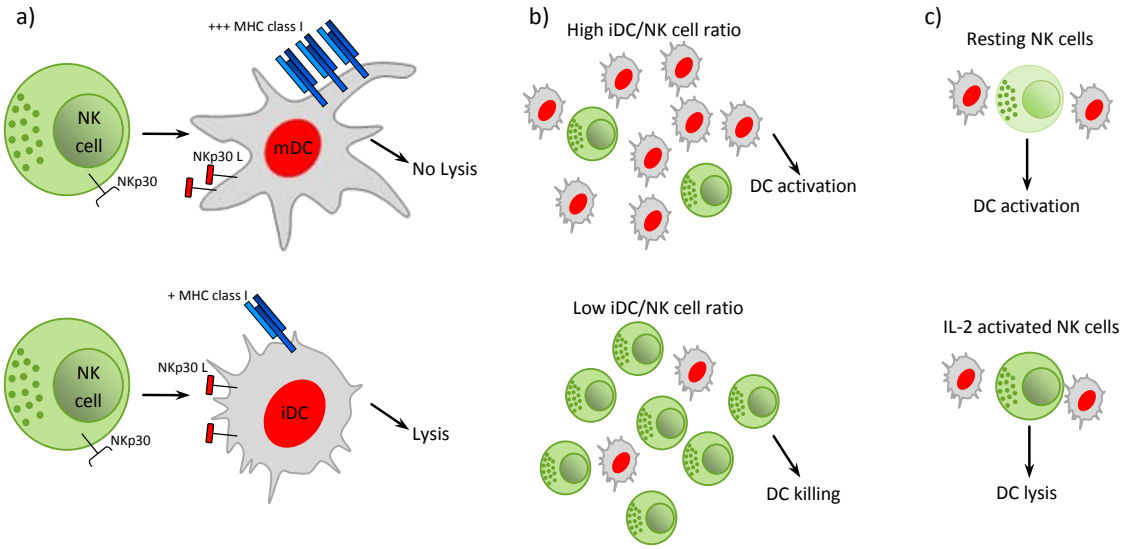


Figure 21 – Editing process. The decision might depend **a)** on the activation status of Dendritic Cells, **b)** on the relative cell numbers involved, or **c)** on the activation status of Natural Killer cells.

NK cells can also induce progression of DC maturation via cytokines released upon direct NK-DC contact (*Moretta et al., 2005*). Similar to the iDC killing process, the ability to induce DC maturation is also dependent on NKp30 (*Vitale et al., 2005*). After NK-DC interaction and NKp30 engagement, NK cells produce TNF-α (and INF-γ) a cytokine that induces DC maturation. Also in this case, the ability to promote maturation is confined to NK cells expressing the KIR^{-}NKG2A^{dull} phenotype (*Della Chiesa et al., 2003*). This process could complement the NK-mediated editing of DCs leading to selection of mDCs. This DC maturation may be relevant in adaptive immune response against cancer cells, since the absence of pathogen-related molecules and of inflammation does not lead to DC maturation and, as result, to an effective tumour antigen presentation. Similarly, NK cell-mediated DC maturation should be crucial in infections caused by viruses unable to trigger DC maturation.

4 NK CELLS IN HEALTH AND DISEASE

4.1 *Natural Killer cells in infections*

Virus infections

NK cells function as important mediators of innate immune defence against viruses especially during the early phases of infection (*Trinchieri, 1989*).

Activating receptors, such as NCRs (NKp46, NKp30, NKp44) or NKG2D, provide "on signals" for triggering NK cell activation and killing in their interaction with nonself target cells (*Moretta et al., 2001b; Long, 2002*). For instance, NKG2D recognizes MICA/B (*Lanier, 2005*) which are poorly expressed on normal cells but markedly upregulated on infected cells and tumour cells (Fig. 22) (*Lodoen et al., 2003; Yokoyama and Plougastel, 2003*).

NK cells through inhibitory receptors also recognize self MHC class I molecules which became downregulated upon stress following infection or transformation, allowing cytotoxicity by NK cells (Fig. 22) (*Parham, 2005; Akira et al., 2006*). When surface expression of MHC class I is either absent or too low to effectively engage inhibitory receptors, NK cell activation signals proceed unopposed and trigger NK cell functions ("missing self recognition") (*Karre et al., 1986*). HCMV encodes several genes that target the expression of MHC class I molecules (*Hengel et al., 1999*) such as US2, US3, US6 and US11 affecting MHC class I expression at the cell surface (Fig. 22). Modulation of MHC class I expression makes infected cells more susceptible to recognition by NK cells (*Huard and Fruh, 2000*).

NKG2D is also involved in the control of infection by other members of the herpes virus family, such as Epstein–Barr Virus (EBV), up-regulating the expression of ULBP1, rendering the infected B cells susceptible to NK cell-mediated lysis (*Pappworth et al., 2007*). The replication of viral DNA has the

potential to activate the DNA damage response. Viruses may also up-regulate NKG2D ligands (*Routes et al., 2005*) through, at least in part, to chromatin remodelling (*Azimi et al., 2006*).

NK cells also mediate noncytolytic suppression of viral replication by secreting several chemokines, such as CCL3, CCL4 and CCL5, and cytokines such as IFN-γ, TNF-α and GM-CSF (Fig. 22) (Cerwenka and Lanier, 2001).

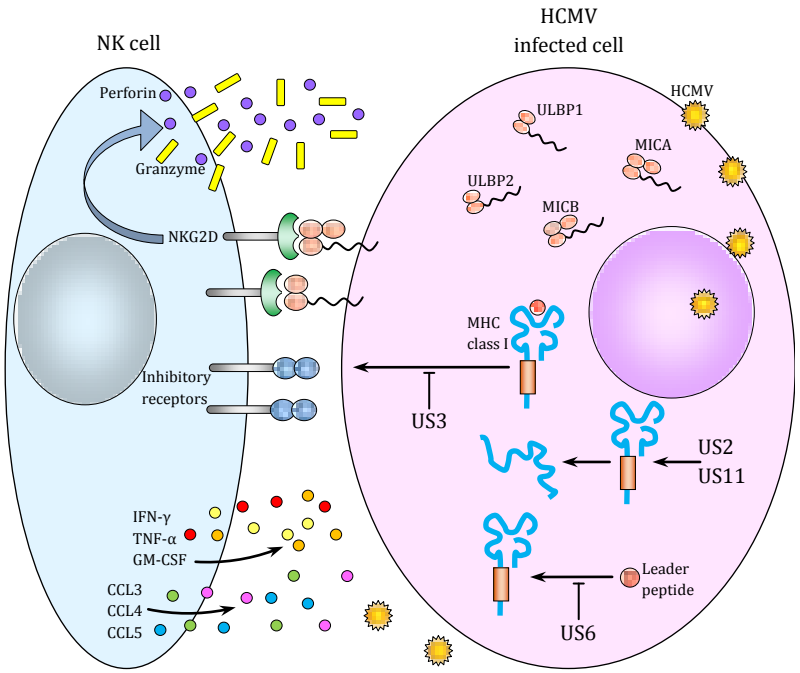


Figure 22 – Human Citomegalovírus recognition by Natural Killer cells.

NK cells can control Cytomegalovirus (CMV) infections by the secretion of antiviral cytokines (*Orange et al., 1995*) or by direct lysis of virus-infected cells by using perforin (Fig. 22) (*Shellam et al., 1981; Tay and Welsh, 1997*) depending on organ-dependent mechanisms used by NK cells to control virus infection.

The antiviral cytokines (IFN-γ and TNF-α) may limit virus replication and viral antigen presentation in hepatocytes without killing them, thereby minimizing potential killer cell damage by direct lysis. IFN-γ, followed by TNF-α, are probably the most important cytokines produced by NK cells with anti-infection activity (*Orange et al., 1995; Taylor et al., 2000*).

Exogenous microbes such as viruses, bacteria and yeasts have conserved motifs termed PAMPs, lipids, lipoproteins, proteins and nucleic acids (*Lee and Kim, 2007*). Generally, various PRRs (including TLRs) have an essential role in the recognition of PAMPs by immune cells (*Iwasaki and Medzhitov, 2004*). Detection of PAMPs by PRRs induces production of several inflammatory cytokines and chemokines,

upregulation of costimulatory molecules and initiates adaptive immune responses (*Akira and Takeda, 2004*).

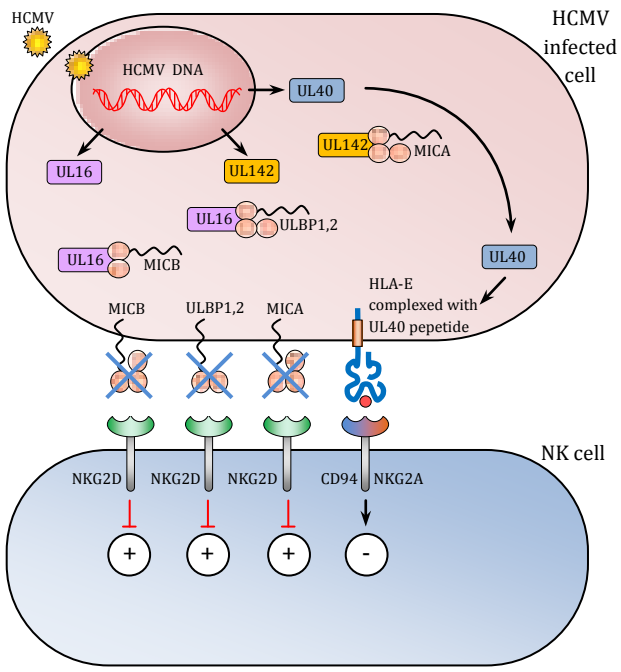


Figure 23 – Mechanisms of HCMV evasion from NK cell immunesurveillance. HCMV-infected cells express several UL viral proteins that retain NKG2D ligands in the interior of the cell and upregulate the CD94/NKG2A ligand (HLA-E).

However, CMV uses immune evasion mechanisms to ensure that viral replication proceeds successfully (Fig. 23). HCMV UL16 glycoprotein binds and retains MICB, ULBP1 and ULBP2 intracellularly, interfering with the NKG2D mediated response (*Dunn et al., 2003; Vales-Gomez et al., 2003; Welte et al., 2003*). Likewise, the HCMV protein UL142 is also able to target full length MICA by downregulating its cell surface expression and leading to protection from NK cytotoxicity (*Chalupny et al., 2006*).

Since direct recognition of CMV-encoded components by NK cells is one mechanism ensuring that an antiviral response is generated, CMV is capable to encode a number of proteins that interfere with the surface expression of stress ligands, protecting them from NK cell recognition.

NK cells are also supposed to play a significant role in preventing and controlling HIV-1 infection. In theory, NK cells should eliminate HIV-1 infected target cells by direct lysis or by ADCC. However, several NK cell functions in individuals infected with HIV-1 are highly impaired: high levels of viral replication induce a depletion of the cytolytic $CD56^{dim}CD16^{+}$ NK cell subset and an expansion of the $CD56^{neg}CD16^{+}$ NK cell subset (with high levels of inhibitory NK receptors and low levels of activating NCRs).

The virus is able to disrupt the NK cell-mediated cytolytic activity through the binding of its gp120 envelope with the α4β7 integrin (*Kottilil et al., 2006; Arthos et al., 2008*). This selective HIV-1 mediated down-regulation of MHC-I surface levels on infected cells should, in theory, inhibit the interaction

between inhibitory NK cell receptors and specific HLA alleles and allow NK cell-mediated lysis. However, HIV-1 is also able to modulate the expression of ligands for activating NK cell receptors (*Ward et al., 2007*).

The presence of a highly dysfunctional $CD56^{neg}$ NK cell subset expressing almost undetectable levels of NKp46 and NKp30, contributes to the reduction of NK cell-mediated killing of endogenously HIV-1 infected $CD4^+$ T cell blasts (*Fogli et al., 2008*). By other side, although ULBPs are detected in HIV-infected $CD4^+$ T cells (*Ward et al., 2007*), HIV-1 has evolved to escape from this NKG2D-mediated cytotoxic response, since HIV-1 Nef protein down-modulates cell-surface expression of MICA, ULBP1 and ULBP2 (*Cerboni et al., 2007*). Nevertheless, the residual NK cell-mediated killing occurs mainly through the NKG2D activation pathway (*Ward et al., 2007*).

NK cells can suppress endogenous HIV replication by cell-to-cell contact as well as by soluble factors. The chemokines CCL3, CCL4 and CCL5, which are ligands for CCR5, can block entry of R5 viruses into target cells by competitive inhibition of receptor binding (*Alkhatib et al., 1996; Choe et al., 1996; Dragic et al., 1996*). In fact, chemokines are the main soluble factors for NK cell-mediated HIV suppression. However, it is also suggested that HIV-induced inhibition of NK cell function involves mechanisms that lead to diminished secretion of chemokines (*Kottilil et al., 2003*).

Bacterial and other infections

The most likely way that NK cells control bacterial infections in vivo is by producing cytokines that activate macrophages to degrade the bacteria (*Kaufmann, 1993; Tripp et al., 1993*). Little information exists concerning the importance of NK cell cytotoxicity with respect to bacterial infection. Significantly, IFN-γ production by NK cells is critical in the prevention of overwhelming infection by obligate intracellular microbial pathogens in several experimental animal models, and monocyte-derived IL-15 is critical for optimal NK cell production of IFN-γ (*Carson et al., 1995*). Where bacteria-infected cells are lysed directly by NK cells it is likely that perforin is relevant. Nevertheless, Lipopolysaccharides (LPS) and bacterial carbohydrates induce NK cell cytotoxicity by increasing FasL expression on NK cells (*Halaas et al., 1998*), and therefore some bacterial infections may be controlled by Fas-mediated cytotoxicity.

NK cells are also recognized as major effectors of innate resistance to *Toxoplasma gondii, Leishmania major* and *Schistosoma mansoni*. The principal mechanism by which NK cells control the growth of these pathogens is indirect, involving cytokine production (IFN-γ) rather than cytolytic activity (*Scharton-Kersten and Sher, 1997*). Cytokine production limits parasite replication and promotes the development of adaptive cell-mediated immunity. In the case of *Eimeria papillata*, resistance to reinfection does not require IFN-γ and appears to be mediated at least in part by a perforin-dependent mechanism (*Schito and Barta, 1997*).

A role for NK cells has also been found in other infections. Murine adenovirus infection results in significant lymphocyte infiltration (both NK and T cells), liver injury, and increased hepatocellular enzymes (*Liu et al., 2000*). Elimination of NK cells by antibody depletion or use of nude mice (NK cell deficient) suppresses hepatocellular enzyme elevations and reduces apoptosis of hepatocytes. $CD8^+$ T lymphocyte responses induced by viral infection were severely depressed after NK cell depletion or in IFN-γ-deficient mice. Therefore, NK cells play a role in the induction of the virus-specific T cell responses in adenovirus infection. NK cells are also implicated in control of *Cryptococcus neoformans* infections. Infection of mice with targeted deletion of IL-12 resulted in a higher number of *Cryptococcus neoformans* organisms in both brain and lung compared to wild-type controls (*Kawakami et al., 2000*). Neutralization of IFN-γ or infection in IFN-γ-deficient mice resulted in more severe infections, suggesting a role for IL-12–induced.

4.2 *Natural Killer cells in tumour biology*

Another important function of NK cells is the elimination of tumour cells (*Smyth et al., 2002*). NK cells control several types of tumours by limiting their growth and dissemination (*Vivier et al., 2008*). The lack of even a single MHC class-I allele, a frequent event in cancer cells, sensitizes them to NK cell cytotoxicity (*Bottino et al., 2004; Moretta et al., 2004*) and, thus, tumour cells are recognized as NK cell targets (Fig. 24) (*Trinchieri, 1989*). In the absence of inhibitory signals, NK cell cytotoxicity must be activated by a set of triggering receptors as NKG2D, DNAM-1 and NCRs, whereas CD16 mediates ADCC (*Trinchieri, 1989;*

Bottino et al., 2004; Moretta et al., 2004). NKG2D and DNAM-1 have been suggested to have a role in tumour immunity (*Costello et al., 2004; Chang and Ferrone, 2006; Moretta et al., 2006a*).

NKG2D ligands are frequently over-expressed on tumour cells from several origins (*Cerwenka et al., 2001; Diefenbach et al., 2001*) and their expression renders tumour cells susceptible to NK cell-killing even if the transformed cells have normal MHC class I expression. In humans, MICA/B proteins are frequently expressed in epithelial tumours of multiple origins, but less frequently found in hematopoietic malignancies. In contrast, ULBPs are not usually expressed in epithelial tumours, but are expressed in leukaemias. It is thought that the main mechanism involved in the upregulation of NKG2D ligands (MICA/B and ULBPs) on cancer cells is cellular stress, as heat shock (*Groh et al., 1998*), oxidative stress (*Yamamoto et al., 2001*), genotoxic stress and stalled DNA replication (*Gasser et al., 2005*). Additionally, DNA damage also activates the expression of NKG2D ligands and some oncogenes may also up-regulate the expression of NKG2D ligands (*Boissel et al., 2006; Cebo et al., 2006*).

The NCRs also play a role in NK cell-mediated lysis of various human tumour cell lines, including melanomas, carcinomas, neuroblastomas, myeloid or lymphoblastic leukaemias and EBV-transformed B cells (*Bottino et al., 2005*). Moreover, NK cells can be activated by various stimuli such as contact with DCs, MHC class I-negative cells, binding of IgG immunocomplexes, direct engagement of NK receptors by stress-induced tumour-associated molecules or pathogen-derived products, and several cytokines (Fig. 24).

Anti-tumour responses can be triggered by NK cells through different effector mechanisms (*Wallace and Smyth, 2005*) as granule exocytosis (*van den Broek et al., 1995; Smyth et al., 1999; Trapani and Smyth, 2002*) and death receptor stimulation (*Medvedev et al., 1997; Zamai et al., 1998; Johnsen et al., 1999; Mirandola et al., 2004; Screpanti et al., 2005*).

NK cells can also produce many different cytokines and chemokines, at least some of which having a direct effect on tumours. Among activatory cytokines, IL-15 has a role in the differentiation, proliferation, survival, and activation of NK cells and synergizes with Flt3-L and SCF inducing the human $CD56^{bright}$ NK cell subset (*Mrozek et al., 1996; Waldmann et al., 2001; Farag and Caligiuri, 2006*). IL-7 is an early-acting

cytokine responsible for the generation of a pool of CD56^{bright} NK cells that respond to the activating action of IL-15 (*Di Santo, 2006*).

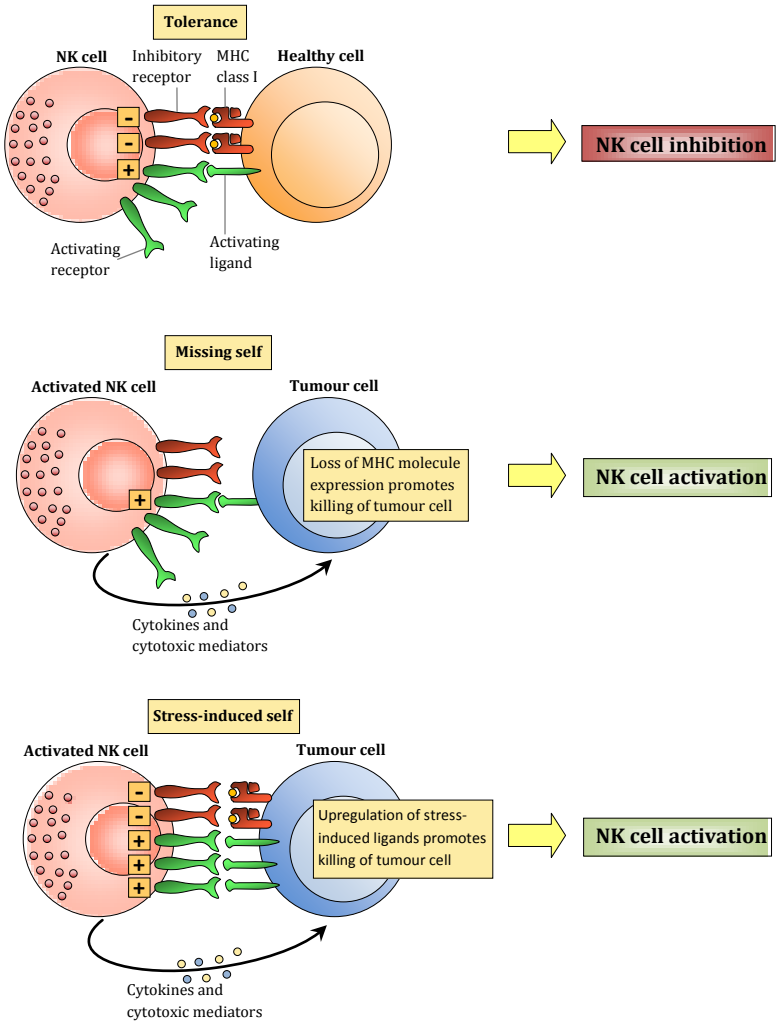


Figure 24 – a) NK cells are tolerant to healthy host cells, as the strength of the activating signals is dampened by the engagement of inhibitory receptors; **b)** Tumour cells may lose expression of MHC class I molecules and NK cells become activated, as they are no longer held in check by the inhibitory signal. **c)** NK cells are selectively activated by 'stressed' cells, which upregulate activating ligands for NK cells and thereby overcome the inhibitory signalling delivered by MHC class I molecules.

IL-2 induces the production of NK effector molecules, enhancing NK lytic activity (*Rosenberg et al., 1993*) and IL-12 and IL-18 enhance cytotoxicity against tumour targets and IFN-γ production by NK cells (*Bennett et al., 1996; Golab, 2000; Lauwerys et al., 2000*). IFN-γ decreases proliferation, enhances autophagy, limits metabolic activity of tumour cells and inhibits angiogenesis (*Hayakawa et al., 2002*). IFN-γ also plays a role in the regulation of killing by DRs, either by downregulating anti-apoptotic proteins, or by upregulating caspases. Finally, IL-21 favours the most cytotoxic CD56^{dim}CD16^{+} NK cell subset and enhances its cytotoxicity (*Parrish-Novak et al., 2000; Brady et al., 2004*).

NK cells are not found in large numbers in advanced human neoplasms, indicating that they do not normally home efficiently to malignant tissues (*Albertsson et al., 2003; Esendagli et al., 2008*) and so, the immune infiltration of NK cells on tumours represents a positive prognostic marker in different carcinomas (*Coca et al., 1997; Ishigami et al., 2000; Villegas et al., 2002*).

It was observed a specific phenotype of tumour-associated NK cells, suggesting that tumour induces alterations of activating NK cell receptor expression, promoting tumour progression. NK cells that infiltrate tumors show, in some cases, downregulation of activating receptors and in others, overexpression of inhibitory ones. Tumour-associated NK cells were refractory to CD16 receptor stimulation, resulting in diminished ADCC against autologous tumour cells (*Carlsten et al., 2009*). The cytolytic potential of NK cells isolated from cancer tissues was lower than that of NK cells from PB or normal lung tissue, with no difference observed in their capability to produce cytokines (*Carrega et al., 2008*). It was also observed a significantly lower percentage of NK cells expressing CD16, NKp30, and NKp46 activating receptors with no substantial differences in NKG2D expression (*Konjevic et al., 2009*). These alterations reduce the NK cells ability to recognize and eliminate tumour cells in Multiple Myeloma (MM) patients (*Markel et al., 2009*). Likewise, the frequency of NK cells expressing the activating receptors NKp30, NKp44, NKp46, NKG2D, and NKG2C was significantly decreased in Acute Myelogenous Leukemia (AML) patients compared to the NK cells of normal controls (*Szczepanski et al., 2010*).

Tumour evasion

Although NK cells play an important role in host anti-tumour responses (Fig. 25-a) (*Kim et al., 2000; Wu and Lanier, 2003; Hayakawa and Smyth, 2006*), advanced tumours may evade NK-mediated surveillance by downregulating their expression of NKG2D ligands (*Eisele et al., 2006*) (Fig. 25-b,c).

Thus, although the expression of MICA/B may result in the elimination of the tumour, the detaching of MICA, from the surface of the cells to the plasma, is a common characteristic of many tumours expressing this protein (*Groh et al., 2002; Salih et al., 2002; Doubrovina et al., 2003*), serving as decoy to subvert NK cell immune responses. The presence of soluble MICA on multiple primary tumours leads to a reduced amount of NKG2D ligand at the membrane of the tumour cells. This situation causes endocytosis and degradation of the NKG2D receptor (*Groh et al., 2006*) with consequent diminished expression of NKG2D on circulating NK cells (*Groh et al., 1999*) (Fig. 25-d).

The chronic exposure to tumour cells expressing NKG2D ligands alters NKG2D signalling and may facilitate the evasion of cancer cells from NK cell responses (*Oppenheim et al., 2005*).

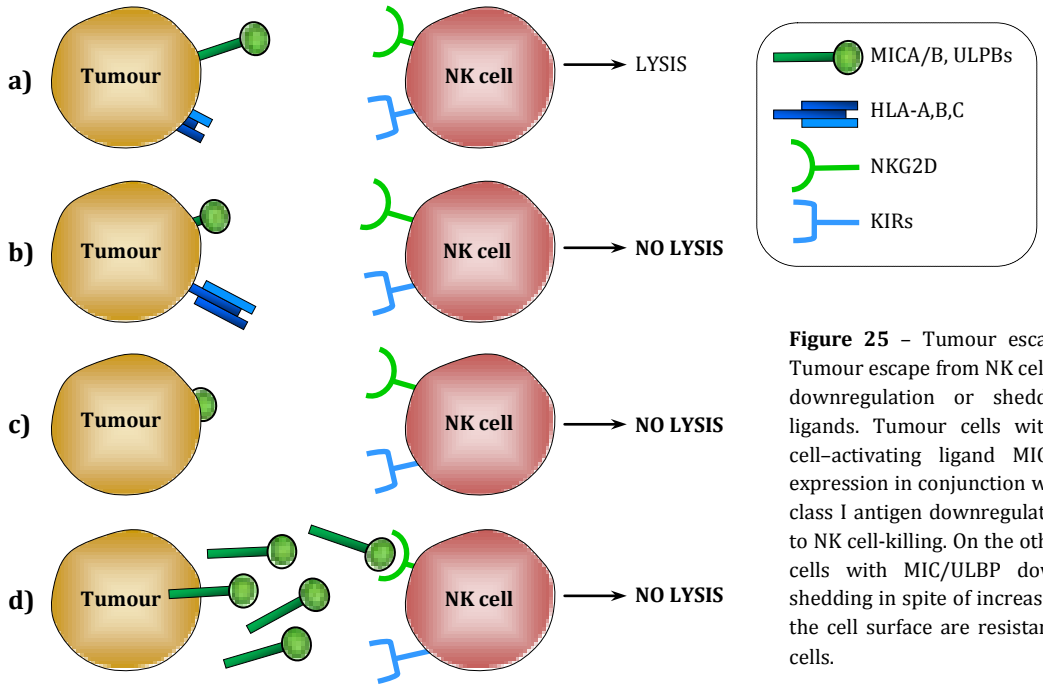


Figure 25 – Tumour escape mechanisms. Tumour escape from NK cell control through downregulation or shedding of NKG2D ligands. Tumour cells with increased NK cell–activating ligand MIC and/or ULBP expression in conjunction with classical HLA class I antigen downregulation are sensitive to NK cell-killing. On the other hand, tumour cells with MIC/ULBP downregulation or shedding in spite of increased expression on the cell surface are resistant to lysis by NK cells.

