DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Immunobiology of Natural Killer Cells:

Subpopulations, phenotypes and functional properties useful for immunotherapy

Débora Tânia Basílio Queirós

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Dr Paulo Rodrigues Santos (Universidade de Coimbra) e da Doutora Ana Luísa Carvalho (Universidade de Coimbra)

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Index of Abbreviations

ACT	Adoptive Cell Transfer
ADCC	Antibody-Dependent Cell Cytotoxicity
APC	Antigen-Presenting Cell
BM	Bone Marrow
CML	Chronic Myeloid Leukaemia
DAMPs	Danger-Associated Molecular Patterns
GrzB	Granzyme B
HPCs	Hematopoietic precursor Cells
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-15	Interleukin-15
ILC	Innate Lymphoid Cells
LCR	Lectin-C receptor
LPS	Lipopolysaccharides
MAMPs	Microbe-Associated Molecular Patterns
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B

MFI	Median Fluorescence Intensity
NCR	Natural Cytotoxicity Receptor
NK Cells	Natural Killer Cells
PAMPs	Pathogen-Associated Molecular Patterns
PMA	Phorbol 12-myristate 13-acetate
Poly (I:C)	Polyriboinosinic polyribocytidylic acid
PRRs	Pattern Recognition Receptors
TCR	T-Cell Receptors
Th 17 Cells	T Helper 17 Cells
TKIs	Tyrosine-kinase inhibitors
TLRs	Toll-like receptors
TNF	Tumour Necrosis Factor
TRAIL	Tumour-necrosis factor related apoptosis-inducing ligand

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Abstract

Over the years, the study of Natural Killer (NK) cells made it clear that this cells are not simply large granular lymphocytes but instead, a highly complex population of cells, with versatile characteristics, that can be used in immunotherapy. The expression of CD8 by NK cells has not been fully understood but a very important purpose for this molecule is believed to exist.

Peripheral NK cells from healthy individuals, lung cancer patients, chronic myeloid leukaemia patients and umbilical cord blood, were characterized by flow cytometry according to the expression of CD8. Additional characterization of important cell types in the integration of signals between innate and acquired immunity, such as γδ T cells and Natural Killer T cells as accomplished.

Here, it was demonstrated that $CD8^+$ NK cells may be a more mature and cytotoxic subset than their counterparts (showed by the higher expression of CD57 and CD7) and that $CD8^-$ NK cells seem to be more of a cytokine secretor subset (IL-10 and TGFβ). Umbilical cord blood $CD8^+$ NK cells responds to stimuli prompter than their equivalents in peripheral blood. Lung cancer patients' NK cells showed an augmented expression of CD57.

Both $CD8^+$ and $CD8^-$ subpopulations of CML patients' iNKT cells reveal increased activation when compared to healthy individuals. $CD8^+$ iNKT cells are more rapidly activated both in lung cancer and in healthy individuals than their $CD8^-$ counterparts. In addition, iNKT cells from lung cancer patients show an increased early activation profile than healthy individuals. γδ T cells were effectively stimulated by Poly (I:C) in order to produce IFN-γ in healthy individuals demonstrating the presence of *Toll*-like receptors.

A similar role for CD8 in NK cells and T cells may be proposed. Further elucidation of the role played by CD8 in NK cells and others may be a very important tool in its application in adoptive cell transfer.

Key-words: Natural Killer Cells, $CD8^+$ Natural Killer Cells, Innate Immunity, cancer, Immunotherapy, Adoptive Cell Transfer.

Ao longo dos anos tornou-se claro que as células Natural Killer (NK) não representam apenas uma população de linfócitos granulares de grandes dimensões, mas que se trata de uma população celular extremamente complexa, com varadas características, que podem ser usadas em imunoterapia. A expressão de CD8 nas células NK não é totalmente compreendida, mas pensa-se que poderá ter um papel muito importante na função destas células.

Células NK de sangue periférico de indivíduos saudáveis, leucemia mielóide crónica, cancro do pulmão a sangue do cordão umbilical foram caraterizadas por citometria de fluxo de acordo com a expressão de CD8. Uma caraterização suplementar de células capazes de integrar de sinais da imunidade inata e adquirida, tal como as células T γδ e células Natural Killer T (NKT) foi realizada.

Foi demonstrado que as células NK $CD8^+$ poderão ser uma subpopulação mais madura e mais citotóxica comparativamente às células NK $CD8^-$ (demonstrado pela maior expressão de CD57 e CD7). A subpopulação $CD8^-$ aparenta ter um maior perfil de secreção de citocinas (IL-10 e TGF-β). As células NK do sangue do cordão umbilical $CD8^+$ respondem mais rapidamente a estímulos comparativamente aos seus equivalentes no sangue periférico. As células NK de cancro do pulmão demonstram uma elevada maturidade.

Ambas as subpopulações de células iNKT $CD8^+$ e $CD8^-$ de doentes de leucemia mielóide crónica demonstraram um perfil de ativação superior ao de indivíduos saudáveis. As células iNKT $CD8^+$ poderão ser ativadas mais rapidamente em indivíduos saudáveis bem como em cancro do pulmão do que as células iNKT $CD8^-$. Ainda, as células iNKT demonstraram um perfil de ativação prematura em relação a indivíduos saudáveis. Estímulos com Poly (I:C) desencadearam a produção de IFN-γ pressupondo assim, a presença de *Toll-like receptors* nas células T γδ.

Um papel semelhante para o CD8 em células NK e linfócitos T pode ser proposto. Esclarecimentos adicionais sobre o papel do CD8 nas células NK e noutras, pode revelar-se de extrema importância para a aplicação destas em transferência adotiva de células.

Palavras-chave: células Natural Killer, células Natural Killer CD8+, imunidade inata, cancro, imunoterapia, tranferência adotiva de células.

Theoretical Background

Immune System

The immune system is an extremely versatile defence system that has evolved to protect multicellular organisms from pathogens and tumour cells. This system is extremely adaptable, defending the body from invaders. The immune system is composed of a wide variety of cells and molecules, all working together in a dynamic network to recognize and eliminate substances that can cause harm to the organism (Figure 1). Besides being able to discriminate between foreign molecules and molecules from the body itself, it is also capable of discriminating the body's own cells (discrimination self/non-self). The immune system also has the ability to recognize altered body cells that can lead to the development of cancer. A more recent definition of the immune system is based on the idea of discrimination between what is and what is not dangerous for the organism (Moretta and Moretta 2004; Finlay and McFadden 2006; Vivier, Raulet et al. 2011).

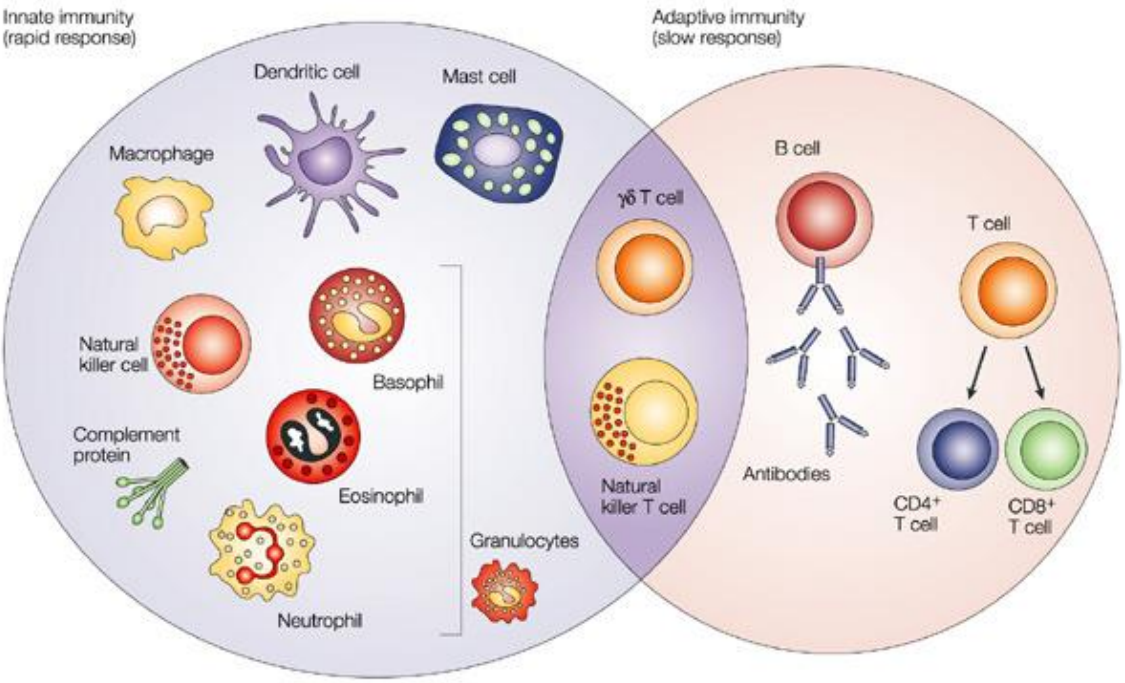


Figure 1 - Cellular constituents of both branches of the immune system (innate and acquired) and the cells that may link the two branches of the immune system (Dranoff 2004).

The various components of the immune system are able to convert the initial recognition event into a variety of effector responses; each response is uniquely suited to remove a particular type of pathogen. Although it is simply referred to as the immune system, it should be noted that there are two types of immune response: innate and acquired (Moretta and Moretta 2004; Caligiuri 2008; Vivier, Raulet et al. 2011).

Innate Immunity

The innate immune system encompasses molecular and cellular mechanisms that help prevent and contain the infection (Finlay and McFadden 2006; Caligiuri 2008; Moretta, Locatelli et al. 2008).

This is the first line of defence, it is highly effective, preventing most early stage infections and therefore eliminating them a few hours after initial contact with the innate immune system. The innate immune system can precisely distinguish between the organism and pathogenic agents, however it is unable to distinguish small differences found between the pathogens (Bewick, Yang et al. 2009).

In Vertebrates, innate immunity is largely dependent on myeloid cells (immunocytes that destroy pathogens). In general, these cells are able to carry out their functions in isolation but they have evolved to work best in conjunction with cells and proteins of the acquired immune system. The myeloid cells include mononuclear phagocytes and polymorphonuclear phagocytes. The mononuclear phagocytes include monocytes, macrophages (monocyte-derived) and dendritic cells, all of which have an elevated capacity for phagocytosis and play an important role in the presentation of antigens to the cells of the acquired immune system. The polymorphonuclear phagocytes include the granulocytes: neutrophils, basophils and eosinophils (Chaplin 2010).

Another cellular component of innate immunity that is of high importance is the Natural Killer (NK) cells. These cells are the only lymphocytic cells belonging to the innate immune system. They originate in the bone marrow and are under the influence of interleukin-2 (IL-2) and interleukin-15 (IL-15) as well as other bone marrow cells. Although they are classified as lymphocytes, these cells do not have receptors for specific antigens (Chaplin 2010).

Even though the cells of the immune system have a limited range of receptors, they are still able to recognize many important molecular components found on microorganisms, but only if these components do not suffer any mutations. For many

years, Immunologists have focused their study on the capacity of the immune system to distinguish between antigens that do not pose a threat from antigens found on hostile invaders, recognized by TLRs. They focused on Pattern Recognition Receptors (PRRs) which are able to recognize Pathogen-Associated Microbial Patterns (PAMPs), those are molecules associated with groups of pathogens. In addition, Danger-Associated Molecular Patterns (DAMPs) (Bianchi 2007) and non-pathogenic Microbial-Associated Molecular Patterns (MAMPs) (Pel and Pieterse 2013) are also recognized by PRRs. The discovery of Toll-like receptors (TLRs) changed the focus of immunological research. It is now known that TLRs have the ability to recognize liposaccharides, glycoproteins and nucleic acids and when activated they can trigger a rapid immune response (Beutler 2004; Bewick, Yang et al. 2009).

Acquired Immunity

The key feature of this type of immunity is focused on its ability, after appropriate stimulation, to provide effector capability against a specific antigen and not cells, and simultaneously initiate mechanisms that allow a more effective response in future infections, even if these infections occur decades later (Chaplin 2010).

Adaptive immunity exhibits a specific response to a particular pathogen and has memory capacity; this memory develops in response to an infection and adapts to recognize, eliminate and remember the invading pathogen in case of future encounters with the same agent. Adaptive immunity follows innate immunity, starting a few days after the initial infection. It provides a complete and a second line of defence, which

eliminates pathogens that escape the innate response or persists despite it. An important consequence of an adaptive response is memory. In a second encounter with the same pathogen, memory cells provide the mechanism for the adaptive immune system to carry out a fast and effective attack against these invading agents (Moretta and Moretta 2004; Finlay and McFadden 2006; Caligiuri 2008). B and T cells are part of the adaptive immune response and express an almost unlimited set of recombinant Ig receptors and T-cell receptors (TCR), respectively, which are able to recognize any antigen and once activated retain a specific long-term memory (Cooper and Alder 2006).

The main characteristics of B and T cells, and consequently the acquired immune system, are both its specificity and memory. This specificity allows the immune system to recognize subtle differences among antigens. After recognition of a specific antigen occurs, B and T memory cells formed by clonal expansion react quicker during a second contact than the first interaction (Dempsey, Vaidya et al. 2003). On first contact with the antigen, effector B and T cells and memory cells are only able to respond after a few days, so it has become clear that the innate immune system is essential for limiting or eradicating pathogens during the first phase of infection, i.e. before the B and T cells are capable of achieving an efficient response (Moretta and Moretta 2004; Finlay and McFadden 2006; David, Morvan et al. 2009; Chaplin 2010).

Innate Lymphoid Cells

Natural killer (NK) cells are an important component of the innate immunity and they belong to the family of innate lymphoid cells (ILC) (Montaldo, Vacca et al.

2014). Even though some aspects of NK cells remain unclear, it is believed that they derive from a common lymphoid progenitor cell type, that later becomes committed either to the B, T, dendritic or NK lineage. This common progenitor has been identified in human foetal liver, bone marrow and umbilical cord blood (Carayol, Robin et al. 1998). Innate lymphoid cells represent a group of cells that share common phenotypic and functional characteristics. All ILC have morphological characteristics of lymphoid cells, however, they do not have modified antigen receptors, i.e. they lack the somatic rearrangement of Ig and T cells receptors, as this is representative for cells of the acquired immune system (Yu, Freud et al. 2013). The ILC respond to a wide variety of signals and have important roles in the formation of lymphoid tissue, repair damaged tissue, tissue homeostasis and immunity against infectious microorganisms and tumours cells. They are thought to be developmentally related once their derive from a common precursor expressing the transcription factor ID2 (Spits and Cupedo 2012; Montaldo, Vacca et al. 2014).

Although NK cells have been studied for close to four decades, other ILC have been better characterized only in the last few years. ILC plays an extremely important role not only in innate defence but also in lymphoid tissue organization mainly during foetal life.

There are three different functional groups of ILC (ILC1, ILC2 and ILC3) accordingly to the cytokine array that they produce and the transcription factors needed for their differentiation. The characteristics of the three groups are summarized in Figure 2.

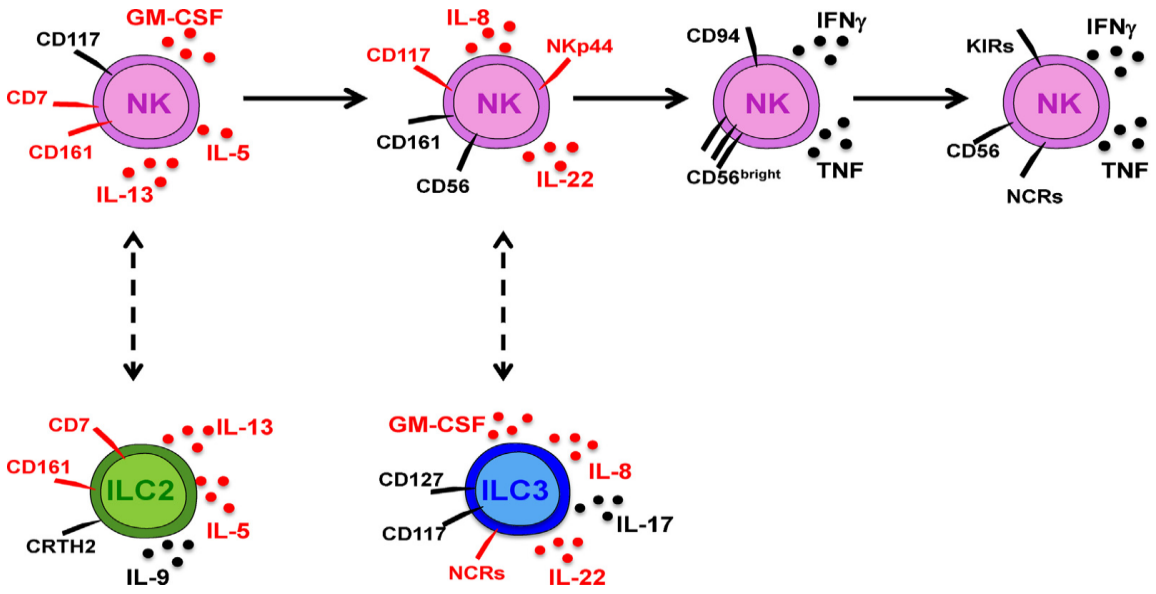


Figure 2 - Developmental liaison between NK cells and other groups of ILC.

The upper row shows the different stages of human NK cell differentiation process (from left to right); the lower row shows ILC2 and ILC3 and the similarities of some immature stages of NK cells with other ILCs namely from group 2 and 3. Dashed arrows indicate common phenotypic and/or functional properties (also shown in red) (Montaldo, Vacca et al. 2014).

Innate Lymphoid Cells type 1 (ILC1)

The cells belonging to these group are characterized by their ability to produce type 1 cytokines and that have the ability to eliminate target cells, which includes NK cells (other similarities of ILCs and NK cells are represented in Figure 3), and more recently, cells that can produce IFN-γ, although it is still not clear if these cells represents a different population or a specialized group of NK cells, located in specific tissues (Fuchs, Vermi et al. 2013).

Innate Lymphoid Cells type 2 (ILC2)

Limited information is available about ILC2, especially in humans. Cells belonging to this subset were originally described as innate helper cells, due to their ability to

produce type 2 cytokines. ILC type 2 are involved in the production of cytokines associated with Th2 cells, in particular IL-5 and IL-13 (Spits and Cupedo 2012; Montaldo, Vacca et al. 2014).

Innate Lymphoid Cells type 3 (ILC3)

The transcription factor RORγt is a landmark of ILC3 involved in the formation of lymphoid tissues that can produce Th17, IL-17 and IL-22 cells. Besides, ILC3 share common markers with NK cells such as CD56 and some NCRs (NKp44, and NKp46) (Montaldo, Vacca et al. 2014).

ILC group	Cells	Main transcription factors	Main effector cytokines	Functions
ILC1	NK cells iILC1	NFIL3 Tbet Eomes	IFNγ TNF	Cytotoxicity Viral defenses Anti-tumor activity
ILC2	Nuocytes Natural helper cells Innate helper cells	GATA3 RORα	IL-13 IL-5 IL-9	Helminth defenses Airway inflammation
ILC3	LTi LTi-like NCR⁺ILC3	RORγt AHR	IL-17 Il-22 IL-8	Lymphoid organogenesis Tissue remodeling Extracellular pathogen defenses

Figure 3 – Main features of Innate Lymphoid Cells (ILCs), that distinguishes them into three different groups (Montaldo, Vacca et al. 2014).

Natural Killer cells

Development and maturation

NK cells originate from Hematopoietic Precursor Cells (HPCs) that are $CD34^{+}$, throughout a sequential acquisition of receptors and functions. The commitment of NK cells lineage requires not only the expression of specific transcription factors but also an appropriate cytokine microenvironment (Carayol, Robin et al. 1998; Freud, Yokohama et al. 2006)

Nowadays it is clear that NK cell development occurs not only in bone marrow but also in peripheral lymphoid and non-lymphoid organs. This findings have mainly being achieved by the characterization of the surface expression of some receptors namely, CD34, CD117, CD94 and CD56 (Freud, Yokohama et al. 2006)

In early stages, NK cells were shown to be derived from $CD34^{+}CD45RA^{+}CD10^{+}$cells present in the bone marrow but this type of cells can also give rise to T and B cells (Yu, Freud et al. 2013). Further studies have shown that peripheral blood have $CD34^{low}CD45RA^{-}CD10^{-}\alpha4\beta7^{hi}CD7^{+/-}$ cells able to further differentiate in NK cells (Freud and Caligiuri 2006).

With this, differentiation stages of NK cells development were described as: stage I, as the most immature ($CD34^{+}CD45RA^{+}CD117^{-}CD94^{-}CD56^{-}$), stage II and stage III as intermediate stages ($CD34^{+}CD45RA^{+}CD117^{+}CD161^{-/+}CD94^{-}CD56^{-}$ and $CD34^{-}CD117^{+}CD161^{+}CD94^{-}CD56^{-/+}$, respectively), stage IV or pre-mature ($CD34^{-}CD117^{-}CD94^{+}CD56^{bright}CD16^{-}$), and stage V, as the most mature state ($CD34^{-}CD117^{-}CD94^{-/+}CD56^{dim}CD16^{+}$), where the acquisition of functions and receptors (Freud and Caligiuri 2006). NK cells development, beyond occurring in BM

is possible in peripheral organs trough a migration of HPCs and/or NK cell committed precursors (NKPs) from bone marrow to peripheral locations such as it is believed to occur in decidual and endometrial tissues (Di Santo 2006; Montaldo, Vacca et al. 2014).

Functional properties

NK cells are part of the innate immune response, they are morphologically characterized as large granular lymphocytes that have cytotoxic activity against tumour cells and cells infected by certain types of viruses, they are also involved in autoimmune diseases and allogeneic responses (Moretta and Moretta 2004; Yawata, Yawata et al. 2008). In fact, these effector cells are able to enter and defend the tissues almost immediately after infection occurs (Vivier, Raulet et al. 2011). These cells are able to recognize normal or aberrant cells through multiple receptors, which detect normal host molecules, ligands induced in stressful situations and patterns displayed by pathogenic agents. The role of NK cells in the innate immune response appears to be their rapid recruitment to sites of inflammation, due to the production of pro-inflammatory cytokines and their cytolytic response without prior sensitization (Stewart, Laugier-Anfossi et al. 2005).

Recently, new data revealed that these cells may be the link between innate and acquired immunity since they have characteristics of both branches of the immune system (innate and acquired) exhibiting the ability to develop an antigen- dependent memory (Stewart, Laugier-Anfossi et al. 2005; Vivier, Raulet et al. 2011). Moreover, due to their interaction with dendritic cells, B cells and T cells, NK cells significantly influence the magnitude and quality of the subsequent adaptive response (Moretta and Moretta 2004; Stewart, Laugier-Anfossi et al. 2005). NK cells have also come to be

recognized as cells that express a repertoire of activating and inhibitory receptors that have been calibrated to ensure tolerance of the body's own constituents, while allowing a high efficacy against attacks such as viral infections and tumour development as cited above (Moretta and Moretta 2004; Stewart, Laugier-Anfossi et al. 2005; Vivier, Raulet et al. 2011).

In humans, NK cells account for about 5-20 % of circulating lymphocytes and they are also found in the lymph nodes, spleen and peripheral tissues (Lopez-Verges, Milush et al. 2011). Due to the fact that they produce early cytokines and chemokines (regulators of inflammatory responses) and for their ability to lyse target cells without prior sensitization, NK cells are crucial components of the innate immune system (Moretta and Moretta 2004; David, Morvan et al. 2009; Cheng, Zhang et al. 2012). The ability of NK cells to readily elicit an immune response is possible, as they are involved in the early production of interferon-γ (IFN-γ) required for the control of certain viral, bacterial and parasitic infections; and by the production of TNF-α, interleukin-10 (IL-10) and growth factors (Lanier 1998; Cheng, Zhang et al. 2012). NK cells require two signals to produce IFN-γ. One of these signals is always the cytokine IL-12. The second signal can be provided by IL-2, IL-15 or IL-18 (Dranoff 2004; De Maria, Bozzano et al. 2011).

Subpopulations and phenotypes

In humans, the NK cells can be divided into two subsets according to the density of CD56 expressed on their surface (glycoprotein expressed on the surface of these cells); the first is $CD56^{bright}CD16^{-}$ (has a regulatory activity for producing increased amounts of cytokines) and the second is $CD56^{dim}CD16^{+}$ (exhibit greater cytotoxic

activity, although it is now clear that they also are able to produce large amounts of cytokines) (Montaldo, Del Zotto et al. 2013). Both groups have different phenotypic properties; unique functional attributes and therefore different roles in the immune response (Cooper, Fehniger et al. 2001; Yawata, Yawata et al. 2008; Lopez-Verges, Milush et al. 2011; Cheng, Zhang et al. 2012; Konjevic, Jurisic et al. 2012). The CD56 antigen is an isoform of human neuronal adhesion molecule its role in NK cells remains unknown although it is thought that it may be involved in the adhesion between this cell type and its target cells. The majority of NK cells in humans (about 90 %) are $CD56^{dim}CD16^{+}$ while only 10% are $CD56^{bright}CD16^{-}$ (Cooper, Fehniger et al. 2001).

Currently, it is known that there is a differential expression of receptors in these two subpopulations of NK cells ($CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$). It is believed that the differential expression of inhibitory receptors probably provides the ability to regulate the cytotoxicity of these cells (Cooper, Fehniger et al. 2001).

More recently new NK cells subpopulation has been described. For example, a $CD7^{+}$ NK cell subset was reported. Even though CD7 is also expressed in T cells and pre-B cells, the expression of this receptor at the cell surface of NK cells was related to the co-expression of NK cell-associated receptors such as KIRs and NCRs and the lack of CD7 was related with monocytes/dendritic cells markers such as HLA-DR (Milush, Long et al. 2009). Additionally, the expression of CD7 on NK cells was associated to a higher cytokine production and cytotoxicity. After stimulation, either with target cells or cytokines, the $CD7^{+}$ NK cell subset showed a greater production of IFN-γ, perforin release and CD107a expression, exhibiting increased effector functions (Milush, Long et al. 2009).

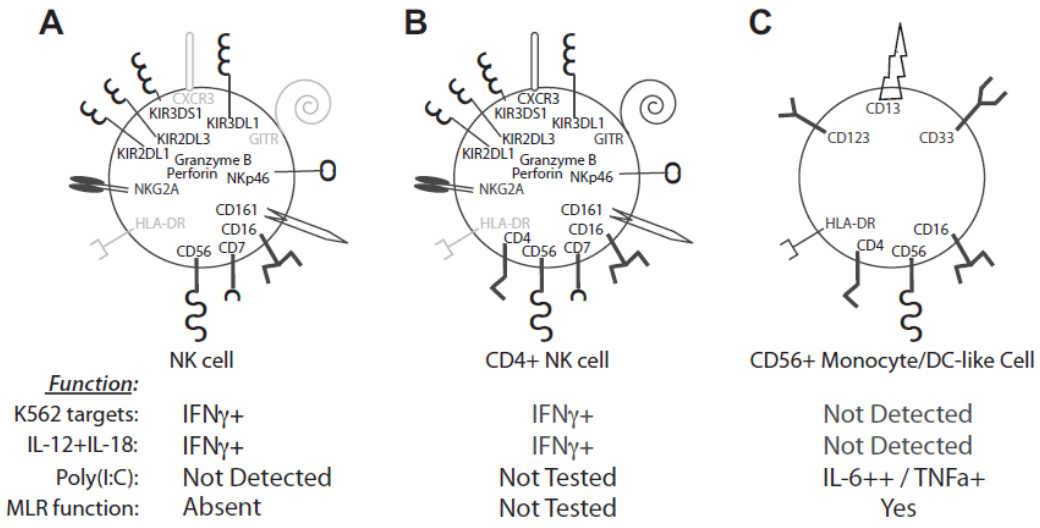


Figure 4 - Differences in receptor expression between $CD56^+$ immune cell subsets.

Model depicting the distribution of receptors on (A) $CD4^-$ NK cells, (B) $CD4^+$ NK cells, and (C) $CD56^+$ monocyte/DC-like cells. Receptors expressed on the majority of cells of that subset are represented in black. No expression or lower than 15% of cells expressing a receptor is indicated in grey (Milush, Long et al. 2009).

Contrary to cells of the acquired immune system, NK cells lack the antigen-specific receptors present on B and T cells. CD4 and CD8 are normally expressed in T cells playing an important role in assisting in the recognition of MHC molecules, even though these receptors can also be expressed in other hematopoietic cells, which is the case of NK cells. Despite the fact that CD4 has been largely studied in $CD4^+$ T cells, not much is known in other cell types. The function of CD4 in NK cells begins to be understood (Bernstein, Plasterer et al. 2006). Some studies report that CD4 can efficiently mediate NK cells cytotoxicity and that $CD4^+$ NK cells are more likely to produce cytokines such as IFN-γ and TNF-α (Bernstein, Plasterer et al. 2006). Others

defend that the function of CD4 in innate cells could be related to FcR-dependent responses and suggests a potentiation role for this receptor (Gibbings and Befus 2009).

In T cells, CD8 has been related to the enhancement of CTL cytokine release and cytotoxic activity, due to an amplification of TCR-MHC CLASS I binding (Gibbings and Befus 2009). In NK cells particular case, besides the expression of CD56 and CD16, it has also been described that, in healthy individuals, NK cells express about 50% of the CD8 α-homodimer at the cell surface, and that this expression can be regulating some NK functions, similar to what occurs with T cells (Spaggiari, Contini et al. 2002).

More recently, four novel subsets of NK were identified accordingly to the expression of CD11 and CD27 (Figure 3). The gain and loss of these receptors may show a maturation path and distinct functional properties. The double negative NK cells display an immature phenotype and a potential for differentiation. This cell population was found in large numbers in Umbilical Cord Blood, in line with a more immature developmental stage. The double positive population was shown to have the best ability to secrete cytokines. Furthermore, single positive $CD11b^{+}$ proved to be highly cytolytic (Fu, Wang et al. 2011; Desbois, Rusakiewicz et al. 2012).

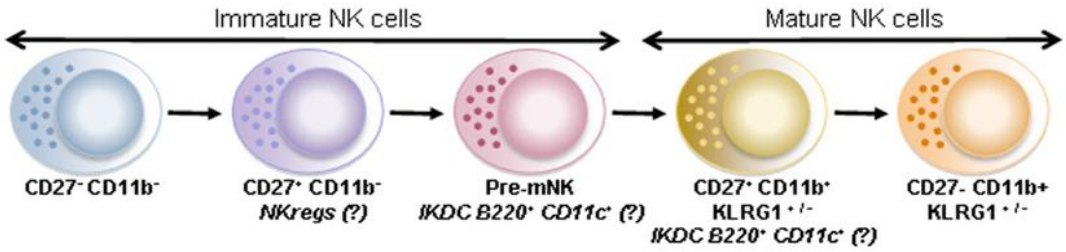


Figure 5 - Maturation stages of NK cells.

$CD19^{-}CD3^{-}CD56^{+}$ NK cells are generally defined by high expression (bright) or intermediate (dim) expression of the CD56 molecule. Several studies have been suggesting a maturation path according to the expression of CD27 and CD11b. (a) The most immature state of NK cells is shown ($CD27^{-}CD11b^{-}$). (b) NK cell start their maturation having a low expression of CD27 ($CD27^{low}CD11b^{-}$). (c) An intermediate maturation state of NK cells referred as pre-mature NK cells. (d) Along the maturation, NK cells gain CD11b becoming $CD27^{low}CD11b^{low}$. (e) At the final stage of maturation NK cells lose CD27 and gain even more CD11b, becoming $CD27^{-}CD11b^{+}$ (Desbois, Rusakiewicz et al. 2012).

Moreover, NK cells have been shown to be able to acquire antigen-specific memory, an immune adaptive feature, related to the expression of CXCR6, on human liver cells. The presence or absence of this receptor create a new subset of NK cells, in which $CXCR6^{+}$ NK cells have the ability to respond more effectively to a recall challenge, even though the presence of this receptor alone is not sufficient to induce or maintain memory (Paust, Gill et al. 2010).

Natural Killer Receptors

NK cells do not rearrange their coding genes for antigen recognition receptors, but they have the ability to recognize target cells through activating or inhibitory receptors expressed on the cell surface, the Killer Immunoglobulin-like receptors

(KIRs). The balance between activating and inhibitory signals will determine the level of NK cell activation, lysis of the target cell will occur only when activating signals prevail in respect of inhibitors (Lanier 2005). The family of KIRs comprise now a total of six activating and seven inhibitory receptors, besides KIR2DL4 that can function both as inhibitory and activating (Ivarsson, Michaelsson et al. 2014).

The first point of control is related to the expression the Major Histocompatibility Complex (MHC) class I (Karre, Ljunggren et al. 1986). A low expression of MHC class I is observed during tumour transformation and viral infection which prevents the binding by inhibitory receptors of the NK cell (Langers, Renoux et al. 2012). Simultaneously, ligands for activating receptor have to be expressed in the target cell to trigger cytotoxicity by NK cells. These ligands are absent or expressed at low levels in normal cells whereas its expression is enhanced in potentially dangerous cells (Lanier 2005).

The mechanism by which NK cells recognize their target cells and their subsequent activation or inhibition is still not completely understood. However, it is believed that the MHC class I receptors are crucial in this process (Cooper, Fehniger et al. 2001). The process by which this mechanism occurs was proposed after the observation that NK cells lysed cells containing no MHC class I (Stewart, Laugier-Anfossi et al. 2005).

NK cells can eliminate pathogens by different mechanisms of recognition. The greatest advances in the understanding the true role of NK cells in the body's defence has been related to the discovery and molecular characterization of numerous surface receptors that play a crucial role in the function of these cells (Cheent and Khakoo 2009).

Three mechanisms of recognition by NK cells have been proposed. The "non-self recognition", "stress induced recognition" and "lack of self-recognition" (Cheng, Zhang et al. 2012).

NK cells recognize MHC class I via surface receptors that transmit signals that inhibit (rather than activate) NK cell cytotoxicity (Moretta and Moretta 2004; Ruggeri, Mancusi et al. 2005; Yawata, Yawata et al. 2008; Cheng, Zhang et al. 2012). This recognition of MHC class I further demonstrates the interaction between the innate and adaptive immune system (Stewart, Laugier-Anfossi et al. 2005).

A key example is represented by the specific immunoglobulin MHC class I receptors that plays an important role in the regulation of NK cell function and are also involved in the recognition of receptors from subpopulations of NK cells (Moretta, Locatelli et al. 2008). Some of the surface receptors involved in the process of MHC class I recognition by NK cells are the so-called killer immunoglobulin-like receptors. They are expressed by NK cells and a subpopulation of T cells and represent specific inhibitory receptors that act on allelic forms of MHC class I, Human Leukocyte Antigen (HLA) in humans (Moretta and Moretta 2004; Ruggeri, Mancusi et al. 2005; David, Morvan et al. 2009; Purdy and Campbell 2009). After the recognition of HLA class I molecules, KIR block the activation of NK cells (Moretta and Moretta 2004; Moretta, Locatelli et al. 2011). In the absence of interaction between HLA class I and KIR, immediate lysis of the target cell occurs, since the predominant signals in this situation are activating signals (Moretta and Moretta 2004; David, Morvan et al. 2009).

Various scenarios resulting from the contact between NK cells with other cell types may exist. Examples of this are shown in Figure 6.

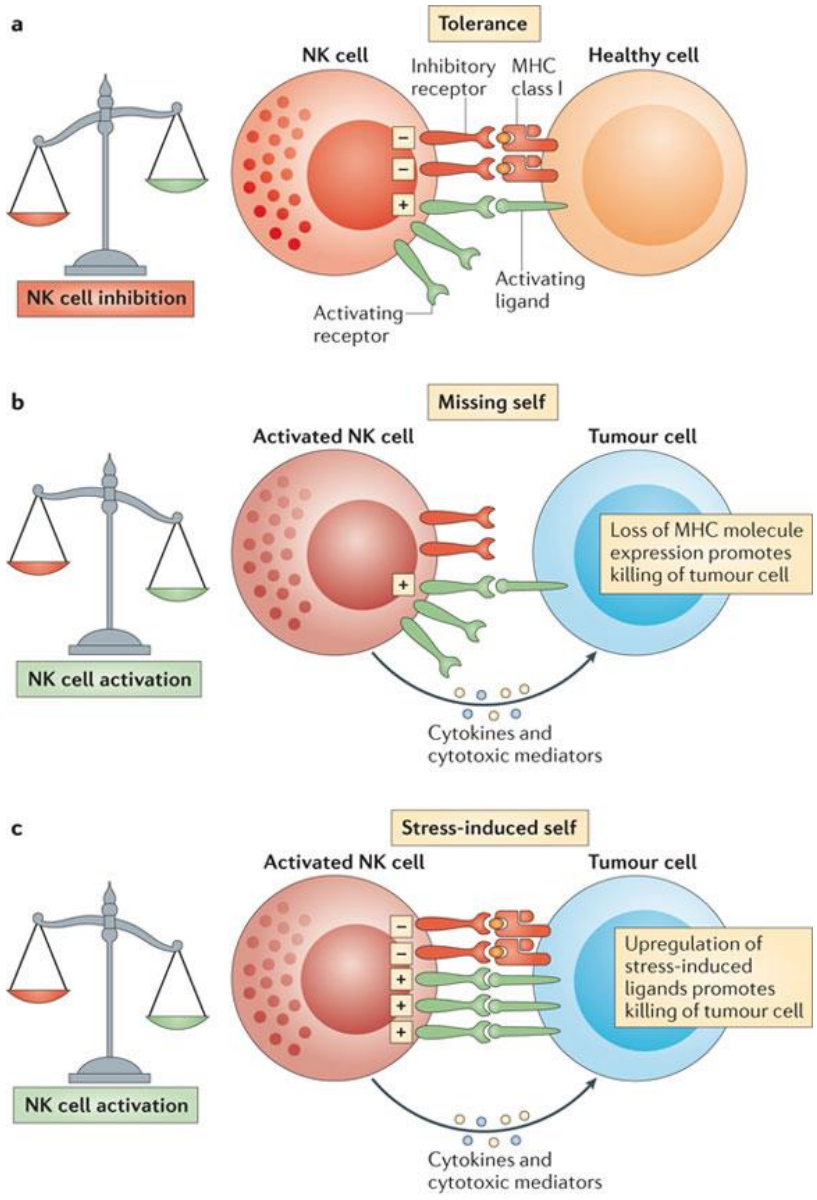


Figure 6 - Representation of the interaction between NK cells and potential target cells and the possible outcomes of this meeting according to the receptors present on the surface of potential targets.

In (A) activation of NK cells does not occur since inhibitory signals prevail over activating signals. In (B), the loss of MHC class I expression during cellular transformation is responsible for a prevalence of activating signals, resulting in NK cells killing of target cell. In (C) an increased expression of activating receptors will lead to an higher frequency of activating receptors, culminating in lyse of target cell (Vivier, Ugolini et al. 2012).

The lack of interaction between these receptors with MHC class I molecules can result in the death of the target cells (Moretta and Moretta 2004; Ruggeri, Mancusi et al. 2005; Yawata, Yawata et al. 2008). The absence of this interaction occurs when the

target cells fail to express or express an insufficient amount of MHC class I molecules, which occurs during tumour transformation or infection with certain viruses (Moretta and Moretta 2004; Purdy and Campbell 2009; Cheng, Zhang et al. 2012). The various immediate reactions carried out by the innate immune system against invading pathogens can lead to the end of the infection without needing to involve the acquired immune system and without disease manifestation (Moretta and Moretta 2004).

The only rule that seems to exist with respect to the expression of receptors is that all NK cells have, at least, one inhibitory receptor specific for MHC class I molecules in order to avoid self-reactivity (Langers, Renoux et al. 2012). Some of the receptors present on NK cells are shown in Figure 7.

In addition to expressing inhibitory and activating KIRs, NK cells also express activating receptors that have been well characterized. These include C-type lectins (CLEC) and Natural Cytotoxicity Receptors (NCRs) (de Rham, Ferrari-Lacraz et al. 2007; Cheng, Zhang et al. 2012).

The main activating receptors involved in cell lysis are CD16, NKG2D and NCRs (Bauer, Groh et al. 1999; Bryceson, March et al. 2005; Biassoni 2008).

The activating receptors have charged residues in their transmembrane domain, which is required for its interaction with adapter proteins. These proteins have small extracellular domains and therefore do not participate in the interaction with ligands. The adapter proteins have in their intracellular domain docking sites for signalling molecules that play an important role in stimulating downstream reactions (Olcese, Cambiaggi et al. 1997; Lanier, Corliss et al. 1998).

The CD16 (low affinity Fc receptor III) has a transmembrane domain and is found on the surface of NK cells but also on some dendritic cells, T cells, monocytes and macrophages (Moldovan, Galon et al. 1999). When IgG molecules recognize

specific antigens on the surface of tumour cells, NK cells, via the CD16 receptor are able to bind opsonized tumour cells and trigger processes that will induce tumour cell death. This process is known as Antibody-Dependent Cellular Cytotoxicity (ADCC), it is dependent on the action of perforin and granzyme molecules, which are capable of damaging the cell membrane of the target cell (Nimmerjahn and Ravetch 2007).

The NKG2D receptor is expressed on most NK cells and is associated with the adapter protein DAP10 (in humans) (Rosen, Araki et al. 2004; Champsaur and Lanier 2010). Ligand binding protein DAP10 and also MICA or MICB lead to an increase in proliferation, cytotoxicity and production of cytokines and chemokines (Sutherland, Chalupny et al. 2002).

Major NCRs expressed in NK cells are NKp46, NKp44 and NKp30. They were collectively named as NCRs for their ability to strongly activate NK cell cytotoxic activity (Sivori, Carlomagno et al. 2014).

NKp30 and NKp46 are expressed both on resting NK cells as well as in activated NK cells, while NKp44 is only expressed on activated NK cells (Cantoni, Bottino et al. 1999). The quantity of NCRs expressed on the surface of NK cells differs from individual to individual and this level of expression may determine the ability of these cells to eliminate tumour cells (Costello, Sivori et al. 2002).

NKp80 is a particular case. It is virtually expressed by all freshly isolated NK cells from peripheral blood and it may function as a co-receptor rather than a classical receptor. NKp80 appears to function synergistically to NCRs where it has been described that the strength of the activating signal may be reinforce, in part, by NKp80 (Moretta, Bottino et al. 2001).

NKp80 was identified as a dimer, and it is present on the surface of any new or activated NK cell. This co-receptor appears to play a key role in certain types of pathologies, since their inhibition with monoclonal antibodies triggered partial mediated inhibition of NK cell to lyse a type of blastoma, while having no effect on other tumour (Vitale, Falco et al. 2001).

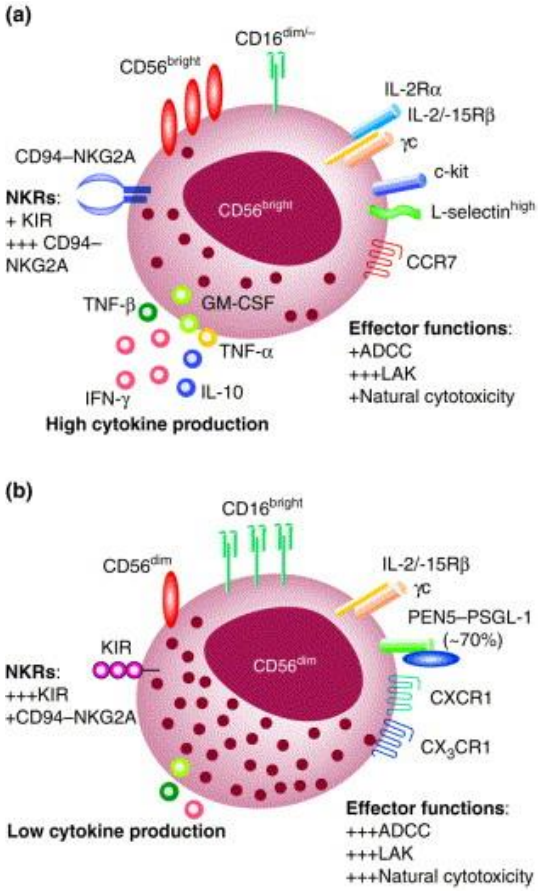


Figure 7 - Representation of different cell surface receptors expressed by NK cells.

It is found that there is a differential expression of these receptors in the two NK cell subpopulations (NK $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$) (De Maria, Bozzano et al. 2011).