Moreover, it was also observed bilateral transfer of NKG2D and ligands (MICB) between NK cells to target cells (*Roda-Navarro et al., 2006*), leading to a marked reduction in the capacity of the NK cells to mediate NKG2D-dependent cytotoxicity.

The repression of the NKG2D ligand transcription (MICA/B or ULBPs) by epigenetic mechanisms (*Stern-Ginossar et al., 2007; Lopez-Soto et al., 2009*) or of the NKG2D ligand expression by Transforming Growth Factor (TGF)-β1 (*Friese et al., 2004; Eisele et al., 2006*) are other relevant immune evasion mechanisms, promoting cancer progression and immune evasion.

5 RELEVANCE OF NK CELLS IN TREATMENT OF HUMAN MALIGNANCIES

NK cells play a significant role in the surveillance and elimination of malignant transformed cells. These cells regulate HLA class I expression making them susceptible to NK cell-mediated lysis (*Khong and Restifo, 2002*). Evidence for the involvement of NK cells in destroying human tumour cells in vivo derives from allogeneic BM transplantation.

Anti cancer effects of NK cells include, among others, the ability of NK cells to lyse tumour cells. The lack of even a single MHC class I allele, a frequent event in cancer cells, leads to tumour cells be recognized as NK cell targets (*Trinchieri, 1989*) sensitizing them to NK cell cytotoxicity *(Bottino et al., 2004; Moretta et al., 2004*). Moreover, NKG2D ligands are frequently over-expressed on tumour cells from several origins (*Cerwenka et al., 2001; Diefenbach et al., 2001*) and their expression renders tumour cells susceptible to NK cell-killing even if the transformed cells have normal MHC class I expression.

The presence of NK cells within the tumours also represent an anti-cancer effect observed by NK cells. Typically, NK cells are not found in large numbers in advanced human neoplasms, indicating that they do not normally home efficiently to malignant tissues. However, the immune infiltration of NK cells on tumours represents a positive prognostic marker in several carcinomas (*Coca et al., 1997; Ishigami et al., 2000; Villegas et al., 2002*). By other side, this migratory process of NK cells can be further enhanced by immunotherapeutic regimens that enhance NK recruitment to the tumour microenvironment (*Choi et al., 2007*).

Another evidence showing that NK cells participate in tumour defence is their increased function and anti-tumour response in individuals treated with IL-2. In fact, endogenous or ex vivo activated NK cells with IL-2, increases the density of surface expression of activation molecules and consequently NK cell cytotoxicity (*Fujisaki et al., 2009*). This will enhance the immunotherapeutic action of NK cells in the elimination of tumours (*Fehniger et al., 2002*).

The correlation of decreased NK cell function with tumour progression (*Albertsson et al., 2003*) represents another predictive factor involving NK cells in immunological response to tumours. Suppressive factors, such as MIC, which are downmodulating receptors on NK-cells, have been hypothesized to be responsible for this impairment of NK-cell function in malignant disease (*Brittenden et al., 1996; Doubrovina et al.,*

2003). Furthermore, stress and surgical interventions as well as chemotherapy may account for disturbances of NK-cells in cancer (*Ben-Eliyahu et al., 1999; Koda et al., 2003*).

Two principle strategies for therapeutic use of NK cells exist: the activation of endogenous NK cells and the adoptive transfer of NK cells (*Albertsson et al., 2003; Costello et al., 2004*). A third strategy could be the Graft *versus* Tumour (GvT) effect mediated by developing donor NK cells following Hematopoietic Stem Cell Transplantation (HSCT) (*Velardi et al., 2002*).

5.1 *Principles of autologous NK cell immunotherapy*

The use of autologous activated NK cells in cancer therapy dates back to the 1980s, when the first therapeutic trials using adoptive immunotherapy were performed by several groups testing autologous lymphokine-activated killer (LAK) cells to treat a variety of malignancies (*Rosenberg et al., 1987*). Since then, the clinical benefit appeared to be marginal for most tumours. However, subcutaneously administration of IL-2 was demonstrated to be safety and feasibility to stimulate endogenous NK cell activity in patients with cancer (*Miller et al., 1997; Robinson et al., 1997; Meropol et al., 1998; deMagalhaes-Silverman et al., 2000*).

Two main strategies for the therapeutic use of NK cells have been considered, although they have the same biological significance. NK cells can be endogenously cytokine-activated or used in terms of adoptive transfer in which NK cells from the patient are collected, ex vivo activated and placed back into the patient. In both settings, patient and donor are the same person and NK cells are cytokine activated

Activation of endogenous Natural Killer cells

The therapeutic efficacy of endogenous NK cells depends on the effectiveness of NK-activating agents to mobilize sufficient numbers of these cells to metastatic sites. Various clinical protocols for endogenous

NK-cell stimulation have been initiated based on cytokine-therapy regimens. Because IL-2 is still considered to be among the most highly potent immunostimulatory compounds, most clinical trials use this agent (usually at high-dose) as monotherapy or together with other biological-response modifiers (BRMs).

Importantly, in the metastatic tissues of untreated patients, tumour infiltrating NK cells are either not detectable or present only in very small numbers (*Vujanovic et al., 1996*). The ability of NK cells to migrate to tumour sites appears to be tightly linked to their stage of activation (*Hagenaars et al., 1998; Hokland et al., 1999*). Although the number of NK cells in malignant tissues is usually small, systemic treatment with BRMs, such as IL-2 (*Hagenaars et al., 1998; Hokland et al., 1999*) or Poly I:C (Polyinosinic:polycytidylic acid) (*Wei and Heppner, 1987*), increases accumulation of these cells around and even inside tumour nodules. Although the general NK-cell:tumour cell ratio inside a tumour nodule is promising, it is far below that necessary to induce NK-cell elimination of tumour cells in vitro. However, it is possible that fewer NK cells activated by IL-2 might be needed in vivo to exert significant anti-tumour effects, especially against smaller tumours. In this respect, it is important to bear in mind that almost all of the clinical studies accomplished to-date have been performed in patients with disseminated disease and large tumour burdens. Under such conditions, treatment-induced upregulation of endogenous NK-cell activity and frequency might not be sufficient to induce tumour regression.

The use of IL-2 allowed the in vitro expansion and enhanced the cytotoxic activity of NK cells and broadened the spectrum of NK-susceptible tumour cells to include solid tumors as well as hematologic malignancies. This led to a plethora of clinical trials using IL-2 in order to improve the anti-tumor effect of NK cells by either endogenous activation of the patient's own NK cells by IL-2 or of ex vivo expanded autologous NK cells (*Rosenberg et al., 1987*). Although these clinical trials demonstrated the safety of activated NK cells, only infrequent clinical responses were reported, with some durable long term complete responses largely in patients with metastatic melanoma or renal cancer. This led the Food and Drug Administration (FDA) to approve the use of high dose IL-2 for patients with metastatic renal cancer in 1992 and for patients with metastatic melanoma in 1998 (Fig. 26-a).

The very recent availability of GMP grade IL-15, now in phase I clinical trials by the National Institutes of Health (NIH) (*Geller et al., 2011*) may circumvent the use of IL-2, providing a better activation signal for NK cells. The recent excitement surrounding IL-15 concerns its ability to stimulate NK and CD8+ T cells without inducing capillary leak syndrome, decreasing the risk for organ failure in some patients. Also, unlike IL-2, IL-15 doesn't trigger regulatory T (Treg) cells that might otherwise put the brakes on its therapeutic benefits. Thus, if the in vivo administration of IL-15 proves to be less toxic compared to IL-2, this may allow the use of IL-15 in combination with lower dose IL-2 in future clinical trials to enhance the NK anti-tumor effects in cancer patients.

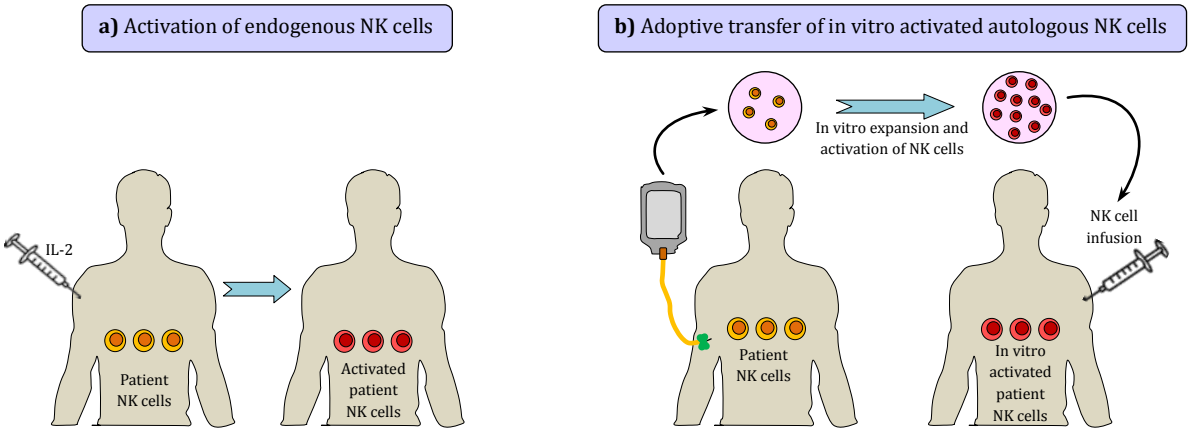


Figure 26 – Natural Killer cell therapies in autologous settings.

Adoptive transfer of in vitro IL-2-activated NK cells

Adoptive immunotherapy with in vitro IL-2-activated NK (A-NK) cells is based on the inoculation of autologous effector cells, which are thus ready to migrate to and infiltrate metastases (Fig. 26-b). From a clinical perspective, cancer treatment using adoptive immunotherapy with NK cells started with LAK-cell therapy. This course of therapy rapidly advanced into the clinic because promising results were obtained in preclinical experiments.

IL-2 was used to expand the number of circulating NK cells in vivo in patients who recovered after autologous transplant, resulting in NK cell expansion, increase of NK differentiation from BM progenitors and IL-2 dependent delay in NK cell death (*Fehniger et al., 2000*).

However, IL-2 treatment is associated with life-threatening toxicity, essentially represented by capillary leak syndrome (*Fehniger et al., 2002*). Moreover, IL-2 activated NK cells increase their sensitivity to apoptosis when in contact with vascular endothelium (*Rodella et al., 2001*), likely causing a decrease in NK

cell migration toward the cancer area. Thus, toxicity of systemic cytokine administration and cytokine-activated NK cell apoptosis are two important limitations of cytokine-mediated (and NK adoptive) immunotherapies for cancer treatment. Thus, the prospective candidate must be in fairly good health to qualify for this drug and the IL-2 administration must be given in a hospital setting where the patient can be closely monitored by physicians and staff who have significant experience in its use. Although protocols using IL-2 treatment resulted in activation of NK cell cytotoxicity (*Phillips et al., 1987*), this effect is dependent on the dose and schedule of IL-2 administration (*Gratama et al., 1993*). The significant toxicity of the capillary leak syndrome induced by high-dose IL-2 led to trials using low-dose subcutaneous IL-2, either alone or in combination with activated NK cells. The NK cell-based autologous immunotherapy has the advantage to prevent relapse, however these strategies failed to show efficacy in patients with lymphoma and breast cancer (*Burns et al., 2003*).

IL-2 injection may increase NK-cell lifespan and activity, but also can generate outgrowth of Treg cells that may hamper the overall response to the tumour as shown in pilot clinical trials (*Barkholt et al., 2009; Geller et al., 2011*).

These initial results have prompted several groups to embark on the large-scale expansion of highly purified, "good manufacturing practice" (GMP) grade NK cells after longer-term in vitro expansion. Some of the protocols have reached small-scale phase I clinical trials and have demonstrated that high numbers of infused NK cells are safe in humans (*Barkholt et al., 2009; Fujisaki et al., 2009*). Moreover, large-scale expansion method was already been possible for human NK cells (*Spanholtz et al., 2010; Sutlu et al., 2010*).

By other side, in the clinical setting, the purity of NK cells to be used, is also a the key factor to consider. Simple purification of NK cells by a single-step or two-step procedure may be enough for some applications. Various groups have evaluated the adoptive transfer of autologous NK cells for cancer immunotherapy in the clinical setting. Escudier *et al.* have demonstrated that the infusion of autologous NK cells that have been expanded and activated *ex vivo* for 13–21 days, greatly improves clinical responses in patients with metastatic Renal Cell Carcinoma (RCC) who have previously achieved partial remission through IL-2 infusions (*Escudier et al., 1994*). Moreover, the high level of IL-2Rα expression in long-term expanded NK cells (*Luhm et al., 2002; Clausen et al., 2003*), as compared with endogenous

$CD56^{dim}$ or $CD56^{bright}$ NK cells and short-term activated NK cells, may provide a higher benefit from subsequent IL-2 administration to the patients.

5.2 *Natural Killer cells in allogeneic transplantation*

NK cells play a prominent role in determining the outcome of allogeneic HSCT. This is based on the concept of "missing self", whereby NK cells recognize and eliminate foreign target cells due to their lack of expression of self MHC class I. In this line, autologous NK cell therapy failed in some clinical settings due to inhibitory receptors that recognize MHC on tumour cells (*Moretta et al., 1993; Karre, 1995; Raulet and Held, 1995*) thus making allogeneic cell transfer (ACT) more attractive.

Allogeneic HSCT (from a nonidentical donor) is a complex clinical procedure, with considerable differences in the nature and origin of the graft, as well as in pregraft treatments (conducted to remove recipient hematopoietic cells and thereby allow the graft to implant) and postgraft treatments [to prevent Graft *versus* Host Disease (GvHD) caused by donor T cells]. Initial results with the use of mismatched allografts led to limited enthusiasm due to GvHD and infectious complications resulting in unacceptable treatment-related morbidity and mortality.

The use of allogeneic NK cells is tentatively alluring, given the current comprehensions of NK cell regulation. A provisional prerequisite for NK cell alloreactivity is that the recipient lacks one or more KIR ligands present in the donor. The donor may in such situations have NK cells that express inhibitory KIR for which there is no ligand on recipient cells. Therefore, a KIR ligand-mismatched donor is likely to give the best chances for clinical response.

Among the well-characterized KIR ligands are the HLA-C group 1 (Asn80) and group 2 (Lys80) alleles, HLA-Bw4 epitope and HLA-A3/A11 Ag. Although NK cells are self-tolerant, they can recognize allogeneic cells because of the lack of relevant HLA inhibitory ligand ("missing self"). The two HLA-C groups of alleles

defined by the epitopes Asn80 and Lys80, respectively, are ligands for activating and inhibitory receptors. The inhibition consequences of KIR epitopes mismatches have been exploited in haploidentical (half-matched) HSCT: when the patient/donor are KIR epitope-mismatched, say the patient is homozygous for HLA-C S77/N80 group 1 ligand and donor is heterozygous for group 1 and group 2 ligands, the donor NK clones with KIRs recognizing group 2 HLA-C alleles will fail to exert an inhibitory action because of the lack of HLA-C group 2 epitope on patient's cells. In this case, such NK clones exert a beneficial alloreactivity against host leukemic cells (*Weisdorf et al., 2002*).

Haploidentical HSCT provides an opportunity for nearly all patients to benefit from HSCT when a HLA genotypically matched sibling is not available. In this context, Velardi and co-workers announced a new era in the exploitation of NK cells for cancer immunotherapy. In 2002, they published that KIR-ligand mismatch between patients and their donors was associated with improved outcomes in AML after T-cell deplete (TCD) haploidentical hematopoietic cell transfer (HCT) (*Ruggeri et al., 2002*). They tested the effect of the intentional KIR-ligand mismatch to treat haematological malignancies, demonstrating a benefit of NK cells alloreactivity with improved rated of BM engraftment, less relapse (better overall survival) and suppression of T-cell mediated GvHD.

The Graft *versus* Leukaemia (GvL) effect correlates with the generation of alloreactive NK cells characterized by potent anti-leukaemia activity. The reduced rate of GvHD is also likely to be the result of the NK cell-mediated killing of recipient APCs. Alloreactive NK cells cannot mediate a DC editing process because they kill all DCs of the host, independent on their stage of maturation. This, in turn, would be essential to prevent donor T cell priming and subsequent generation of GvHD. Unlike allogeneic T cells that can cause GvHD disease in the recipient, allogeneic NK cells may have a better capacity to discriminate tumour cells from normal healthy tissues (Fig. 27).

Later studies have shown that NK cells from healthy donors and cancer patients show higher cytotoxic activity against various KIR-ligand mismatched tumour cell lines when compared with KIR-ligand matched targets (*Igarashi et al., 2004*). After the ground-breaking retrospective analysis of haplotype

mismatched HSCT, within the setting of NK cell-based immunotherapy, the KIR-ligand mismatch phenomenon has attracted great attention (*Ruggeri et al., 2006*). Thus, in certain donor-recipient combinations, chances for missing-self may prevail, providing better possibilities for anti-tumour reactivity.

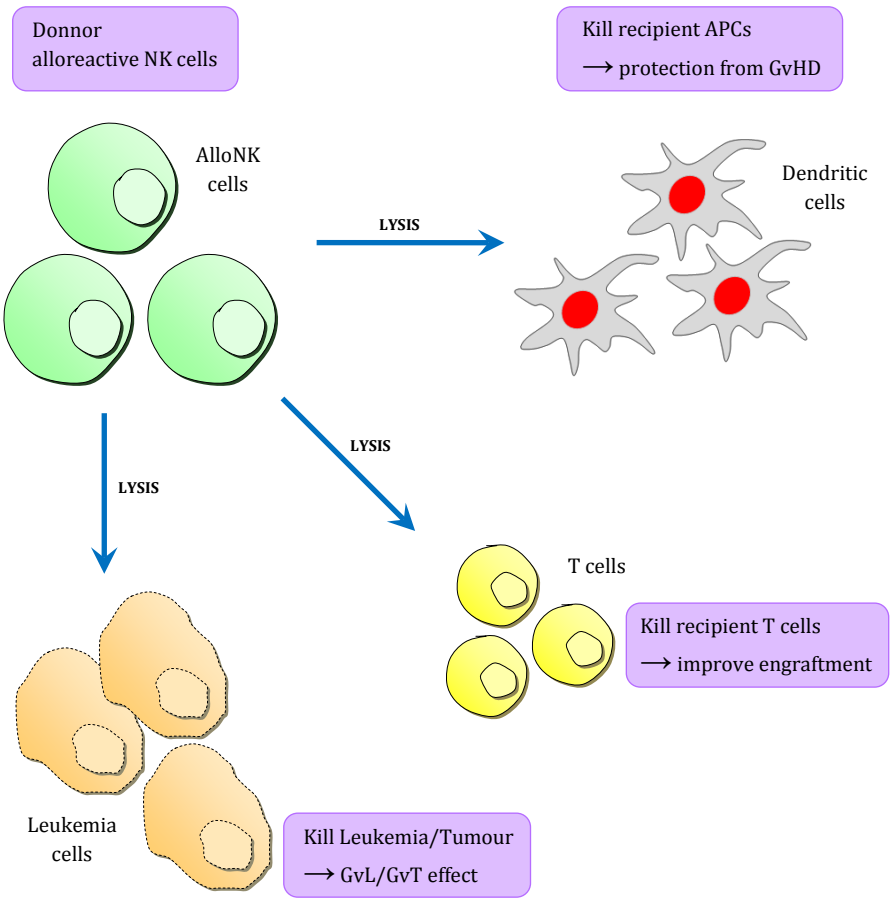


Figure 27 – NK cells in allogenic stem cell transplantation. Allogenic NK cells from donor can directly kill recipient T cells as well as inhibit T cell mediated GVHD through killing of recipient antigen presenting cells such as dendritic cells that may initiate GVHD. Furthermore, NK cells can provide anti-tumor effects by killing residual cancers in the recipient.

In fact, in TCD, HLA-matched, but KIR-mismatched BM transplants (in which not all donor-derived NK cells are inhibited by the recipient HLA or educated by donor or recipient HLA), the number of inhibitory KIR-HLA mismatches (that is, missing inhibitory ligands in the recipient) correlates with positive outcomes, including fewer leukemic relapses and improved graft acceptance (*Symons et al.; Hsu et al., 2005; Clausen et al., 2007; Sobecks et al., 2007*).

Reciprocally, in TCD BM grafts, HLA-mismatched, positive outcomes correlate with lack of HLA ligands in the recipient for inhibitory KIRs on donor-derived NK cells (*Ruggeri et al., 2002; Miller et al., 2007*). Thus, in the case of NK cell-mediated GvL, expression of inhibitory KIRs on donor-derived NK cells that are

reactive with recipient HLA limited the effectiveness of donor NK cells in clearing leukaemias and promoting graft acceptance (*Yokoyama et al., 2010b*).

Since the initial data from haploidentical HSCT, a number of retrospective studies in allostransplantation have been published, sometimes leading to different clinical outcomes (*Witt, 2009*). These conflicting results may be explained primarily by three factors: the degree of T cell alloreactivity, the definition of "KIR mismatch" and misclassification. Initially, alloreactive NK cells were simply defined by having KIRs that were only incompatible with the host MHC, and several studies have identified such alloreactive NK cells that are effective against AML blasts. NK cell alloreactivity occurs when a subset of NK cells express KIR specific for an allelic epitope that is absent on (recipient) allogeneic cells, leading NK cells to kill these allogeneic cells. An HLA class I mismatch between NK cells and target cells does not lead necessarily to NK-mediated target cell killing. KIRs recognize allotypic determinants that are shared by groups of HLA class I alleles. KIR mismatch is necessary to induce activity against MHC-positive cells (we will refer to these cells as potentially alloreactive) but not entirely sufficient, as they must have undergone an education process. Thus "alloreactive" NK cells must not only express KIR that are not engaged by any of the HLA class I alleles present on allogeneic target cells (*Vitale et al., 1995*) but also should not express CD94/NKG2A, as HLA-E is present on all HLA class I^+ cells (*Pende et al., 2006*).

The licensing model predicts that developing NK cells that fail to receive a signal through an inhibitory receptor are hyporesponsive to activating stimuli. However, licensing can be bypassed in a proinflammatory environment (*Kim et al., 2005*), as is seen following myeloablative conditioning. Leung and colleagues argue that alloreactivity may be predicted by considering only the expression of donor-inhibitory KIR and recipient ligand (*Leung et al., 2004*). In the setting of allo-HCT, higher numbers of activating KIR genes have been correlated with increased frequency of acute and chronic GvHD. In 448 unrelated transplants for AML, it was found that the presence of a donor, KIR-B haplotype, which contains a higher number of activating KIR genes than the A haplotype, predicted improved relapse free and overall survival in which there was no KIR ligand mismatch (*Cooley et al., 2009*). This benefit was not observed in patients with KIR ligand mismatched transplants. An interesting recent study showed that in the setting of T-cell depleted (TCD) haploidentical transplantation with KIR-L mismatch, NK cells co-expressing the activating KIR2DS1 with inhibitory KIR2DL2/3 or NKG2A were able to kill recipient leukaemia blasts,

highlighting that recognition by activating KIR might be able to overcome inhibitory signals (*Pende et al., 2009*). NK cell effector capacity is also influenced by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands (*Pfeiffer et al., 2007; Yu et al., 2007*).

Generally, there are two main scenarios:

(1) In haploidentical HSCT, the harsh conditioning regimen and high CD34-positive cell content allow the donor NK cells to mature with a recognition of the "self" MHC type on the donor hematopoietic cells, and therefore become truly alloreactive against residual recipient blast cells, whereas normal host tissues are spared because of lack of NK-stimulatory ligand expression (*Pende et al., 2009; Haas et al., 2010*).

(2) In nonhaploidentical situations, education of NK cells on donor HLA may be lacking in some graft preparation and pregraft regimens, which might account for the neutral effects seen (cells remain potentially alloreactive). Conflicting results in nonhaploidentical situations (*Davies et al., 2002; Giebel et al., 2003*) may be also explained by different treatments resulting in different T-cell levels in grafts and consequently different levels of GvHD (*Cooley et al., 2009*). This hypothesis is further supported by protocols where the graft origin is umbilical cord blood (UCB), a situation with few mature T cells in the graft, which results in a beneficial outcome (*Willemze et al., 2009*).

UCB is a promising source of NK cells because these cells have enhanced sensitivity to stimulation, decreased potential to cause GvHD and are available from cord banks throughout the world.

GvHD is a common side effect of patients receiving stem cell transplants, which results when the T cells in the transplanted blood react against the patient's own cells. This disease can become fatal if it's unable to be controlled. NK cells operate differently from T cells, leaving normal cells alone while targeting and killing the cancerous cells. Historical transplants used a matched donor's PB or BM to transplant to a patient. However, in 1988, researchers found UCB to be another source for stem cell transplantation (*Gluckman et al., 1989*). These immature stem cells were easier to match to patients, especially those from non-Caucasian ethnicities, and could be stored for use as needed. These NK cells demonstrate significant cytotoxic activity against human AML and Acute Lymphoblastic Leukaemia (ALL) cell lines and patient

leukaemia blasts, supporting the evaluation of UCB-derived NK cells as a potential immuno-therapeutic approach in acute leukaemias.

Overall, NK cell products for adoptive transfer can be prepared from adult donor lymphapheresis products, from UCB units or from cell lines, and can be expanded either in vivo or ex vivo.

5.3 *Monoclonal antibodies approaches*

Retrospective studies of KIR/HLA mismatched stem cell transfer (SCT) in AML patients showed that the lack of KIR engagement on donor NK cells by patient MHC class I molecules was associated with a significant reduced risk for leukaemia relapse (*Ruggeri et al., 2002*). NK cell activity can be augmented with cytokines and immunomodulatory drugs such as thalidomide, TLR-agonists or vaccines. However, the manipulation of NK cell reactivity in these settings implies haploidentical HSCT, which are associated with considerable adverse effects, including GvHD mediated by allogenic T cells. A safer strategy is to block NK cell inhibitory receptors in an autologous setting, and is currently tested in phase II clinical trials with a fully human anti-KIR monoclonal antibodies (mAbs) (*Romagne et al., 2009; Sola et al., 2009*) (Fig. 28). Accordingly, autologous NK cells can be directed against tumours by blocking inhibitory receptors, increasing the expression of activating receptor ligands on tumours and by targeting NK ADCC against those receptors with specific antibodies.

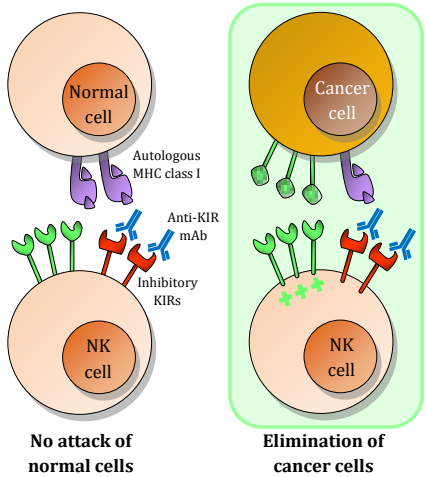


Figure 28 – Anti-KIR therapy in autologous settings.