Natural Killer Cells and Cancer

Different mechanisms by which NK cells can eliminate cancer cells are known:

a) Cytotoxicity mediated by Perforin/Granzyme:

The release of cytotoxic granules containing perforin and granzyme is the fastest and the most powerful way of eliminating tumour cells. NK cells create synapses with their target cells, into which perforin and granzyme are released, resulting in the death of target cell (Bryceson, March et al. 2005).

b) Apoptosis mediated by death receptors:

The death of the target cell is induced by apoptosis via ligands from the Tumour Necrosis Factor (TNF) family, Fas ligand and Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL), an alternative way for granule release. This second mechanism is slower, with duration of a few hours, and it is often less effective than the mechanism previously mentioned (Takeda, Hayakawa et al. 2001).

c) Effector functions of Interferon-γ:

Upon activation, NK cells secrete various cytokines such as interferon-γ, TNF-α, Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), IL-10 or IL-13 and its anti-tumour activity can be mediated by IFN-γ. In fact, this cytokine inhibits tumour cell proliferation *in vitro* and *in vivo* it indirectly inhibits through the induction of anti-angiogenic factors. It has been stated that IFN-γ is capable of enhancing the cytotoxic activity of NK cells either by increasing the expression of adhesion molecules, by increasing the sensitivity of tumour cells to granule-mediated cytotoxicity or

through the involvement of death receptors (Trinchieri 1995; Street, Cretney et al. 2001).

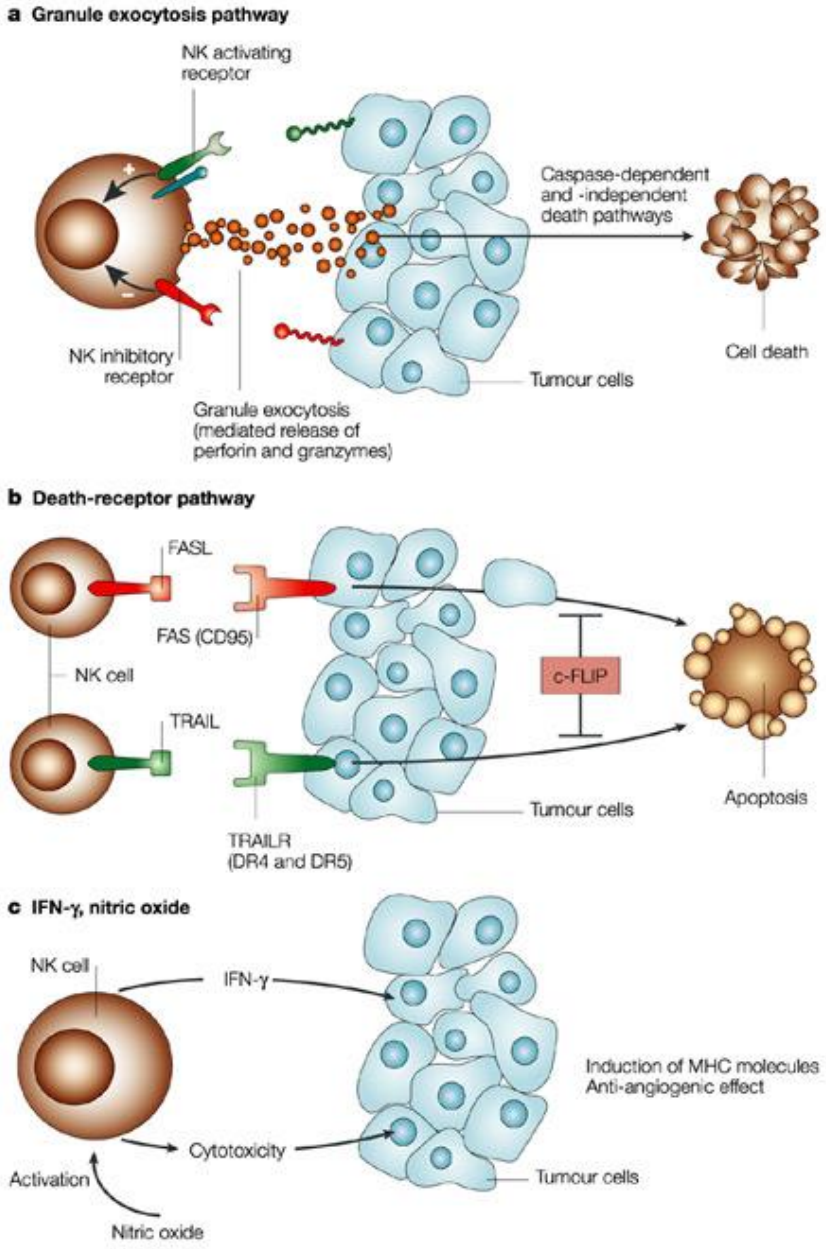


Figure 8 - Mechanism of NK cell effector functions.

There are three main mechanisms by which NK cells can eliminate the target cells: dependent of granules which may be triggered by (a) signalling of activating receptors and secretion of granzyme and perforin into the intercellular cleft between NK cell and tumour cell. Perforin disturbs the tumour cell membrane which allows the entry of Granzyme, (b) FAS ligand or tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL) expressed by some NK cells

bind to their ligands present at the surface of tumour cells inducing apoptosis of tumour cell, and (c) by the secretion of effector molecules such as IFN-ɣ that can be regulated by Nitric Oxide (NO) (Smyth, Hayakawa et al. 2002).

Natural Killer Cells and Infection

Normally, NK cells are associated with an immune response against tumour cells and virus-infected cells, however, they may also participate in immune responses against bacteria, fungi and protozoa (Culley 2009; Chiche, Forel et al. 2011).

In the case of infection with the *Influenza* virus both in animal models and in humans, it is possible to see a mobilization of NK cells to the lungs resulting in the deletion of these cells culminating in increased mortality only a few days after infection. NK cells and adaptive immune response are regulated by each other since NK cells are needed for the activation of cytotoxic T lymphocytes and IL-2 production by these lymphocytes leads to an increased secretion of IFN-γ by NK cells in subsequent responses. The fight against infection involves several receptors. In the specific case of the activating receptor NKp46, critical in the protection against lethal infection in mice, is one of the few known cases in which there is a direct interaction between viral glycoprotein and a NK cell receptor. In addition, there is a possibility of direct interaction with TLRs by molecular structures derived from pathogens, such as LPS, RNA or DNA. It has also been suggested that the activating receptors NCR may be involved, not only in indirect recognition, but also in the recognition of direct structures associated with pathogens (Galluzzi, Vacchelli et al. 2012).

Invariant Natural Killer T Cells

As with NK cells, invariant Natural Killer T (iNKT) cells are thought to play an important role in tumour immunosurveillance and their function is starting to be uncovered (Vivier, Ugolini et al. 2012).

iNKT cells are characterized by the expression of an invariant T cell receptor α chain encoded by a Vα24Jα18 rearrangement (iNKT1 cells). They are believed to be the optimal tool to an ideal anti-tumour immunotherapy once this cell type is able to make the connection between the two branches of the immune system by recognizing α-galactosylceramide conjugated with CD1d (MHC class I-like), typically expressed by APCs and that have the ability to bind a wide variety of lipids, augmenting the immune response. Besides the expression of TCR, iNKT cells also express a diversity of receptors from both NK and conventional T cells (Monteiro and Graca 2014) Due to their ability to rapidly produce IFN-γ they are able to, in one hand, recruit NK cells which will eradicate MHC class I negative tumour cells, and in the other and, recruit T CD8 lymphocytes that will eliminate MHC class I positive tumours, that would escape NK cells killing, pursuing a complete removal of tumour cells (Figure 9) (Fujii, Shimizu et al. 2013). The role of this cell type is not only related to tumour immunosurveillance but also with a wide variety of other diseases related to transplantation, allergy, autoimmunity and other inflammatory pathologies (Monteiro and Graca 2014).

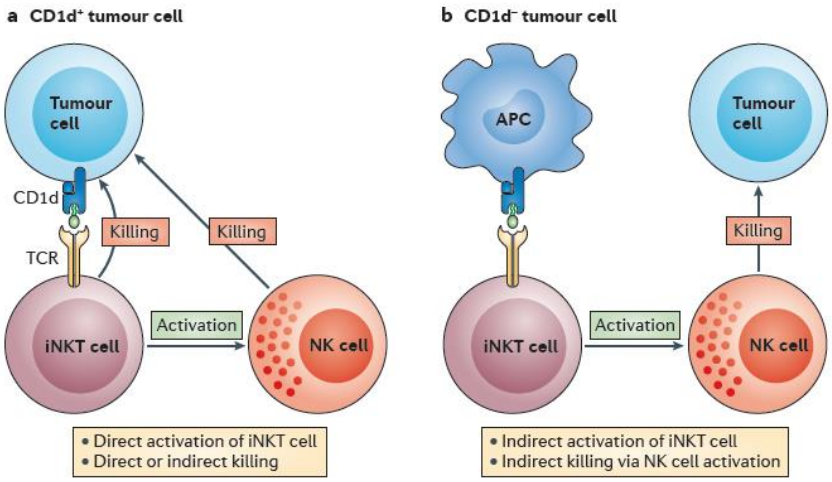


Figure 9- Killing strategies of iNKT cells.

(a) iNKT cells are able to eliminate CD1d tumour cells by one of two strategies. They can either kill directly malignant cells and/or recruit NK cells. (b) Even though some tumour cells do not express CD1d, iNKT cells can be indirectly activated by CD1d expressing-Antigen Presenting Cells. This will lead to the promotion of NK cells and lysis of the target cell. (Adapted from (Vivier, Ugolini et al. 2012).

Gamma delta T cells

In healthy adults, γδ T cells represent only about 0.5 to 5% of circulating T cells. The importance of this cell type is related to their ability to both share features, depending on a particular context, of innate and adaptive systems and they have been considered more than a third player in the adaptive system but a third branch of the immune system (Hayday 2000).

γδ T cells, besides expressing TCR, also express NK cells receptors, as NKG2D and heterodimeric complex CD94/NKG2A and increasing evidences reported that they may also express TLRs (Barakonyi, Kovacs et al. 2002; Wesch, Beetz et al. 2006). In this way, they are able to target stress-induced or infected cells as well as tumour cells (Ferreira 2013). With interest, γδ T cells not only have the capability to directly present antigens to naïve αβ T cells but they also influence DC maturation by an intensive production of TFN-α (Brandes, Willimann et al. 2005; Anderson, Gustafsson et al. 2012).

Adoptive Cell Transfer

Immunotherapy is a strategy that uses certain components of the immune system to combat diseases such as cancer. This may be achieved in different ways:

- Stimulation of the immune system itself so that it can more effectively attack tumour cells.

- Administration of immune system constituents, such as proteins from this system.

Immunotherapy includes a variety of treatments that may function differently. While some appear to strengthen the immune system indiscriminately, others strengthen the immune system so that it becomes more reactive and effective against specific cells (Kirkwood, Butterfield et al. 2012).

Adoptive cell transfer (ACT) involves the (re) introduction of large amounts of lymphocytes with anti-tumour activity in a patient. When possible, the starting material for ACT is obtained through surgery or a biopsy, from this the infiltrating lymphocytes

in tumours are isolated and/or selected for its specificity for TCR. Before the reinfusion of lymphocytes, these processes suffer *ex vivo* expansion in the presence of certain interleukins and growth factors so that immunostimulatory compounds may also be activated. In situations where the starting material cannot be obtained by surgery or biopsy, there is the possibility of using genetic engineering in order to convert lymphocytes obtained from peripheral blood into lymphocytes with the ability to eliminate malignant cells. In this situation, the lymphocytes are genetically engineered so as to express TCRs that recognize antigens of tumour cells with high affinity (Parkhurst, Riley et al. 2011).

Current NK cell-based immunotherapy, aims to overcome the quiescence of these cells in patients. One strategy focuses on activation or proliferation of the patient's own NK cells or on the induction of NK cells with greater cytotoxicity. These objectives have been met with success in the treatment of certain cancers, by administering cytokines and type I interferons. These compounds are involved in the differentiation and activation of these cells, being that these cytokines are part of interleukins-2, -12, -15, -18 and -21 (Cheng, Zhang et al. 2012). Another strategy uses expanded allogeneic NK cells. In this case, allogeneic NK cells are *in vivo* or *ex vivo* activated or expanded and are not inhibited by self-histocompatibility antigens in the way autologous NK cells are. Other approach uses a stable allogeneic NK cell line that favours large-scale production. Here, cells are transferred after *ex vivo* expansion. Alternative approaches rely on genetic modification of NK cells or NK cell lines in order for them to present an higher expression of cytokines, Fc receptors or chimeric tumour-antigen receptors following different approaches, for instance, silencing of inhibitory receptors, overexpression of activating receptors or cytokine transgene (Cheng, Chen et al. 2013).

In addition to the recognition of MHC class I, NK cells also exhibit other forms of recognition. Unlike T cells, NK cells do not express specific receptors for antigens; as mentioned before, the function of NK cells is mediated by a complex balance of activating and inhibitory signals that are recognized by a variety of surface receptors. As such, the manipulation of this cell type in order to make them readily reactive against tumour cells may be an important tool in combating and eradicating tumours in combination with other types of conventional treatments such as chemotherapy (Parkhurst, Riley et al. 2011).

Moreover, it has recently been described that NK cells are required for dendritic cell-based therapies at the time of tumour challenge. Dendritic cells therapies take advantage of their role as APCs. As NK cells tumour destruction can lead to an increased release of tumour antigens within the lymphatic system, the antigens presented lead to an induction of T cell response amplifying DC function (Bouwer, Saunderson et al. 2014).

Aims

Natural Killer cells were dubbed, back in 1975, according to their ability to promptly kill cancer cells without prior sensitization (Kiessling, Klein et al. 1975). Since then, much has been discovered about this cell type and a great increase in the knowledge of NK cells tools has been achieved. NK cells have been described as being professional killer cells with license to kill target cells (Nash, Teoh et al. 2014). Although, mechanisms by which target cells are able to escape NK killing strategies have also arise. In this way, characterization of NK cells subsets besides the classical $CD56^{bright}$ and $CD56^{dim}$, that can more efficiently eradicate target cells and/or more easily be manipulated in a concerned manner are needed.

Thus, we propose to characterize subpopulations, phenotypes and functional properties in Natural Killer cells that can be useful for immunotherapy. We will focus on CD8 subpopulations of NK due to a large expression of this adhesion molecule in this cell type and because of the importance of CD8 in the effector functions of cytotoxic T lymphocytes. Peripheral blood NK cells from healthy individuals and umbilical cord blood will be analysed as NK cells from both cells sources show similar characteristics. Further, healthy individuals NK cells will be compared with some neoplastic pathologies, such as lung cancer and chronic myeloid leukaemia. In addition, important cell types related to the communication among innate and adaptive immune system that share common features with NK cells, iNKT and γδ T cells, will be studied, also in regard to the expression of CD8.

Material and Methods

Study Populations

A total of 31 peripheral blood samples from healthy individuals, with ages comprised between 18 and 65 years old, were collected in leukocyte depletion bags, provided from *Instituto Português do Sangue e Transplantação (CST Coimbra).*

Peripheral blood from 4 lung cancer patients and 6 umbilical cord blood were included. Lung cancer samples were stabilized for 24h in Transfix® (Caltag, Buckingham, UK), a solution that prevents cellular degradation in human and animal blood for analytical testing purposes. Additionally, 22 patients of chronic myeloid leukemia under tyrosine-kinase inhibitors (TKIs) therapy were studied. All the patients were attended at *Centro Hospitalar e Universitário de Coimbra*.

Blood donors and patients signed informed consent and this study was previously approved by the Ethics Committee of *Faculdade de Medicina da Universi*dade de Coimbra and *Centro Hospitalar e Universitário de Coimbra*.

Peripheral Blood Mononuclear Cells (PBMCs) Isolation

The most widely used technique to obtain PBMCs refers to the separation of the different cell types present in peripheral blood by density gradient separation, normally using Ficoll-Paque®. Briefly, peripheral blood was diluted 1:3 with PBS 1x EDTA 0.1mM. Diluted blood was added to a 50 mL Falcon® prepared with 10 mL of Ficoll-Paque®. The tube was then centrifuged at 400g for 20 minutes at room temperature. PBMCs were collected and washed twice with PBS 1X EDTA 0.1 mM. After this, cells

were ressuspended in 10 mL of complete medium (RPMI 1640 with glutamax supplemented with 10% of heat inactivated Foetal Bovine Serum and 1% of Penicillin/Streptomycin)

Cell Counting

After isolation cells were counted in the COULTER AC•T diff Analyzer (Beckman Coulter) using 12 μL of leukocytes in complete medium. The method used in COULTER AC•T diff Analyzer accurately counts and distinguishes cells by detecting and measuring changes in electrical resistance when a particle in a conductive liquid passes through a small orifice.

Cell Viability

Trypan blue was used to assess cell viability. This assay rests on the assumption that viable cells have intact cell membranes, impermeable to certain dyes, such as Trypan Blue, whereas death or senescent cells lose membrane integrity and get stained (Strober 2001). To 10 μL of cell suspension were added 10 μL of Trypan Blue (0,2%). 10 μL were then transferred to a Neubauer chamber and counted in a Leitz microscope.

Natural Killer cells Enrichment – Magnetic Activated Cell Sorting

NK cells were isolated from PBMCs using a negative selection magnetic activated cell sorting (MACS) Human NK cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) accordingly to the manufactures instructions. Briefly, cell number was determined. According to the desired number of cells, a certain volume of cell suspension was centrifuged (10 minutes at 300g) and ressuspended in appropriate amount of buffer, 40 µL of MACS buffer (PBS 1x pH 7.2, 0.5% Foetal Bovine Serum and 2 mM EDTA) per 10^7 total cells. 10 µL of Biotin-Antibody cocktail was added per 10^7 total cells. Cells were incubated at 4ºC for 5 minutes. 30 µL of MACS buffer was added per 10^7 total cells followed by the addition of 20 µL of Micro Beads per 10^7 total cocktail and incubated at 4ºC for 10 minutes. Cells were then passed onto a depletion column whereas the flow through represents the negative portion of the cells suspension i.e. NK cells fraction. LS Depletion columns were hydrated previous to use with 3 mL of MACS buffer and washed twice with 3 mL of MACS buffer after cell flow through of labelled fraction.

In vitro stimulation of PBMCs

In vitro leukocyte stimulation was performed with polyriboinosinic polyribocytidylic acid, also know as Poly (I:C), IL-2, IL-12, IL-18, LPS and phorbol 12-myristate 13-acetate (PMA) and Ionomycin. 1 to 2 million of cells were cultured in 24 well plate for 24h in complete medium (RPMI 1640 with glutamax plus 10% of foetal calf serum and 1% antibiotic/antimicotic) supplemented with different stimuli: Poly (I:C) (10 ug/mL), a

combination of IL-2, IL-12, IL-18 (100 ng/mL each), LPS (100 ng/mL) and PMA (2.5 µg/mL) plus Ionomycin (0.5 µg/mL).

Degranulation assay

$1x10^6$ NK cells were co-cultured with target cells (at an effector:target ratio 3:1) for 5 hours on 96 well U-bottom plates.

CD107a was added at the beginning of the experiment. After 1 hour of co-culture, Brefeldin A was added to the well and the incubation proceeded for 4 hours. At the end, cells were harvested and staining with monoclonal antibodies was performed. Data acquisition was achieved using BD FACSCanto II.

Surface and Intracellular staining for Flow Cytometry

1 million cells were added to a staining tube. Monoclonal antibodies were added following manufacturers recommendations. Cells were then incubated in the dark, at room temperature. After that cells were incubated with 2 mL of BD Lysing Solution® (for surface staining) or 100 µL Fix and Perm Medium A® (for intracellular staining) for 10 minutes in the same conditions as previously. Cells were then washed with 2 mL of PBS 1x EDTA 0.1 mM and centrifuged at 300g for 5 minutes. Supernatant was disposed. For intracellular staining, 100 µL of Fix and Perm Medium B® and at the same time intracellular mAbs were added and incubated for 20 minutes in the conditions previously described. Cells were washed with PBS 1X EDTA 0.1 mM and

centrifuged at 300g 5 minutes. Cells were ressupendend in 300 µL of PBS 1X and acquired in the flow cytometer.

Table 2- Summary of the monoclonal antibodies reactivities, conjugates and respective clones.

mAb reactivity	Conjugate	Clone
CD3	PerCpCy5.5	HIT3a
CD4	V500	RPA-TA
CD7	PE	CD7-6B7
CD8	APC	HIT8a
CD14	PerCpCy5.5	M5E2
CD16	APCCy7	3G8
CD19	PerCpCy5.5	HIB19
CD28	FITC	CD28.2
CD56	PeCy7	HCD56
CD57	PB	HCD57
CD69	PECy7	FN50
CD107a	PE	H4A3
CD137	APC	4B4-1
CD137L	PE	5F4
TCR Vα24-Jα18 (iNKT cell)	PE	6B11
TCRγδ	PE	B1
TGFβ	FITC	TW4-6H1
IL-10	PE	JES3-9D7
Granzyme B	FITC	GB11
Perforin	PB	dGg

Flow Cytometry Analysis

All sample data was acquired in FACSCanto II from BD Biosciences with FACS Diva software version 6.1.3. Data from flow cytometry were analysed using FlowJo version 10.6 (Tree Star Inc, Ashland, USA).

Compounds

The following compounds were used: PBS, RPMI 1640 with Glutamax, Heat Inactivated Foetal Bovine Serum, Penicillin, Streptomycin, Fungizone® (amphotericin B) (GIBCO, Invitrogen, Carlsbad, CA, USA); Lipopolysaccharide (LPS) and Poly (I:C) (InvivoGen, San Diego, CA, USA); All antibodies and human recombinant IL-2, IL-12 and IL-18 used were purchased from Biolegend (San Jose, CA, USA); PMA and Ionomycin (SIGMA-Aldrich St. Louis, MO, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5 software. Mann-Whitney non-parametric test, One-way ANOVA and Two-Way ANOVA tests were used. Tukey's post-test was performed when needed. Statistically significant P values are annotated as follows: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Results

Natural Killer cells from Healthy Individuals

In order to elucidate the role of novel NK cells subpopulations, healthy individuals were studied and characterized accordingly to the surface expression of CD8 using Flow Cytometry. Since CD8 in T cells is described to synergistically enhance ligation MHC class I-ligand, the presence of CD8 in NK cells might be related to the same process.

First of all, lymphocytes were gated according to their size and complexity by Side Scatter (SSC) and Forward Scatter (FSC) parameters (Figure 10). Within this gate, NK cells were then selected as CD3/CD14/CD19 negative population.