In fact, modulation of NK cell recognition of tumours may be achieved by blockade of inhibitory KIR as it was recently demonstrated using a humanized antibody (1-7F9) to KIR2DL1, KIR2DL2, and KIR2DL3 as well as the activating receptors KIR2DS1 and KIR2DS2 (*Romagne et al., 2009*). While the monoclonal 1-7F9 should be valuable to block the inhibition of NK cells (and mediating lysis of leukemic cells), other products are now available that can enhance the activation of NK cells.

However, there are always a risk of generating a strong reactivity against normal self-tissues and/or to interfere with NK cell education. The development of anti-KIR-therapeutic mAbs that block NK inhibition may allow the use of autologous cells as an easier source of cell material, by inducing alloreactivity of NK cells that would otherwise be MHC-tolerant.

Administration of mAbs to tumour-associated Ags can also promote NK cell-mediated ADCC response against tumour targets (*Clynes et al., 2000; Caligiuri et al., 2004*).

Despite the lack of true specificity and the limited efficacy, this approach has a unique mechanism of action that does not produce cross-resistance or overlapping toxicities with conventional agents (*Caligiuri et al., 2004*) and that can therefore be combined with cytokine-based immunotherapies. NK cells are important effectors in the mAb-driven immune response to tumours, and data continue to accumulate on their importance (*Beano et al., 2008*).

Multiple clinically successful mAbs utilize NK-mediated ADCC as a mechanism of action (Fig. 29). Rituximab (anti-CD20), Herceptin (anti-HER2/neu), Cetuximab [anti-Epidermal Growth Factor Receptor (EGFR)], and the anti-disalganglioside (GD2)-mAbs 3F8 and ch14.18 are examples of tumour-specific mAbs whose clinical activity can be attributed, at least in part, to NK cells.

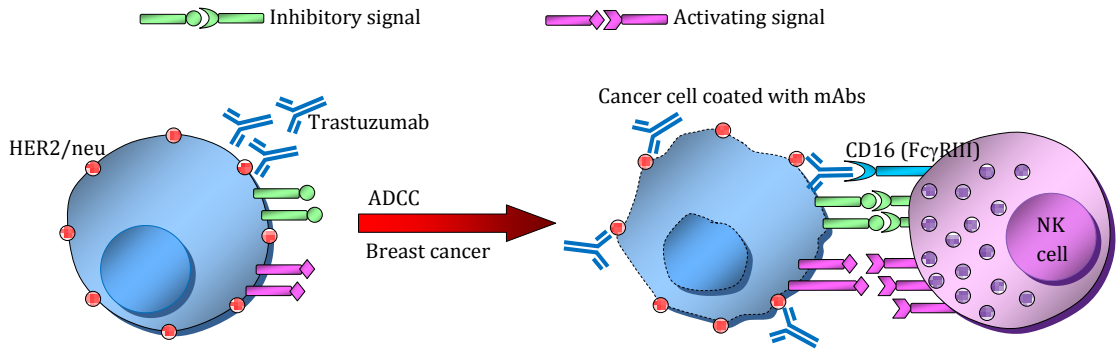


Figure 29 – Example of combinatorial approach of NK cell immunotherapy with mAb therapy (e.g., the anti-HER2/neu mAb trastuzumab). NK cells express an activating Fc receptor (CD16) that recognizes the constant region of IgG and allows them to kill antibody-coated target cells via ADCC.

Herceptin, a humanized antibody against HER2/neu, to normal resting NK cells without IL-2 activation significantly enhanced killing of breast cancer targets. GD2 is overexpressed on tumors of neuroectodermal origin, such as neuroblastoma and melanoma, and minimally expressed in normal

tissues making it a good target for tumour-specific mAb. Anti-GD2 mAbs work through NK cell-mediated ADCC and have demonstrated clinical benefit for children with neuroblastoma (*Yang and Sondel, 2010*). These examples use mAbs targeting tumour antigens antibody-facilitated NK cell-mediated cancer immunotherapy strategies.

Antibodies with high affinity Fc regions for FcγRIIIa are better at activating NK cells and can be used at lower concentrations than traditional antibodies and maintain anti-tumour activity (*Bowles et al., 2006*). These mAbs maybe beneficial in a clinical setting by reducing the amount of antibody necessary to produce an antitumor response and therefore reduce mAb-related toxicities (*Weiner et al., 2010*).

The combination of mAb therapy with cytokines is another strategy used to increase their activity. Combination of Herceptin with IL-12, an important cytokine to NK cell responsiveness and IFN-γ production, increases the response of NK cells to HER2-expressing breast tumour cells in a mouse model of breast cancer (*Parihar et al., 2002*). IL-2-activated LAK cells have increased ADCC activity against mAb-coated tumour cells (*Hank et al., 1990; Schultz et al., 1990*) by diminishing the required amount of antibody necessary for NK cells to effectively lyse antibody-coated tumour targets (*Watanabe et al., 1993*).

Additional approaches to augment NK cell activity include the utilization of Immunocytokines (ICs). ICs are antibodies with linked cytokines at the Fc terminal end. The anti-GD2 IC hu14.18-IL-2 is a humanized mAb developed via fusion of two molecules of IL-2 to the mAb, 14.18, that recognizes GD2, expressed on high risk melanoma and neuroblastoma (Fig. 30) (*Gillies et al., 1993; Hank et al., 1996*). ICs may have certain advantages over traditional mAbs (*Yamane et al., 2009*).

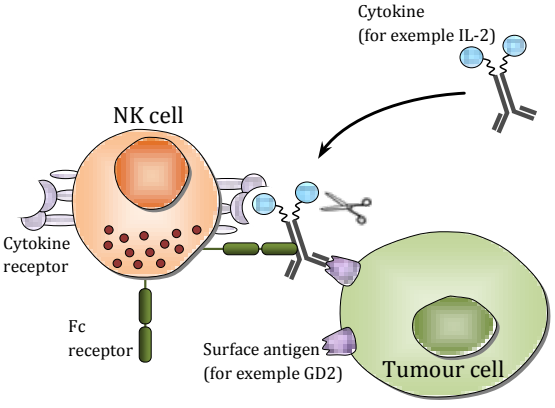


Figure 30 – Monoclonal antibody specific for a tumour-associated antigen allows the enrichment of cytokines in the tumour microenvironment. In the case of interleukin-2 (IL-2) it enhances antibody-dependent cellular cytoxicity mediated by F_c-receptor positive effector cells such as Natural Killer cells.

ICS are fusion proteins that genetically fuse immunologically reactive mAbs to cytokines. The goal is to retain the functions of both the cytokine and the antibody components in a single bifunctional molecule, and to ultimately expand the biologic activities of one component (the antibody) with the biologic function of the other component of the IC (the cytokine).

In several preclinical models, using 3 different ICs, the IC provided far greater antitumor effects than the same amount of the naked mAb infused with the same amount of IL-2 (but infused simultaneously as separate molecules rather than as the IC fusion protein). ICs transport cytokine to the site of tumour and can support an ongoing local anti-tumour immune response (*Lode et al., 1997; Johnson et al., 2008*). Direct delivery of IC into the tumour itself elicits a more potent local effect (*Johnson et al., 2008*). The potential benefit of IL-2 containing ICs in activating and assisting NK cells in tumour cell destruction is a relatively new research area for clinical NK-mediated tumour immunotherapy.

More recent findings show that polymorphisms in genes encoding FcγRs are associated with clinical responses to other Abs (*Musolino et al., 2008; Bibeau et al., 2009*). ADCC enhancement through Fc domain modification has shown promise in the development of next generation mAbs. NK cells containing a valine at position 158 have a higher affinity for IgG than those containing a phenylalanine in the same position (*Koene et al., 1997*). NK cells with FcγRIIIa158v receptors may have a twofold advantage in the setting of mAb-mediated cancer immunotherapy: enhanced ability to recognize and bind to tumour cells coated with mAb molecules and the release of more granules for each tumour cell they encounter. Patients with an FcγRIIIa158v genotype respond better to therapy that utilizes an ADCC-mediating mAb. The treatment with Herceptin (Trastuzumab), Rituximab, and Cetuximab has correlated an FcγRIIIa158v receptor genotype with better response to therapy (*Cartron et al., 2002; Zhang et al., 2007; Musolino et al., 2008*).

5.4 *Pharmacological agents used to modulate Natural Killer activity*

In addition to some already mentioned compounds, there are also some therapeutic agents that are being developed and tested in order to stimulate NK cell activity.

In fact, it has been shown by several groups that certain drugs, already available in the therapeutic arsenal, can increase the expression of NK-activating ligands on the tumour, and therefore increase NK tumour lysis in vivo. Initially, it was shown that some chemotherapy (5-FU, Ara-C, cisplatin) and radiation or ultraviolet therapy targeting the DNA damage pathway can increase expression of the NK-stimulating ligand NKG2D on tumour cells, and lead to enhanced NK lysis of tumours (*Romagne et al., 2009*) (Fig. 31-a).

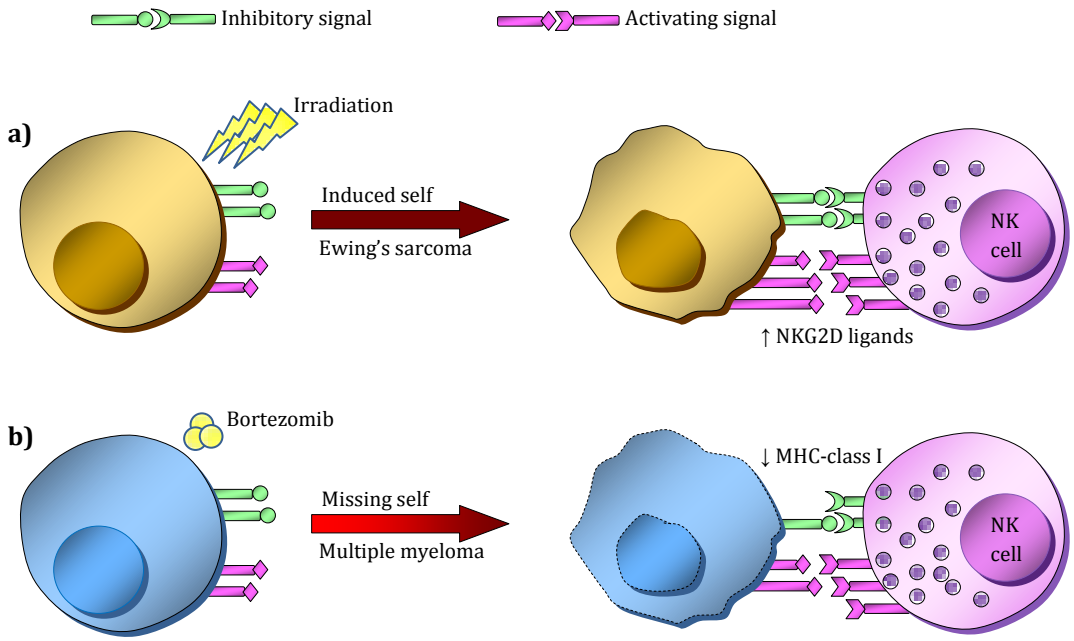


Figure 31 – Examples of combinatorial approaches of Natural Killer cell immunotherapy with (A) chemotherapy (e.g., bortezomib); and with (B) radiation therapy.

More recently, new drugs targeting proteasome inhibitors, such as bortezomib, which is now registered for the treatment of MM, have also been shown to induce NK-stimulatory ligands (*Gasser et al., 2005; Ames et al., 2009*) (Fig. 31-b).

Finally, lenalidomide (Revlimid), a drug which has been shown to be active in MM and to have promising preliminary results in other hematological malignancies, has been shown, in addition to having a direct antitumor effect, to upregulate NK-cell function through induction of cytokines (*Butler et al., 2009*) and to induce NK-stimulatory ligands on tumour cells.

Some of these drugs, such as bortezomib or chemotherapies (*Davies et al., 2001; Markasz et al., 2007*), can also have inhibitory effects on NK cells so their use must be carefully evaluated, but their clinical availability opens the door to multiple combination possibilities, either sequentially or concomitantly, with cell therapy and anti-KIR antibodies. Such combinations are beginning to be tested in the clinic (phase I/II for anti-KIR in combination with lenalidomide, and cell therapies in combination with bortezomib) (*Berg et al., 2009*).

5.5 *Genetic engineering of Natural Killer cells*

Although the risk of adverse events may be low at the cytokine dosages required to sustain NK cell survival, genetic modifications generating autocrine cytokine signals may be a mechanism to avoid the consequences of systemic administration.

Gene transfer into NK cells may open new possibilities for the immunotherapy of cancer in both autologous and allogeneic settings. Such investigations have began with the optimization of NK cell genetic modification via various methods including electroporation (*Grund and Muise-Helmericks, 2005; Schoenberg et al., 2008*), nucleofection (*Trompeter et al., 2003; Maasho et al., 2004*) and transduction by chimeric adenoviral (*Schroers et al., 2004*), chimeric EBV/retroviral (*Becknell et al., 2005*), retroviral (*Guven et al., 2005; Alici et al., 2009*) and lentiviral (*Tran and Kung, 2007*) vectors.

Despite A-NK cells (activated NK cells) leads to a significant reduction in tumour burden and to prolonged survival of the host, it is necessary to genetically alter them so that they acquire better target recognition or target-killing capabilities, or so that they can secrete cytokines that either stimulate the NK cells or other antitumour effector arms of the immune system. For example, direct infusions of NK-92 cell line (which does not express any KIR but still has a broad spectrum of activating receptors) in patients, may be safe and potentially beneficial (*Gong et al., 1994; Maki et al., 2001; Tonn et al., 2001*).

IL-2 has been successfully transduced into NK cell lines, resulting in increased cytotoxicity as well as proliferation independent of supplementation (*Nagashima et al., 1998*). IL-15 has also been introduced into NK-92 and NKL cell lines (*Zhang et al., 2004; Jiang et al., 2008*) resulting in increased proliferation and cytotoxicity. IL-15 transduction also increases natural cytotoxicity and survival (*Zhang et al., 2004*). Thus far, there is limited described experience with cytokine transduction into primary or expanded NK cells (*Alici et al., 2009*), but such modifications could allow increased NK cell survival or proliferation without the restrictions associated with using transformed cell lines.

Transgenic production of IL-12 by NK cells (A-NK12 cells) would also be beneficial as it enhances IFN-γ production, upregulates CD25 (IL-2Rα) expression by NK cells (*Rabinowich et al., 1993*) and reduces requirement for IL-2. Also, IL-12 produced by the tumour-infiltrating A-NK12 cells (*Basse et al., 1991*) is able to maintain survival of not only the transduced cells themselves, but of neighbouring nontransduced A-NK cells as well (*Goding et al., 2007*). It is important however that IL-12 being produced at the tumour site on the survival of nontransduced A-NK cells (*Yang et al., 2003*).

The co-presence of IL-12 and IFN-γ leads to therapeutic effect, but, if IL-12 is present while IFN-γ is absent from both host and adoptively transferred cells, a fatal but yet poorly understood toxicity is induced. Although IL-12 has been successful in enhancing A-NK cell therapy without generating toxicity, this does not seem to be sufficient to improve long-term, adaptive host immunity.

TNF-α is a very early initiator of inflammation, which activates DCs and promotes CTL generation (*Gorelik et al., 1995; Gorelik et al., 1996*). Thus, in addition to other beneficial effects, like enhancing LAK activity, increasing vascular permeability, and direct tumour killing, TNF-α is an attractive choice to add to the A-NK12 therapy to further augment its antitumour effect. The addition of TNF-α gene transduction does not improve the A-NK cell treatment to an extent that can be measured with survivability as endpoint. Additionally, the number of A-NK cells present within the lung tumours does not always correlate with the therapeutic outcome, that is, the survival of recipients. Although the mechanisms involved in the double-transduced A-NK cells persistence to such a high degree without mediating greater therapy have not been elucidated, in any case, the increased survival of A-NK cells, provides a great deal of promise for future use of transduced A-NK cells.

One possibility to make autologous NK cells lyse the patient's own cancer cell is by engineering them to express ligands that recognize specific antigens on the tumour, thereby overriding any inhibitory KIR activation by the "self " MHC. This strategy has been employed with NK cells that express chimeric antigen receptors (CARs) against HER2/neu (*Uherek et al., 2002; Demirtzoglou et al., 2006; Meier et al., 2008*) or the lymphoid antigens CD19 (*Boissel et al., 2009*), CD20 (*Muller et al., 2008*) and CD33 (*Schirrmann and Pecher, 2005*). The transfer of cells expressing a chimeric receptor against HER2/neu in primary NK cells (*Kruschinski et al., 2008*) resulted in high level of cytotoxic activity and also could inhibit tumour progression. Thus, these data indicate that the adoptive transfer of chimeric antigen-specific bearing NK cells might be an efficient approach in cancer immunotherapy.

As discussed herein, gene transduction of A-NK cells can be a powerful antitumour tool, which can be used to improve/change target recognition by the NK cells (*Pegram et al., 2008*) or to deliver combinations of cytokines, and possibly in the future, chemokines and/or danger signals also, to the microenvironment.

Moreover, strategies to manipulate the balance between inhibitory and activating receptor signalling in favour of activation are now emerging from the vast research into the molecular basis of target-cell recognition [reviewed in (*Farag et al., 2003*)]. However, manipulation of NK cells to make them more efficient killers is only part of the task. To be highly efficient in vivo, NK cells must also be capable of bringing themselves into close contact with malignant cells. Therefore, a better understanding of how NK cells find and infiltrate tumours is crucial, particularly with regard to tumour microenvironment, migration of NK cells in this environment, and NK cell interaction with the extracellular matrix components of the tumour.

Future work will focus on NK cell engineering to generate cells with improved activity or survival. One limitation to current approaches to genetic modification is the reliance on gene transfer primarily by retroviral transduction. However, electroporation offers a mechanism of transient expression without the time, expense or risks of clinical-grade viral transduction (*Li et al., 2010a*).

5.6 *Perspectives*

Due to several recent findings that give more insight in the biology of NK cells in health and disease, this type of cells moved in the focus of cellular therapy, i.e. in human malignancies over the past two decades. Therefore, it is of outmost importance to understand the biological principals of interaction of NK cells with tumour cells and/or regulatory cells.

In addition, for therapeutic approaches large numbers of functionally competent NK cells are necessary and need to be generated, expanded and activated in vitro. Thus, it is important to understand differentiation processes and mechanisms involved in the NK cell differentiation pathway. The possibility to recapitulate in vitro the ontogeneic steps from early progenitors to mature effector cells provides a tool to investigate mechanism which lead to NK cell functionality.

Umbilical cord blood (UCB) has been shown to be a valuable source of stem and progenitor cells with the potential to be differentiated in vitro in fully maturated NK cells, becoming a reservoir for possible use in adoptive cellular immunotherapy. These in vitro differentiated NK cells might induce an antileukemic effect in the state of minimal residual disease after hematopoietic stem cell transplantation. Accordingly, functional NK cells obtained by differentiation of $CD34^+$ progenitor cells from UCB may provide a promising source for immunotherapy.

We proposed an in vitro model that mimics the ontogeny of human NK-cell development, defining the differentiation stages of mature and functional NK cells. With these experiments, we will be able to successfully generate and differentiate NK cells starting from $CD34^+$ hematopoietic stem cells. Knowledge of certain transcription factors and differentiation/activation pathways in process of in vitro NK-cell development may lead to additional therapeutic tools of NK-application allogeneic cellular immunotherapy settings.

AIMS

AIMS:

Our first aim was the recapitulation of Natural Killer (NK) cell ontogeny by in vitro differentiation of NK cells from CD34+ human Umbilical Cord Blood Hematopoietic Stem cells. For this purpose, we induced CD34+ human Umbilical Cord Blood Hematopoietic Stem cells to differentiate in mature NK cells. During the differentiation period we analysed NK cell phenotype with NK specific molecules and receptor markers to get inside the differentiation steps. We have also analysed the functionality of these in vitro differentiated NK cells by means of cytotoxicity tests against the K-562 cell line.

MARIA JOÃO PINHO, MICHAEL PUNZEL, MÁRIO SOUSA and ALBERTO BARROS. **Ex vivo differentiation of natural killer cells from human umbilical cord blood CD34+ progenitor cells** (2011). *Cell Communication & Adhesion 18(3):45-55.*

The second objective was to investigate the expression of several Transcription Factors involved in NK cell commitment, differentiation and maturation. For this proposal, we did a quantitatively analysis of a number of Transcription Factors that were referred in literature as important or determinant either in lineage commitment or in differentiation steps of NK cell maturation.

MARIA JOÃO PINHO, CRISTINA JOANA MARQUES, FILIPA CARVALHO, MICHAEL PUNZEL, MÁRIO SOUSA and ALBERTO BARROS. **Genetic regulation on ex-vivo differentiated natural killer cells from human umbilical cord blood CD34+ cells** (2012). *Journal of Receptors and Signal Transduction 32(5):238-49*

PAPER I

"Ex vivo differentiation of natural killer cells from human umbilical cord blood CD34+ progenitor cells"

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FULL LENGTH RESEARCH ARTICLE

Ex vivo differentiation of natural killer cells from human umbilical cord blood CD34+ progenitor cells

MARIA JOÃO PINHO[1], MICHAEL PUNZEL[2], MÁRIO SOUSA[1,3,4] and ALBERTO BARROS[1,4]

[1]*Department of Genetics, Faculty of Medicine, University of Porto, Porto, Portugal*
[2]*MediaPark Klinik Cologne, Center for Bone Marrow and Stem Cell Donation, Cologne, Germany*
[3]*Lab Cell Biology, ICBAS-Institute of Biomedical Sciences Abel Salazar, University of Porto, Porto, Portugal*
[4]*Center for Reproductive Genetics ABarros, Porto, Portugal*

Abstract

Natural Killer (NK)-cells are peripheral blood lymphocytes that represent an important arm of the innate immune system. NK-cells play a critical role in the immune surveillance against tumors and virally infected cells in a major histocompatibiliy complex (MHC)-unrestricted fashion. We have explored such capacities of NK-cells after differentiation from hematopoietic stem and progenitor cells derived from human umbilical cord blood. Several culture conditions have been established supporting proliferation and subsequent differentiation of these cells in terms of receptor expression and specific lysis depending on the growth conditions in the presence and absence of supportive stromal feeders. We show that acquisition of Killer Immunoglobulin Receptor (KIR) as well as NK Cytotoxicity Receptor expressions is independent of culture condition whereas absence of stromal feeders did not support acquisition of CD94/NKG2A expression. Such KIR-positive/NKG2A-negative cells generated under different culture conditions showed strong and specific cytolytic activity which could have impact on further immunotherapeutic strategies.

Keywords: natural Killer cells, NK receptors, ex vivo culture, immunotherapy

Introduction

Natural Killer (NK)-cells, originally described as non-T, non-B cells, are large granular lymphocytes that were identified in mice by the innate capacity to rapidly lyse some tumor cells. Unlike T-cells, they can kill targets without prior sensitization and exhibit spontaneous cytotoxicity activity towards cells that do not express class I molecules of the major histocompatibility complex (MHC) (Borrego et al., 2002; Orange & Ballas, 2006). NK cells express a large variety of activating receptors on their surface capable of recognizing target cells (Moretta et al., 2002). They also express inhibitory receptors whose signals are able to override basal activation signals (Hallett & Murphy, 2006; Lanier, 2008). The ligands for the predominant inhibitory receptors are major histocompatibility complex (MHC) class I molecules that are expressed by most normal cells. As originally proposed by the "missing self" hypothesis, down-regulation by tumor transformation or infection can make cells susceptible to NK lysis due to partial or complete loss of class-I MHC expression (Garcia-Lora, Algarra & Garrido, 2003; Orange, Fassett, Koopma, Boyson, & Strominger, 2002). These cell modifications are perceived by NK-cells and, in that event, the normal balance between activating and inhibitory signals provided by diverse receptors is compromised and can make cells susceptible to a NK-cells-response. In humans, the predominant NK inhibitory receptors interacting with MHC class I molecules are the heterodimeric CD94/NKG2A, the killer immunoglobulin-like receptors (KIRs) and the leukocyte immunoglobulin-like receptors (LIRs or ILTs). While the ligands for the type I transmembrane glycoproteins KIR and LIRs are mainly the classical human leukocyte antigen (HLA) class I molecules (HLA-A, B and C), the ligand for NKG2A is the nonclassical class I molecule HLA-E (Borrego, Ulbrecht, Weiss, Colugan, and brooks, 1998; Brooks, Posch, Scorzelli, Borrego, & Coligan, 1997).

NK cells are characterized by the absence of conventional antigen receptors, and are phenotypically identified by the presence of CD56 and absence of CD3 surface antigens (Robertson and Ritz, 1990). The majority of human NK cells (≈90%) have low density expression of CD56 ($CD56^{dim}$) whereas ≈10% of NK cells are $CD56^{bright}$ (Cooper, Fehniger, & Caligiuri, 2001). While $CD56^{bright}$ NK cells appear to have an important immunoregulatory role, they are less effective mediators of antibody-dependent cellular cytotoxicity (ADCC) and natural cytotoxicity. $CD56^{dim}$ NK cells are more naturally cytotoxic against NK-sensitive targets (Farag and Caligiuri, 2006). In spite of that, $CD56^{bright}$ and $CD56^{dim}$ NK cells show similar levels

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Address correspondence to Dr Maria João Pinho, Department of Medical Genetics, Faculty of Medicine, University of Porto, Alameda do Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. Tel: 00351 225513647. Fax: 00351 225513648. E-mail: mjpinho@med.up.pt

of cytotoxicity when treated with IL-2 both *in vitro* and *in vivo* (Caligiuri et al., 1990; Nagler, Lanier, & Phillips, 1990; Robertson et al., 1992). Although there is no direct functional significance of high- or low-level expression of CD56 (Lanier, Testi, Bindl, & Phillips , 1989), Cooper and colleagues reviewed a number of other cell-surface markers that confer unique phenotypic and functional properties to $CD56^{bright}$ and $CD56^{dim}$ subsets (Cooper et al., 2001).

Several studies have been engineered to enhance NK-cell activity, however the molecular mechanisms and regulation of the NK cell alloreactivity in humans is yet largely unknown and the repertoire of the receptors that are involved in alloreactivity (KIRs) is quite variable. In fact, *in vitro* differentiation of NK cells from $CD34^+$ hUCB progenitors has been already studied *in vitro* under different conditions (Carayol et al., 1998; Grzywacz et al., 2006; Kao et al., 2007; Lewis & Verfaillie, 2000; Miller & McCullar, 2001). However, these experiments have generated conflicting results. Feeder layers were either shown to have no beneficial effects simultaneously on the NK cell differentiation, expansion and maturation (Lewis & Verfaillie, 2000), to increase $CD56^+$ expansion but not maturation (Carayol et al., 1998), or to promote $CD56^+$ expansion and maturation (Grzywacz et al., 2006, Miller and McCullar, 2001). Overall, the possibility to generate/differentiate NK-cells from $CD34^+$, with and without stroma was already demonstrated (Papamichail, Perez, Gritzapis, & Baxevanis, 2004). However, the possibility to generate NK-cells from UCB without stroma/adhesive microenvironmental factors for a possible clinical application in terms of adoptive cellular therapy, was never been demonstrated so far.

We attempted to recapitulate the development of functional NK-cells from multipotent hematopoietic UCB-stem cells *in vitro* reconstituting the complete process of NK-cell development and maturation from HSPC to functionally competent effector cells. We also investigate the possibility to generate a specific NK cell population ($CD56^+$ KIR^+ NKG2A-) from UCB without stroma/adhesive microenvironmental factors.

In summary, multipotent hematopoietic stem and progenitor cells were retrieved from human umbilical cord blood (hUCB) samples and induced to proliferate and mature *in vitro* into NK cells, with determination of the temporal expression of the specific surface receptors and analysis of the functional effector characteristics of NK cells. To analyze the role of the niche created by support cells in the transition of primitive stem cells to less primitive cells, the established AFT024 cell line was used in the *in vitro* culture system (Moore, Emma, & Lemischka, 1997) and compared to conditioned medium and standard medium enriched with specific growth factors and interleukins.

Methods

Cell samples

In accordance to guidelines of the local Ethic Committee, human umbilical cord blood (hUCB) was harvested by umbilical vein puncture after normal full-term deliveries under previous informed consent from pregnant woman before delivery. Samples were collected in 150 cc sterile bags, containing 21 ml of citrate-phosphate-dextrose (CPD) (MacoPharma, Tourcoing, France), and kept at room temperature for less than 12 h. In total, 18 hUCB were included, with at least 5 hUCBs being used in each experiment.

Isolation of mononuclear cells

Mononuclear cells (MNCs) from a total of 18 hUCBs were isolated using 1.077 g/ml Ficoll-Histopaque (Sigma, St. Louis, MO, USA) by gradient centrifugation (400 g, 30 min, 20°C). Low density MNCs were collected, washed twice (1600 rpm, 6 min, 4°C) and resuspended in Dulbecco's phosphate buffered saline (DPBS; Sigma), supplemented with 0.5% of fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah, USA) and 2 mM of ethylene diamine tetraacetic acid (EDTA; Sigma). When necessary, lysis of residual red cells was performed with ice-cold lysis buffer consisting of 8.29 g NH_4Cl, 1 g $KHCO_3$ (Merck, Darmstadt, Germany; Sigma) and 0.2 ml of 0.5 M EDTA in 1 L H_2O. After 15 min, cells were washed twice and resuspended in DPBS supplemented with 0.5% of FBS and 2 mM of EDTA.

Selection of $CD34^+$ cells

The $CD34^+$ cell fraction from MNCs was immunomagnetically selected using a Magnetic Activated Cell Selection (MACS) system (Miltenyi Biotec, Auburn, CA, USA). The Progenitor Cell Isolation Kit uses antibodies recognizing the CD34 epitope QBEND/10 and was performed according to manufacturer's instructions with minor alterations. Briefly, MNCs were incubated with 75 µl MACS microbeads $CD34^+$ /10^8 total cells. Cell clumps were removed by passing the cells through a pre-separation Filter and labeled cells were separated using a high gradient magnetic separation column in a strong magnetic field. Magnetically retained cells were released by flushing down the column with a plunger and thereafter eluted into a 15 ml Falcon tube. Cell aliquots were taken for cell viability using Trypan blue exclusion method (Sigma) and an Improved Neubauer Haemocytometer for count. The purity of the $CD34^+$ fraction was determined by flow cytometry, being consistently above 90%.

AFT024 feeder layers

AFT024 cells, a stromal cell line derived from murine fetal liver (Heinrich Heine University, Duesseldorf, Germany), were used as feeder layers. Cells were cultured in 75 cc flasks with Dulbecco's modified Eagles medium (DMEM) containing 1000 mg glucose/L (Gibco, Paisley, Scotland, UK), and supplemented with 20 % FBS, 100 U/ml Penicilin and 100 µl/ml Streptomicin (Pen/Strep; Gibco), 2 mM L-Glutamin (Gibco) and 24 µM β-mercaptoethanol

(Gibco). Cells were cultured for one week at 33°C with 5 % CO_2 in humidified air. When a confluent layer formed, cells were recovered by digestion (5 min, 37°C) with 10 ml of a trypsin/EDTA solution (trypsin/EDTA; Gibco). To increase cell number, cells were cultured again in 2, 3 or 4 new 75 cc-culture flasks until confluency. AFT024 cells were then plated in 24-well plates (10^5 cells/1 ml/well). When confluent, cells were uniformly irradiated (2000 rad) using a cobalt irradiator (Theratron 780C; Atomic Energy of Canada Ltd., Chalk River, Ontario, Canada). After irradiation, plates were incubated at 37°C and the culture medium changed the day after.

Cell culture

$CD34^+$ cells were used in a two-step protocol, consisting of 1 week of proliferation and 3 weeks of differentiation. Cells were plated at different concentrations (4×10^3 cells/well, 2×10^3 cells/well, 1×10^3 cells/well and 0.1×10^3 cells/well) and incubated in three different conditions: 12 samples with supplemented $medium^+$ AFT024 feeder layers (AFT), 5 samples with supplemented medium (C), and 5 samples with supplemented medium conditioned from AFT024 (CM). Cells were cultured (5% CO_2 in air at 37°C) with the medium being changed once a week. In the first week, the medium consisted of a 2:1 proportion of DMEM containing 4500 mg glucose/L (Sigma) and HAMs F12 (Biochrom AG, Berlin, Germany), supplemented with 20 % of heat inactivated human AB serum (BioWittaker, Walkersville, USA). This was then supplemented with Pen/Strep (100 IU/100 µl/ml), β-mercaptoethanol (24 µM), ascorbic acid (20 mg/L), selenium selenate (50 µ/L), ethanolamine (50 µM) (Sigma), L-Glutamine (2 mM) (Gibco) and a cocktail of recombinant human cytokines, 1000 IU/ml IL-2, 5 mg/ml IL-3, 20 mg/ml IL-7, 20 mg/ml stem cell factor (SCF) and 10 mg/ml Flt3-L (PeproTech, London, UK). At the end of the first week, the medium was changed and the cells were cultured with a 2:1 proportion of DMEM containing 4500 mg glucose/L and HAMs F12, supplemented with 10 % of heat inactivated human AB serum, and the cocktail of cytokines described above, with the exception of IL-3 that was replaced by IL-15 (10 mg/ml) and the addition of IL-21 (10 mg/ml) (PeproTech).

Cell phenotyping

Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated and phycoerythrin-Cy5 (PE-Cy5)-conjugated monoclonal antibodies (MoAbs) were used for the analysis of controls and specific cell surface markers. MoAbs anti-mouse IgG1 (clone 679.1Mc7) -FITC, anti-mouse IgG2a (clone 7T4-1F5)-PE, anti-CD34 (clone 581)-FITC, anti-CD56 (clone N901)-PECy5, anti-CD158a (clone EB6B)-PE, anti-p50.3 (clone FES172)-PE, anti-NKp30 (clone Z25)-PE, anti-NKp44 (clone Z231)-PE, anti-NKp46 (clone BAB281)-PE and anti-NKG2a (clone Z199)-PE were purchased from Beckman Coulter (Immunotech, Marseille, France). MoAbs anti-mouse IgG1 (clone MOPC-21)-FITC/PE/PECy5, anti-CD45 (clone HI30)-FITC/PE/PECy5, anti-CD94 (clone HP-3D9)-FITC, anti-CD3 (clone UCHT1)-FITC, anti-NKB1 (clone DX9)-PE and anti-KIR-NKAT2 (clone DX27)-PE were acquired from Beckton & Dickinson (BD PharMingen, San Diego, CA). Once a week, a sample of the cultured cells was harvested for immunophenotyping. Briefly, cells were resuspended and collected in a 15 cc Falcon tube, washed in DPBS supplemented with 0.5 % FBS and 2 mM EDTA, and divided into aliquots to evaluate defined surface markers and stain cells with isotype-matched antibodies ensuring the sample control. All aliquots were incubated with 5 l of MoAbs per $5x10^5$ cells for 30 min in dark conditions at 4°C, and washed twice in DPBS supplemented with 0.5 % FBS and 2 mM EDTA, according to the manufacturer's instructions. Flow cytometry was performed in a FACScalibur apparatus equipped with Cell Quest Pro software, and data were analyzed with the Paint-a-Gate software (Becton Dickinson, Oxford, UK). Each sample was analyzed at least for 20000 events. Results are presented as the percentage of cells in a selected lymphocyte region.

Cytotoxicity tests

After 4 weeks, $CD56^+$ cells were purified from cultures (C, AFT) (n = 5) by MACS and used as effector cells. K562 cells, a human erythroleukemia cell line (ICLC HTL94001; Interlab Cell Line Collection, Genova, Italy), were used as target cells. Cytotoxic activity was assessed using a non-radioactive cytotoxicity assay (CytoTox 96; Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, effector cells were incubated with 1×10^4 K562 cells and 4 replicates were performed for each experiment in 96-well round bottom plates at different effector:target rates, 10:1, 5:1, 2.5:1, 1.25:1, 0.63:1, 0.31:1 and 0.16:1. Plates were maintained for 4 h in a humidified chamber at 37°C, 5 % CO_2, and cytotoxicity was determined by lactacte dehydrogenase (LDH) release from target cells. Culture medium or 0.8 % Triton X-100 (CytoTox 96) was added to target cells for calculation of spontaneous (S) and maximum release, respectively. Supernatants were collected and LDH was assayed using an ELISA plate reader (Stat Fax 2100 Microplate Reader; Awareness Technology, Inc., Palm City, FL, USA). Cytotoxicity was calculated using the following formula:

% Cytotoxicity = [Experimental – (Effector Spontaneous + Target Spontaneous)/Target maximum - Target Spontaneous] × 100.

Statistics

Results of experimental points from different experiments were reported as the mean ± standard deviation

(SD). Significance levels for cytometry results were determinate by two-sided Pearson Chi-Square analysis and statistical significance for cytotoxicity values was tested by two-sided Student's t-test analysis.

Results

Proliferation

Positively selected $CD34^+$ cells (Figure 1a) were cultured in a defined medium containing IL-2, IL-3, IL-7, SCF and Flt3-L (C) (n = 5), C^+ AFT024 feeder layers (AFT024) (n = 12) (Figures 1b & 1c) and C^+ AFT024 conditioned medium (CM) (n = 5). Cobblestone-like areas became visible at the end of the first week of culture in all three culture conditions, suggesting an effective proliferation of the original $CD34^+$ cells (Figure 1d). Quantitatively, the percentage of $CD34^+$ cells was significantly higher ($p < 0.001$) in AFT (26.89 ± 12.46%) than in CM (11.76 ± 5.96%) or C (13.16% ± 6.79%), and in C in relation to CM, which suggests a positive effect of direct cell contacts but not of a factor released by the feeder layers. As expected, in the subsequent three weeks of culture using a modified mixture directed to NK cell differentiation (IL-2, IL-7, IL-15, IL-21, SCF and Flt3-L), the pool of $CD34^+$ cells progressively decreased (0.01 ± 0.02% in AFT, 0.15 ± 0.30 % in CM and 0.00 ± 0.00 in C) (Figure 1e; Table 1). In this period, the same type of significance was observed between the three culture conditions ($p < 0.001$), with exception of day 28, where no differences were found between AFT and C ($p = 0.374$).

Differentiation

During the same differentiation period, the relative numbers of NK cells ($CD56^+ CD3^-$) progressively increased as $CD34^+$ cells decreased (Figures 2a-c, 3a &3b).

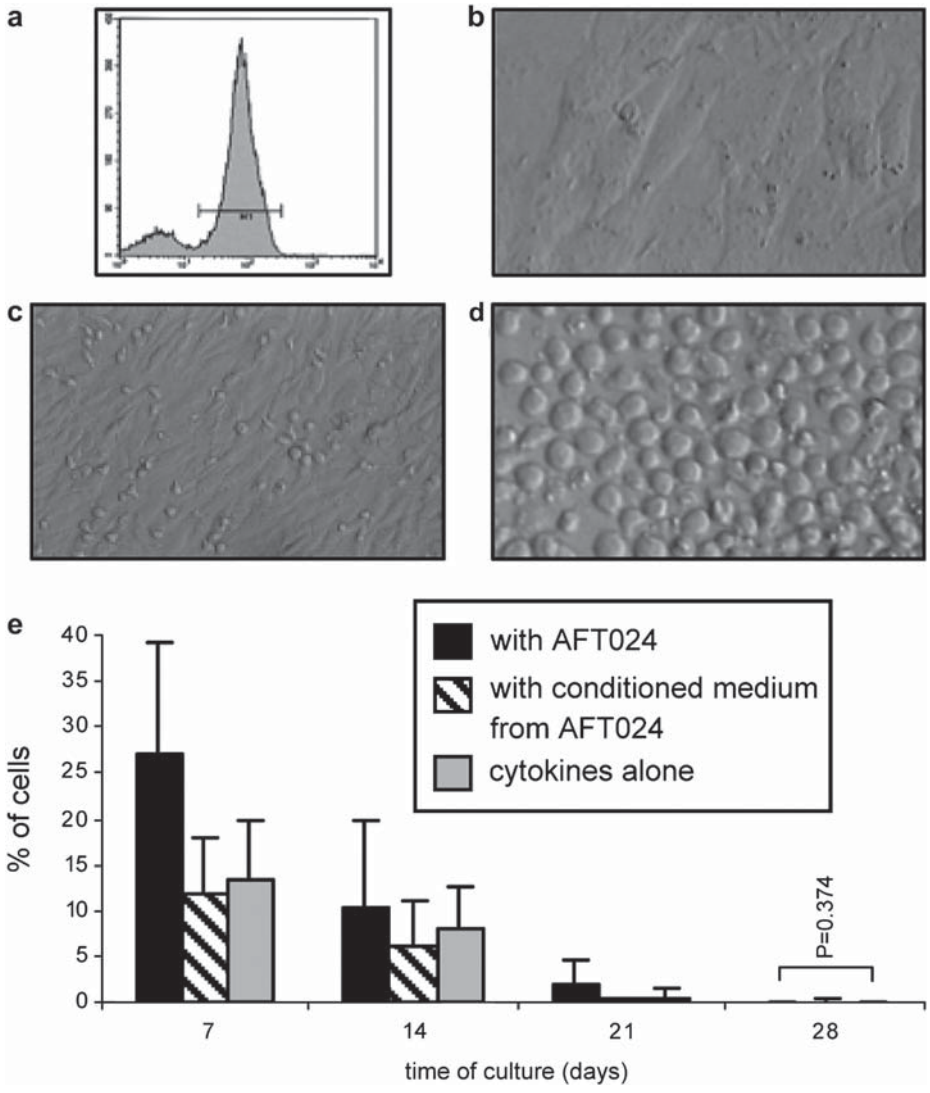

Figure 1. Establishment of hUCB $CD34^+$ cell co-cultures with AFT024 (a-c), proliferation of progenitor cells (d) and phenotypic behavior along the 4 week period (e). a) $CD34^+$ cells from hUCB after MACS enrichment; b) monolayer of stroma cells after irradiation (day 0); c) $CD34^+$ enriched cells over stroma AFT024 (day 0); d) Cobblestone-like areas over feeder layer (day 7); e) percentage of $CD34^+$ cells obtained by flow cytometry after each week of culture; $p < 0.001$.

Table 1. Percentage of $CD56^+CD3^-$ and proportion of $CD56^+CD3^-KIR^+$, $CD56^+CD3^-NCR^+$ and $CD56^+CD3^-NKG2A/CD94^-$ cells generated by $CD34^+$ cells grown in the described three conditions. Kinetics of expression along the time can be better visualized in fig.2.

Culture days	Culture	d7	d14	d21	d28
$CD34^{+a)}$	AFT	26.89 ± 12.46	10.31 ± 9.69	1.79 ± 2.66	0.01 ± 0.02
	CM	11.76 ± 5.96	6.14 ± 4.87	0.28 ± 0.26	0.15 ± 0.30
	C	13.16 ± 6.79	7.88 ± 4.70	0.48 ± 0.87	0.00 ± 0.00
$CD56^+CD3^{-a)}$	AFT	3.59 ± 2.41	20.37 ± 13.13	53.56 ± 21.23	81.38 ± 13.43
	CM	4.03 ± 1.77	23.42 ± 5.45	42.11 ± 23.19	83.89 ± 10.58
	C	4.15 ± 1.74	32.28 ± 5.41	54.57 ± 28.25	68.69 ± 19.52
$CD56^+KIR^{+b)}$	AFT	49.51 ± 17.02	80.65 ± 12.91	95.15 ± 6.46	97.15 ± 2.32
	CM	72.97 ± 23.42	95.19 ± 2.09	97.59 ± 2.90	95.98 ± 2.54
	C	81.97 ± 12.36	96.12 ± 2.27	92.43 ± 10.32	96.90 ± 4.15
$CD56^+NCR^{+b)}$	AFT	71.01 ± 21.58	84.02 ± 15.13	95.72 ± 6.69	98.89 ± 1.11
	CM	82.37 ± 24.91	99.44 ± 0.32	98.51 ± 1.71	99.45 ± 0.47
	C	89.03 ± 13.10	82.53 ± 37.80	98.78 ± 1.32	99.01 ± 1.65
$CD56^+NKG2A^{-c)}$	AFT	67.60 ± 22.89	65.01 ± 17.75	57.57 ± 22.57	43.35 ± 14.49
	CM	58.80 ± 20.64	49.39 ± 10.03	54.55 ± 22.66	66.38 ± 15.90
	C	57.58 ± 23.92	42.10 ± 17.78	40.12 ± 17.03	78.49 ± 13.90

Results are expressed as mean ± SD
a)Percentages were calculated in the lymphoid gate (see fig.1)
b)Percentages were calculated in the $CD56^+CD3^-$ gated region (see fig.1b)
c)Percentages were calculated in the $CD56^+CD3^-$ gated region (data not showed).

Generally, this increase was significantly higher ($p < 0.001$) in controls up to day 21, whereas on day 28, it was significantly higher ($p < 0.001$) in AFT (81.38 ± 13.43%) and CM (83.89 ± 10.58%) in relation to C (68.69 ± 19.52%). Specifically, the significant differences were found between all culture conditions in all weeks of the experiment period ($p < 0.001$). Two exceptions with no significant differences were found between C and CM at day 7 ($p = 0.466$) and between AFT024 and CM at day 28 (Figure 4a; Table 1). This data thus suggest that NK cell differentiation is first delayed by contact with feeder layers, but then stimulated after 14 days of culture by some factor(s) released from the stroma.

KIR acquisition

Analysis of surface receptors during NK cell maturation revealed the expression of the most important KIRs (NKAT2, NKB1, CD158a, p50.3) and the known Natural Killer Cytotoxicity Receptors, NCRs (NKp30, NKp40, NKp46). Acquisition of at least one KIR was found in almost all $CD56^+CD3^-$ cells (>95%) at the end of the culture period, with data suggesting that KIRs expression is initially delayed due to direct cell contacts with feeder layers, followed by a stronger stimulation under these conditions. Generally, the presence of KIRs was detected very early during cultures, being significantly higher ($p < 0.001$) in C than in CM or AFT, both at day 7 (81.97 ± 12.36% in C, 72.97 ± 23.42% in CM and 49.51 ± 17.02% in AFT) and day 14 (96.12 ± 2.27% in C, 95.19 ± 2.09% in CM and 80.65 ± 12.91% in AFT), thereafter becoming significantly higher ($p < 0.001$) in AFT (95.15 ± 6.46% at day 21 and 97.15 ± 2.32% at day 28) and CM (97.59 ± 2.90% at day 21 and 95.98 ± 2.54% at day 28) in relation to C 92.43 ± 10.32% at day 21 and 96.90 ± 4.15% at day 28). Specifically, Table 1 shows detailed data of expression of this receptors with significant differences between all culture conditions in all weeks of the experimental period ($p < 0.001$) with exception for day 21 and day 28 where no significant differences were found between AFT024 and CM ($p = 0.568$) and between AFT024 and C, respectively (Figures. 3c& 4b).

NCR acquisition

The majority of the cells (>50%) also expressed at least one NCR very early during cultures (day 7)

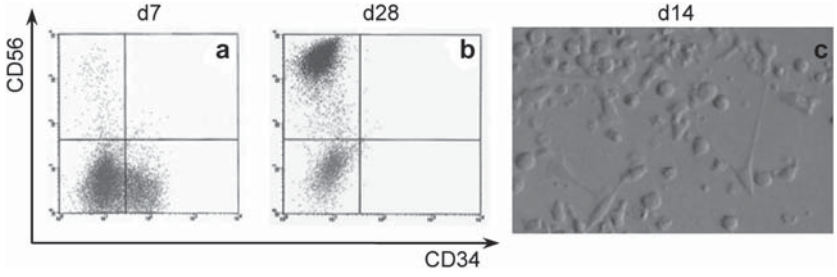

Figure 2. Phenotype of in vitro cultured hUCB $CD34^+$ cells. Flow cytometry analysis after 7 (a) and 28 (b) days of culture. Note that the $CD34^+$ cells almost become extinct between day 7 (d7) and day 28 (d28) with the simultaneous appearance of $CD56^+$ phenotype. Differentiation areas can be seen in c) at day 14 (d14) of culture. Detailed data is shown in table 1.

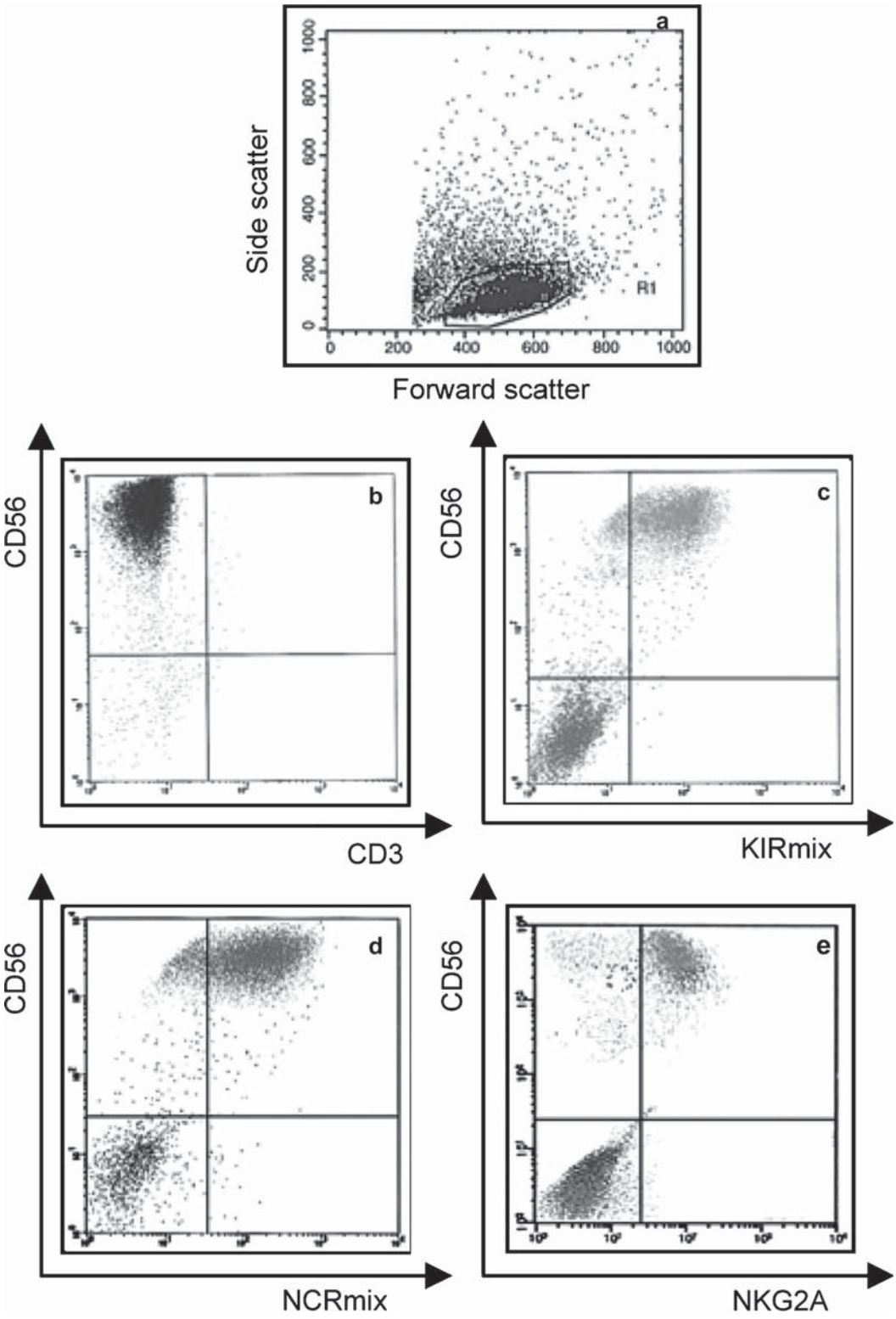

Figure 3. Flow cytometry analysis of differentiated hUCB CD34$^+$ cells at the end of the experimental period. Cells were analysed by flow cytometry and positivity for the different mAbs was defined in the morphological gate represented in a). NK cells population is shown in b) and the expression several receptors are shown in c) for the KIRmix, in d) for the NCRmix and in e) for NKG2A.

(89.03 ± 13.10% in C, 82.37 ± 24.91% in CM and 71.01 ± 21.58% in AFT), and most of the CD56$^+$ CD3$^-$ cells had these receptors at the end of the 4 week maturation period (>98%) (99.01 ± 1.65% in C, 99.45 ± 0.47% in CM and 98.89 ± 1.11% in AFT), with data suggesting that NK cell differentiation is first delayed by contact with feeder layers and then stimulated by some factor(s) released from the stroma. The mean percentage of NCR

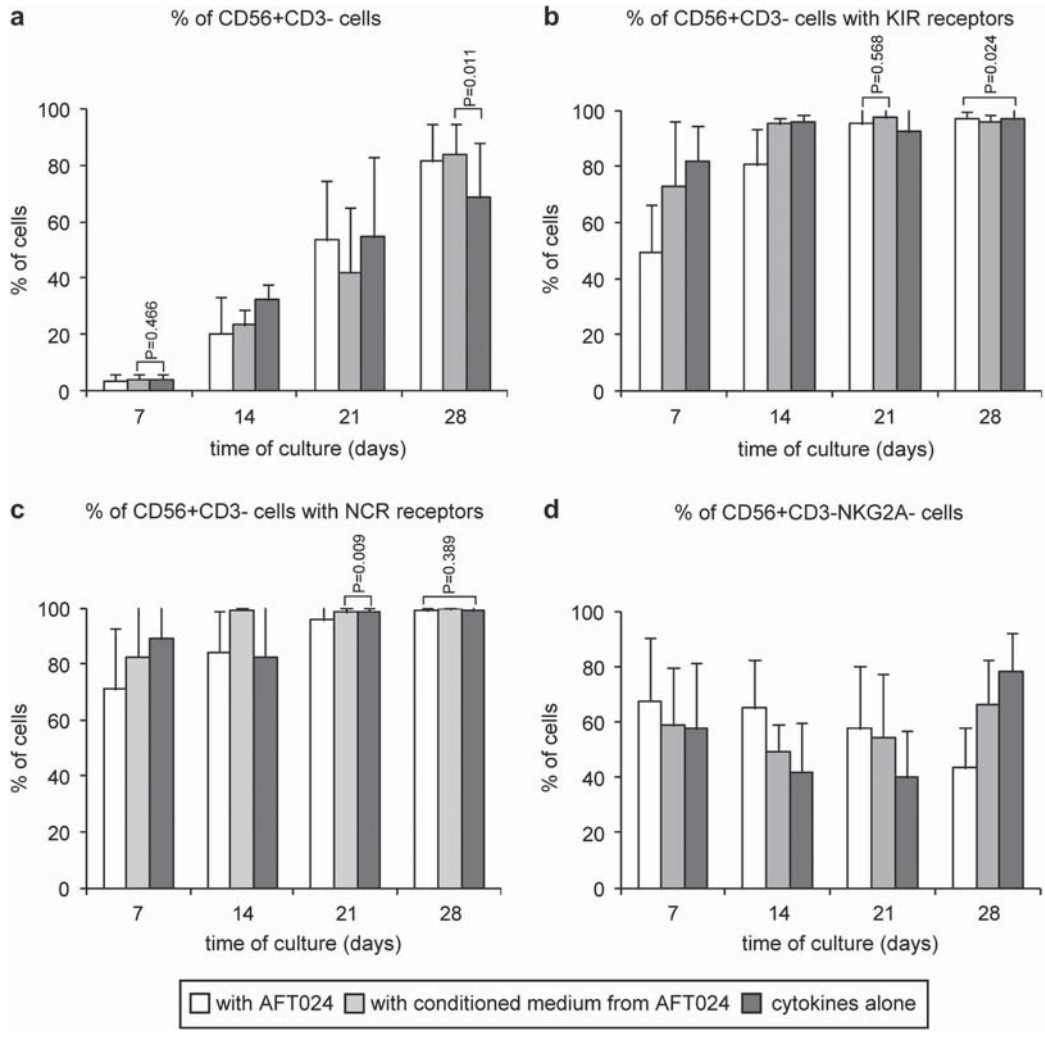

Figure 4. hUCB CD34$^+$ cells differentiation into NK cells along the 4-week period. Cells were analysed by flow cytometry and positivity for the different mAbs was defined in the morphological lymphoid gate defined in Fig.1a. Results from a) and b) were additionally defined in the CD56$^+$ CD3$^+$ gated region (see fig.1b) and results from d) were also defined in the CD56$^+$ CD94$^+$ gated region (data not showed). The graphics show the CD56$^+$ CD3$^+$ cells generated in vitro from CD34$^+$ progenitors (a) and the kinetics of expression of KIRs (b), NKps (c) and CD94/NKG2A$^-$ (d) ($p < 0.001$). See table 1 for detailed data.

is specified in Table 1 where all cultures present significant differences ($p < 0.001$) with exception for day 21 and day 28 where no differences were seen between C and CM and between AFT024 and C ($p = 0.389$), respectively (Figures 3d & 4c).