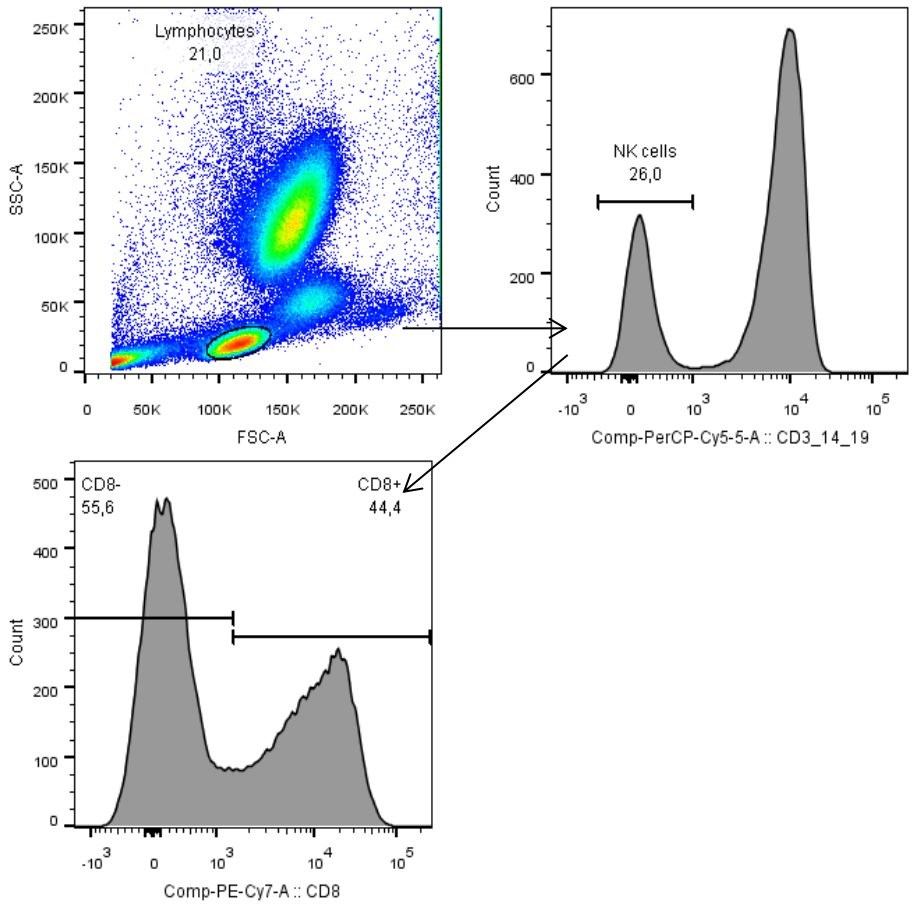
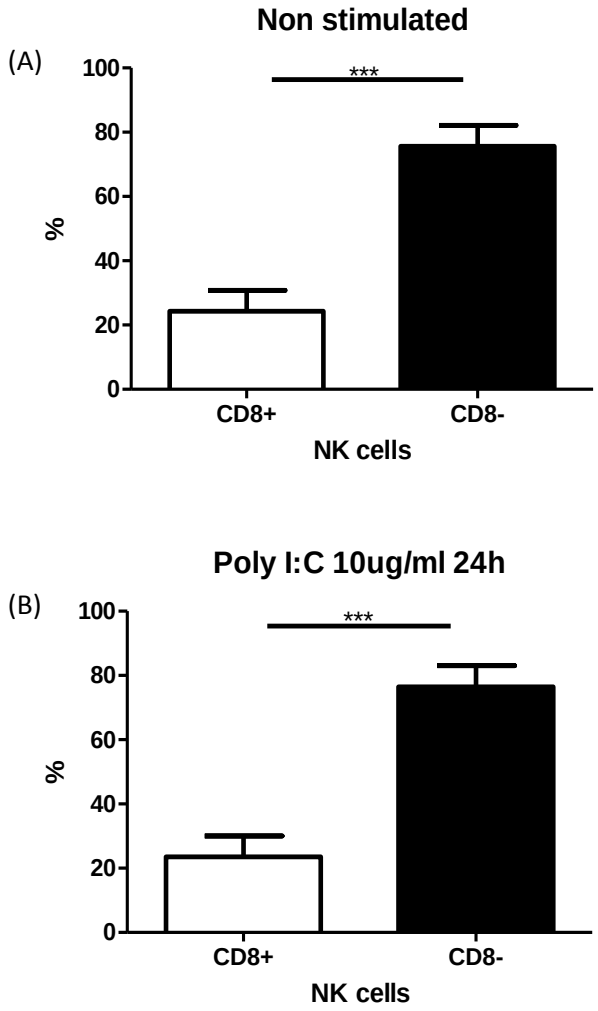


Figure 10 - Illustration of the gating strategy for Natural Killer cells selection.

NK cells show two different subsets related to the expression of CD8

The main focus in the present work was to describe significant differences in NK cells that could be useful for the characterization of new subsets, and how these differences can be favourable for the use of NK cells in immunotherapy. Here we found that the expression of CD8 by NK cells leads to two different subsets and that the differences in these subsets are not altered by stimuli (Figure 11).



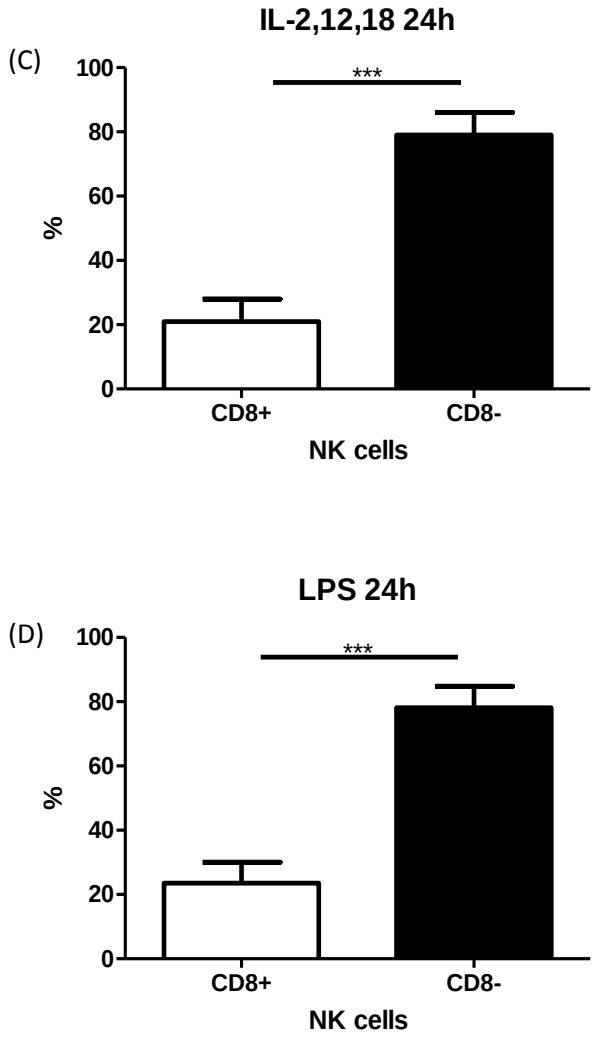


Figure 11 - Analysis of NK cells CD8 expression in Healthy Individuals samples.

In (A) Non stimulated samples (n=14), (B) stimulated with 10µg/mL Poly (I:C) (n=12), (C) stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each (n=12) and (D) 100 ng/mL lipopolysaccharides (n=12). Cells were incubated for 24h at 37ºC. Cell expression of CD8 was assessed by flow cytometry and NK cells were selected as CD3, CD 4 and CD19 negative population. Non parametric Mann-Whitney showed consistent significance among the four conditions ($p<0.001$).

Taking in account the results shown in Figure 11, it is possible to admit that the expression of CD8 is not affected by different stimuli, being the relative expression CD8+/CD8- persistent despite of the stimuli.

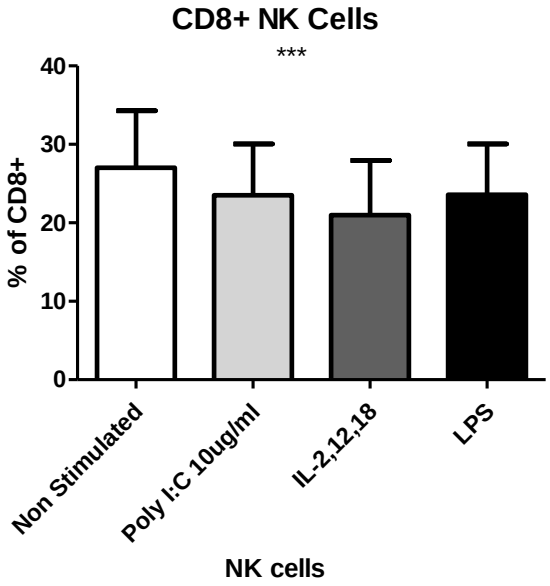


Figure 12 - Analysis of CD8+ subpopulation of NK cells with different stimuli.

(A) Non stimulated samples, (B) stimulated with 10 µg/mL Poly (I:C), (C) stimulated with a cocktail of IL-2, IL-12 and IL-18, 100 ng/mL each and (D) 100 ng/mL lipopolysaccharides (n=12). Cells were incubated for 24h at 37ºC. Cell expression of CD8 was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. One-way ANOVA showed significant differences among the data set ($p<0.001$) though Tukey's post-test did not reveal significances between each column.

Taking in account the results shown in Figure 12, it is possible to observe slight overall differences in the percentage of $CD8^+$ NK cells when cells are subjected to different stimulus however there are no differences when each stimuli is compared with the others.

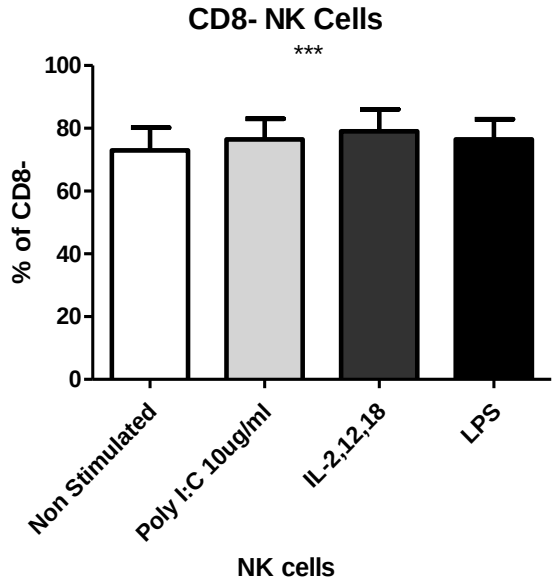


Figure 13 - Analysis of CD8- subpopulation of NK cells with different stimuli in Healthy Individuals.

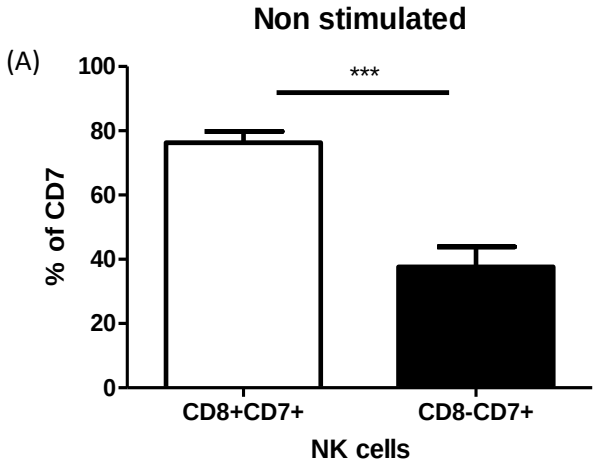
In (A) Non stimulated samples, (B) stimulated with 10 μg/mL Poly (I:C), (C) stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and (D) 100 ng/mL lipopolysaccharides (n=12). Cells were incubated for 24h at 37ºC. Cell expression of CD8 was assessed by flow cytometry and NK cells were selected as CD3, CD 4 and CD19 negative population. One-way ANOVA showed significant differences among the data set ($p<0.001$) though Tukey's post-test did not reveal significances between each column.

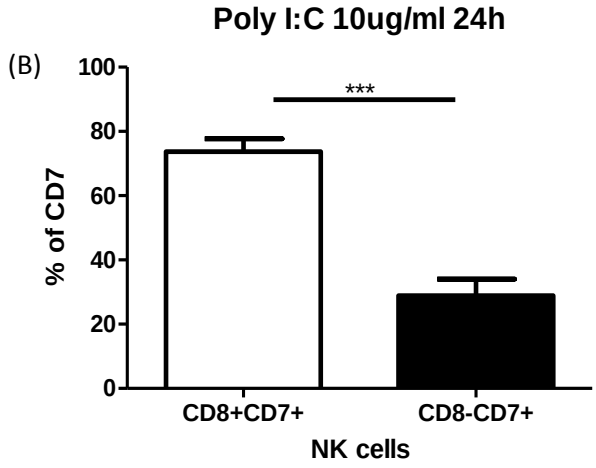
As it occurs for the $CD8^+$ subset, by the observation of Figure 13, it is possible to perceive the same tendency for the CD8- subpopulation. Minor changes are observed in the percentage of CD8- NK cells in the presence of different NK cells stimuli.

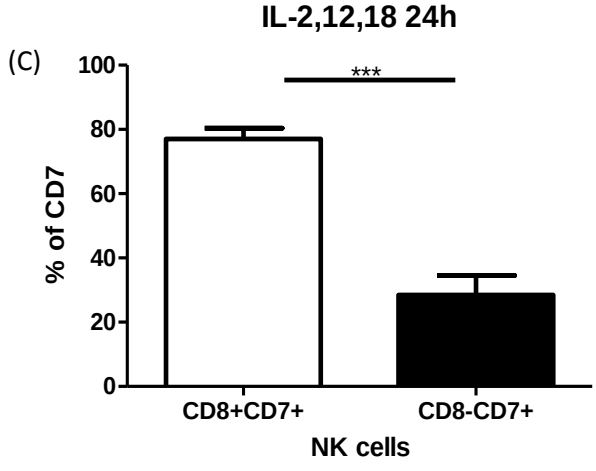
CD8+ NK cells subset show an higher expression of CD7

The expression of CD7 has been related to the production of effector molecules by NK cells (Milush, Long et al. 2009). Therefore, a differential expression in CD8

subsets can highlight for their functional role and some of their properties. Also, possible differences due to different stimuli were tested.







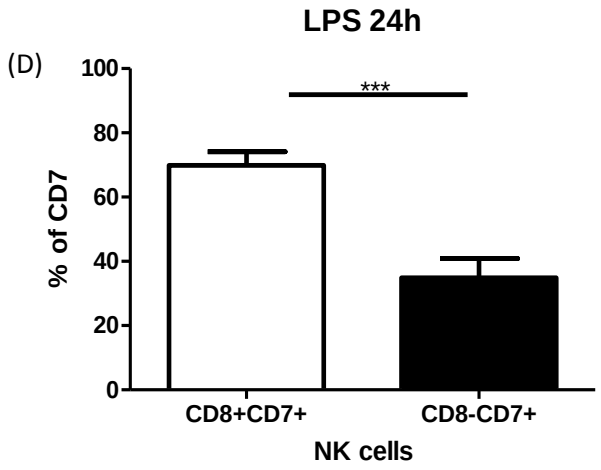


Figure 14 - Analysis of the relative expression of CD7 by $CD8^+$ and $CD8^-$ NK cells in Healthy Individuals.

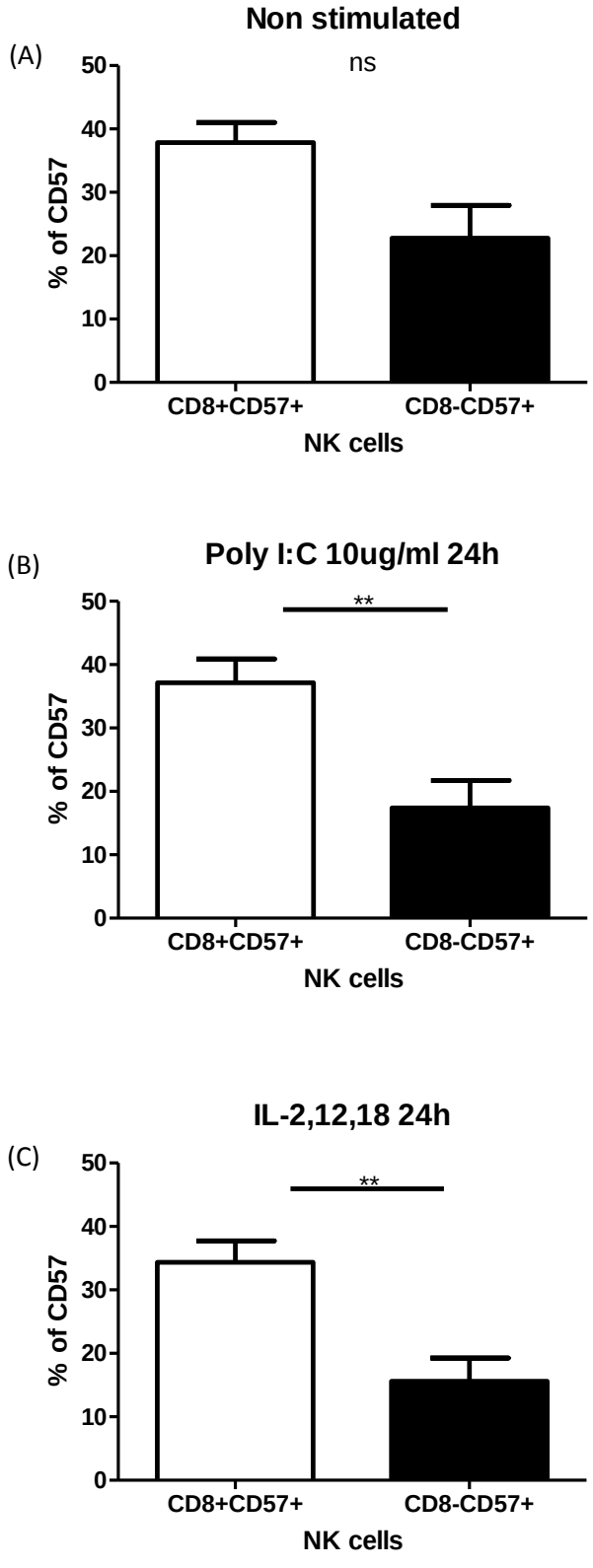
Showed in (A) Non stimulated samples, (B) stimulated with 10 µg/mL Poly (I:C), (C) stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and (D) 100 ng/mL lipopolysaccharides (n=12). Cells were incubated for 24h at 37ºC. Cell expression of CD8/CD7 was assessed by flow cytometry and NK cells were selected as CD3, CD 4 and CD19 negative population. One-way ANOVA showed significant differences among the data set ($p<0.001$) though Tukey's post-test did not reveal significances between each column.

Regarding Figure 14, it is clear that the $CD8^+$ subset has an higher expression of CD7 and that this relative expression is not altered when cells undergo different conditions. These data suggests that $CD8^+$ NK cells might be an effector population.

$CD8^+$ NK Cells represent a more mature subset

CD57 expression has been considered as a maturity marker, i.e., as a marker of terminal differentiation and possibly a senescence indicator (Sze, Giesajtis et al. 2001). Its expression has been reported to increase with age and associated with some clinical

conditions. Additionally, new-born NK cells lack this receptor (Nielsen, White et al. 2013).



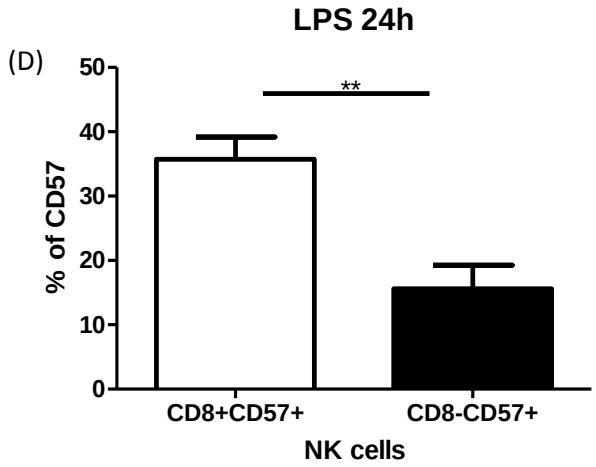


Figure 15 - Analysis of the relative expression of CD57 by $CD8^+$ and $CD8^-$ NK cells in Healthy Individuals.

Illustrated in (A) Non stimulated samples, (B) stimulated with 10 µg/mL Poly (I:C), (C) stimulated with a cocktail of IL-2, IL-12 and IL-18, 100 ng/mL each and (D) 100 ng/mL lipopolysaccharides (n=12). Cells were incubated for 24h at 37ºC. Cell expression of CD8/CD57 was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney analysis showed consistent significances in the expression of CD57 among $CD8^+$ and $CD8^-$ NK cells ($p<0.01$).

From the observation of Figures 15, we are able to say that $CD8^+$ NK cells may be a more mature subset, and even though the cell stimulation conditions, these stimuli were not effective in promote the maturation of the $CD8^-$ NK subset.

NK cells regulate the expression of CD137 according to stimuli but express few or none CD137L

The targeting of immunomodulatory mAbs to promote anti-tumour activity can be the next step in immunotherapy. One example of this strategy might be the use of CD137 (4-1BB). CD137 is an inducible T-lymphocyte surface molecule belonging to the Tumour Necrosis Factor Family. Its biding to a ligand promotes the co-stimulation

of CD4 and CD8 T lymphocytes (Simeone and Ascierto 2012). CD137 is expressed by a variety of cells, including NK cells, and it is believed to modulate an adaptive response against cancer cells. This molecule can be revealed as an high priority in the modulation of T cells using innate cells in immunotherapy (Lin, Liu et al. 2010).