CD94/NKG2A acquisition

CD94/NKG2A was the less acquired surface receptor: its expression decreased along all period of culture in presence of AFT024 (67.60 ± 22.89% at day 7 to 43.35 ± 14.49% at day 28). In the absence of stroma, its expression decreased until 2nd week with conditioned medium (58.80 ± 20.64% at day 7 to 49.39 ± 10.03% at day 14) and until 3rd week with control medium (57.58 ± 23.92% at day 7 to 40.12 ± 17.03% at day 21), starting to increase from there. Minimal expression was achieved at the end of 3rd week in absence of any factors released from the stroma. Specifically, the mean percentage of CD94/NKG2A expression is shown in Table 1 where all cultures have significant differences between them ($p < 0.001$).

Cytotoxicity

To evaluate the functionality of the *in vitro* derived mature NK cells, cytotoxicity assays were performed against K562 target cells with CD56$^+$ CD3$^-$ effector cells derived from AFT and C day 28 cultures (Figure 5). Significant higher cytotoxicity levels were found for cells from AFT at the different E:T ratios tested, 70.7% (C) and 90.6% (AFT) at 10:1, 59.6% vs. 70.2% at 5:1, 32.7% vs. 50.4% at 2.5:1, 18.5% vs. 27.4% at 1.25:1, 9.8% vs. 16.8% at 0.62:1 ($p < 0.001$), 8.2% vs. 11.4% at 0.31:1 ($p = 0.004$), 3.4% vs. 5.7% at 0.16:1 ($p = 0.013$), 3.9% vs. 5.5% at 0.08:1 ($p = 0.025$), with no significant differences at 0.04:1 (1.7% vs. 2.5%) and at 0.02:1 (2.0% vs. 2.9%).

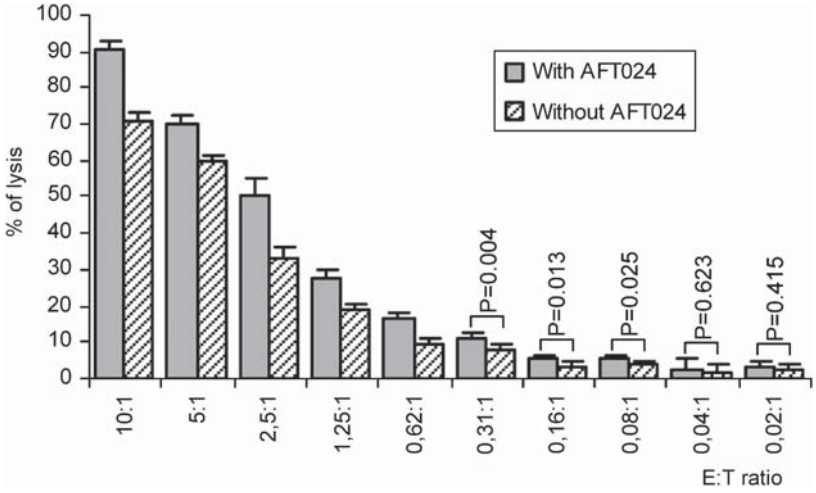

Figure 5. Cytotoxicities mediated by in vitro differentiated NK cells derived from hUCB-HSCs (effector cells) against K-562 target cells. These activities were measured using a Non-Radioactive Cytotoxicity test at the effector:target (E:T) cell ratios indicated. Each data bar represents a mean SD percentage value of five separate experiments, each performed in quadruplicate ($p < 0.05$).

Discussion

Since it was previously thought that the addition of stromal cells was important for the expansion of NK cells, we investigated if the presence of supporting stroma or alternatively, stroma-conditioned medium, could improve the generation and subsequent functionally maturation of NK cells from undifferentiated HSPC. The murine stromal cell line AFT024 was selected for its described capacity to support and amplify the differentiation of $CD34^+$ cells (Moore et al., 1997). NK cell maturation is a dynamic and continuous process as NK cells respond differently to external signals to secrete cytokines and to destroy target cells. To define the phenotype profile and functional activity of NK cells differentiated *in vitro*, different cultures were performed combining cytokines with or without stroma cells or using conditioned medium released from the stroma. All culture conditions showed proliferative capacity but in spite of the cytokines included in the medium and the factors released from the stroma, it seems that the direct contact with stroma have beneficial effects on $CD34^+$ expansion and on the capacity to avoid cell commitment. This indicates that stroma is a more potent inducer on proliferation and preservation of hUCB progenitor cells than the two other conditions. During the 4-week period, the cytometry results showed an exponential decrease of $CD34^+$ cells with a simultaneous increase of $CD56^+$ $CD3^-$ in all culture conditions. However, in the first weeks of culture in contact with stroma, cells yielded a lower proportion of $CD56^+$ $CD3^-$ cells compared with the other conditions. In contact with stroma, cells maintained for longer period the immature status. This can explain the initial delay in the commitment of NK phenotype. On the other hand, our results did not reveal any major differences in the capacity of $CD34^+$ cells collected from hUCB to generate high numbers of NK cells after 4 weeks in the presence of stroma cells or in conditioned medium from the stroma. However, in absence of stroma, there is a reduced production of the relative number of $CD56^+$ cells meaning that in spite of IL-15 in combination with Flt3-L, SCF, IL-2, IL-7 and IL-21 be sufficient to induce human $CD34^+$ cells to differentiate into NK cells, it seems that the addition of AFT024 cells did not qualitatively influence NK differentiation from $CD34^+$ cells. In spite of that, Miller and colleagues verified that cytokines alone are inefficient to support NK cell differentiation and the conditioned medium only supports differentiation partially (Miller & McCullar, 2001). However, we observed that the factors produced by stroma cells may improve the initial development of NK cell phenotype. Our results are supported by others that concluded that cytokines together with stromal cells clearly increase the number of generated NK cells but didn't qualitatively alter NK cell differentiation (Carayol et al., 1998) and that stromal cells do not bring any beneficial effect compared to conditioned medium, once released factors and/or cytokines are sufficient to induce NK cell differentiation (Lewis & Verfaillie, 2000). Additionally, during the process of maturation, NK cells establish a characteristic cell-surface phenotype and the capacity to elicit effector functions. Several markers of mature NK cells were used including a number of KIRs such NKAT2, NKB1, CD158a and p50.3, NCRs such NKp30, NKp40 and NKp46 and the inhibitory receptor CD95/NKG2A. The maturity of NK cells, detected by the presence of receptors, was checked and, in independent conditions, we found that the vast majority of differentiated $CD56^+$ $CD3^-$ cells co-expressed at least one of the KIRs and NCRs. Since $CD56^+$ phenotype was delayed in presence of stroma, the same occurred for the appearance of such receptors when in contact with stroma. Nevertheless, having in mind the results of the end of culture period, it seems that the appearance of these receptors is independent of the culture condition. The high levels of KIRs and NCRs might be consistent with a mature NK phenotype.

We know that the activation of immune responses depends on a tight balance between activating and inhibitory signals. For cell therapeutic purposes, it would be desirable to downregulate NKG2A expression in order to decrease the activation threshold of effector cells and to allow for more efficient lysis of target cells. In all our tested conditions, the complex NKG2A/CD94 is present in NK cells in spite of its expression being markedly lower than the other receptors. There are some reports of *in vitro* differentiated $CD56^+$ cells that express $CD94^+$ on its majority independently of the presence or not of stroma (Carayol et al., 1998). Simultaneously, less than 5% of $CD56^+$ cells present KIRs expressed after the CD94/NKG2A. With our system, the presence of stroma induced a continuous increase of surface expression of this complex along the time. Curiously, NKG2A/CD94 positive cells in conditions CM and C got its maximums earlier than condition AFT024. However, we also saw a strong decline since then suggesting that the lack of cells to establish contact with NK cells may diminish the stimuli to express this receptor. We thought that the lower expression of the complex CD94/NKG2A compared with that of the other receptors could be explained by the fact that maturation of NKG2A is dependent on the association with CD94 by glycosylation requiring much more metabolic complexity. Thus, it appears that there are some factors released by stroma that could be important for the first steps of induction towards NK cells lineage but acquisition of receptors seems to be independent of stroma signals. Some published data refers that NK cells receptors are acquired in an orderly and nonrandom manner during *in vitro* human NK cell differentiation (Miller & McCullar, 2001; Mrozek, Anderson, & Caligiuri, 1996; Grzywacz et al., 2006) and, differing from our results, indicated that the first inhibitory receptor to be surface-expressed is CD94/NKG2A while KIRs appear only later and in low percentages (Briard, Brouty-Boye, Azzarone, & Jasmin, 2002; Iizuka et al., 1999; Grzywacz et al., 2006; Miller & McCullar, 2001). Cytotoxicity results were consistent with the phenotypic behavior implying that these NK cells can demonstrate specificity in the recognition process. In fact, our cytotoxicity results of *in vitro* differentiated NK cells against classical NK cell target, K562, was especially higher than published NK cell activities from fresh isolated NK cells of cord blood or peripheral blood. Condiotti et al. demonstrated that cytotoxic $CD56^+$ cells could be produced from human UCB (Condiotti, Zakai, Barak, and Nagler, 2001). Nevertheless the intent of their experiments was to prove the cytotoxic capabilities of NK cells in UCB, we went farther starting with $CD34^+$ cells. Our studies showed that NK cell activity can be significantly enhanced during $CD34^+$ differentiation in controlled conditions. Although K562 cells do not carry HLA-E, it was proposed by Figueiredo. et al. that this increased cytolytic activity is partially mediated by the natural cytotoxicity receptor NKp30 (Figueiredo, Settsam, & Blasczyk, 2009) since it was recently shown that the ligand of NKp30 is expressed in K562 cells (Byrd, Hoffmann, Jarahian, Momburg, & Watzl, 2007). In general, the stroma was a more potent inducer for cord blood differentiation into functional NK cells than the other experimental conditions. However, in the presence of suitable cytokines and factors released from the stroma, $CD34^+$ cells rapidly acquired large quantities of NCRs and KIR receptors, becoming cytotoxic at this level. In spite of some reports referring that stromal cells play an important role in NK cell maturation (Colucci, Caligiur, & Di Santo, 2003; Miller & McCullar, 2001) and others reporting that $CD34^+$ HSC cannot give rise to mature NK cells in absence of stroma (Lewis & Verfaillie, 2000), with our well-defined and highly reproducible culture conditions we were able to generate fully competent NK cells *in vitro* with or without stroma cells, demonstrating that NK cells functionality can be increased under appropriate conditions. The use of cytokines reveals itself as a remarkable tool for the production of functional mature cells. From the present findings we can argue that IL-15 in combination with Flt3-L, SCF, IL-2 and IL-21 were sufficient to induce human $CD34^+$ cells to differentiate into mature cytotoxic NK cells *in vitro*. However, we cannot exclude the possibility that other factors produced by the stroma may have influenced the development of NK cells. Additionally, based on the work of others (Kato et al., 2007) and using specific drugs, downregulation of the NKG2A ligands expression can be attempted on targets. This could also be an attractive therapeutic strategy to induce susceptibility of leukemic cells to the cytotoxicity of NKG2A-lacking cells.

Conclusions

These results represent strong indications that we are in presence of mature NK cells generated *in vitro* and it seems that these receptors are indispensable for NK cell maturity. In fact, the capacity of hUCB $CD34^+$ cells to acquire CD56 may therefore provide a cytotoxic cell population that may be of therapeutic potential in the treatment of hematopoietic malignancy. These data together with the possibility to achieve NK cells negative for CD94/NKG2A receptor will allow us to generate alloreactive NK-cells for clinical applications in adoptive cellular therapies. This study also extended our knowledge of the effects of different cytokines on the proliferation of NK cells from human cord blood progenitor cells. One of the reasons for the lower incidence of GVHD following UCB transplant may be the reduced cytotoxic potential of CB-derived NK cells. However, we can increase NK cells functionality under appropriate conditions. The reactivity of NK cells results from an integration of activating and inhibitory stimuli which are transmitted through their receptors. The capacity to manipulate this balance on NK cells may open new perspectives in immunotherapy. Thus, this balance can be altered in favor of activation by the induction of activating

receptor expression on NK cells through the use of the correct cocktail of cytokines. This matter is not yet well understood and there are only a few preliminary studies in this field with no conclusive results. In fact, there are many reports with mixed conclusions difficult to reconcile. An improved understanding of these processes will continue to drive the clinical applications for NK cells as a tool against infection and tumors as well as the establishment of NK cell lines with "specific" characteristics will provide favorable tools for culture models.

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PAPER II

"Genetic regulation on ex-vivo differentiated natural killer cells from human umbilical cord blood CD34+ cells"

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RESEARCH ARTICLE

Genetic regulation on *ex vivo* differentiated natural killer cells from human umbilical cord blood CD34⁺ cells

Maria João Pinho[1], Cristina Joana Marques[1], Filipa Carvalho[1], Michael Punzel[2], Mário Sousa[1,3], and Alberto Barros[1]

[1]Department of Genetics, Faculty of Medicine, University of Porto, Portugal, [2]MediaPark Klinik Cologne, Center for Bone Marrow and Stem Cell Donation, Cologne, Germany, and [3]Lab Cell Biology, ICBAS-Institute of Biomedical Sciences Abel Salazar, University of Porto, Portugal

Abstract

Natural killer (NK)-cells are a lymphocyte population playing a critical role in the immune surveillance against tumors and virally infected cells. The development of human hematopoietic stem cells (hHSC) into fully differentiated NK-cells pass through discrete stages of differentiation involving a variety of factors such as cytokines, membrane factors, and transcription factors (TFs). Because there is lack of studies in this field, we decided to perform an extended analysis of TFs during *in vitro* differentiation of NK-cells. At several points of differentiation, cells were characterized by their mRNA expression either for NK-cell cell markers, for a number of mature NK-cell receptors or a large panel of TFs. Our data suggests that some TFs (ID2, EGR-2 and T-BET) play a role in NK-cell commitment, differentiation and maturation. Although delayed on its expression, BLIMP1 also seems to be involved in differentiation and maturation of NK cells, but not in NK-cell commitment. E4BP4 and TOX are more related with initial stages of NK-cell commitment. PU.1, MEF, Ikaros, EGR-1, BCL11B and IRF-2 revealed less involvement in maturation and were more associated with NK-cell commitment and pNK cell production. GATA-3 showed a differential role during the ontogeny of NK-cells. We show that assessment of the transcripts present in the differentiating NK-cells demonstrated, a pattern of preserved and differential gene expression remarkably similar to that seen in mice except for E4BP4 that showed constant downregulation throughout the culture period. A thorough understanding of NK-cell developmental mechanisms is important as it may enable future therapeutic manipulation.

Keywords: NK cell ontogeny, NK cell receptors, transcription factors

Introduction

NK cells are large, granular lymphocytes that mediate critical functions in innate immunity by directly eliminating infected or transformed cells. Additionally, they are also involved in indirect elimination via recruitment of other elements of the immune system, by releasing cytokines upon stimulation (1,2). In humans, this specific elimination is dependent on the inhibitory CD94-NKG2A complex and the killer cell immunoglobulin-like receptors (KIRs). Upon stimulation, NK cells release perforin, granzymes and several tumor necrosis factor (TNF) family ligands leading to the apoptosis of the transformed or virus-infected cells (3). NK cell activating receptors (NKp30, NKp44 and NKp46) are also essential for the activation of NK cell functions, which results in either cytotoxicity and/or cytokine production (4,5). Activating signals are also triggered by the association of NKG2D homodimer with DAP10 or DAP12 adaptor molecules (6). Binding of NK cell inhibitory receptors with their ligands renders the target cells to be protected from NK cell-mediated cytotoxicity. When target cells lack self-MHC class I molecule, NK cells no longer receive inhibitory signal via MHC class I molecules and kill the target cells, in a process known as missing self recognition (7).

NK cells are broadly defined as $CD56^{+}CD3^{-}$ lymphocytes derived from $CD34^{+}$ hematopoietic stem

Address for Correspondence: Maria João Pinho, Department of Medical Genetics, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal. Phone: 00351 225513647, Fax: 00351 225513648. E-mail: mjpinho@med.up.pt

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cells (HSCs) in the human bone marrow (BM) (1). HSCs differentiate into common lymphoid progenitors (CLPs) which are generally thought to give rise to all lymphocytes (8) which in turn can develop ultimately into mature NK (mNK) cells (1,9), passing through three main stages: lineage commitment, NK receptor repertoire selection and functional maturation (10–16). In fact, it is known that during this process NK cells acquire characteristic cell surface markers and the capacity to elicit effector functions (1,9). NK cell development, differentiation and maturation are also dictated by a variety of factors such as cytokines, membrane factors, and transcription factors (TFs) in addition to BM microenvironment. Concerning TFs, mouse knockout models have been a very useful tool for studying which ones control murine NK cell development and, therefore, our current understanding of NK cell development stems primarily from findings in mice.

The first group of TFs includes E-twenty six (Ets) family members such as PU.1 and Ets-1, and Ikaros family (17,18). These TFs have been reported to be essential for the generation of mouse NK cells *in vivo* by specification or maintenance of pNKs via regulation of key cytokine receptors required for NK cells (19–22).

The second group of TFs including Gata-3, IRF-2 and T-bet is involved in the process of final maturation of NK cells; thus, their absence leads to incomplete development of functional NK cells or immature phenotypes of NK cells (23–26). Mice deficient in these TFs exhibit similar phenotypes despite the TF involved (23–25,27–30).

The third group of TFs includes MEF, MITF, and CEBP-γ which are known to regulate the cytolitic effector functions of fully-matured NK cells. Mice lacking these TFs exhibit normal development of NK cells, but with reduced cytotoxic capacity and cytokine production (31).

Additional TFs were also reported to have a role in the developmental process of NK cell differentiation. For example, the E-proteins are basic helix-loop-helix transcriptional regulators that are important for coordinating cell proliferation and differentiation (32) since a decreased number of NK cells has been observed in Id knockout model (33,34). TOX has also been recently implicated in regulation of human NK cells differentiation since it was found to be highly up-regulated in immature NK cells with loss of mature NK cells in absence of TOX (35). The basic leucine zipper transcription factor E4BP4 was implicated either in NK cell specification and commitment or in NK cell development (36,37). However, although pNK cell numbers in E4BP4$^{-/-}$ mice are unchanged, they present no detectable iNK or mNK cells and no NK cell-mediated cytotoxicity. Concerning early growth response factors 1 (EGR-1) and 2 (EGR-2), a recent series of studies showed that Egr-2 plays an important role in iNKT development, while EGR-1 is apparently dispensable (38). The transcriptional repressor B-lymphocyte-induced maturation protein 1 (Blimp1) is suggested to play a crucial role in the post-activation phenotype of NK cells by negatively regulating cytokine transcription in a coordinate manner, without compromising perforin-mediated cytotoxicity. Its increasing expression during functional maturation of NK cells identify this TF as an important player in the transcriptional network governing NK-cell differentiation and homeostasis. Such a mechanism may have important implications in innate immunity and tumor surveillance (39). In contrast, it was recently demonstrated that NK cells express Blimp1 constitutively from a very early point in their development (40). Both experiments indicate that IL-15 signals are also required for the induction of Blimp1 in early NK-cell development. Recent works attributes BCL11B an important role for the cancer treatment research since it was proposed to be possible to reprogram T cells into induced T-to-natural killer (ITNK) cells, by BCL11B deletion, with promising applications in cancer immunotherapy and other cell-based therapies (41).

As the collaborative interactions of these factors have been identified in many processes and referred to contribute to the conventional NK cells development.

NK cell development is far less understood compared with that of T and B cells. However, the critical importance of NK cells in innate immunity lead us to recapitulate the development of functional NK-cells from multipotent hematopoietic UCB-stem cells, reconstituting the complete process of NK-cell development and maturation from the naive stem cell to the functional effector cells. Because of rapid cytolitic function without previous priming against broad range of targets, NK cells may be candidates for cancer therapy emerging to apply as therapeutic agents against a broad range of malignancies (42,43).

As lineage decisions always involve changes in gene expression programs and being these decisions ultimately controlled by TFs, here we characterize human developing NK cells at molecular level based on mRNA expression of several TFs that have been already described to have important roles, at least on mouse NK cell lineage. To our knowledge, this is the first work that follows-up the *in vitro* human NK cell development evaluating a set of TFs during lineage commitment and maturation. Simultaneously, we established a relation between gene expression and the detection of the functional receptor on the surface of the NK cells.

Methods

Cell samples

Human umbilical cord blood (hUCB) was harvested by umbilical vein puncture after normal full-term deliveries under previous informed consent from pregnant woman before delivery, in accordance to the guidelines of the local Ethic Committee. Samples were collected in 150 cc sterile bags, containing 21 ml of citrate-phosphate-dextrose (CPD) (MacoPharma, Tourcoing, France), and kept at room temperature for less than 12 h. In total, three hUCB (biological replicates) were included in this study.

Isolation of mononuclear cells

Mononuclear cells (MNCs) from a total of three hUCB were isolated using 1.077 g/ml Ficoll-Histopaque (Sigma, St. Louis, MO, USA) by gradient centrifugation (400 g, 30 min, 20°C). Low density MNCs were collected, washed twice (1600 rpm, 6 min, 4°C) and resuspended in Dulbecco's phosphate buffered saline (DPBS; Sigma), supplemented with 0.5% of fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah, USA) and 2 mM of ethylene diamine tetraacetic acid (EDTA; Sigma). When necessary, lysis of residual red cells was performed with ice-cold lysis buffer [8.29 g of 53,49M NH_4Cl, 1 g of 100,1M $KHCO_3$ (Merck, Darmstadt, Germany) and 0.2 ml of 0.5 M EDTA in 1 L H_2O]. After 15 min, cells were washed twice and resuspended in DPBS supplemented with 0.5% of FBS and 2 mM of EDTA.

Selection of CD34+ cells

The CD34+ cell fraction from MNCs was immunomagnetically selected using a magnetic activated cell selection (MACS) system (Miltenyi Biotec, Auburn, CA, USA). The Progenitor Cell Isolation Kit uses antibodies recognizing the CD34 epitope QBEND/10 and was used according to manufacturer's instructions with minor alterations. Briefly, MNCs were incubated with 75 µl MACS microbeads CD34+/10^8 total cells. Cell clumps were removed by passing the cells through a Pre-Separation Filter and labeled cells were separated using a high gradient magnetic separation column in a strong magnetic field. Magnetically retained cells were released by flushing down the column with a plunger. Cell aliquots were taken for cell viability using Trypan blue exclusion method (Sigma) and an Improved Neubauer Haemocytometer for count. The purity of the CD34+ fraction was determined by flow cytometry, being consistently above 90%.

AFT024 feeder layers

AFT024 cells, a stromal cell line derived from murine fetal liver (Heinrich Heine University, Duesseldorf, Germany), were used as feeder layers. Cells were cultured in 75 cc flasks with Dulbecco's modified Eagles medium (DMEM) containing 1000 mg glucose/L (Gibco, Paisley, Scotland, UK), and supplemented with 20% FBS, 100 U/ml Penicilin and 100 µl/ml Streptomicin (Pen/Strep; Gibco), 2 mM L-Glutamin (Gibco) and 24 µM β-mercaptoethanol (Gibco). Cells were cultured for one week at 33°C with 5% CO_2 in humidified air. When a confluent layer formed, cells were recovered by digestion (5 min, 37°C) with 10 ml of trypsin/EDTA solution (1×) (trypsin/EDTA; Gibco). AFT024 cells were then plated in 24-well plates (10^5 cells/ml/well). When confluent, cells were uniformly irradiated (2000 rad) using a cobalt irradiator (Theratron 780C; Atomic Energy of Canada Ltd., Chalk River, Ontario, Canada). After irradiation, cells were incubated at 37°C and the culture medium changed on the day after.

Cell culture

CD34+ cells were used in a two-step protocol, consisting of 1 week proliferation and 3 weeks differentiation, as previously described (44). Cells were plated at different concentrations (4×10^3 cells/well, 2×10^3 cells/well, 1×10^3 cells/well and 0.1×10^3 cells/well) in 24-well plates with supplemented medium, in direct contact with irradiated AFT024 stromal cells. Cells were cultured (5% CO_2 in air at 37°C) with the medium being changed once a week. In the first week, the medium consisted of a 2:1 proportion of DMEM containing 4500 mg glucose/L (Sigma) and HAMs F12 (Biochrom AG, Berlin, Germany), supplemented with 20% of heat inactivated human AB serum (BioWittaker, Walkersville, USA). This was then supplemented with Pen/Strep (100 IU/100 µl/ml), β-mercaptoethanol (24 µM), ascorbic acid (20 mg/L), selenium selenate (50 µ/L), ethanolamine (50 µM) (Sigma), L-Glutamine (2 mM) (Gibco) and a cocktail of recombinant human cytokines, 1000 IU/ml IL-2, 5 mg/ml IL-3, 20 mg/ml IL-7, 20 mg/ml stem cell factor (SCF) and 10 mg/ml Flt3-L (PeproTech, London, UK). At the end of the first week, the medium was changed and the cells were cultured with a 2:1 proportion of DMEM containing 4500 mg glucose/L and HAMs F12, supplemented with 10% of heat inactivated human AB serum, and the cocktail of cytokines described above, with the exception of IL-3 that was replaced by IL-15 (10 mg/ml) and the addition of IL-21 (10 mg/ml) (PeproTech).

Once a week, a sample of the cultured cells was harvested: the initial 4×10^3 well harvested in the first week, the 2×10^3 well in the second week, the 1×10^3 well in the third week and the 0.1×10^3 well in the fourth week. Cell pellets were frozen at –80°C until processed.

RNA isolation and reverse transcription (RT) reaction

After thawing the frozen pellets, cells were lysed on ice with 1000 µl of TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) and passed several times through a syringe and needle. The total mRNA was then extracted according to the associated protocol. At the end, RNA pellet was ressuspended in 40 µl of diethylpyrocarbonate (DEPC)-treated RNase-free water (Promega, Wisconsin, USA) and incubated for 1 h on ice. RNA was then quantified in NanoDrop 2000c (Wilmington, DE, USA). 100 ng of RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Carlsbad, CA, USA), with random hexamers as the priming method.

Gene expression analysis by quantitative real-time PCR (q-RT-PCR)

After initial screening with Ubiquitin B (UBB), β-actin (ACTB), 60S Acidic Ribosomal Protein P0 (RPLP0) and β-2-microglobulin (β2M), the results obtained suggested β2M as the best housekeeping gene for q-RT-PCR analysis.

All primers (both for the housekeeping gene and for experimental transcripts) were designed to be CDNA specific (at least one of the primers is exon-spanning or the primer pair is separated by at least one intron on the corresponding genomic DNA) and are listed on Table 1.

Table 1. Primer sequences used for quantitative Real-Time PCR technique. Information about the gene name, accession number and product length is also given. Reference Sequences (RefSeq) are accordingly to accession numbers of GenBank.

Primer sequences used for quantitative Real-Time PCR			
Gene	Accession Number	Primer sequence (5′- 3′)	Product length (bp)
B2M	NM_004048.2	TGC CTG CCG TGT GAA CCA TGT	97
		TGC GGC ATC TTC AAA CCT CCA TGA	
CD34	NM_001025109.1	ACC GCG CTT TGC TTG CTG AGT	85
		GGG TAG GTA ACT CTG GGG TAG CAG T	
CD56	NM_001242607.1	TCA AGC AGA CAC CCC CTC TTC ACC	141
		TCA CCA ACT GCT CTC CAC TCA GC	
CD94	NM_002262.3	ACG AAA GTC GGC ATC TCT GTG C	135
		AGG CGG TGT GCT CCT CAC TGT A	
CD117	NM_000222.2	TGC ATT CAA GCA CAA TGG CAC GG	117
		GTG TGG GGA TGG ATT TGC TCT TTG T	
CD122	NM_000878.2	GAG GGT GCT GTG CCG TGA GG	88
		GGG GGC CAT CAG GCG AAG GTT	
PRF1	NM_005041.4	TTG CAG CCC AGA AGA CCC ACC A	82
		ACC ACA TGG AAA CTG TAG AAG CGG C	
GZMB	NM_004131.4	GAG AGC AAG GAG GAA ACA ACA GCA	136
		TTG GCC TCA TGT CCC CCG ATG A	
NKG2A	NM_002259.4	ACC TGG CCT CTC CAC TAA AGG	143
		TCG TTG CTG CCT CTT TGG GTT	
NKG2D	NM_007360.3	AAA TGG ATC TTG GCA GTG GGA A	84
		GCA CAG TCT CCC TTC TGC AT	
NCR1	NM_004829.5	CAG AAA GAC CAT GCC CTC TGG GA	145
		AAG CTC TGC TGG CTC GCT CT	
NCR2	NM_004828.3	AGC GCA CAG GAA AAG GAC CA	80
		TGA GAG CCT GGG AAC AGC A	
NCR3	NM_147130.2	GCT GGT GGT GGA GAA AGA ACA T	117
		TGG TAA TAG ACG GTG CTG CCC A	
E4BP4	NM_005384.2	GGC CCG AGA GCA GGA ACA CGA TAA	80
		TAC CGT CTG GGA TAA ATC CGT CAG G	
MEF	NM_001421.3	GGA AGA CCA AGG GCA ACC GAA GT	90
		TGG TGC TGC CTT TGC CAT CCT	
TOX	NM_014729.2	TAT GTG CCA GCC AGC CAG TCC TA	92
		TGG TCT GGG AGG GAA GGA GGA GTA A	
IKAROS	NM_006060.4	AGC TCG GCT TTG TCG GGA GTT	130
		GCC GTT CTC CAG TGT GGC TTC T	
GATA-3	NM_001002295.1	AGC ACA GAA GGC AGG GAG TGT	148
		TTC GCT TGG GCT TAA TGA GGG GC	
IRF-2	NM_002199.3	CCT ATG CAG AAA GCG AAA CGA CTG A	122
		TCG AGT CCC CAT GTT GCT GAG GT	
EGR-1	NM_001964.2	GCA CCT GAC CGC AGA GTC TTT TCC	147
		TTG CCA CTG TTG GGT GCA GG	
EGR-2	NM_000399.3	AGG CCC CTT TGA CCA GAT GAA CG	122
		TAG GTG CAG AGA CGG GAG CAA A	
ID2	NM_002166.4	AGC CTG CAT CAC CAG AGA CC	98
		TCA GAA GCC TGC AAG GAC AGG A	
PU.1	NM_001080547.1	CCC TCA GCC ATC AGA AGA CCT	112
		GTA ATG GTC GCT ATG GCT CTC C	
BLIMP1	NM_001198.3	TGT GGG TAC GAC CTT GGC TG	109
		ATC CGC ATC CTC CAT GTC CA	
T-BET	NM_013351.1	GGA TGC GCC AGG AAG TTT CA	149
		CTC TGG CTC TCC GTC GTT CA	
BCL11B	NM_138576.2	CTC TCA CCC ACG AAA GGC AT	137
		GCA CGC AGA GGT GAA GTG AT	

Primer sequences were designed and optimized using a specific software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

RNA expression levels were analyzed by quantitative Real-Time PCR (q-RT-PCR) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). q-RT-PCR were performed in a volume of 20 µl, using cDNA corresponding to a 5 ng transcribed RNA, 20 pM of each primer and 10 µl of PerfeCta® SYBR® Green FastMix®, ROX™ (Quanta Biosciences, Gaithersburg, MD, USA), with a Fast Protocol according to manufacturer instructions. Briefly, after initial enzyme activation for 30 sec at 95°C, 40 cycles were performed, each one consisting of 1 sec at 95°C and 30 sec at 60°C. Melting curves were also performed to verify PCR specificity. Standard curves were performed with five points in duplicates and each PCR for relative quantification was run in triplicate (technical replicates). Three hUCB (biological replicates) were analyzed for each time of culture.

Data analysis and statistics

Data was firstly analyzed with StepOne Software version 2.2.2 (Applied Biosystems) to establish the threshold and baseline for each sample. Then, relative expression of mRNA was calculated for each gene using the β2M gene as endogenous reference and normalized to day 0 of culture using the 2–ΔΔCT method (45). Results of experimental points from different experiments were reported as the mean +/– standard deviation (SD). Significance levels for mRNA expression between different time points were determined by the Relative Expression Software Tool (REST 2009) (46) that estimates up and downregulation for gene expression (http:// www.qiagen.com/rest).

Results

CD34+ cells isolated from hUCB were co-cultured with AFT024 feeder layer in the presence of IL-2, IL-3, IL-7, SCF and Flt3-L. At the end of the first week, IL-3 that was replaced by IL-15 and IL-21 was also added. With this system we have previously demonstrated, by flow cytmometry, that *in vitro* derived human NK cells express NK cell markers together with NK cell specific receptors. In the same study and using cytotoxicity tests, we have also shown that derived NK cells are also capable of killing K562 cell line, therefore showing high cytotoxic capacity (44). In fact, in our culture system, hHSCs rapidly commit, differentiate and acquire NK cell features. To test whether these cells express mRNA corresponding to previous observed extracellular markers and receptors associated with NK cells, we performed q-RT-PCR analysis of cells at time zero (d0) and of the resultant cells after each week of culture (d7, d14, d21 and d28). HSCs and differentiating NK cells were also tested for mRNA expression of several genes encoding important proteins associated with NK cell maturation and cytotoxicity (NKG2A, NKG2D, PRF1, GZMB, NCR1, NCR2 and NCR3). To further characterize differentiating steps of human NK cell development at transcriptional level, we analyzed the expression of a wide range of TFs suggested to be important or crucial, at least in mice, for the healthy NK cell development, including PU.1, T-BET, MEF, TOX, IKAROS, IRF-2, EGR-1, EGR-2, ID-2, BLIMP1, GATA-3, BCL11B and E4BP4.

All PCR products exhibited dissociation plots indicating the amplification of a single product (data not shown). Standard curves of all genes examined showed high efficiency. Some transcripts have virtually no expression in some stages, resulting in offscalle measurements. Data presented here was obtained from one single experiment (Figures 1 and 2) and is representative of three independent biological replicates with the same tendency (see supplemental files).

NK cell differentiation

Confirming our previous observations using flow cytometry, we found increased expression of CD56 along the all culture period with mRNA levels being expressed since the first week of culture (d7). The marker of excellence of NK cells (CD56) started to be expressed immediately

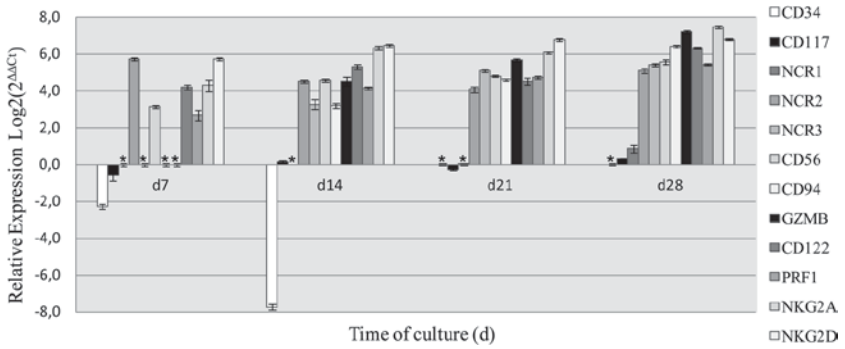

Figure 1. hUCB CD34+ cells differentiation into NK cells along the 4-week period. Natural killer cell specific markers and receptors expression was analyzed by q-RT-PCT expression during NK cell differentiation. Relative transcript levels were determined by comparing expression values obtained from cells at different point times of culture (d7, d14, d21 and d28) to those obtained for hHSC and corresponding to the starting population (d0), after internal normalization to β2M transcript levels. Results are the mean ± SD of data representative of three biological replicates, each measured in triplicate. Some transcripts are marked with asterisks corresponding to, virtually, no expression in those stages.

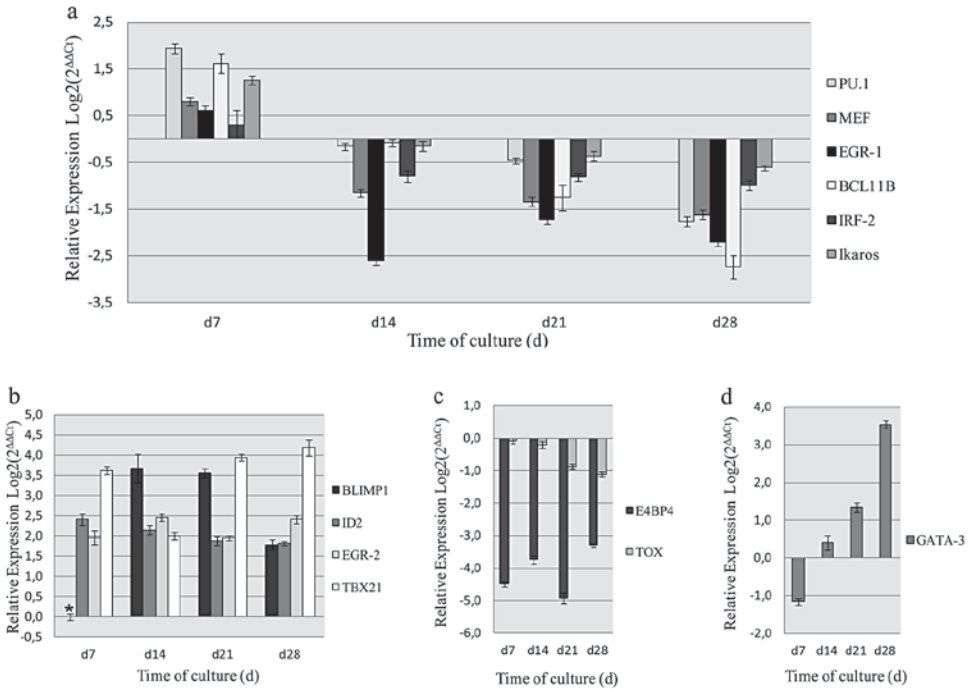

Figure 2. Transcription factors (TFs) expression during NK cell differentiation along the 4-week period using hUCB CD34⁺ cells as a starting population. TFs expression was analyzed by q-RT-PCT using the ddCt method. Relative transcript levels were determined by comparing expression values obtained from cells at different point times of culture (d7, d14, d21 and d28) to those obtained for hHSC and corresponding to the starting population (d0), after internal normalization to β2M transcript levels. Resulting data was grouped according to a given pattern and thus the graphics show the mRNA relative levels for a) PU.1, MEF, EGR-1, BCL11B, IRF-2 and Ikaros, for b) BLIMP1, ID2, EGR-2 and T-BET, for c) E4BP4 and TOX and for d) GATA-3. Results are the mean ± SD of data representative of three biological replicates, each measured in triplicate. Some transcripts are marked with asterisks corresponding to, virtually, no expression in those stages.

after one week of culture together with CD122 (IL2/15Rβ). These two markers gradually manifested along the remaining three weeks of culture (Figure 1). Expression of CD94 only appeared at the second week of culture with a continuous increment of expression (65- to 86-fold) until the end of culture period. Simultaneously, we found a decreased expression of CD34 with no detectable levels of mRNA above d14–21 of culture. CD117 marker (also known as c-KIT) showed a subtle expression in these cultures. In fact, its variations during the culture period had no statistical significance. Other mature-NK cell markers became expressed at d7 of culture such as PRF1, NKG2A and NKG2D. These markers had a significantly increase of mRNA expression until the end of culture (d28). Thus, between d0 and d28, PRF1 increased its levels in 27- to 83-fold, NKG2A in 142- to 211-fold and NKG2D in 49- to 110-fold. No detectable levels of GZMB were seen at d7. Concerning natural cytotoxicity receptors (NCRs), only NCR2 (NKp44) expressed mRNA at d7-14, also with a constant increase of its expression until d28 (34- to 174-fold). At the second week of culture (d14), the expression of GZMB was observed, demonstrating constant increasing expression since there. In fact, GZMB had an increment of 88- to 228-fold from d7 to d28. The cytotoxic receptor NCR3 (NKp30) also became visible after the second week of culture (d14) with increments of its mRNA levels of 62- to 240-fold since it appeared and until the end of culture period. The generated NK cells only expressed mRNA for NCR1 (NKp46) after the third week of culture (2- to 21-fold) with no further increased in expression at the fourth week.

Transcription factors

Concerning expression of TFs, PU.1 showed, at the first week, an increment of its expression of around the 4-fold, followed by a sharp decline of 10- to 19-fold until the end of culture period (day 28) where it reached its minimum (Figure 2a). Ikaros had a increase of 1- to 2-fold in its expression at day 7 followed by a strong decline in 3- to 4-fold since there, until the end of culture period. IRF-2 increased its expression at day 7 in 1- to 2-fold with further downregulation in 2- to 3-fold until the end of culture. MEF doubled its expression on day 7 showing upregulation but rapidly fell down in 3- to 5-fold through the remaining culture period. Similarily, EGR-1 showed a minor increase of its mRNA levels at day 7 followed by a high downregulation of 4- to 17-fold at day 14 which lasted with minor oscillations until the end of culture period. BCL11B, follows the same pattern of the aforementioned TFs and demonstrated upregulation in 2- to 3-fold in the first week of culture with further significant downregulation until the end of culture (13- to 21-fold).

On the other hand, a number of TFs followed a somewhat different pattern of expression (Figure 2b). For example, T-bet (TBX21) had an evident and strong increment of its mRNA levels in the first week of culture. That

represented 10- to 14-times more expression regarding d0. Despite its decline at d14, its expression never came above the initial values. On the contrary, mRNA levels of T-bet rapidly increased again at d21, with a strong and evident increment, with no further significant variations until the end of culture. EGR-2 had also a significant increase of 4- to 33-fold just after one week of culture (d7). In the following weeks, although there are some oscillations, it was always upregulated until the end of culture. Likewise, there was a significant increment of ID2 expression in the first week of culture in 4- to 7-fold. Notwithstanding some oscillations during the remaining culture period, its expression levels were maintained always upregulated. Despite BLIMP1 not being expressed at detectable levels of mRNA in the first two weeks of culture, this TF showed high upregulation at d14 of culture in 5- to 13-fold, showing a slight decrease of its expression until d28. However, BLIMP1 showed high upregulation since d14 until the end of culture. In short, the latter four TFs showed a similar upregulated pattern of expression.

A different profile was identified for E4BP4 and TOX (Figure 2c). E4BP4 showed a strong downregulation during all culture period with small oscillations during that period. TOX had no significant variations of mRNA levels at the first week of culture. Since there, it showed downregulation until the end of culture period.

Regarding GATA-3, it was the only analyzed TF with evident oscillations with up and downregulations during the process of *in vitro* differentiation of NK cells (Figure 2d). GATA-3 showed a decline in its expression in the first week of culture, showing 0.3- to 0.5-fold times less mRNA expression compared to the beginning of culture. In the following weeks, GATA-3 increased its expression which was accentuated from d21 to d28. Despite its short decline between d0 and d 7, GATA-3 was high upregulated since there in 10- to 26-fold.

Discussion

After previous results demonstrating that human NK cells with cytotoxic capacity leading to effective effector functions can be successfully differentiated (44), we started a new approach, characterizing the resulting cells of the NK cell cultures at molecular level, based on mRNA expression of several molecules present in mature and functional NK cells: CD56, CD94, NKG2A, NKG2D, PRF1, GZMB and NCRs (NCR1, NCR2 and NCR3). Expression levels for CD34, CD122 and CD117 were also analyzed.

Within this culture system, CD122 (IL-2/15Rβ) and CD56 mRNA expression started to express at d7. CD122 acquisition is, in fact, an essential step in the commitment of HSCs to the NK cell lineage. As precursor NK cells (pNK) express IL-2/15Rβ, its ligand IL-15 has a vital role in the maturation of NK cells. In the first week, it was also visible the expression of PRF1, NKG2A and NKG2D but not CD94 or GZMB showing that these molecules are much more responsive in the final steps of NK cell maturation. Also, in the first week of culture, despite NKG2A showing some upregulation of its mRNA levels, supposedly it does not have yet any function as effective receptor once it acts as heterodimer associated with CD94, and this receptor does not have any expression yet at this time. NKG2D is also upregulated at the first week of culture indicating that this activating receptor may be in the forefront of importance of NK cell development. Despite NKG2D having a role in activation or inhibition of killing activity in mature NK cells, it is known that this receptor is expressed in pNK cells but its function on these precursor cells is not known yet (47). NCR2 was the only cytotoxic receptor that showed some expression in the first week of culture. It is known that this molecule (NKp44) is progressively expressed by all NK cells *in vitro* after culture with IL-2 (48). As this culture system includes IL-2 since the beginning of experiments, this early expression is not surprising. In spite NK differentiating cells having no function at this stage, this initial differentiation of NCR2 and PRF1 may be a initial preparation of these cells, that once committed, start to prepare for rapidly responding to adversities.

At the second week of culture, at the same time that CD56 and CD122 increase their expression, other markers start to appear namely CD94 and GZMB as well as the cytotoxic receptor NCR3. These new expressions may have been potentiated by the replacement of IL-3 by IL-15 at the beginning of first week. It should also be noted that while NKG2D did not increase its mRNA levels, NKG2A had a significantly increase of its expression. Thus, at same time that CD94 molecules are produced, they recruit NKG2A molecules to form heterodimers and so more NKG2A molecules are needed, justifying the increment of NKG2A expression. The appearance of CD94 expression also marks the beginning of functional maturation of NK cells since this stage is marked by the expression of the NK receptor CD94-NKG2A. NKG2D mRNA expression was maintained once they had already a much higher expression in the first week compared to NKG2A, allowing them to produce protein in sufficient amount to form NKG2D homodimers. At this time point, NCR3 also became expressed its mRNA. Once NK forming cells became more and more differentiated, their cytotoxic potential started to appear. NKp30 together with NKG2D and NKp46 represents a major triggering receptor involved in the induction of NK cells cytotoxicity. It should be also noted that simultaneously to this new expression, the mRNA levels for NCR2 had have an increase too. The expression of CD34 marker was gradually decreased along the culture period with no longer expression since day 14 of culture. This fact is in agreement with the loss of stem status of hHSCs.

At third week of culture, the natural cytotoxicity receptor NCR1 finally starts to be expressed, with no visible variation until the end of culture period. The increase of activity of NK cells is associated with enhanced expression of NKp46 and/or NKG2D. Thus, in the final steps of NK cell maturation, the acquisition of its functions is associated to the increment of these markers that will

make the difference in NK cell capacities. At the same time, in spite NCR3 having an increment at this time point of culture, no further increment was seen until the end of experiment period. On the contrary, the maintenance of NCR2 high expression levels at third week of culture may reveal that, at this point, NK cells are fully competent given that this NCR is specific for activated human NK cells.

At d21 of culture, CD94, CD56, CD122, PRF1, GZMB, NKG2A and NKG2D had a significantly increase in their mRNA expression promoting an increment of NK cell maturity and function capacities. The upregulation of these markers were more pronounced at d28 of culture where NK cells are probably full of its functional capacities, expressing cytotoxic granules containing perforin and granzyme B.

These results confirmed that with our culture system, well differentiated and mature NK cells can be obtained. Additionally, by analysis of these markers we could predict in which differentiation step NK cells are. They become to commit, at least, immediately after one week of culture and, after the replacement of IL-3 by IL-15 (at d7), cells rapidly start to acquire irreversible NK cell features.

The mRNA expression of a wide range of TFs known to have a role in the development of NK cells, was also tested. Mostly are known to have a function on NK cells by experiments on mice. Thus, we investigated first the expression of PU.1, T-BET, MEF, TOX, IKAROS, IRF-2, EGR-1, EGR-2, ID-2, BLIMP1, GATA-3, BCL11B and E4BP4 in hHSCs (d0) and further at different time points during the NK cell differentiation period (d7, d14, d21 and d28).

Our results confirmed Ikaros and PU.1 as important TFs for precursor NK specification and maintenance since their values of expression rich their maximums at the first week of culture with a decrease of their expression since there, demonstrating a minimal significance of these factors along the remainder culture period of differentiation and maturation of NK cells. An increased expression of MEF also at the first week of culture was accompanied by the expression of PRF1 gene. In fact, this Ets protein is thought to have a critical role in PRF1 gene expression during the development of NK cells (31). However, the increment of PRF1 gene expression along the time culture period was not accompanied with an increment of MEF. In fact, MEF was downregulated since the d7 of culture, and so it seems that this TF might have been replaced by another TF also implicated on PRF gene activation. The role of EGR-1 is compared with that published in the literature in mice (38). It revealed an increment of its mRNA levels at first week of culture but despite this upregulation, EGR-1 showed a significant decrease of its expression in the remaining culture period. Our observations are in agreement with the role of this TF in the initial steps of NK cell lineage with no further visible significance for the maturation process of NK cells. As IRF-2 deficiency is associated with apoptosis, this TF may not be implied directly on the NK differentiation but in the maintenance of NK cell pool. In fact, our results suggest a discrete function of this factor during all NK cell development. After an initial discrete increment of mRNA expression, its values were maintained low during the culture period. BCL11B follows the same pattern as the previous described TFs. Despite the upregulation in the first week of culture, the continuous decreasing levels of BCL11B along the culture period are in agreement with the fact that deletion of BCL11B gene induces production of NK cells (41). T-BET is an example of a TF with a constant upregulation in all culture period. This is in accordance to its attributed role in the differentiation and effector functions on NK cells (24). Although it has been suggested that this TF is not sufficient to complete late stages of NK cell differentiation, there is no doubt that this factor has an indispensable role in all NK cell differentiation steps including the final functional maturation, as it is implicated in regulation of perforin and granzyme B expression (49). There are strong evidences that TFs as EGR-2 and ID2 are of enormous importance during all differentiation steps of NK cell development and maturation, once they were highly upregulated during all the culture period. Although the literature gives more importance to EGR-2 for generation of immature NK (iNK) cells (38), our results strongly emphasis the EGR-2 role during all the NK cell differentiating steps. ID2 overexpression is also in accordance of data reported on mice that showed that ID2 stimulates NK cell development at different levels (34,50). In fact, ID2 expression was always upregulated throughout the culture period showing importance during NK development.

Notwithstanding, no expression levels were detected for BLIMP1 at d0 and d7 in cultured NK cells, at d14 this TF showed high significant upregulation. Despite its small decrease after d14, BLIMP1 was always upregulated during the final steps of NK cell differentiation and maturation. This fact is in agreement to the important role of this TF in the transcriptional network governing NK-cell differentiation and homeostasis of NK cells (39,40). T-BET, ID2, EGR-2 and BLIMP1 seemed to be important regulators of all processes of NK cell differentiation once they are highly upregulated during all stages of NK cell development.

The low expression levels of E4BP4 until third week of culture may indicate that this TF is important in the very beginning of NK cell commitment. At the same time, the increment at the end of culture period may reflect some importance of this TF in NK-cell mediated cytotoxicity. Despite results published on mice (37), our dta strongly suggest that E4BP4 has a role in the commitment of HSCs to the NK cell lineage. This regulator does not seem, to be IL-15 dependent or indispensable to transition of pNK cells to mNK cells, since it did not reveal any expression increment after addition of IL-15 to the cultures. However, the expression increment in the last week of culture may be explained by its necessity in developing cytotoxicity.

According to literature, TOX is highly upregulated in immature NK cells and no mNK cells are seen in mice in the absence of this TF. Here we show strong evidence that we got mature NK cells in spite of downregulation since the d7 of culture. Probably TOX is important to act in the very beginning of culture where the commitment or the first immature NK cells begins. However, TOX did not show any additional visible importance in NK cell maturation. These TFs, E4BP4 and TOX, showed a similar pattern of expression in developing NK cells. Both were downregulated through the culture period, suggesting that these two factors are important in the very beginning of NK cell commitment.

In spite GATA-3 expression having a decrease at d7, the increment of its levels in the following weeks are in accordance with the literature observations (23,30). In fact, the increment of GATA-3 values were accompanied by NKG2A gene expression that is thought to be regulated by this TF. Our results are consistent with published results on mice as we got high levels of GATA-3 expression even at the end of culture confirming its essential role in NK cell maturation and in specifying the distinct effector phenotypes.

Assessment of the transcripts present in these hHSCs induced to differentiate in mature NK cells, showed a pattern of preserved and differential gene expression remarkably similar to that seen in mice, except for E4BP4 that is downregulated since the beginning of culture period being less expressed as time progressed.