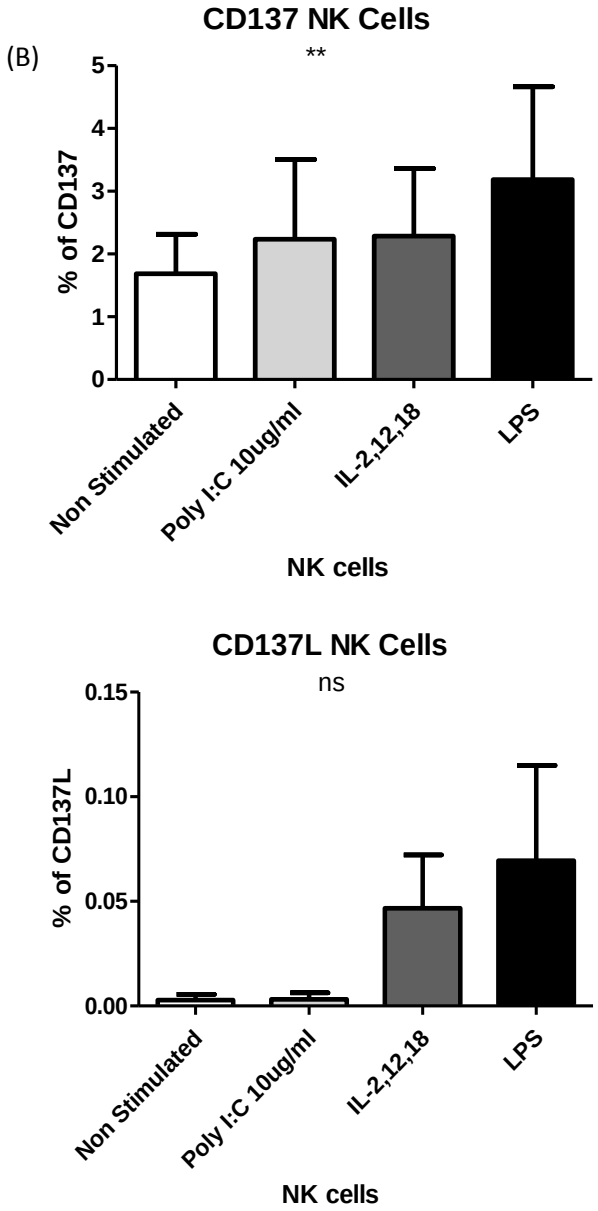


Figure 16 – Analysis of the expression of CD137 (A) and CD137L (B) in Healthy Individuals NK cells that have undergone different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL LPS (n=12)).

Cells were incubated for 24h at 37ºC. Cell expression of CD137/CD137L was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. One-way ANOVA revealed significances among the different conditions groups ($p<0.01$) in (A) whereas in (B) no differences were found.

From the observation of Figure 16 it is possible to conclude that the expression of CD137 and its ligand may be induce upon stimulation, even though the lack of significance for the expression of CD137L, it is shown that an overall change occurs in the expression of CD137 and stimuli with LPS seems to be the more effective.

CD8+ NK cells express none or few TGFβ as well as IL-10

IL-10 and TGFβ, as immunosuppressive cytokines are very important immunoregulatory players in a wide range of diseases including cancer. The production of these cytokines my lead to a suppression of adaptive immune response or to a skew toward a Th2 response, which is significantly less effective in tumour elimination (Zagury and Gallo 2004; Cheng, Chen et al. 2013). In this way, understanding the

producer population of these molecules will impact therapy strategies.

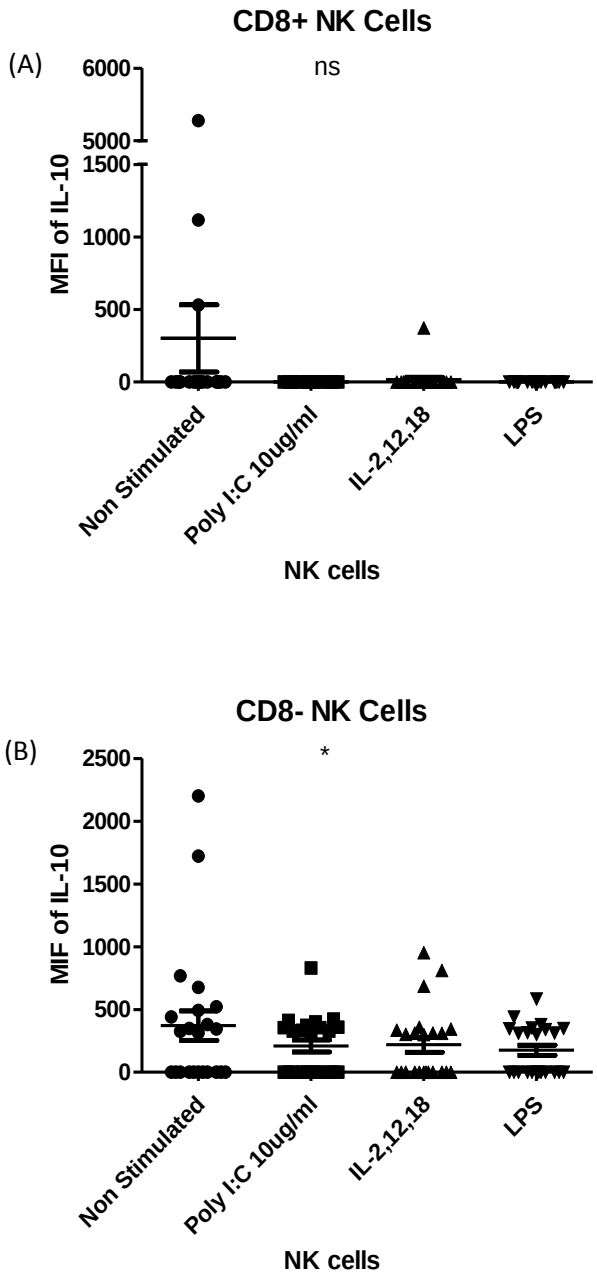


Figure 17 – Median Fluorescence Intensity (MFI) of IL-10 in $CD8^+$ (A) and $CD8^-$ (B), in Healthy Individuals NK cells that have undergone different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL LPS (n=23)).

Cells were incubated for 24h at 37ºC. Cell expression of CD8 and production of IL-10 was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. One-way ANOVA revealed significances among the different conditions groups in (B) ($p<0.01$), whereas in (A) no differences were found.

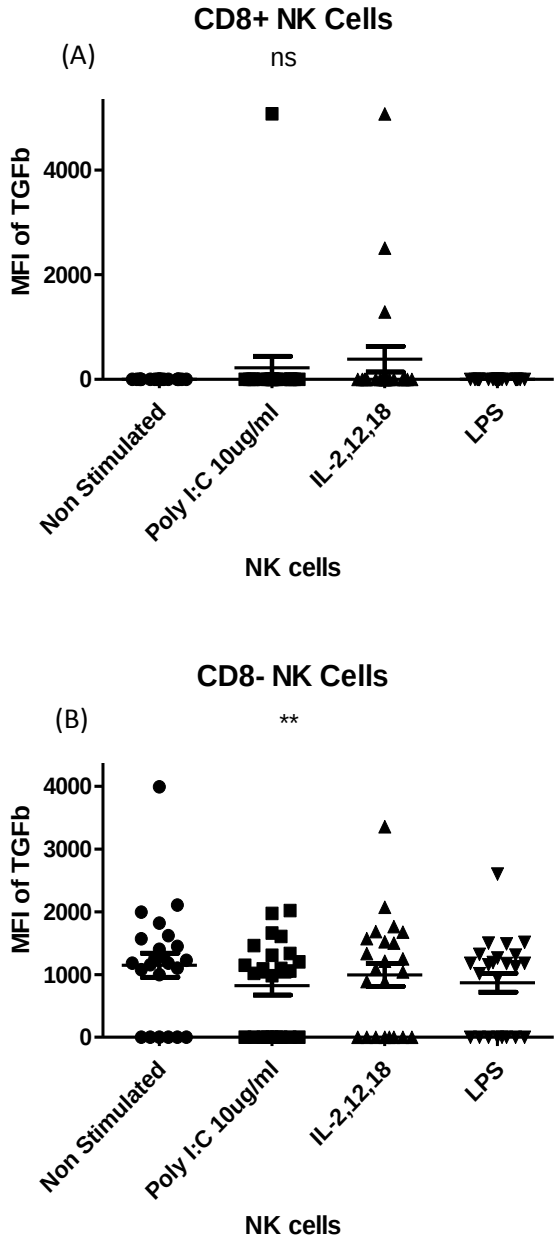


Figure 18 – Median Fluorescence Intensity of TGFβ in $CD8^+$ (A) and $CD8^-$ (B) NK cells subsets in Healthy Individuals samples that have undergone different stimuli (Non stimulated samples, stimulated with 10 μg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL LPS (n=23)).

Cells were incubated for 24h at 37ºC. Expression of TGFβ was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. One-way ANOVA revealed significances among the different conditions groups ($p<0.01$) in (B) whereas in (A) no differences were found.

Giving the graphs from Figure 17, $CD8^-$ NK cells are able to produce more IL-10 than $CD8^+$ subpopulation. Regarding the production of TGF-β (Figure 18), once again, $CD8^-$ subset shows a greater production comparatively to their equivalent $CD8^+$ subset.

The observation of this figure suggests that the $CD8^-$ population may have a more regulatory function as their equivalent $CD8^+$.

CD8+ NK cells tend to produce more Granzyme B than CD8- NK cells

As a cytotoxic granule, Granzyme B is a very important component of the killing machinery of NK cells.

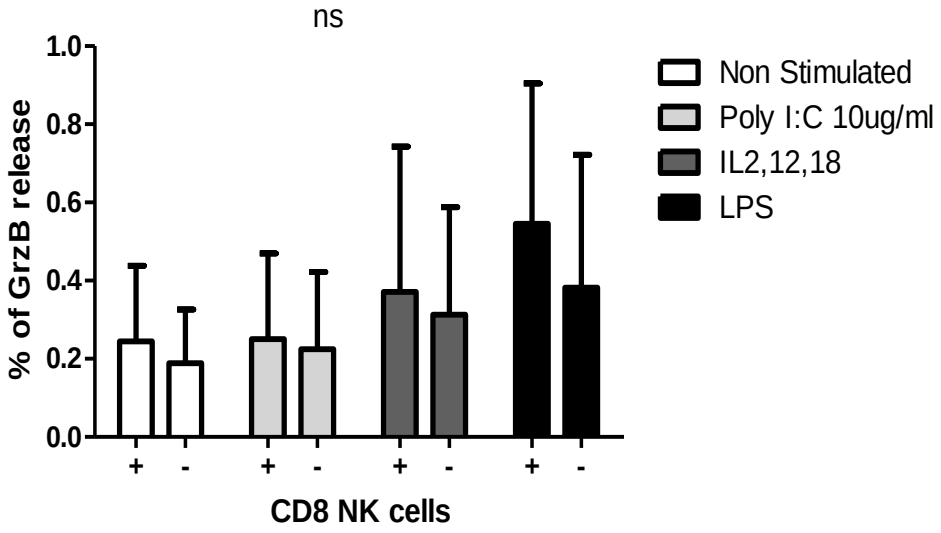


Figure 19 – Analysis of Granzyme B production by $CD8^+$ (A) and $CD8^-$ (B) NK cells when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL LPS (n=23)).

Cells were incubated for 24h at 37ºC. Expression of Granzyme B release was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. One-way ANOVA revealed no significant differences.

The observation of Figure 19 shows that even there were no statistical differences, CD8+ subsets is there more effective regarding to the production of Granzyme B. All the stimuli revealed to increase the production of Granzyme B but this production is even more effective when cells undergone a stimulus with a cocktail of cytokines and with LPS.

Cytotoxicity Assay

In this assay, the effector skills of NK cells were assessed by the labelling of effector granules that are released at time of encounter with a target cell. Here, K562 cell line (Leukaemia cells) was used.

By the time degranulation occurs, secretory lysosomes are released. The lysosome associated membrane protein-1 (LAMP-1 or CD107a) is transported to the surface of NK cells. In this way, CD107a antibodies enable the identification of NK cells that have undergone activation for degranulation (Aktas, Kucuksezer et al. 2009).

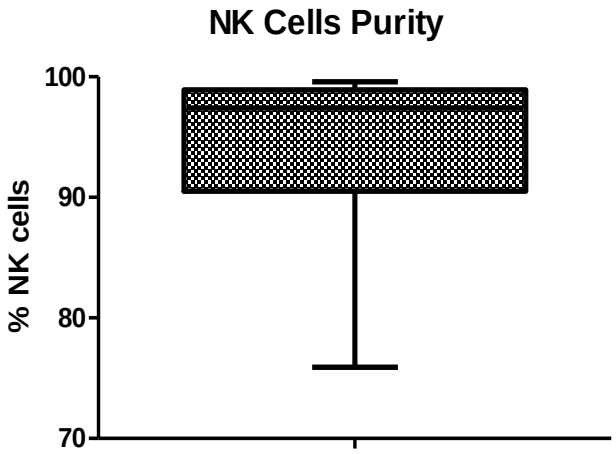


Figure 20 – Analysis of NK cells purity after manual Magnetic Isolation from PBMCs (n=13).

NK cells were isolated from PBMCs with a mean purity of 93.9% of total lymphocytes.

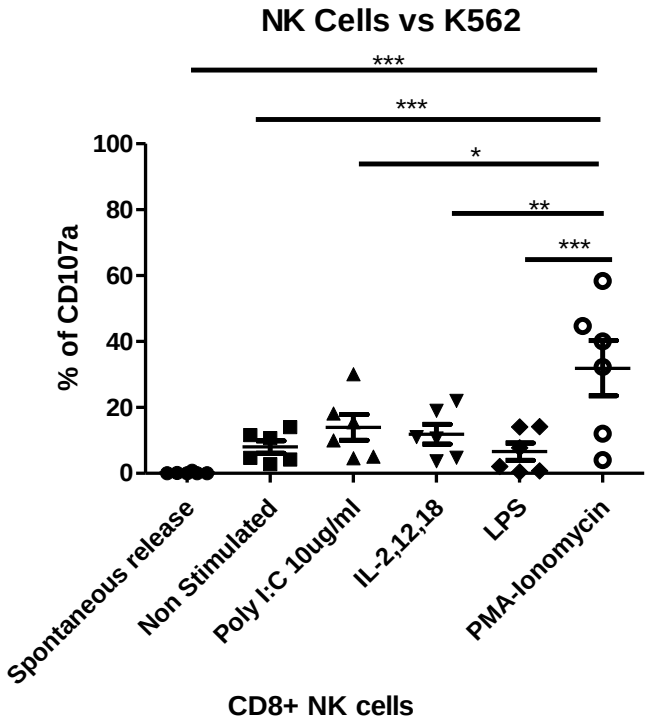


Figure 21 – Comparison between CD107a spontaneous release by healthy donors NK cells and release promoted by the interaction of NK cells with target cells in $CD8^+$ subset when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated

with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^{-}CD56^{+}$ population. One-way ANOVA revealed significant differences among all the groups when compared with PMA-Ionomycin.

In this assay the spontaneous release was assessed (last column of Figure 21). Since the spontaneous release was close to zero (~0,31%), this column was unvalued. Stimulus with PMA-Ionomycin was used as positive control.

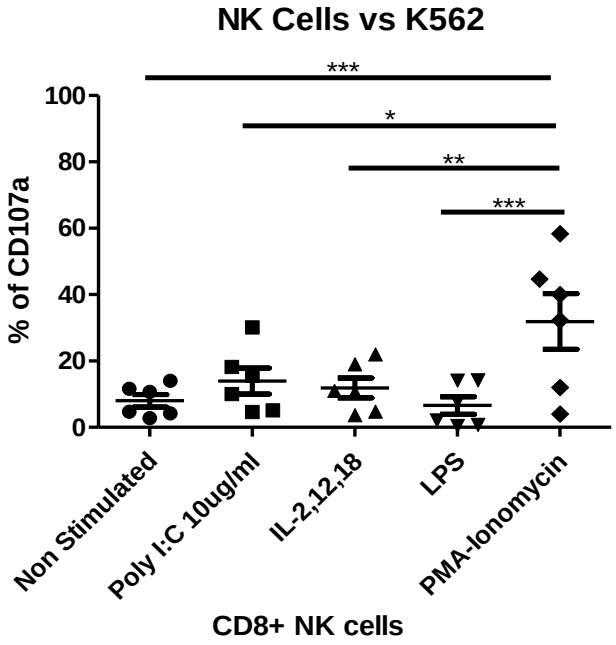


Figure 22 – Comparison between CD107a spontaneous release by healthy donors NK cells and release promoted by the interaction of NK cells with target cells in $CD8^{+}$ subset when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^{-}CD56^{+}$ population. One-way ANOVA revealed significances with $p<0.001$ for PMA-Ionomycin vs Non Stimulate and LPS; significances with $p<0.01$ for PMA-Ionomycin vs IL-2, IL-12,IL-18 and significances with $p<0.05$ for PMA-Ionomycin vs Poly (I:C) 10µg/ml.

By the observation of Figure 22 it is possible to conclude that the stimulus with LPS was ineffective in the promotion of cell cytotoxicity, showing a similar effect as the unstimulated cells. Stimuli with a cocktail of cytokines and Poly (I:C) revealed to be the more effective since they produce more approximate results as the positive control, which reveals a potentiated effect.

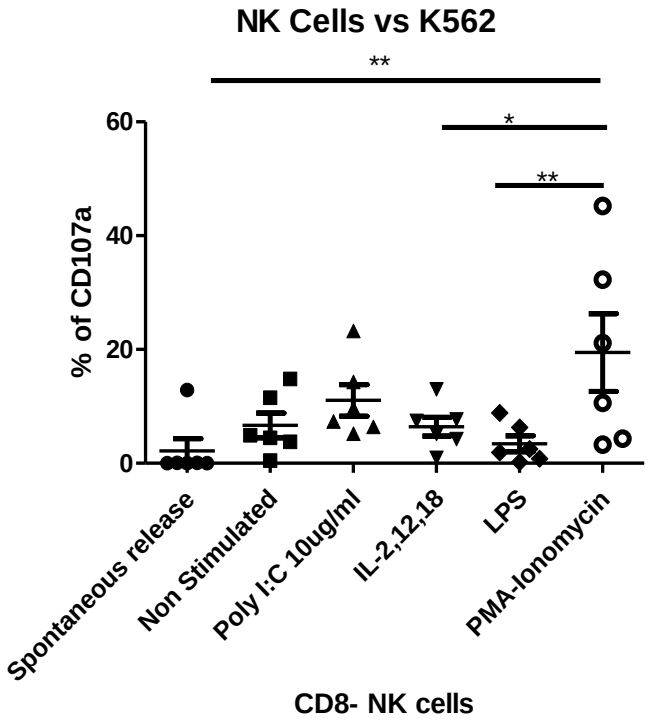


Figure 23 - Comparison between CD107a spontaneous release by healthy donors NK cells and release promoted by the interaction of NK cells with target cells in $CD8^-$ subset when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100ng/mL LPS and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^-CD56^+$ population. One-way ANOVA revealed differences with $p<0.01$ for PMA-Ionomycin vs Spontaneous Release and LPS and significances with $p<0.05$ for PMA-Ionomycin vs IL-2, IL-12, IL-18.

Once again, spontaneous release was assessed for $CD8^-$ subpopulation, although in this case the spontaneous release was greater (~4.3%) it was still unvalued.

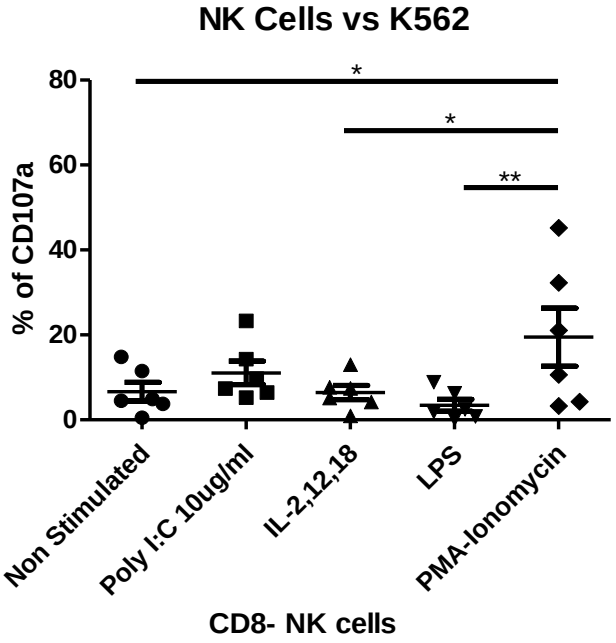


Figure 24 - Comparison between CD107a spontaneous release by healthy donors NK cells and release promoted by the interaction of NK cells with target cells in $CD8^-$ subset when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^-CD56^+$ population. One-way ANOVA revealed differences with $p<0.01$ for PMA-Ionomycin vs LPS and significances with $p<0.05$ for PMA-Ionomycin vs IL-2, IL-12, IL-18 and Non Stimulated.

In Figure 24 it is possible to comprehend that $CD8^-$ cells do not respond as effectively to stimuli, in particular to LPS, that shows the higher statistical difference comparatively to PMA-Ionomycin (Positive Control).

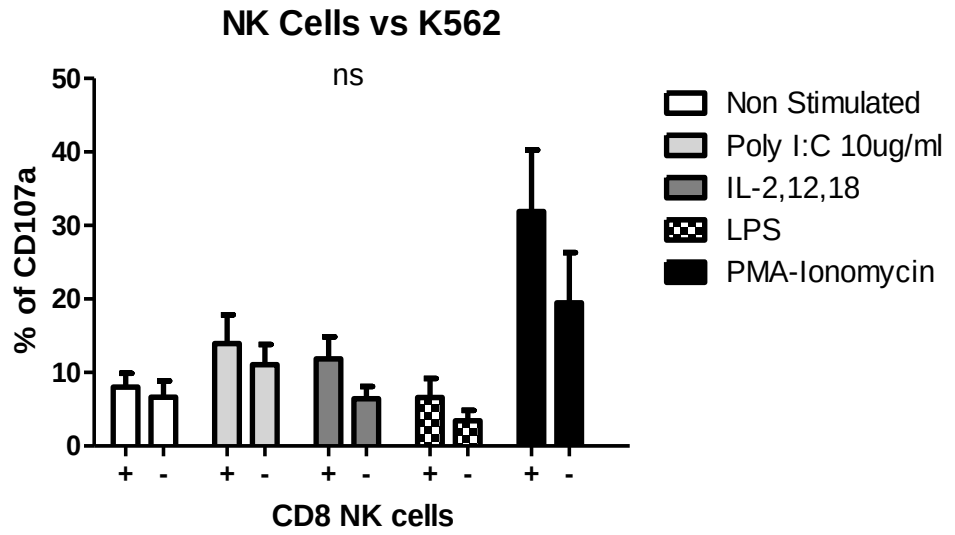


Figure 25 – Comparison of CD107a release in the two CD8 subsets of NK cells exposed to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^{-}CD56^{+}$ population. Two-way ANOVA revealed no differences within the different groups.