Quantitative real-time PCR (q-RT-PCR) measurements of the relative levels of transcripts for several genes revealed that some (ID2, EGR-2 and T-BET) were highly upregulated during all the culture period, with a final increment of T-bet expression in the final 2 weeks of culture. These findings suggests that these TFs may have a marked role in all process of NK cell commitment, differentiation and maturation. BLIMP1 is also expressed at high levels after the second week of culture suggesting its importance in differentiation and maturation of NK cells but not in NK cell commitment. BCL11B also shows a somewhat constant high expression during all culture period but in a more softened way. On the other hand, transcripts like E4BP4 and TOX showed downregulation since d7 and d14 of culture, respectively, representative of their relation with the initial stages of NK cell commitment or pNK cells production. IRF-2 did not show any relevant significance in all NK cell differentiation process since its expression hardly varied. Many of the transcripts revealed a differential pattern of expression (PU.1, MEF, Ikaros, EGR-1 and ID3) with an increase at d7 and a following decrease from there suggesting that these transcripts are less related with maturation and final acquisition of functional capacities of NK cells and more associated with NK cell commitment and pNK cell production. It is interesting to note the role of GATA-3 in these cultures: this TF showed a downregulation of mRNA levels at d7 and then a upregulation until the end of culture. This suggests a distinct differential role of GATA-3 during the ontogeny of NK cells.

Continuous research concerning NK cell fate and ontogeny is still needed, at least at molecular level. In fact NK cells have been demonstrating a enormous potential for its use on cancer immunotherapy. It is well known that IL-2 infusions used to augment cytotoxicity of endogenous NK cells is associated with life-threatening toxicity, essentially represented by capillary leak syndrome (51). Moreover, additional NK cell-based immunotherapy trials show to be ineffective, thus, leading scientists to try other positive approaches using NK cells for adoptive cell transfer. Indeed, strategies that use NK cell donors mismatched for inhibitory NK receptors and MHC-I ligands, present in some allogeneic settings, have been more successful (42,52). In fact, an important antitumor role for alloreactive NK cells has been shown in patients with acute myeloid leukemia either after stem cell transplantation or adoptive transfer of haploidentical NK cells (4). Based on these evidences, monoclonal Antibodies (mAbs) have been used to block NK inhibitory interactions with MHC-I on tumor cells. Also, multiple clinically successful mAbs utilize NK-mediated ADCC as a mechanism of action. Rituximab (anti-CD20), Herceptin (anti-Her-2/neu), Cetuximab (anti-EGFR), and the anti-GD2-mAbs 3F8 and ch14.18 are examples of tumor-specific mAbs whose clinical activity can be attributed, at least in part, to NK cells (53). Moreover, genetic modifications of NK cells for cancer immunotherapy also open new possibilities in the use of these cells.

Conclusion

We used a established culture system to characterize NK cell progenitors at different steps in development and examined the relation between time of culture and the expression of several TFs. Here, we examined the role of Transcription Factors on *in vitro* NK cell differentiation and found that E4BP4 and TOX have a role in the very beginning of hHSC commitment; PU.1, MEF, EGR-1, BCL11B, IRF-2 and Ikaros are more specific to lineage specification; GATA-3 is implicated in maturation steps and effector functions and, finally, BLIMP1, ID-2, EGR-2 and TBX21 are undoubtedly the more important TFs during all culture period, playing an essential role in NK cell differentiation. Although the identification of developing intermediates have helped enormously to provide insight into the mechanisms controlling NK development, the molecular events that govern NK lineage commitment still remain unclear. However, altogether, these findings are of great interest to determine whether the switch in the biological properties is accompanied by a change in their gene expression profile leading to modifications during ontogeny of human NK cell differentiation.

The results presented here allow either to facilitate the identification of a variety of components needed for NK cell development or to provide clues on determination of NK cell lineage commitment. However, we are aware that it has been difficult to establish a perfect model to study the NK cell ontogeny. Although we know that

knock-out models are a useful tool to study the effect of several factors they have the limitation of being tested in mice, letting us to wonder whether the effect is the same in humans. In fact, although our model to study NK cell differentiation was based on *in vitro* culture system in which the differentiation could be manipulated by the addition or subtraction of exogenous cytokines and growth factors, we were able to follow and characterize human NK cells along the time. It should be referred that we observed some differences in our results compared to what was published in mice.

This is the first approach that follows-up the expression of several TFs during human NK cell induced differentiation, in parallel with NK cell markers enabling the identification of the differentiation step of NK cell development, including early stages of human NK cell ontogeny. This pathway is characterized by different surface markers and, as observed, it involves specific gene expression profiles. However, the specific developmental stage at which these factors have a role, will need to be addressed in the future. A new *in vitro* differentiation model should be drawn using for example human embryonic stem cells deficient in specific receptor genes providing insight on these mechanisms in humans.

The ultimate proof for the instructive capacity of a TF is the demonstration that it can reprogram a committed cell into another lineage by perturbing its TF network, deconstructing the old one and reconstructing it into a new one. A well-described example of this was already shown, for example, with BCL11B (41). It is crucial a thorough understanding of NK cell developmental mechanisms as it may enable future NK cell manipulation for adoptive cell-based cancer immuntherapy.

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Declaration of interest

The authors declared no conflict of interest.

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DISCUSSION AND CONCLUSIONS

The use of unrelated umbilical cord blood (UCB) as a graft source in allogeneic hematopoietic stem cell (HSC) transplantations is a safe and successful treatment preferably in children (*Rocha and Broxmeyer, 2010*). UCB has several advantages over bone marrow (BM) or peripheral blood (PB) stem cell grafts such as rapid availability, lower risk of viral disease transmission as well as lower risk of severe Graft *versus* Host Disease (GvHD) despite Human Leukocyte Antigen (HLA) mismatches (*Laughlin, 2001; Gluckman, 2009*).

However, the clinical use of UCB is limited due to the low number of cells as well as due to its primitive biological features. Therefore, the parameter that has emerged to be most crucial for the clinical outcome is the cell dose (*Gluckman and Rocha, 2006; Brunstein et al., 2007*). In addition, another obstacle for the clinical success of UCB-transplantation is the reduced Natural Killer (NK) cell activity of UCB compared to adult stem cell sources (*Webb et al., 1994; El Marsafy et al., 2001; Nomura et al., 2001*). This may contribute to the lower incidence of GvHD but could also be one reason for the increased morbidity and mortality after UCB transplantation. The importance of the NK cells in the allogeneic transplant setting is further underlined by studies that have demonstrated, that donor alloreactive NK cells eliminate not only residual leukemic cells but also T cells and antigen presenting cells (APCs) from the host due to the interactions of host major histocompatibility complex (MHC) class I molecules with the Killer Cell Immunoglobulin-like Receptors (KIRs) of the donor NK cells (*Ruggeri et al., 2002; Miller et al., 2007; Ruggeri et al., 2008; Vago et al., 2008*).

Graft *versus* Leukaemia (GvL) effect, that relates to decreased relapse risk, has been associated with a survival advantage as a result of NK cell activity (*Ruggeri et al., 2007*). In fact, the therapeutic potential of targeted NK cell therapies has been addressed not only in hematopoietic malignancies but also in some selective solid tumors (*Caligiuri, 2008; Terme et al., 2008*).

NK cells are Large Granular Lymphocytes (LGL) that were identified in mice by the innate capacity to rapidly lyse some tumour cells. Unlike T-cells, they can kill targets without prior sensibilisation and exhibit spontaneous cytotoxic activity towards cells that do not express class I molecules of the MHC (*Borrego et al., 2002; Orange and Ballas, 2006*) and express a large variety of activating receptors on their surface capable of recognizing target cells (*Moretta et al., 2002*). They also express inhibitory receptors

whose signals are able to override basal activation signals (*Hallett and Murphy, 2006; Lanier, 2008*). The ligands for the predominant inhibitory receptors are MHC class I molecules that are expressed by most normal cells. As originally proposed by the "missing self" hypothesis, downregulation by tumour transformation or infection may turn cells susceptible to NK lysis due to partial or complete loss of class I MHC expression (*Orange et al., 2002; Garcia-Lora et al., 2003*). These cell modifications are perceived by NK cells and, in that event, the normal balance between activating and inhibitory signals provided by diverse receptors is compromised taking NK cells to a response.

In order to recapitulate the development of human NK cells, primitive progenitor candidates from hUCB were isolated by immunomagnetic selection of $CD34^+$ cells from the mononuclear cell fraction. Using a two-step protocol of proliferation and differentiation, cells were plated at different conditions and at different concentrations.

The supplemented medium included a selected cocktail of cytokines chosen based on basic requirements for NK cell differentiation and/or activation. Some factors were included as activator factors (*Miller et al., 1999*) and others in favour of further requirements of NK cells phenotype concerning to cell survival, proliferation, maturation and cytotoxicity (*Silva et al., 1994; Carson et al., 1997; Miller et al., 1999; Wang et al., 1999; Briard et al., 2002; Sivori et al., 2003; Leonard et al., 2008*). Moreover, it was already established that the combination of IL-21 with IL-7, IL-15, SCF and Flt3L augments the generation of NK cells in vitro (*Sivori et al., 2003*) and IL-7 and/or IL-15 might abolish the absolute requirement of stroma to induce NK cell differentiation (*Silva et al., 1994; Mrozek et al., 1996; Yu et al., 1998*). IL-21 seems, as well, to augment the maturation state of NK cells (*Sivori et al., 2003; Leonard et al., 2008*).

Also, as it was previously thought that the addition of stromal cells was important for the expansion of NK cells, we asked if the presence of these supporting cells or the conditioned medium from them, could improve the growth properties of NK cells under our culture conditions. Feeder layers were either shown without beneficial effects on the NK cell expansion, differentiation and maturation (*Lewis and Verfaillie, 2000*), but also with increased $CD56^+$ expansion but not maturation (*Carayol et al., 1998*), or alternatively, to promote $CD56^+$ expansion as well as maturation (*Miller and McCullar, 2001; Grzywacz et al., 2006*).

Therefore, in order to analyze the role of the niche created by supportive cells in the transition of primitive stem cells to less primitive cells, the established AFT024 cell line was used in this in vitro culture system (*Moore et al., 1997*) and compared to conditioned medium from the stroma and also to supplemented medium.

At the end of the first week of culture, areas of primitive progenitor proliferation (so called "cobblestone-like-areas") became visible in all three culture conditions, suggesting an effective proliferation of the original $CD34^+$ cells (Fig. 1d) (paper I) (***Pinho et al., 2011***). The ratio of $CD34^+$ cells was significantly higher ($P<0.001$) when cells were cultured directly over AFT024 feeder layers ($26.89 \pm 12.46\%$) than with conditioned medium ($11.76 \pm 5.96\%$) or with supplemented medium ($13.16\% \pm 6.79\%$). These results suggested that there is a positive effect of direct cell contact without influence of factors released by feeder layers. The murine stromal cell line AFT024 was selected for its described capacity to support and amplify the differentiation of $CD34^+$ cells (*Moore et al., 1997*). In fact, this capacity of AFT024 was also observed in our culture system but, interestingly, direct contact with stroma has beneficial effects on expansion of primitive cells without differentiation and lineage commitment, indicating that stroma-contact alone is a potent inducer on proliferation and preservation of hUCB progenitors at primitive stage compared to other conditions (***Pinho et al., 2011***).

Spanholtz *et al,* successfully expanded $CD34^+$ stem and progenitor cells without feeder cell layers or animal serum, just with the use of cytokines (*Spanholtz et al., 2010*). Here we found, in agreement to previously reported studies, that cells still grow better in presence of a feeder layer (*Miller et al., 1992; Lotzova et al., 1993; Mrozek et al., 1996; Carayol et al., 1998; Lewis and Verfaillie, 2000; Miller and McCullar, 2001; Yu et al., 2001; Perez et al., 2003; Grzywacz et al., 2006; Kao et al., 2007; Vitale et al., 2008*). However, most of these culture systems are unsuitable for clinical applications because of the use of animal sera, animal-derived proteins and supportive feeder cell lines. Nevertheless, the possibility to generate NK cells from UCB without stroma/adhesive microenvironmental factors for a possible clinical application in terms of adoptive cellular therapy, was never been demonstrated so far. To our knowledge, the most promising work was the work recently published by Spanholtz and colleagues revealing itself the best approximation of the requirements concerning cell therapies proposes (*Spanholtz et al., 2010*).

In addition, it will be important for cellular therapy that the generated NK cells express KIRs and do not express NKG2A molecules on its surface as it was shown in conditions without animal sera.

To surmount these shortcomings, we have established a two-step protocol in which we developed a cytokine-based method for differentiation of mature and functional $CD56^+$ NK cells from hematopoietic stem and progenitor cells. As expected, with our culture conditions, in the subsequent three weeks of culture using a modified mixture directed to NK cell differentiation (IL-2, IL-7, IL-15, IL-21, SCF and Flt3-L), the pool of $CD34^+$ cells progressively decreased (Fig. 1e, Figs. 2a,b, Table 1) (paper I) **(*Pinho et al., 2011*)**. In this period, the same type of significance was observed between the three culture conditions ($P<0.001$). Interesting, all cocultures established with AFT024 maintained its viability until the end of culture period, contrarily to the other conditions that have supported only 80% of cultures until the end of 4th week, suggesting a more supportive capacity of cultures when in contact with AFT024.

In order to evaluate the phenotype of the differentiating NK cells, a sample of the cultured cells was harvested for once a week, stained with the defined surface markers-matched antibodies and read by flow cytometry. The results obtained were presented as the percentage of cells in a selected lymphocyte region (Fig. 3a) (paper I) **(*Pinho et al., 2011*)**. We observed that NK cell-phenotype ($CD56^+CD3^-$) progressively increased as $CD34^+$ cells decreased (Figs. 2a-c, Fig. 3b) (paper I) **(*Pinho et al., 2011*)**. Generally, this increase was significantly higher ($P<0.001$) in controls (supplemented medium) up to day 21, whereas at day 28 it was significantly higher ($P<0.001$) in contact with stroma and with conditioned medium. Specifically, the significant differences were found between all culture conditions in all weeks of the experiment period ($P<0.001$). Two exceptions with no significant differences were found between the supplemented medium and conditioned medium at day 7 and between the contact with stroma and conditioned medium ($P=0.011$) at day 28 (Fig. 4a, Table 1) (paper I) **(*Pinho et al., 2011*)**. These data suggest that NK cell differentiation is first delayed by contact with feeder layers, but then stimulated after 14 days of culture by some factor released from the stroma.

During the first weeks of culture in contact with stroma, cells yielded a lower proportion of $CD56^+CD3^-$ cells compared with the other conditions. In fact, in contact with stroma, as cells maintained for longer period the “stem status”, this can explain the initial delay in the commitment of NK phenotype. On the

other hand, our results did not reveal any major differences in the capacity of $CD34^+$ cells collected from hUCB to generate high numbers of NK cells after 4 weeks in the presence of stroma cells or in conditioned medium from the stroma. However, in absence of stroma, there is a reduced production of $CD56^+$ cells meaning that in spite of IL-15 in combination with Flt3-L, SCF, IL-2, IL-7 and IL-21 be sufficient to induce human $CD34^+$ cells to differentiate into NK cells, it seems that the addition of AFT024 cells did not qualitatively alter NK differentiation from $CD34^+$ cells (paper I) **(*Pinho et al., 2011*)**. In spite of that, Miller and colleagues verified that cytokines alone are inefficient to support NK cell differentiation and that conditioned medium only supports differentiation partially. However, we saw that the factors produced by stroma cells may improve the initial development of NK cell phenotype. Our results are supported by others that concluded that cytokines together with stromal cells clearly increase the number of generated NK cells but did not qualitatively alter NK cell differentiation (*Carayol et al., 1998*) and that stromal cells do not bring any beneficial effect compared to conditioned medium, once released factors and/or cytokines are sufficient to induce NK cell differentiation (*Lewis and Verfaillie, 2000*).

Additionally, during the process of maturation, NK cells establish a characteristic cell-surface phenotype and the capacity to elicit effector functions. Analysis of surface receptors during NK cell maturation revealed the expression of the most important KIRs (NKAT2, NKB1, CD158a, p50.3) and of the known NCRs (NKp30, NKp40, NKp46) (Figs. 3c-d, 4b-c, Table 1) (paper I) **(*Pinho et al., 2011*)**. Although it was reported that expression of KIRs occurs at later stages and are hardly observed in vitro (*Freud and Caligiuri, 2006*), with our culture conditions, acquisition of at least one KIR was found in almost all $CD56^+CD3^-$ cells (>95%) at the end of the culture period. Generally, the presence of KIRs was detected very early during cultures (day 7), being significantly higher ($P<0.001$) in supplemented medium than in conditioned medium or in cococulture with AFT024 up to day 14, thereafter becoming significantly higher ($P<0.001$) in contact with AFT024 and in conditioned medium in relation to control medium. With exception for day 21 and day 28 no differences were found between the direct contact with stroma and the conditioned medium and between the direct contact with stroma and the supplemented medium ($P=0.024$), respectively (Fig. 3c, Fig. 4b, Table I) (paper I) **(*Pinho et al., 2011*)**. The majority of the cells (>50%) also expressed at least one Natural Cytotoxicity Receptor (NCR) very early during cultures (day 7), and most of the $CD56^+CD3^-$ cells had these receptors at the end of the 4 week maturation period (>98%). This suggested again that NK cell differentiation is first delayed by contact with feeder layers and

then stimulated by factors released from the stroma (Fig. 3d, Fig. 4c, Table I) (paper I) **(*Pinho et al., 2011*)**.

We found that the vast majority of differentiated $CD56^{+}CD3^{-}$ cells co-expressed at least one of the KIRs and NCRs. Since $CD56^{+}$ phenotype was delayed in presence of stroma, the same occurred for the appearance of such receptors when in contact with stroma. Nevertheless, having in mind the results of the end of culture period, it seems that the appearance of these receptors is independent of the culture condition. The high levels of KIRs and NCRs might be consistent with a mature NK phenotype.

Our conditions downregulated NKG2A/CD94 and its expression decreased along all period of culture in presence of AFT024 (Fig. 3e, Fig. 4d, Table I) (paper I) **(*Pinho et al., 2011*)**. It is known that activation of immune responses depends on a tight balance between activating and inhibitory signals, thus downregulation of NKG2A expression decreases activation threshold of effector cells and could allow for more efficient lysis of target cells. There are some reports of in vitro differentiated $CD56^{+}$ cells that express $CD94^{+}$ on its majority independently of the presence or not of stroma (*Carayol et al., 1998*). Simultaneously, less than 5% of $CD56^{+}$ cells present KIRs expressed after the CD94/NKG2A. With our system, the presence of stroma induced a continuous increasing of surface expression of this complex along the time. Curiously, NKG2A/CD94 positive cells in supplemented and conditioned medium conditions got its maximums earlier than with direct contact with AFT024. However, we also saw a strong decline since then suggesting that the lack of cells to establish contact with NK cells may diminish the stimuli to express this receptor. We thought that the lower expression of the complex CD94/NKG2A compared with that of the other receptors could be explained by the fact that maturation of NKG2A is dependent on the association with CD94 by glycosilation requiring much more metabolic complexity. Thus, it appears that there are some factors released by stroma that could be important for the first steps of induction towards NK cells lineage but acquisition of receptors seems to be independent of stroma signals. However, the fact that only AFT024 had capacity to maintain viability in all cultures until the end of the experience indicates that this feeder layer may have some importance on the maintenance of long term cultures and on the viability of NK cells for longer periods. Some published data refers that NK cells receptors are acquired in an orderly and nonrandom manner during in vitro human NK cell differentiation (*Mrozek et al., 1996; Miller and McCullar, 2001; Grzywacz et al., 2006*) and, differing for our results,

indicated that first inhibitory receptor to be surface-expressed is CD94/NKG2A while KIRs appear only later and in low percentages (*Iizuka et al., 1999; Miller and McCullar, 2001; Briard et al., 2002; Grzywacz et al., 2006*).

Taken together, we can state that we have generated a developmentally mature NK cell population expressing NKG2A, KIR and activating receptors. This was achieved with all culture conditions. AFT024 stroma feeder has the highest capacity to maintain the stem cell status with the ability to promote proliferation of stem and progenitor cells. This knowledge could be used to improve the capacity to yield large amounts of mature and functional NK cells without supportive cells. As we have shown to generate mature and functional NK cells in absence of supportive cells, we are on track to obtain suitable effector NK cells for immunotherapy. Spanholtz *et al* have demonstrated similar results for generating clinically relevant NK cells for the use in NK cell-based immunotherapy (*Spanholtz et al., 2010*)

Additionally, in order to evaluate the functionality of the in vitro derived mature NK cells we assessed the cytotoxic activity using K562 cells, a human erythroleukaemia cell line as target cells. We observed a significant higher cytotoxicity levels for NK cells derived from cocultures with AFT024. These cytotoxicity results were consistent with the phenotypic behavior implying that these NK cells can demonstrate specificity in the recognition process (Fig. 5) (paper I) **(*Pinho et al., 2011*)**. In fact, our cytotoxicity results of in vitro differentiated NK cells against classical NK cell target, K562, was especially higher than published NK cell activities from fresh isolated NK cells of cord blood or peripheral blood. Condiotti et al demonstrated that cytotoxic $CD56^+$ cells could be produced from human UCB (*Condiotti et al., 2001*). Nevertheless the proposal of their experiments was, like us, to prove the cytotoxic capabilities of NK cells in UCB to allay the potential consensus of low GvL effects of UCB, we went farther starting with $CD34^+$ cells.

Our studies showed that NK cell activity can be significantly enhanced during $CD34^+$ differentiation in controlled conditions. Although K562 cells do not carry HLA-E, it was proposed by Figueiredo, C. et al that this increased cytolytic activity is partially mediated by the natural cytotoxicity receptor NKp30 (*Figueiredo et al., 2009*) since it was recently shown that the ligand of NKp30 is expressed in K562 cells

(*Byrd et al., 2007*). In general, the stroma was a more potent inducer for cord blood differentiation into functional NK cells than the other experimental conditions.

The strong cytotoxic activity of our UCB-derived NK cells against the tumour cell line K562 was displayed by specific lysis and these results indicate that UCB-derived NK cells generated by our culture method have the ability to kill (at least) myeloid leukaemia cells.

In the course of our experiments we demonstrated that in the presence of suitable cytokines and factors released from the stroma, $CD34^+$ cells rapidly acquired large quantities of NCRs and KIR receptors, becoming cytotoxic at this level (paper I) **(*Pinho et al., 2011*)**. In spite of some reports referring that stromal cells play an important role in NK cell maturation (*Miller and McCullar, 2001; Colucci et al., 2003*) and others reporting that $CD34^+$ HSC cannot give rise to mature NK cells in absence of stroma *(Lewis and Verfaillie, 2000*), with our well-defined and highly reproducible culture conditions we were been able to generate fully competent NK cells in vitro with or without stroma cells, demonstrating that NK cells functionality can be increased under appropriate conditions (paper I) **(*Pinho et al., 2011*)**. The use of cytokines reveals itself a remarkable tool for the production of functional mature cells. From the present findings we can be argued that IL-15 in combination with Flt3-L, SCF, IL-2 and IL-21 were sufficient to induce human $CD34^+$ cells to differentiate into mature cytotoxic NK cells in vitro. Otherwise, we cannot exclude the possibility that other factors produced by the stroma may have influenced the development of NK cells. Additionally, based on someone work (*Kato et al., 2007*) and using specific drugs, downregulation of the NKG2A ligands expression can be attempted on targets. This could also be an attractive therapeutic strategy to induce susceptibility of leukemic cells to the cytotoxicity of NKG2A-lacking cells.

Overall, these findings exemplify that this culture system could hold great promise for the ex vivo generation of clinical grade NK cell products for cellular immunotherapy against cancer.

In BM, human $CD34^+$ HSCs differentiate into common lymphoid progenitors (CLPs) giving rise to all lymphocytes (*Kondo et al., 1997*). These CLPs in turn can develop ultimately into mature NK (mNK) cells (*Galy et al., 1995; Di Santo, 2006*), passing through three main stages: lineage commitment, NK receptor repertoire selection and functional maturation (*Kim et al., 2002; Colucci et al., 2003; Freud et al., 2005;*

Vosshenrich et al., 2005b; Freud and Caligiuri, 2006; Freud et al., 2006; Mujaj, 2011). During this process NK cells acquire characteristic cell surface markers and the capacity to elicit effector functions (*Galy et al., 1995; Di Santo, 2006*).

In humans, NK cell function is dependent on the inhibitory CD94-NKG2A complex and on the KIRs. Upon stimulation, NK cells release perforin, granzymes and several Tumour Necrosis Factor (TNF) family ligands leading to the apoptosis of the transformed or virus-infected cells (*Maki et al., 2001*). NK cell activating receptors (NKp30, NKp44 and NKp46) are also essential for the activation of NK cell functions, which results in either cytotoxicity and/or cytokine production (*Moretta et al., 2001a; Farag et al., 2002*). Activating signals are also triggered by the association of NKG2D homodimer with DAP10 adaptor molecule (*Cosman et al., 2001*). Binding of NK cell inhibitory receptors with their ligands renders the target cells to be protected from NK cell-mediated cytotoxicity. When target cells lack self-MHC class I molecule, NK cells no longer receive inhibitory signal via MHC class I molecules and kill the target cells, in a process known as missing self recognition (*Ljunggren and Karre, 1990*).

Therefore, after our previous results demonstrating that human NK cells with cytotoxic capacity leading to effective effector functions can be successfully differentiated (paper I) **(*Pinho et al., 2011*)**, we started a new approach, characterizing the resulting cells of the NK cell cultures at molecular level, based on mRNA expression of several molecules present in mature and functional NK cells. To confirm whether these cells express mRNA corresponding to known markers and receptors associated with NK cells (CD34, CD56, CD94), we performed q-RT-PCR analysis of cells at time zero (d0) and of the resultant cells after each week of culture (d7, d14, d21 and d28). HSCs and differentiating NK cells were also tested for mRNA expression of several genes encoding important proteins associated with NK cell maturation and cytotoxicity (NKG2A, NKG2D, PRF1, GZMB, NCR1, NCR2 and NCR3).

Confirming our previous observations using flow cytometry (paper I) **(*Pinho et al., 2011*)**, we found increased expression of CD56 along the all culture period with mRNA levels being expressed since the first week of culture (d7). The key marker of NK cells (CD56) started to be expressed immediately after one week of culture together with CD122 (IL2/15Rβ). These two markers gradually manifested along the remaining three weeks of culture (Fig. 1) (paper II) **(*Pinho et al., 2012*)**. This observed expression is in

agreement with the literature since it is already known that all NK cells express CD122, which is required for IL-15 responsiveness and it is essential for NK cell generation and peripheral survival (*Di Santo, 2006*). CD122 acquisition is, in fact, an essential step in the commitment of HSCs to the NK cell lineage. As precursor NK cells (pNK) express IL-2/15Rβ, its ligand IL-15 has a vital role in the maturation of NK cells.

Human CD94 is a subunit of the disulfide-linked, heterodimeric NK cell surface receptor CD94/NKG2. This receptor participates in regulating NK cell directed lysis through interaction with the major histocompatibility antigen HLA-E. However, the expression of CD94 and its association with NKG2A, NKG2C, and NKG2E subunits is dispensable for NK cell development, education, and many NK cell functions (*Orr et al., 2010b*). In our differentiating NK cells, we found CD94 expression only at the second week of culture with a continuous increment of expression (65- to 86-fold) until the end of culture period (Fig. 1) (paper II) **(*Pinho et al., 2012*)**. Simultaneously, as expected, we found a decreased expression of CD34 with no detectable levels of mRNA above d14-21 of culture (Fig. 1) (paper II) **(*Pinho et al., 2012*)**.

CD117 marker (also known as c-kit) showed a subtle expression in these cultures. In fact, its variations during the culture period had no statistical significance.

Other mature-NK cell markers became expressed at d7 of culture such as PRF1, NKG2A and NKG2D (Fig. 1) (paper II) **(*Pinho et al., 2012*)**. These markers had a significantly increase of mRNA expression until the end of culture (d28). Thus, between d0 and d28, PRF1 increased its levels in 27- to 83-fold, NKG2A in 142- to 211-fold and NKG2D in 49- to 110-fold. No detectable levels of GZMB were seen at d7. The expression of PRF1, NKG2A and NKG2D immediately after the first week of culture contrarily to CD94 or GZMB, may suggest that the latter are much more responsive in the final steps of NK cell maturation than the formers. However, despite some mRNA upregulation showed by NKG2A in the first week of culture, supposedly it does not have yet any function as effective receptor once it acts as heterodimer associated with CD94, and this receptor does not have any expression yet at this time. The upregulation of NKG2D, at the first week of culture, might indicate that this activating receptor may be in the forefront of importance of NK cell development. Despite NKG2D having a role in activation or inhibition of killing activity in mature NK cells, it is known that this receptor is expressed in pNK cells but its function on these precursor cells is not known yet. (*Huntington et al., 2007*).

Concerning NCRs, only NCR2 (NKp44) expressed mRNA at d7-14, also with a constant increase of its expression until d28 (34- to 174-fold) (Fig. 1) (paper II) **(*Pinho et al., 2012*)**. This early expression immediately after the first week of culture may be related to the fact that this molecule (NKp44) is progressively expressed by all NK cells in vitro after culture with IL-2 (*Vitale et al., 1998*). As this culture system includes IL-2 since the beginning of experiments, this early expression is not surprising. In spite NK differentiating cells having no function at this stage, this initial differentiation of NCR2 and PRF1 may be a initial preparation of these cells, that once committed, start to prepare for rapidly responding to adversities.

At the second week of culture (d14), the expression of GZMB was observed, demonstrating constant increasing expression since there. In fact, GZMB had an increment of 88- to 228-fold from d7 to d28. At this point, at the same time that CD56 and CD122 increase their expression, other markers start to appear namely CD94 and GZMB as well as the cytotoxic receptor NCR3. These new expressions may have been potentiated by the replacement of IL-3 by IL-15 at the beginning of first week. It should also be noted that while NKG2D did not increase its mRNA levels, NKG2A had a significantly increase of its expression. Thus, at same time that CD94 molecules are produced, they recruit NKG2A molecules to form heterodimers and so more NKG2A molecules are needed, justifying the increment of NKG2A expression. The appearance of CD94 expression also marks the beginning of functional maturation of NK cells since this stage is marked by the expression of the NK receptor CD94-NKG2A. NKG2D mRNA expression was maintained once they had already a much higher expression in the first week compared to NKG2A, allowing them to produce protein in sufficient amount to form NKG2D homodimers.

Concerning NCR3 (NKp30), it showed with increments of its mRNA levels of 62- to 240-fold since it appeared (d14) and until the end of culture period. In fact, since NK forming cells became more and more differentiated, their cytotoxic potential started to appear. NKp30 together with NKG2D and NKp46 represents the major triggering receptors involved in the induction of NK cells cytotoxicity. It should be also noted that simultaneously to this new expression, the mRNA levels for NCR2 had have an increase too. The expression of CD34 marker was gradually decreased along the culture period with no longer expression since day 14 of culture. This fact is in agreement with the loss of stem status of hHSCs.

The generated NK cells only expressed mRNA for NCR1 (NKp46) after the third week of culture (2- to 21-fold) with no further increased in expression at the fourth week. The increase of activity of NK cells is associated with enhanced expression of NKp46 and/or NKG2D. Thus, in the final steps of NK cell maturation, the acquisition of its functions is associated to the increment of these markers that will make the difference in NK cell capacities. At the same time, in spite NCR3 having an increment at this time point of culture, no further increment was seen until the end of experiment period. On the contrary, the maintenance of NCR2 high expression levels at third week of culture may reveal that, at this point, NK cells are fully competent given that this NCR is specific for activated human NK cells. At d21 of culture, CD94, CD56, CD122, PRF1, GZMB, NKG2A and NKG2D had a significantly increase in their mRNA expression promoting an increment of NK cell maturity and function capacities. The upregulation of these markers were more pronounced at d28 of culture where NK cells are probably full of its functional capacities, expressing cytotoxic granules containing perforin and granzyme B.

These results confirmed that with our culture system, well differentiated and mature NK cells can be obtained. Additionally, by analysis of these markers we could predict in which differentiation step NK cells are. They become to commit, at least, immediately after one week of culture and, after the replacement of IL-3 by IL-15 (at d7), cells rapidly start to acquire irreversible NK cell features.

It is already known that during this process NK cells acquire characteristic cell surface markers and the capacity to elicit effector functions (*Galy et al., 1995; Di Santo, 2006;* ***Pinho et al., 2011***). NK cell development, differentiation and maturation are also dictated by a variety of factors such as cytokines, membrane factors, and Transcription Factors (TFs) in addition to BM microenvironment. Little is known about the role of TFs on human NK cell development, and so, although mouse knockout models have been a very useful tool for studying which TFs control NK cell development, our current understanding of NK cell development stems primarily from findings in mice. As we have described, in the course of our experiments we were able to generate mature and functional NK cells. Now it was important to analyze the TFs involved in each commitment step of NK cell differentiation.

To get inside the molecular mechanisms that intervene in each differentiation step of NK cell ontogeny, we have analyzed some TFs that could be related with NK cell commitment, differentiation and acquisition of

effector functions. As the collaborative interactions of these factors have been identified in many processes, this indicates that a functional network encompassing these transcriptional regulators could be crucial for the development and functioning of NK cells. Among many individual TFs that were already referred to contribute to the conventional NK cells development, we studied the expression of PU.1, Ikaros, Gata-3, IRF-2, T-bet, MEF, Id2, TOX, E4BP4, EGR-1, EGR-2, BLIMP1 and BCL11B.

The transcription factors that control engagement to the NK cell lineage have only recently started to be identified. For example, it is known that PU.1 (purine rich box-1) is expressed on NK cells but its role in NK cell development is not known. In an attempt to search for a contribution of PU.1 on NK cell ontogeny, it was found that, in PU.1 knockout (KO) mice, NK cells displayed reduced expression of the receptors for SCF and IL-7 suggesting a nonredundant role for PU.1 in regulating the expression of these cytokine receptor genes during NK cell development (*Colucci and Di Santo, 2000; DeKoter et al., 2002*).

During our differentiation protocol, PU.1 showed a overexpression of its mRNA in the first week (d7) and a further gradual decline since there, maintaining these low levels of expression until the end of culture period (Fig. 2a) (paper II) **(*Pinho et al., 2012*)**. Our results suggest that PU.1 might have a role in commitment and establishment of NK cells since this transcription factor showed an increment of expression in the beginning of culture period and a downregulation along the remainder culture period of differentiation and maturation of NK cells. In agreement with Colucci and co-workers, we demonstrate that NK cells express PU.1, even if this transcription factor is referred not to be required for the generation of functional NK cells in vivo (Colucci et al., 2001). However, diverging from our results and although these studies have been reported on mice, these authors also showed that NK cells maintain expression of PU.1 throughout differentiation. Taken together, this transcription factor appears to be essential for the generation of NK cells in vivo by specification or maintenance of pNKs via regulation of key cytokine receptors required for NK cells. It appears obvious that PU.1 regulates NK cell differentiation and homeostasis. However, it was also suggested that PU.1 is less required in NK cell development compared to B and T cells. More studies should be addressed in the future concerning this TF due to the lack of an established function for PU.1 together with the disparity of conclusions between mouse and human.

Ikaros (a member of the Ikaros zinc-finger family) expression followed the same pattern of expression as PU.1. It also reached its maximum at the first week, followed by a sharp decline until the end of culture period (day 28) where they got their minimums of expression (Fig. 2a) (paper II) **(*Pinho et al., 2012*)**. So far, there are no data concerning the role of Ikaros on human NK cell development. However, our results are consistent with the suggestions retrieved from studies on mice (*Boggs et al., 1998*). Consistent to our data, it was suggested that a deficiency in Ikaros appears to have a negative effect on NK cell development which may be related to a diminished expression of Flt3 and CD122. Whether Ikaros is required for the generation of NKP or at later stages of NK cell differentiation has not been reported. However, in the same work authors also suggested that the deficiency of mature NK cells in Ikaros mutant mice is related to lack of functional precursors.

Our differentiating NK cells doubled MEF (myeloid elf-like factor) expression on day 7 but rapidly fell down in 3- to 5-fold through the remaining culture period, thus, showing a similar pattern of expression as PU.1 and Ikaros (Fig.2a) (paper II) **(*Pinho et al., 2012*)**. This may suggest that MEF may also have a role in the specification and or commitment of NK cells. Nevertheless, these results are not in accordance with literature that attributes MEF to be responsible for regulate cytolitic effector functions of fully-matured NK cells (*Lacorazza et al., 2002*) since its absence, at least in mice, causes reduced cytotoxic capacity and cytokine production in spite of the normal development of NK cells. In the same work, authors reported that, in MEF-deficient NK cells, Perforin protein expression is severely impaired and that MEF directly regulates transcription of the perforin (PRF1) gene in NK cells (*Lacorazza et al., 2002*). This hypothesis is also not consistent with our results. Although we observed an increased expression of MEF at the first week of culture accompanied by the expression of PRF1 gene (Fig. 1) (paper II) **(*Pinho et al., 2012*)**, the increment observed for PRF1 gene expression along the remaining culture period was not accompanied with an increment of MEF. In fact, MEF was downregulated since the d7 of culture, and so it seems that this TF might have been replaced by another TF also implicated on PRF gene activation.

To our knowledge, EGRs (early growth response factors) was never studied in terms of NK cells ontogeny. Quantitative measure of mRNA expression showed that EGR-2 had a strong upregulation of its mRNA levels just after one week of culture (d7) with a continuous high expression until the end of culture (Fig.2b) (paper II) **(*Pinho et al., 2012*)**. Although EGR-2 has been strongly implicated in certain

lymphocyte development other than NK cells (*Lazarevic et al., 2009; Hu et al., 2011; Seiler et al., 2012*), our results strongly suggest that, due to persistent higher expression of EGR2 during all the culture period, it is also implicated in the early and late stages of NK lineage differentiation. This is not surprising, as EGR-2 also bound the promoter of IL-2Rβ, which encodes the interleukin 2 (IL-2) receptor β-chain, and controlled the responsiveness to IL-15.

Although EGR-1 also play a role in NKT cell specification, the contribution of this transcription factor seems to be more subtle than the attributed to EGR-2 (*Hu et al., 2011*). In fact, EGR-1 (early growth response factor 1) showed a minor increase of its mRNA levels at day 7 followed by a high downregulation at day 14 which lasted with minor oscillations until the end of culture period (Fig.2a) (paper II) **(*Pinho et al., 2012*)**. Although experiments from Hu and co-workers have been done in NKT cells in mice, our results seem to show a quite similar pattern leading to a similar conclusions. However, since there is a lack of knowledge in the human NK cells, the different contribution of these two closely related transcription factors to NK cell development remains an area of interest for future studies.

It is known that IRF-2 deficiency on mice causes NK cell deficiency (*Lohoff et al., 2000; Taki et al., 2005*). Our experiments showed an initial increment of IRF-2 mRNA expression with further downregulation until the end of culture (Fig. 2a) (paper II) **(*Pinho et al., 2012*)** reflecting the same pattern of expression of the aforementioned transcription factors. Lohoff and colleagues initially reported that IRF-2 is required for NK cell development in vivo since it was observed decreased numbers of NK cells in IRF-$2^{-/-}$ mice and the NK cells that were present were immature in phenotype (*Lohoff et al., 2000*). These observations were confirmed later when other group showed that, in IRF-2 deficient mice, NK cells in the periphery were reduced due to selective loss of mNK cells contrarily to BM NK cells that proliferated almost normally but undergoing accelerated apoptosis (*Taki et al., 2005*). Excepting the first beginning, our results suggest a discrete function of this factor during all NK cell development (Fig. 2a) (paper II) **(*Pinho et al., 2012*)** and, thus, contrarily to reported findings on mice, this TF does not seems to be implicated in the process of final maturation of human NK cells (at least in our in vitro derived NK cells). As suggested, if IRF-2 deficiency is associated with apoptosis, this TF may not be implied directly on the NK differentiation but in the maintenance of NK cell pool, thus, giving support to our results. Definitively, further studies on human NK cells are needed to explain this subtle role of IRF-2 on NK cell ontogeny.

BCL11B was also selected for mRNA expression analysis in developing NK cells due to recent exciting discovery concerning reprogramming T-cells into other cell kind similar to NK cells [called induced T-to-natural killer (ITNK)] by simply deleting BCL11B gene from the cells. ITNK cells seem to be morphologically and genetically similar to conventional NK cells, killed tumour cells in vitro, and effectively prevented tumour metastasis in vivo. In our work, BCL11B was used as a negative control for NK cells since it is expressed in all developmental stages of T cells, but not in NK cells. Nonetheless, ITNKs may represent a new cell source for cell-based therapies (*Li et al., 2010b*). As expected, we found downregulation of this transcription factor immediately after the first week of culture which lasted until the end of culture (Fig. 2a) (paper II) **(*Pinho et al., 2012*)**. This is in agreement with the fact that BCL11B suppress NK cell genes (Li et al., 2010b) and hence its absence in NK cells. The work published by Li and co-workers was of particular interest since, although the experiments were performed on mice, the fact that we could modify the developmental fate of immune system cells could be of enormous value in cancer treatments.

Our observations suggest that transcription factors like PU.1, Ikaros, MEF, EGR-1, IRF-2 and BCL11B might be implicated in initial phases of NK cell lineage specification. For sure, more studies are needed even because the present knowledge concerning this issue is based essentially on studies on mice.

ID2 (inhibitor of DNA binding protein) has been shown to have an essential role in the generation of NK cells (*Yokota et al., 1999; Ikawa et al., 2001; Boos et al., 2007*). In fact, we found a significant increment of ID2 expression in the first week of culture and, notwithstanding some oscillations during the remaining culture period, its expression levels were maintained always upregulated, showing importance during NK development (Fig. 2b) (paper II) **(*Pinho et al., 2012*)**. The high expression levels that we detected for ID2 mRNA over the entire differentiation period are in agreement with the data reported on mice showing that ID2 stimulates NK cell development at different levels. The overexpression of mRNA levels all over the entire culture period fits with literature reports. Initial reports suggested that, in absence of ID2, the reduced population of NK cells is caused by an intrinsic defect in NK-cell precursors (*Yokota et al., 1999*) and on the other hand attribute ID2 a role in the restriction of bipotent T/NK progenitors to the NK cell lineage (*Ikawa et al., 2001*). More recently, Boos and colleagues demonstrated that ID2 is not essential for NK cell lineage specification but rather is required for development of mNK cells (*Boos et al., 2007*).

Despite the high expression of ID2 observed by us and the cumulative evidences that ID2 acts at different levels, more human studies are needed to corroborate these conclusions.

T-BET was identified as a key factor in the terminal maturation and peripheral homeostasis of NK cells (*Townsend et al., 2004*). We found that mRNA expression of this TF is high upregulated during all culture period of NK cell development (Fig.2b) (paper II) **(*Pinho et al., 2012*)**. This is in accordance to its attributed role in the differentiation and effector functions on NK cells (*Townsend et al., 2004*). Our results can be perfectly explained based in literature report conclusions since it was proposed that T-bet has an indispensable role in all NK cell differentiation steps including the final functional maturation. Moreover, T-bet was also recently implicated in regulation of perforin and granzyme B expression (*Cruz-Guilloty et al., 2009*) corroborating ours and others results. We cannot disregard, however, that the published results are based on mice and so, future human approaches should be performed.

BLIMP1 (transcriptional repressor B-lymphocyte-induced maturation protein 1) is a critical negative regulator of NK function (*Smith et al., 2010; Kallies et al., 2011*). Nevertheless, it was also shown that BLIMP1, is expressed by NK cells throughout their development (*Kallies et al., 2011*). These statements are in agreement with our observations. Despite no expression was detected for BLIMP1 at the beginning of our cultures (d0 and d7), at d14 and d21 this TF showed high upregulation (Fig. 2b) (paper II) **(*Pinho et al., 2012*)**. The requirement of IL-15 for the early induction of BLIMP1 in NK cells (*Kallies et al., 2011*) might explain the absence of expression in the beginning of the culture period. Additionally, data referring that BLIMP1 expression increases in the most mature human NK cell subsets also are consistent with our observations since we found high upregulation of BLIMP1 at 2nd and 3rd weeks. Our results together with evidences showing BLIMP1 is required for NK-cell maturation and homeostasis and for regulating their proliferative potential, makes BLIMP1 a notably important factor involved in NK cell development. Blimp-1 has also a role in suppressing the release of IFN-γ, TNF-α, and TNF-β and, thus, ablation of Blimp-1 expression leads to enhanced production of IFN-γ and TNF-α whereas overexpression blocks cytokine production (*Smith et al., 2010*). These findings are in agreement with our results as, in the last week of culture, NK cells showed a significant decrease in BLIMP1 expression (Fig. 2b) (paper II) **(*Pinho et al., 2012*)** suggesting a necessity for downregulation of BLIMP1 for cytokine expression by NK cells. Likewise, Smith and colleagues'' conclusions are in agreement with our results since ablation of BLIMP1 does not

alter cytotoxicity of terminal differentiated NK cells (*Smith et al., 2010*) (Fig. 5) (paper I) **(*Pinho et al., 2011*)** .

There are strong evidences that T-BET, ID2, EGR-2 and BLIMP1 are important regulators of all processes of NK cell differentiation and maturation since, apart from the literature, we observed highly upregulation of these factors during all stages of NK cell development.

E4BP4 (E4-binding protein 4) is a basic region leucine zipper transcription factor showed to be essential for generation of the NK cell lineage (*Gascoyne et al., 2009; Kamizono et al., 2009*). This is in agreement with our observations since in vitro differentiated NK cells showed high downregulation of E4BP4 since a very early point of culture (Fig. 2c) (paper II) (***Pinho et al., 2012***). This might indicate that, in fact, this transcription factor is involved in the NK cell lineage commitment.

TOX is a DNA-binding factor recently implicated in the development of NK cells (*Aliahmad et al., 2010; Yun et al., 2011*). We found some mRNA expression for TOX in the beginning of the cultures with further decrease of its levels since d21 (Fig. 2c) (paper II) **(*Pinho et al., 2012*)**. This is consistent with literature since it was reported a considerably upregulation of TOX in iNK cells and a loss of mNK cells in absence of this transcription factor (*Aliahmad et al., 2010*). Our results suggest that this TF also acts early in the developmental process of NK cells. However, we found in the literature some contradictory results giving TOX a role not only in differentiation of NK cells but also suggesting that effector functions of NK cells are dependent of TOX (*Yun et al., 2011*). Our results are clearly in disagreement with these conclusions since we observed a downregulation of TOX since the 3rd week of culture and, thus, showing no additional significance in acquisition of effector functions.

E4BP4 and TOX, showed a similar pattern of expression in developing NK cells. Both were downregulated through the culture period, suggesting that these two factors are important in the commitment and/or differentiation of NK cells with no further major significance in NK cell maturation and/or acquisition of effector functions.

It is known that GATA-3 (GATA binding protein 3) is expressed both in murine pNK cells and mNK cells (*Samson et al., 2003*). This announcement per se is in favour of the results that we have found in human NK cells. We found a decrease of GATA-3 expression in the first week of culture with further continuous high upregulation throughout all the culture period (Fig. 2d) (paper II) **(*Pinho et al., 2012*)**. Since it was reported that NK cells can be generated in the absence of GATA-3 (*Samson et al., 2003*), it is not surprising the initial downregulation of GATA-3 that we observed. In addition, the upregulation and high overexpression of GATA-3 in the remaining culture period (Fig. 2d) (paper II) **(*Pinho et al., 2012*)** is consistent with Samson *et al* work suggesting that GATA-3 promotes NK cell maturation and acts in this lineage to specify distinct effector phenotypes (*Samson et al., 2003*). In the same research, the authors verified that in absence of GATA-3, NK cells produced less IFN-γ compared to control NK cells. Moreover, in an attempt to study the transcriptional regulation of the human NKG2A gene, GATA-3 was also implicated in regulating NKG2A expression (*Marusina et al., 2005*). This is also in agreement with our observations, since the increment of GATA-3 values were accompanied by NKG2A gene expression (Fig. 1) (paper II) **(*Pinho et al., 2012*)**. However, since we observed an upregulation of NKG2A expression at d7 simultaneously with a downregulation of GATA-3 in the first week of culture, other(s) transcription factors other than GATA-3 must exist and be involved in the NKG2A expression.

However, more studies are needed with focus on human NK cells concerning transcription factors network that commands NK cell fate. Indeed, for the first time we did an approach to several transcription factors involved in NK cell specification.

Assessment of the transcripts present in these hHSCs induced to differentiate in mNK cells, showed a pattern of preserved and differential gene expression remarkably similar to that seen in mice, with few exceptions. Our observations based on the relative levels of transcripts for several genes suggest that some (ID2, EGR-2, and T-BET) may have a marked role in all process of NK cell commitment, differentiation and maturation. BLIMP1 is suggested to participate in events during differentiation and maturation of NK cells but not in NK cell commitment. Other transcript factors (PU.1, Ikaros, MEF, EGR-1, IRF-2 and BCL11B) shown to be discrete throughout the process of differentiation except in the beginning which, by its overexpression and upregulation are suggested to play an important role in the NK cell lineage specification, NK cell commitment and pNK cell production being less related with maturation and

final acquisition of functional capacities of NK cells. Transcripts like E4BP4 and TOX showed downregulation since d7 and d14 of culture, respectively, representative of their relation with the initial stages of NK cell commitment or pNK cells production. It is interesting to note the role of GATA-3 in these cultures: this TF showed a downregulation of mRNA levels at d7 and then a upregulation until the end of culture attributing Gata-3 a role in the process of maturation and acquisition of effector capacities of NK cells.

Although many of these transcription factors are shared with other hematopoietic cell lineages, they control unexpected and unique aspects of NK cell biology.

Because of rapid cytolitic function without previous priming against broad range of targets, NK cells may be candidates for cancer therapy emerging to apply as therapeutic agents against a broad range of malignancies (*Shlomchik et al., 1999; Ruggeri et al., 2002*).

The critical importance of NK cells in innate immunity lead us to recapitulate the development of functional NK-cells from multipotent hematopoietic UCB-stem cells in vitro reconstituting the complete process of NK-cell development and maturation from the naive stem cell to the functional effector cells. We also investigate the possibility to generate a specific NK cell population (CD56+KIR+NKG2A-) from UCB without stroma/adhesive microenvironmental factors.

As lineage decisions always involve changes in gene expression programs and being these decisions ultimately controlled by Transcription Factors, we've also characterize human developing NK cells at molecular level based on mRNA expression of several TFs that have been already described to have important roles, at least on mouse NK cell lineage.

The results presented here indicate that NK cells can be generated *in vitro* and it seems that these receptors are indispensable for NK cell maturity. In fact, the capacity of hUCB CD34+ cells to acquire CD56 may therefore provide a cytotoxic cell population that may be of therapeutic potential in the treatment of hematopoietic malignancy. These data together with the possibility to achieve NK cells negative for CD94/NKG2A receptor will allow us to generate alloreactive NK-cells for clinical applications in adoptive cellular therapies.

This study also extended our knowledge of the effects of different cytokines on the proliferation of NK cells from human cord blood progenitor cells. One of the reasons for the lower incidence of GVHD following UCB transplant may be the reduced cytotoxic potential of CB-derived NK cells. However, we can increase NK cells functionality under appropriate conditions, opening new perspectives in immunotherapy.

This matter is not yet well understood and there are only a few preliminary studies in this field with no conclusive results. In fact, there are many reports with mixed conclusions difficult to reconcile. An improved understanding of these processes will continue to drive the clinical applications for NK cells as a tool against infection and tumors as well as the establishment of NK cell lines with "specific" characteristics will provide favourable tools for culture models.

Moreover, to our knowledge, this was the first work that follows-up the in vitro human NK cell development evaluating a set of TFs during lineage commitment and maturation. Simultaneously, we established a relation between gene expression and the detection of the functional receptor on the surface of the NK cells. Although the identification of developing intermediates have helped enormously to provide insight into the mechanisms controlling NK development, the molecular events that govern NK lineage commitment still remain unclear. However, altogether, these findings are of great interest to determine whether the switch in the biological properties is accompanied by a change in their gene expression profile leading to modifications during ontogeny of human NK cell differentiation.

We are aware that it has been difficult to establish a perfect model to study the NK cell ontogeny. Although we know that knock-out models are a useful tool to study the effect of several factors they have the limitation of being tested in mice, letting us to wonder whether the effect is the same in humans. In fact, although our model to study NK cell differentiation was based on in vitro culture system in which the differentiation could be manipulated by the addition or subtraction of exogenous cytokines and growth factors, we were able to follow and characterize human NK cells along the time. It should be referred that we observed some differences in our results compared to what was published on mice. We present the first approach that follows-up the expression of several TFs during human NK cell induced differentiation, in parallel with NK cell markers enabling the identification of the differentiation step of NK cell

development, including early stages of human NK cell ontogeny. This pathway is characterized by different surface markers and, as observed, it involves specific gene expression profiles. However, the specific developmental stage at which these factors have a role, will need to be addressed in the future.

NK cells have been demonstrating a enormous potential for its use on cancer immunotherapy. It is well known that IL-2 infusions used to augment cytotoxicity of endogenous NK cells is associated with life-threatening toxicity, essentially represented by capillary leak syndrome (*Fehniger et al., 2002*). Moreover, additional NK cell-based immunotherapy trials show to be ineffective, thus, leading scientists to try other positive approaches using NK cells for adoptive cell transfer. Indeed, strategies that use NK cell donors mismatched for inhibitory NK receptors and MHC-I ligands, present in some allogeneic settings, have been more successful (*Ruggeri et al., 2002; Miller et al., 2005*). In fact, an important antitumor role for alloreactive NK cells has been shown in patients with acute myeloid leukaemia either after stem cell transplantation or adoptive transfer of haploidentical NK cells *(Farag et al., 2002)*. Based on these evidences, monoclonal Antibodies (mAbs) have been used to block NK inhibitory interactions with MHC-I on tumour cells. Also, multiple clinically successful mAbs utilize NK-mediated ADCC as a mechanism of action (*Alderson and Sondel, 2011*). Moreover, genetic modifications of NK cells for cancer immunotherapy also open new possibilities in the use of these cells, for example, using strategies for expression of interfering RNAs (siRNA) (*Figueiredo et al., 2009*).

In summary, the NK cell is a complex lymphocyte that deserves increased recognition for its contribution to overall host immunity (*O'Connor et al., 2006*).

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