Comparing the percentage of CD107a labelling, once more $CD8^{+}$ NK cells revealed, although not statistically significant, a greater labelling than their equivalent $CD8^{-}$, being the stimuli with Poly (I:C) and the cocktail of cytokines the most effectives.

As mentioned before, NK cells recognize and kill virus-infected and/or transformed cells, through two main pathways, being one of them the release of cytotoxic granules to the surface of target cells (Cullen, Brunet et al. 2010). In this way, Granzyme B and Perforin production, as NK cells cytotoxic granules, was evaluated.

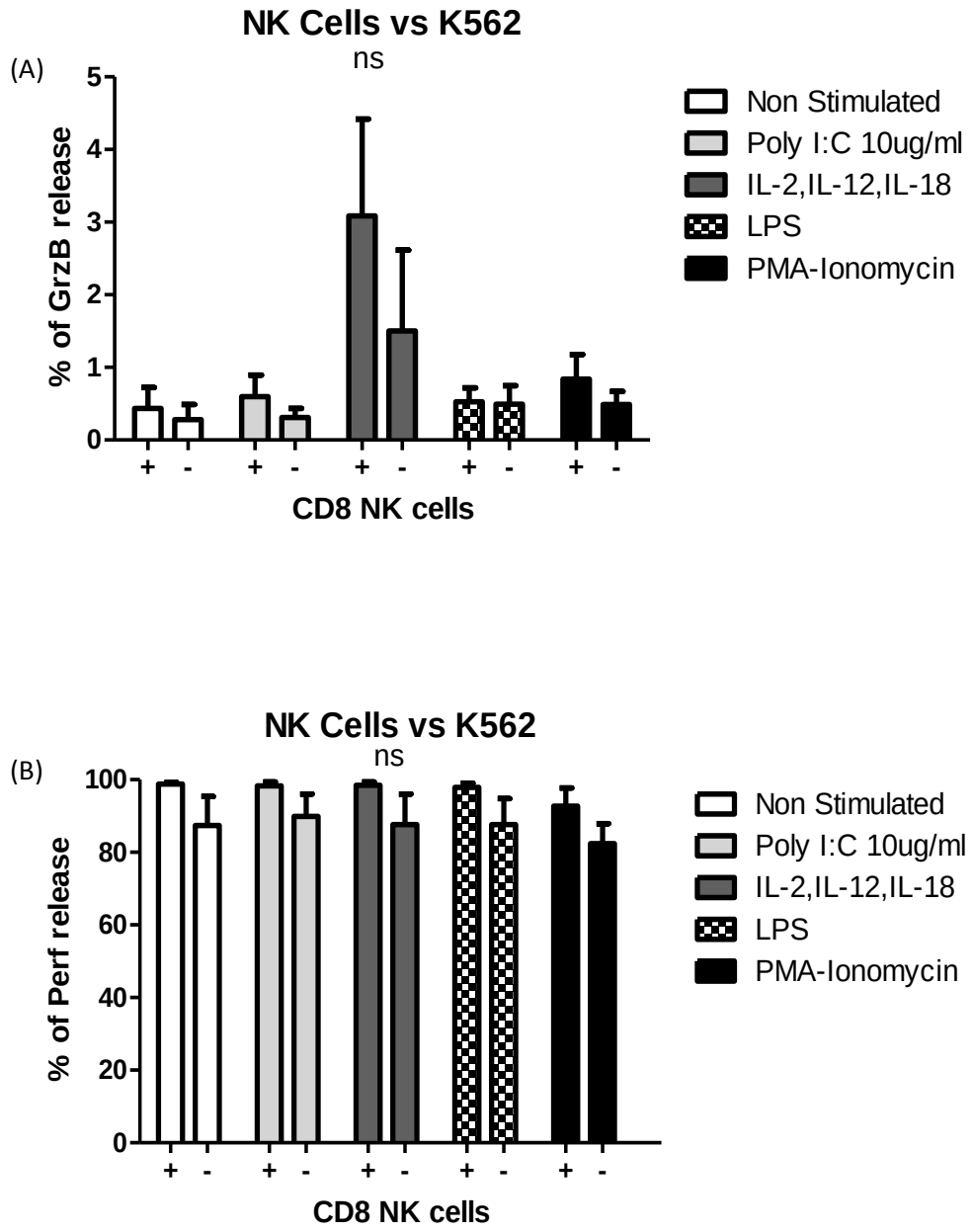
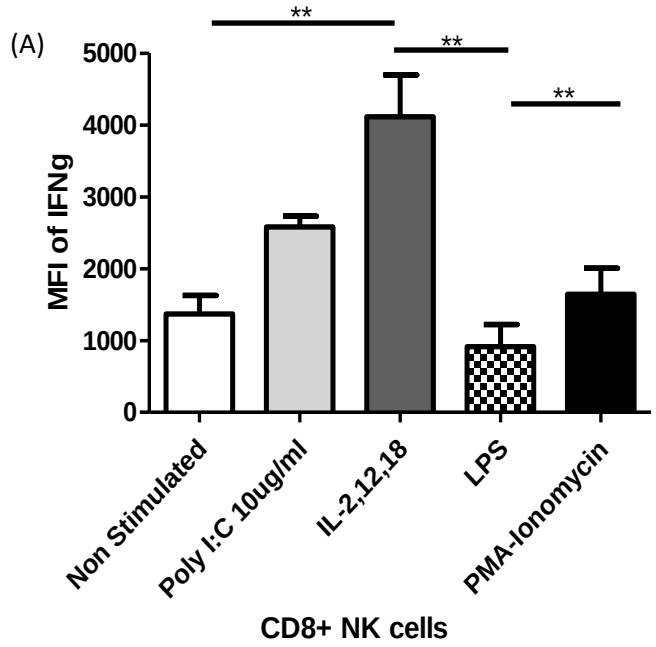


Figure 26 – Analysis of Granzyme B (A) and Perforin (B) release by $CD8^+$ and $CD8^-$ healthy donors NK cells with different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=2)).

Cells were incubated for 24h at 37ºC. CD107a release was assessed by flow cytometry and NK cells were selected as $CD3^-CD56^+$ population. Two-way ANOVA revealed no differences within the different groups.

By the observation of Figure 26, it is possible to determine that the majority of the released granules are Perforin granules and that this release is higher in $CD8^+$, although sample size is reduced and additional assays are essential.

Stimuli with cytokines is the more effective in promoting IFN-γ production



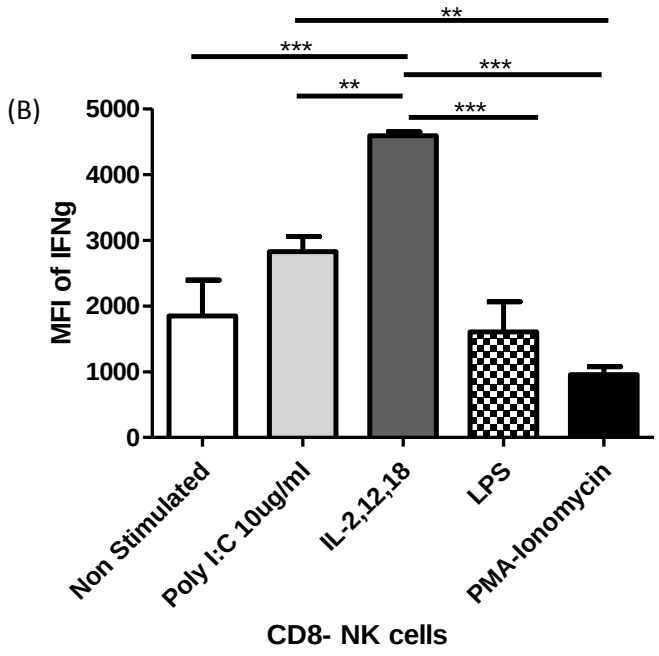


Figure 27 - Analysis of Interferon-γ production by $CD8^+$ (A) and $CD8^-$ (B) NK cells when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=4)).

Cells were incubated for 24h at 37ºC. The expression Interferon-γ production was assessed by flow cytometry and NK cells were selected as $CD3^-CD56^+$ population. One-way ANOVA revealed in (A) significant values for Non Stimulated vs IL-2, IL-12, IL-18 ($p<0.01$), IL-2, IL-12, IL-18 vs LPS ($p<0.01$) and LPS vs PMA-Ionomycin ($p<0.01$). In (B) $p<0.01$ values were found for 10µg/mL Poly (I:C) vs IL-2, IL-12, IL-18 and 10µg/mL Poly (I:C) vs PMA-Ionomycin ; $p<0.001$ values were found for Non Stimulated vs IL-2, IL-12, IL-18; IL-2, IL-12, IL-18 vs LPS and IL-2, IL-12, IL-18 vs PMA-Ionomycin 0.5µg/mL.

Observing Figure 27 it is possible to assess that there are significant differences among the outcome regarding IFN-γ production with different stimuli but no differences were found regarding the comparison between the $CD8^+$ and $CD8^-$ subpopulations of NK cells being both effective in the secretion of this cytokine, mainly when stimulated with a combination of IL-2, IL-12 and IL-18.

Umbilical Cord Blood NK cells

The phenotypic characteristics of NK cells from umbilical cord blood and peripheral blood are similar. Cells from both sources reveal analogous percentages of bright and dim subpopulations (Dalle, Menezes et al. 2005) but some other features, such as the expression of some NK receptors, for instance CD94, NKp46 and NKG2D differ (Verneris and Miller 2009).

The use of umbilical cord blood hematopoietic stem cells has revealed itself in a very exciting approach and many progresses have occurred in recent years (Oran and Shpall 2012). The use of cord blood have shown a growing interest since it includes a rapid availability and less HLA matching requirements among other advantages (Verneris and Miller 2009).

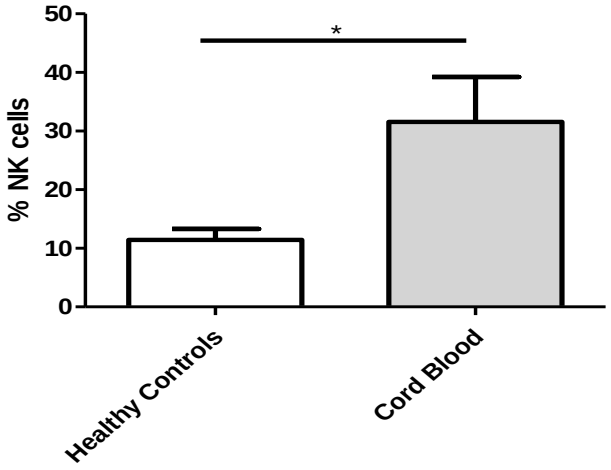


Figure 28 – Analysis of NK cells percentage among Healthy donors (n=7) and Umbilical Cord Blood (n=5).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.05$).

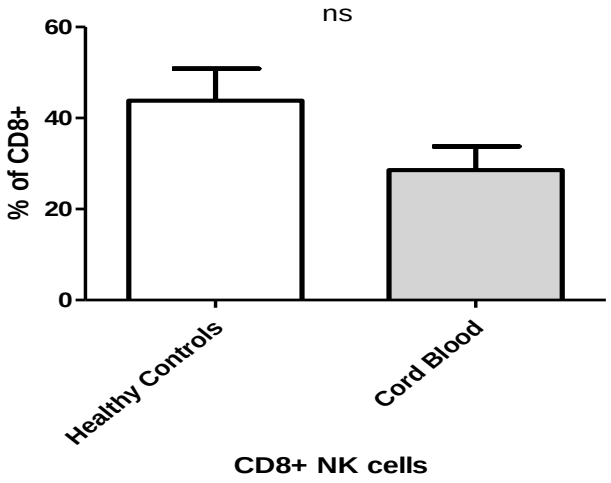


Figure 29 - Analysis of $CD8^+$ NK cells percentage among Healthy donors (n=7) and Umbilical Cord Blood (n=5).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing no significant differences.

The expression of CD8 in cord blood appears to be influenced by stimuli

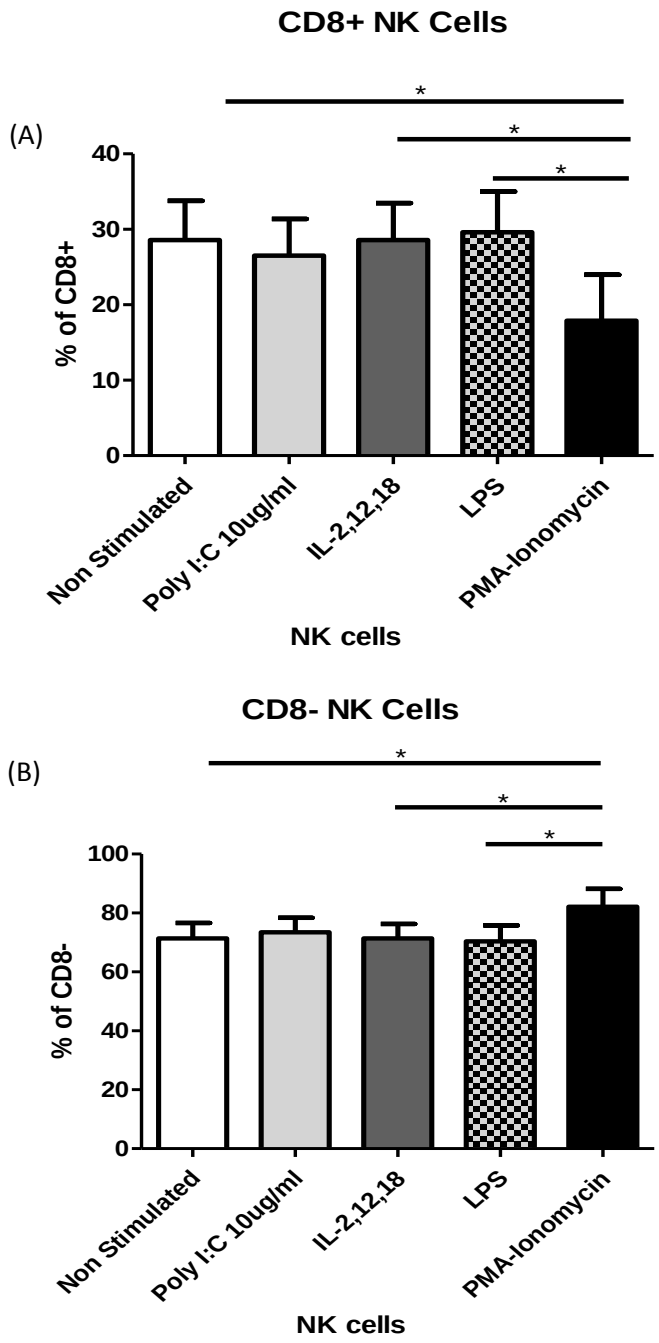


Figure 30 – Comparison of $CD8^+$ (A) and $CD8^-$ (B) NK cells percentages in Umbilical Cord Blood when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2,IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 4h at 37ºC. The expression CD8 was assessed by flow cytometry and NK cells were selected as $CD3^-CD14$ and CD19 negative population. One-way ANOVA revealed in (A) significant values for Non Stimulated PMA-Ionomycin ($p<0.05$), IL-2, IL-12,

IL-18 vs PMA-Ionomycin ($p<0.05$) and LPS vs PMA-Ionomycin ($p<0.05$). In (B) $p<0.05$ values were found for Non Stimulated PMA-Ionomycin; IL-2, IL-12, IL-18 vs PMA-Ionomycin and LPS vs PMA-Ionomycin.

From the observation of Figure 30 it is possible to perceive that the relative expression of CD8 is altered when cells undergo different conditions, for instance, $CD8^+$ subpopulation is diminished, and consequently, $CD8^-$ is augmented upon stimulus with PMA-Ionomycin

Umbilical Cord Blood NK cells may undergo maturation *in vitro*

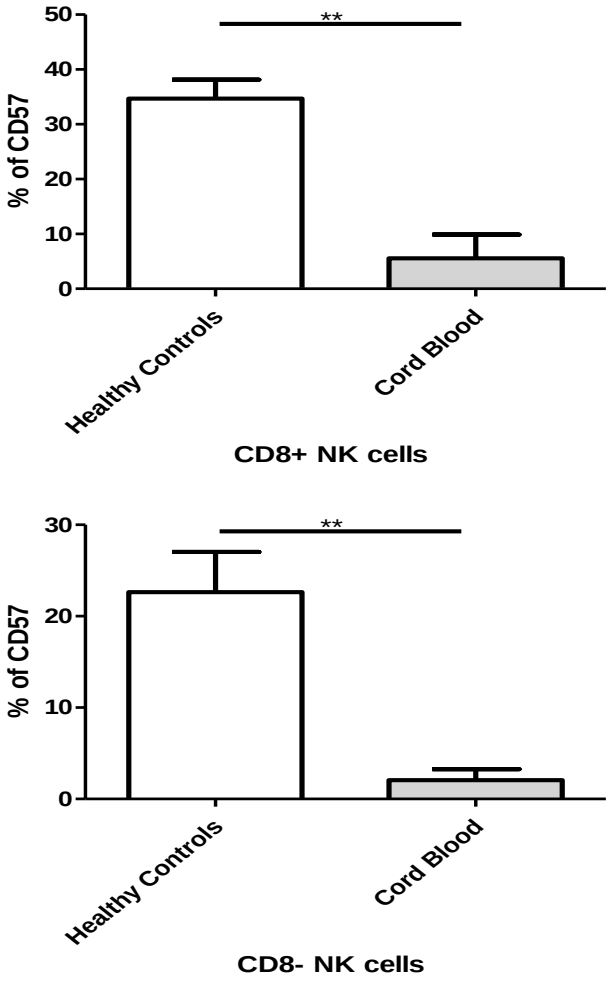
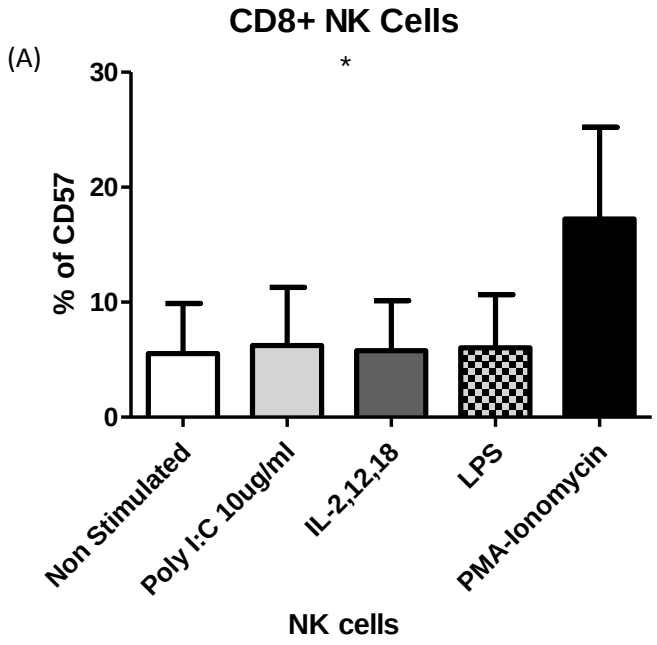


Figure 31– Analysis of the expression of CD57 in (A) $CD8^+$ NK cells and (B) $CD8^-$ NK cells in Healthy Individuals (n=14) and Umbilical Cord Blood (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.01$) for both $CD8^+$ and $CD8^-$.



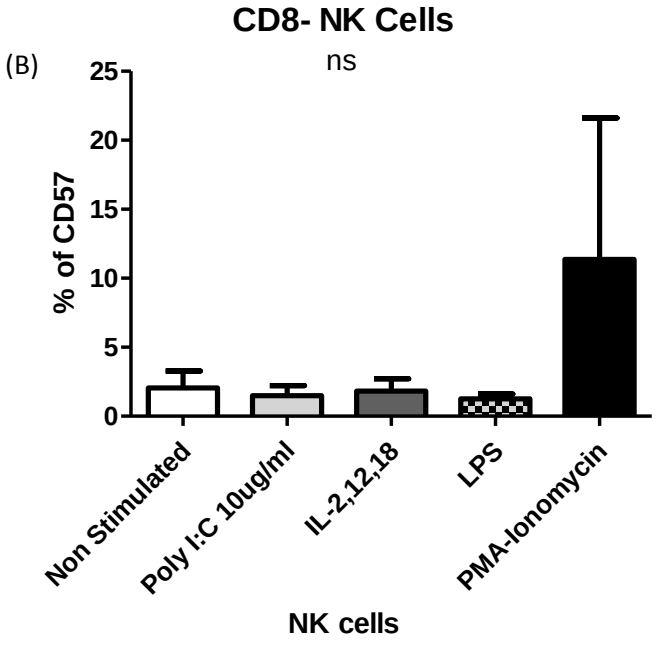


Figure 32 – Analysis of the expression of CD57 by $CD8^+$ (A) and $CD8^-$ (B) NK cells in Umbilical Cord Blood when subjected to different stimuli (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C), stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL lipopolysaccharides and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 4h at 37ºC. The expression CD8 and CD57 was assessed by flow cytometry and NK cells were selected as $CD3^-CD14$ and CD19 negative population. One-way ANOVA revealed in (A) significant overall differences ($p<0.05$), whereas in (B) no differences were found.

An overall maturation is apparent when cells undergo stimulus with PMA-Ionomycin even though in not statistically significant way for $CD8^-$ subset. Also in $CD8^+$ subset an increase in maturity is perceived, where although data is not significantly different in each condition, an overall significance occurs.

Umbilical Cord Blood cells are expected to be irresponsive to stimuli and to do not produce cytokines.

Umbilical Cord Blood NK cells produce few or none IL-10 upon stimulation

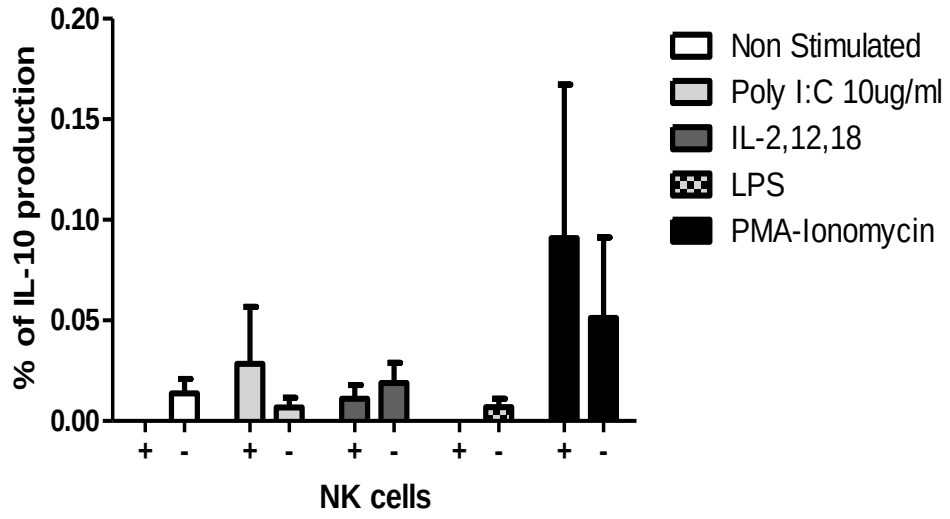


Figure 33 – Comparison of IL-10 production by $CD8^+$ and $CD8^-$ NK cells from Umbilical Cord Blood after stimulation (Non stimulated samples, stimulated with 10 µg/mL Poly (I:C),

stimulated with a cocktail IL-2, IL-12 and IL-18, 100 ng/mL each and 100 ng/mL LPS and PMA-Ionomycin 0.5 µg/mL (n=6)).

Cells were incubated for 4h at 37ºC. The expression CD8 was assessed by flow cytometry and NK cells were selected as CD3, CD14 and CD19 negative population. Two-way ANOVA revealed no significant differences among the conditions.

There were no significant differences among the several groups compared, of note that even though, when there is production of IL-10, $CD8^-$ NK cells produce more IL-10 than $CD8^+$ with the exception of stimuli with Poly (I:C) 10 µg/mL and PMA-Ionomycin which may imply that this subpopulation respond more promptly than their equivalent.

Chronic Myeloid Leukaemia NK cells

There is a wide array of NK cell dysfunctions reported in cancer. They include a decreased cytotoxic activity, down-regulation of activating receptors, poor proliferation, decreased cell count and defective cytokine production (Sabry and Lowdell 2013). Another reason to explain cancer spread is related to ability of some tumour cells to escape NK cells immunosurveillance wherein, in a first phase, tumour cells induce NK cell-tolerance and in a second phase they build up a microenvironment favourable to their development (Kiessling, Wasserman et al. 1999).

Chronic Myeloid Leukaemia (CML) is a haematological disorder resulting from the clonal expansion of pluripotent hematopoietic progenitors containing the active fusion gene BCR/ABL. This mutation displays a constitutive expression of tyrosine kinase activity conferring growth, proliferation and resistance to apoptosis advantages on leukemic cells.

NK cell frequency is increased in CML

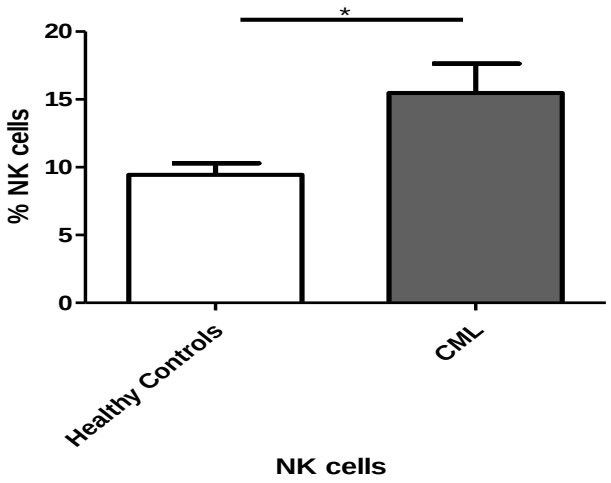


Figure 34 – Comparison among the percentage of NK cells in Healthy Individuals (n=25) and Chronic Myeloid Leukaemia (n=22).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.05$).

CD8 expression by NK cells is similar in health individuals and CML patients

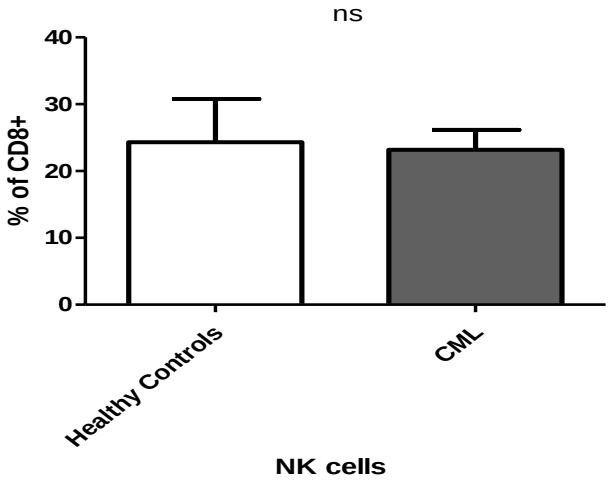


Figure 35 – Comparison among the percentage of $CD8^+$ NK cells in Healthy Individuals (n=25) and Chronic Myeloid Leukaemia patients (n=22).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing no differences between the two groups.

It is possible to observe a significant alteration on NK cells numbers among healthy individuals and CML patients whereas oncologic patients exhibit an increase on NK cells percentage (Figure 34) although no differences are observed regarding the percentage of $CD8^+$ NK cells (Figure 35).

CML $CD8^+$ NK cells show an increased expression of CD7

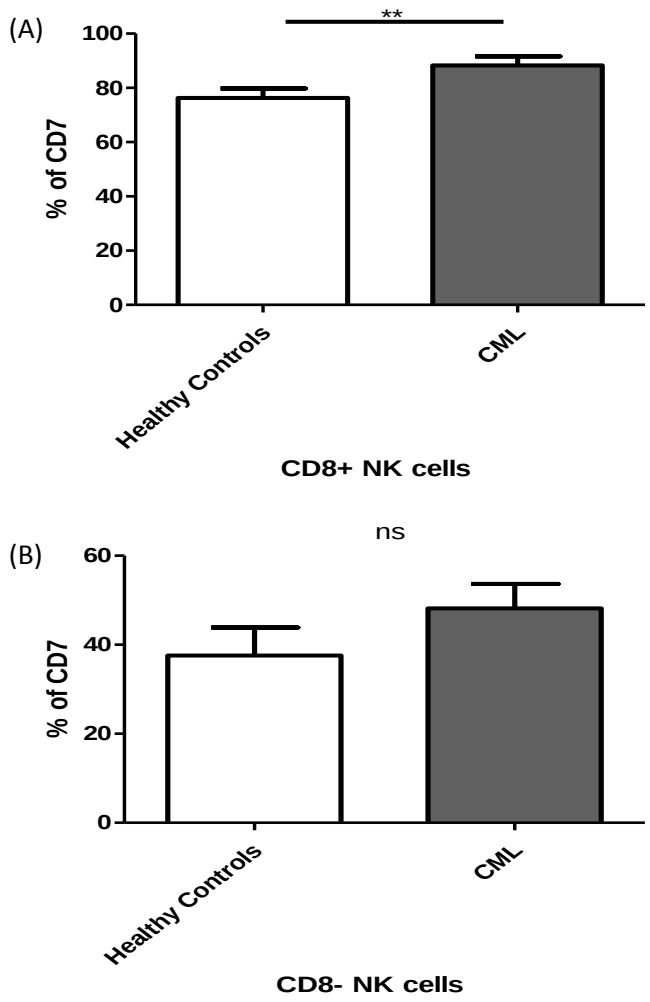
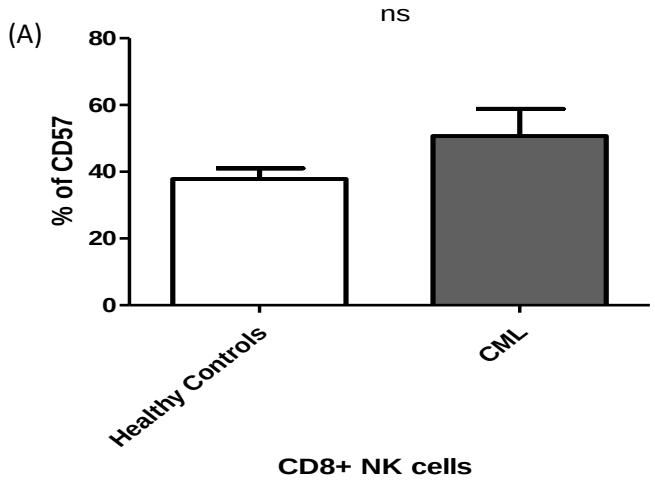


Figure 36 – Comparison of the percentage of NK cells $CD8^+CD7^+$ (A) and $CD8^-CD7^+$ among Healthy Individuals (n=25) and Chronic Myeloid Leukaemia patients (n=22).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was completed showing that the two groups are not significantly different.

No differences in the expression of CD7 were observed in CD8- NK cells however CD8+ NK cells from CML patients appear to be augmented when compared to healthy individuals.

CML NK cells do not show increased maturity



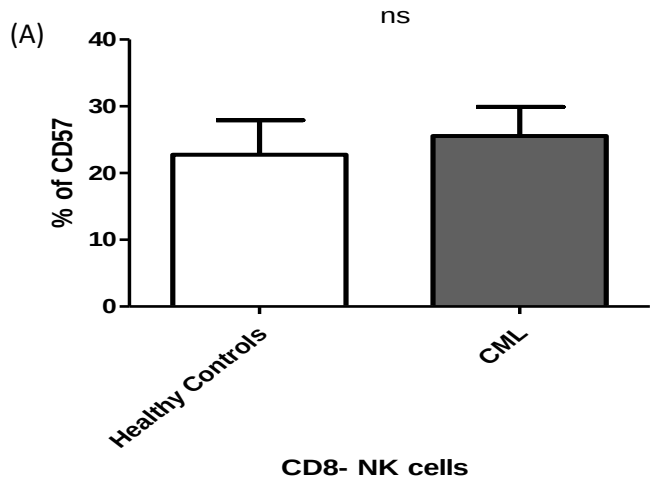


Figure 37 – Comparison of the percentage of NK cells $CD8^{+}CD57^{+}$ (A) and $CD8^{-}CD57^{+}$ among Healthy Individuals (n=25) and Chronic Myeloid Leukaemia patients (n=22).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was completed showing that the two groups are not significantly different.

Interestingly, the expression of CD57, a maturation marker, reveals that NK cells from CML patients and healthy individuals seem to be at a similar developmental stage, assuming very analogous levels of CD57.

Lung Cancer NK cells

As mentioned before, numerous alterations on NK cells can be listed on oncologic disorders. The opportunity to study NK cells in lung cancer patients allow the comparison of CD8 NK cells subpopulations and can represent a great value as NK cells rather than T lymphocytes are thought to constitute the main immunological barrier to the development of lung cancer (Chang, Lin et al. 2013).

Lung Cancer patients show an increase of NK cell frequency

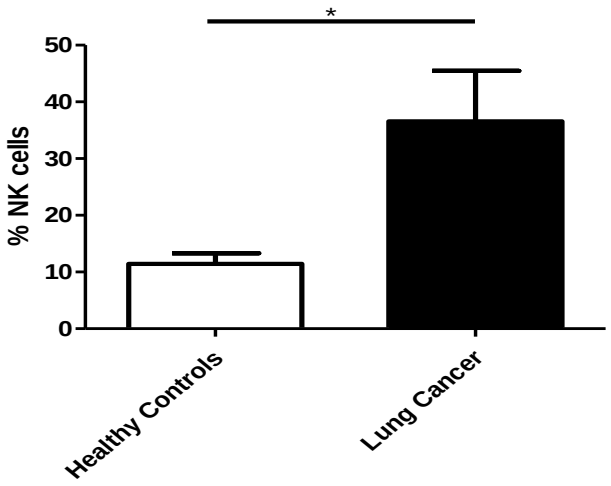


Figure 38 – Comparison among the percentage of NK cells in Healthy Individuals (n=7) and Lung Cancer patients (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.05$).

As shown above in Figure 38, lung cancer patients exhibit a significant increase in NK cell frequency when compared to healthy individuals.

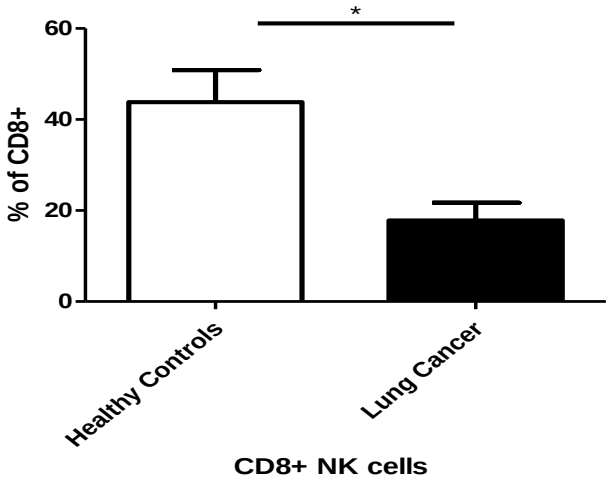


Figure 39 – Comparison among the percentage of $CD8^+$ NK cells in Healthy Individuals (n=7) and Lung Cancer patients (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.05$).

No significant alterations are found in the expression of CD7 in NK cell from lung cancer patients

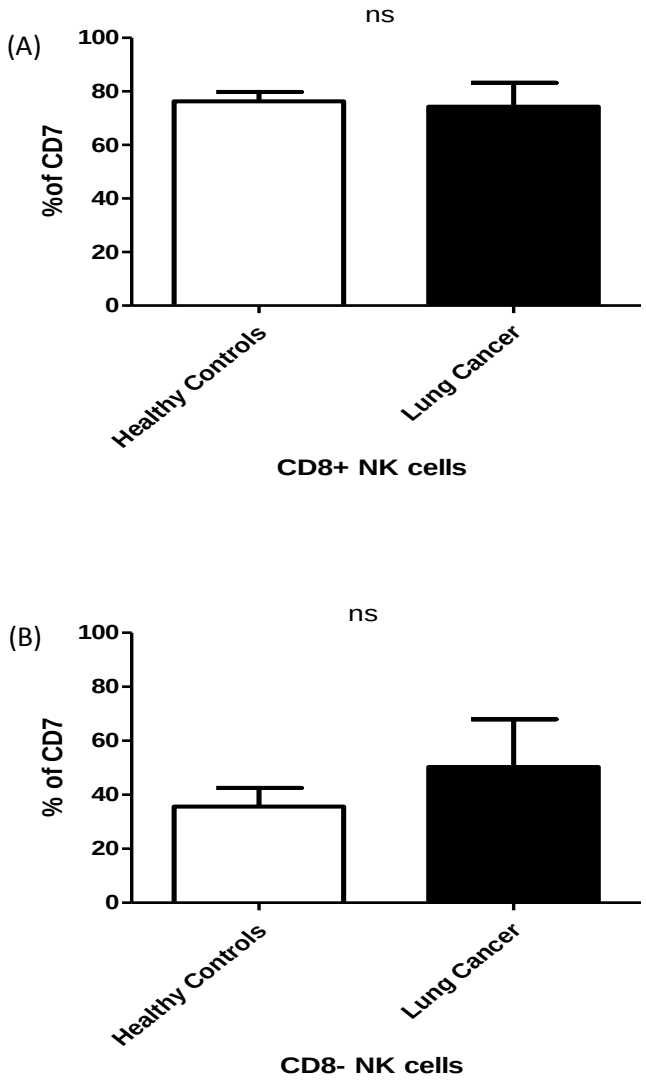


Figure 40 – Comparison of the percentage of NK cells $CD8^{+}CD7^{+}$ (A) and $CD8^{-}CD7^{+}$ among Healthy Individuals (n=14), Lung Cancer (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was completed showing that the two groups are not significantly different.

Once the expression of CD7 is greater in $CD8^+$ NK cells in healthy individuals, a comparison with the equivalent population in lung cancer appears imperative. As shown in Figure 40, lung cancer patients do not exhibit any changes in the expression of CD7. In this way, it is possible to admit that their capacity of secreting effector molecules is not affected.

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Lung Cancer patients have accumulation of end-stage NK cells

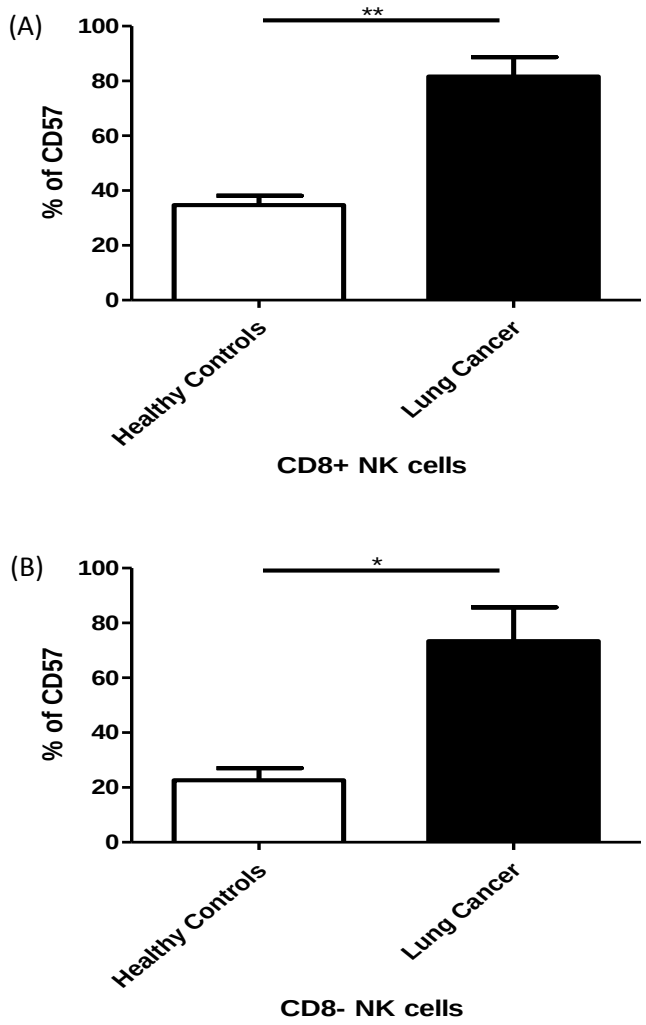

Figure 41– Analysis of the expression of CD57 in (A) $CD8^+$ NK cells and (B) $CD8^-$ NK cells in Healthy Individuals (n=14) and Lung Cancer patients (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are significantly different ($p<0.01$) for $CD8^+$ subpopulation and ($p<0.05$) for $CD8^-$ subpopulation.

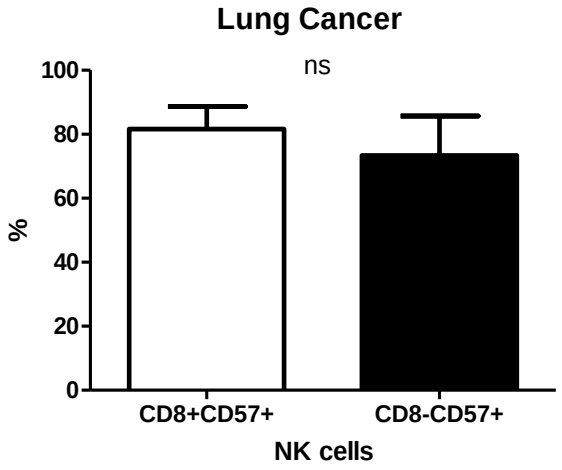

Figure 42– Analysis of the expression of CD57 in $CD8^+$ and $CD8^-$ NK cells in Lung Cancer patients (n=4).

Flow cytometry analysis was performed and NK cells were selected as the CD3, CD14 and CD19 negative population. Non-parametric Mann-Whitney test was performed showing that the two groups are not significantly different.

Comparing Figure 42 with Figure 15, we are able to affirm that both NK cells subpopulations from lung cancer patients are consider mature, opposed to what is observed in healthy individuals, where $CD8^-$ subpopulation express much less CD57 compared to its equivalent $CD8^+$.

Seeing the results shown in Figure 38 and 41, it is possible to say that Lung Cancer patients have an abnormal high number of NK cells, comparatively to healthy individuals although they have an high amount of CD57 in both the CD8 subsets

revealing that even though lung cancer patients have high numbers of NK cells, they belong to a very mature state which in accordance to results shown above, seem to have a more regulatory than effector function.

Invariant Natural Killer T cells and γδ T cells

As mentioned before, iNKT and ɣδ T cells are thought to be cells that are able to link innate and adaptive immunity, besides that fact, they are also able to inhibit tumour progression and development. Whereas iNKT achieve this task in a MHC class I dependent manner (Terabe and Berzofsky 2014), while ɣδ T cells achieve do not require the conventional help of MHC class I. These cells share common functional and phonotypical features of both innate and acquired immune system (Gogoi and Chiplunkar 2013; Wang and Welte 2013). For that reason, a characterization of these cell types as the one performed for NK cells may help in the understanding of their contributions in the elimination of malignancies.

iNKT cells in healthy donors and oncologic patients (CML and lung cancer)

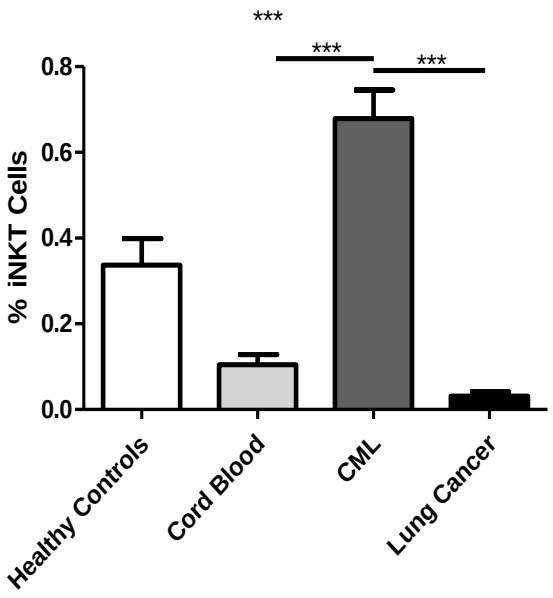

Figure 43 – Comparison of iNKT cells percentage among Healthy Individuals (n=7), Cord Blood (n=6), Chronic Myeloid Leukaemia (n=17) and Lung cancer (n=4).

The expression of TCR Vα24-Jα18 (iNKT) was assessed by flow cytometry and iNKT cells were selected as the $CD3^+$ $TCRV\alpha24\text{-}J\alpha18^+$ population. One-way ANOVA revealed no significant differences among cord blood and Healthy Individuals whereas significance was found among cord blood and CML and among CML and lung cancer ($p<0.001$). Overall differences in data were found ($p<0.01$).

A significant difference is shown in iNKT cell frequency among cord blood and CML patients and among CML patients and lung cancer patients. Cord blood and lung cancer patients show a decreased frequency in this cell type that may imply a deficit in communication between innate and acquired immune branches.

Lung cancer patients show impaired expression of CD8 in iNKT cells

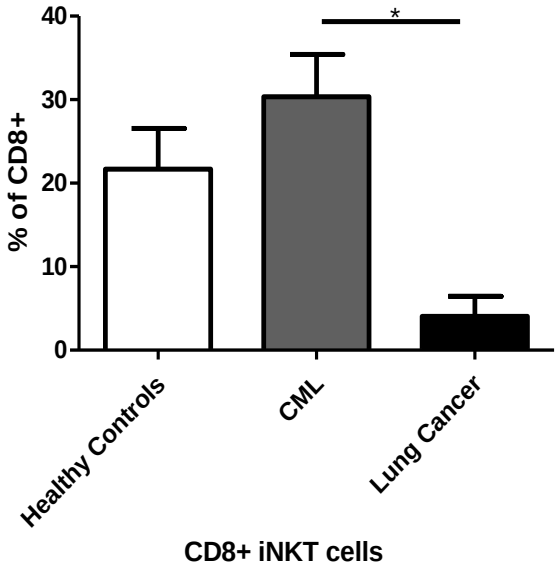

Figure 44 – Comparison of CD8+ iNKT cells in Healthy Individuals (n=14), Chronic Myeloid Leukaemia (n=17) and Lung cancer (n=4).

The expression of CD8 was assessed by flow cytometry and iNKT cells were selected as the TCR Vα24-Jα18 (iNKT cell) and CD3 positive population. One-way ANOVA revealed significant differences among CML and lung cancer ($p<0.05$).

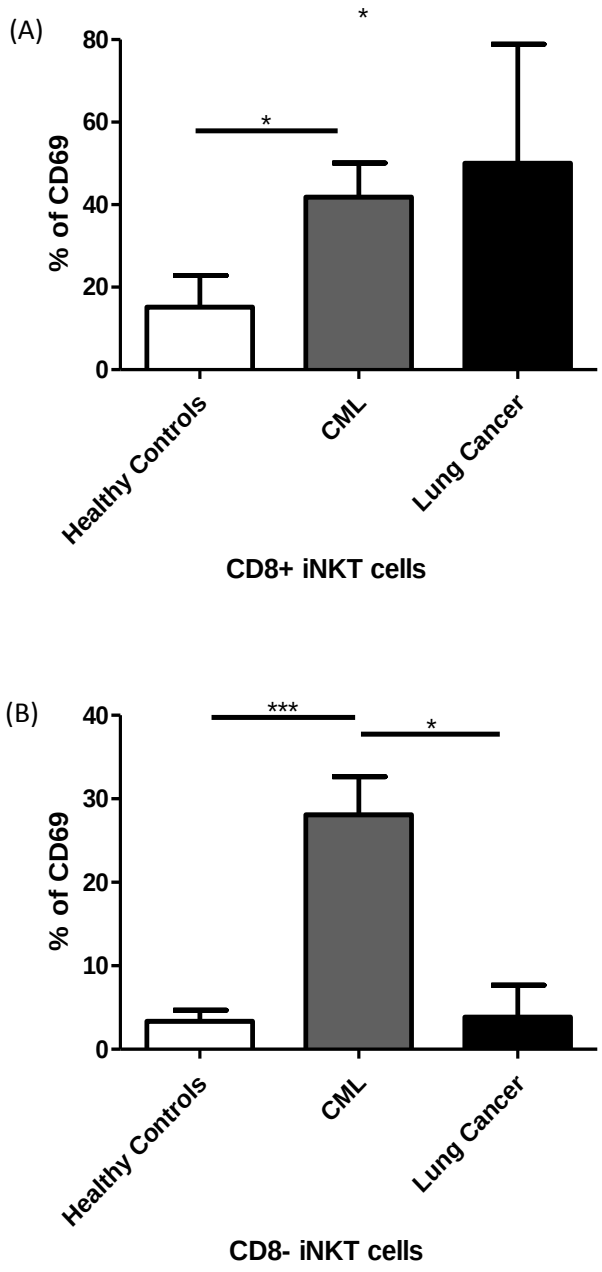

Figure 45 – Comparison of CD69 expression among Healthy Individuals (n=14), Chronic Myeloid Leukaemia (n=17) and Lung cancer (n=4) in $CD8^+$ (A) and $CD8^-$ (B) subpopulations.

The expression of CD69 was assessed by flow cytometry and iNKT cells were selected as the TCR Vα24-Jα18 (INKT cell) and CD3 positive population. Non-parametric Mann-Whitney test was performed, revealing no significant differences in (A) among Healthy Individuals and CML patients ($p<0.05$) and in (B) among Healthy Individuals and CML patients ($p<0.001$) and among CML and lung cancer patients ($p<0.05$). For $CD8^+$ subset, overall differences among data was found ($p<0.05$)

Similarly to the expression on healthy individuals, $CD8^+$ subpopulation in lung cancer patients, show a greater expression of this early activation marker (Figure 45 A) in comparison with its equivalent $CD8^-$ counterpart (Figure 45 B). In regard to CML patients, it is possible to detect that both CD8 subsets show an increased activation relatively to healthy individuals.

γδ T cells in healthy donors and oncologic patients (CML and lung cancer)

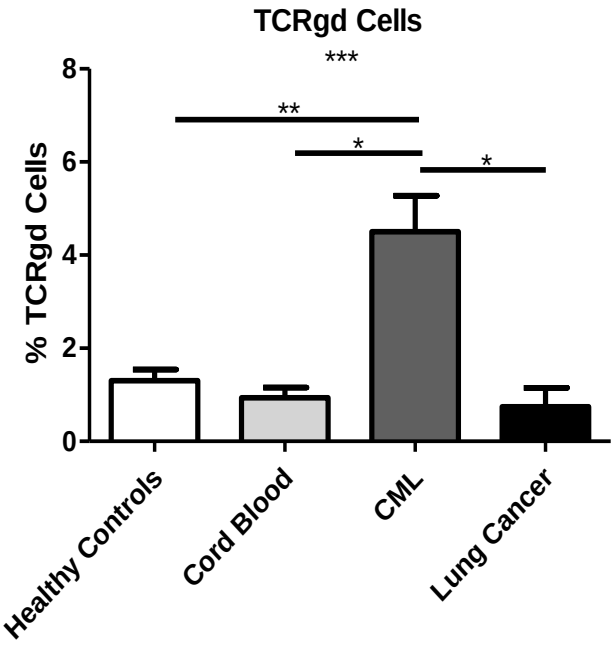

Figure 46 – Comparison of γδ T cells percentage among Healthy Individuals (n=17), Cord Blood (n=6), Chronic Myeloid Leukaemia (n=17) and Lung cancer (n=4).

The expression of TCR γδ was assessed by flow cytometry and γδ T cells were selected as the TCR γδ positive population. One-way ANOVA revealed significant differences among Healthy Individuals and CML patients ($p<0.01$), among cord blood and CML patients ($p<0.05$) and among CML patients and lung cancer patients ($p<0.05$). Overall differences among samples were found ($p<0.001$).

A diminished presence of this cell type (<10%) is in accordance with previously described (Gogoi and Chiplunkar 2013). Alterations in γδ T cells counts were observed

in CML patients. Whereas healthy individuals, cord blood and lung cancer patients show similar percentage of TCRγδ cells, CML patients exhibit differences with these three groups.

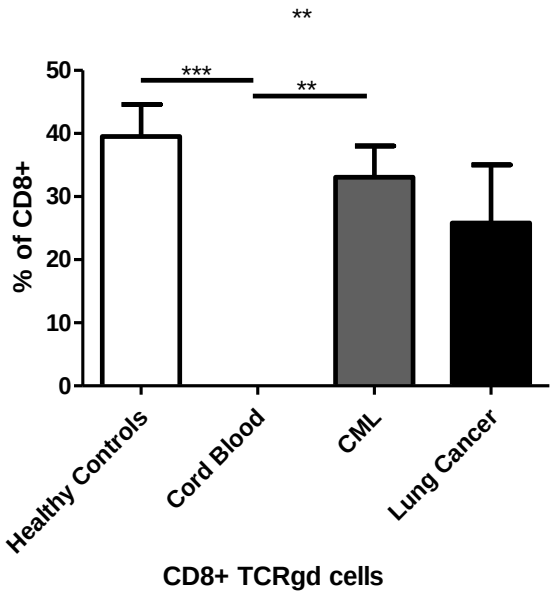

Figure 47 – Comparison of $CD8^+$ γδ T cells percentage among Healthy Individuals (n=17), Cord Blood (n=6), Chronic Myeloid Leukaemia (n=17) and Lung cancer (n=4).

The expression of TCR γδ was assessed by flow cytometry and γδ T cells were selected as the TCR γδ positive population. One-way ANOVA revealed no significant the three groups.

Surprisingly, no CD8 was present in TCRγδ in cord blood. Besides this observation, no differences were observed in the remaining groups analysed.

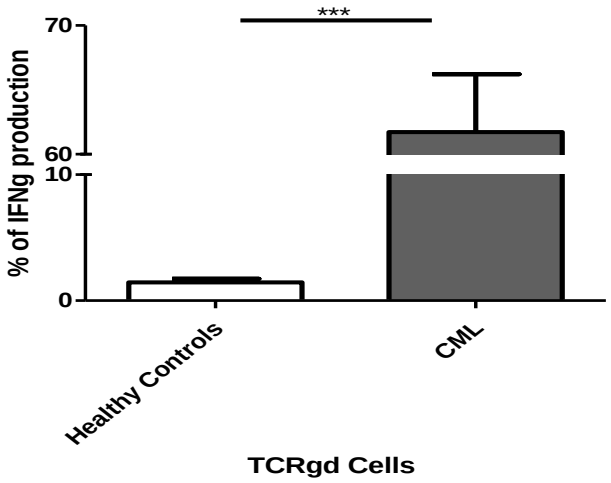

Figure 48 – Comparison of IFN-γ production by γδ T cells in Healthy Individuals (n=6) and Chronic Myeloid Leukaemia (n=12).

The expression of IFN-γ was assessed by flow cytometry and γδ T cells were selected as the TCR γδ positive population. One-way ANOVA revealed significant differences ($p<0.05$) among non-stimulated cells and cells that were stimulated with Poly (I:C) 10 µg/mL and a cocktail of IL-1, IL-12 and IL-18.

γδ T cells are known to be able to produce abundant proinflammatory cytokines such as IFN-γ. The same was observed in the present data and most of the production was observed when cells undergone stimuli with Poly (I:C) and a combination of cytokines.

Increased IFN-ɣ production upon stimulation of γδ T cells with Poly (I:C) and a cocktail of cytokines

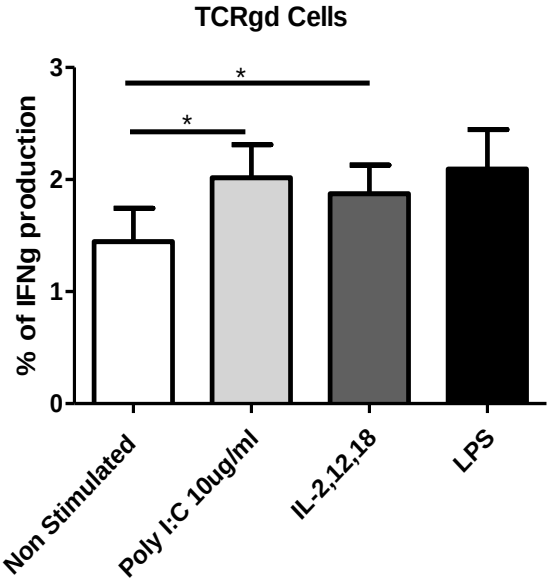

Figure 49 – Comparison of IFN-γ production by γδ T cells in Healthy Individuals (n=6).

The expression of IFN-γ was assessed by flow cytometry and γδ T cells were selected as the TCR γδ positive population. One-way ANOVA revealed significant differences ($p<0.05$) among non-stimulated cells and cells that were stimulated with Poly (I:C) 10 µg/mL and a cocktail of IL-1, IL-12 and IL-18.

Discussion

Since the discovery of NK cells in 1975, and their first identification as effector cells, important insights of their biology and function have been achieved. It is now clear that NK cells are not only $CD3^{-}CD56^{+}$ large granular lymphocytes, but they are a much more complex population composed by a variety of subpopulations (48 distinct subsets identified by Jonges, Albertsson et al. 2001 besides $CD56^{bright}$ and $CD56^{dim}$ and with distinct functions and characteristics even though many of them are still poorly described (Cooper, Fehniger et al. 2001; Cheng, Chen et al. 2013).

From early it was reported that changes in NK cells, such as decreased or absent NK cell numbers or changes in its activity, are related to development or progression of cancer, chronic infection, autoimmune diseases and immunodeficiency (Shereck, Satwani et al. 2007).

Although NK cells have the ability to kill tumour and infected cells without prior sensitization, its target cells have evolved in a way to escape NK cells killing mechanisms (Orange and Ballas 2006) either by the tumour microenvironment cytokines and chemokines or by the down-regulation of activating receptors as a consequence of diverse mechanisms (Moretta, Pietra et al. 2014). The characterization of novel subpopulations might overcome some of the difficulties faced with the use of NK cells in immunotherapy.

Very few studies have been described the expression of CD8 in NK cells. It is known that only αα-homodimer is expressed by this cell type and that the expression of this molecule is presumed to have fundamental significance in NK cells role (Addison, North et al. 2005). One of the assumptions that can be made is the correlation of the presence of CD8 in NK cells with the function that this molecule has in T cytotoxic lymphocytes as an adjuvant of T cell-MHC ligation. As the expression of CD8 by NK

cells is approximately 40% (Figure 11), following this thought, only 40% of NK cells would have effector functions whereas the remaining 60% would have a more regulatory behaviour. In one way, this might explain some of the prevalence of cancer cells over NK cells but in the other way, as it was described by Jonges *et. al*, the expression of CD8 by $CD56^{bright}$ and $CD56^{dim}$ NK cells do not differ significantly. Once $CD56^{dim}$ NK cells represent about 90% (far above 40% of $CD8^{+}$) of total NK cells in peripheral blood, and this subset show cytotoxic functions, this may lead to the question of what is expressed by $CD8^{+}$ NK cells that would increase their cytotoxicity comparatively to their equivalent $CD8^{-}$. Another interesting finding relay on the observation, by Fuchshuber and Lotzova 1992 , that not only the oncolytic activity of these two subsets is different but also, that this may be tumour dependent, i.e., depending on the tumour phenotype, one or another CD8 subset may be the most effective. Interestingly, the expression of CD8 by NK cells is not altered by any of the stimuli used in this work (Figures 12 and 13) being observed only slight changes in its percentage. This may imply that NK cells maintain their profile over time.

The expression of CD7 is one of the first signals of differentiation of hematopoietic progenitor's commitment to NK cell lineage. Even more, CD7 is believed to play an important role in cell adhesion and activation. It correlates with increased cloning efficiency, increased sensitivity to low doses of IL-2 and decreased requirement for stromal contact and increased expression of IL2Rβ (Miller, Alley et al. 1994; Yoo, Kim et al. 2009). Also, this molecule is expressed in both normal mature and immature NK cells and changes in the expression of CD7 were associated with some diseases (Yoo, Kim et al. 2009). Taking these characteristics in account, an higher expression of CD7 by $CD8^{+}$ NK cells (Figure 14) may explain the reason why this subset of cells are more effective than their counterparts.

The expression of the markers previously discussed only enable the assessment of NK cells functional properties, but not on their maturation stage.

CD57 was initially described as marker for NK cells with poor proliferative capacity and probably with some degree of immunosenescence (Brenchley, Karandikar et al. 2003). Later, this theory was revisited and instead of an annergy state, it was described that the gain of CD57 is part of the natural process of maturation (Lopez-Verges, Milush et al. 2010). In fact, acquisition of CD57 has been demonstrated to be a shift towards increased cytotoxicity, greater responsiveness to CD16 signalling and NCRs and decreased sensitivity to cytokines (Lopez-Verges, Milush et al. 2010). Going back to the classical bright and dim NK cells subsets, the bright population is more immature and immunoregulatory whereas the dim population, is more mature and cytotoxic following a similar path of maturation as the previously described for CD57. In this way, we can assume that a higher expression of CD57 by $CD8^+$ NK cells (Figure 15), in accordance to the greater expression of CD7 may reveals a more mature and a more effective population of NK cells. A more mature state does not mean idling but instead, a more accurate path to the final gold: killing the target cell.

In sum, $CD8^+$ NK cells appear to show a greater capacity of effector molecules secretion (expression of CD7) and increased cytotoxicity (CD57).

Transforming growth factor β (TGF-β), a cytokine secreted as a latent complex or in its active form, may influence immune cells (Wahl, Hunt et al. 1987). TGF-β is one of the first players present at the site of inflammation and has for long been established as a tumour promoter at sites of lesion. It is frequently produced by cancer cells to establish a favourable microenvironment, inhibiting NK and T cells immunosurveillance (Medina-Echeverz, Fioravanti et al. 2014). More recently, a biased

role for this cytokine was proposed where dysregulations on TGF-β pathways are related with poor prognosis outcome in some cancers (Akhurst and Derynck 2001). NK cells appear to be the main lymphocyte population to produce substantial amounts of TGF-β regulating adaptive response by influence of T regulatory cells (Horwitz, Gray et al. 1999). Relatively to the present work, it is possible to observe that $CD8^-$ NK cells are the main TGF-β producer (Figure 18), with overall differences among the different stimuli used. Noting that also $CD8^+$ NK cells show some production of TGF-β even though in a much lower manner. It has been reported that NK cells can both produce active and latent TGF-β (Horwitz, Gray et al. 1999), it is possible that a more mature subset of NK cells preferentially produces active TGF-β (reducing the amount of total TGF-β produced) or even that both isoforms are produced, but since $CD8^+$ subset appears to be more mature, the production of cytokines is no longer a priority.

In its turn, IL-10, produced by both immune innate and adaptive cells, is described in most of the studies as having a suppressive role. Nevertheless, this cytokine plays a different role in NK cells. *In vitro*, IL-10 augments NK cells proliferation, cytotoxicity, and IFN-ɣ production (Cai, Kastelein et al. 1999; Mocellin, Panelli et al. 2004). A regulatory subset of NK cells has been described as IL-10 producing cells (Deniz, Erten et al. 2008). The major IL-10 producing NK cells subpopulation characterized here were $CD8^-$ NK cells (Figure 17). Once again, in concordance with data shown above, a possible regulatory part for $CD8^-$ NK cells is characterized by the increased expression of TGF-β and IL-10 and diminished expression of CD57 and CD7.

Another key factor in the characterization of CD8 NK cells relays on their ability to promote anti-tumour immunity.

CD137, formerly known as 4-1BB, a protein related with progressive immunity, was originally discovered on activated T-cells but has now been described in a variety of other cells, including NK cells. It belongs to the family of Tumour Necrosis Factor Receptor (TNFR) and its activation on NK cells can augment IFN-ɣ production, promoting in this way a T CD8 response (Watts 2005). In the present study, the expression of CD137 appears to be modulated by the selected stimuli (Figure 16 A). Constitutive expression of CD137 was described in cytokine activated NK cells (Watts 2005). Interestingly, LPS and Poly (I:C) present similar effects from those of cytokines. In this way, it is possible to admit that TLRs triggers also promote a constitutive expression of CD137 in NK cells. This observation also demonstrates a link between NK cells and T cells. As this receptor is a co-stimulatory molecule of T cells and it is up-regulated on innate cells (NK cells), this receptor comprises a link among the two branches of the immune system in the way that NK cells and T cells act in a concerted manner. Regarding on the expression of CD137L, although no significant differences were shown (Figure 16 B), a minor increased is visible in cytokines and LPS activated NK cells compared to non-stimulated NK cells. The mechanism by which CD137 and its ligand cooperate is not fully understood, but it is believed that receptor-ligand interaction occurs via APCs (Watts 2005) therefore, a minor CD137 expression on NK cells (less than 0.15 % of NK cells) may be in accordance with this theory.

Analysis of intratumoural NK cells and their comparison with NK cells from other locations have shown that the tumour microenvironment influences biological modifications in NK cells (Gillard-Bocquet, Caer et al. 2013). Consequently, exploring NK cells changes in these patients can enlighten possible therapeutic strategies.

Granzyme and perforin are the main elements of the killing machinery of NK cells. Granzymes, a family of serine proteases found in cytotoxic T lymphocytes and

NK cells (Susanto, Trapani et al. 2012) and a pore-forming, Perforin, responsible for a granule-mediated cell death pathway (Voskoboinik, Dunstone et al. 2010). Granzyme was assessed in two different preparations in the present work. The first approach assessed Granzyme B release by NK cells under different stimulation conditions of PBMCs (Figure 19) and when purified NK cells were stimulated (Figure 26). It was possible to observe that besides no statistical differences were observed in non-purified NK cells, there is an apparent greater release of Granzyme B by $CD8^+$ NK cells while purified NK cells do not show a similar behaviour. Of note that none of these results are significant, therefore only some assumptions can be made. One of the possible assumptions is that other cells may signal NK cells in a way for them to produce indistinguishably Granzyme B while isolated NK cells do not receive those signals. Another assumption is related to the quantity of Granzyme B that is secreted. Where NK cells show a greater secretion of this granule when isolated (Figure 26), NK cells from PBMCs do not show an equivalent secretion (Figure 19)

NK cells cytotoxic capacity was evaluated on purified NK cells against K562 cell line. It is possible to perceive that the most effective stimuli are the cocktail of cytokines and Poly (I:C) since they are the two conditions with less statistical significance when compared with the positive control for both $CD8^+$ (Figure 22) and $CD8^-$ NK cells (Figure 23) even though the percentage of CD107a release is distinct among the two subpopulations, no statistical differences were achieved. The same occurs with Perforin and Granzyme B (Figure 26) were no significant alterations are noted even though slight modifications can be observed among $CD8^-$ and $CD8^+$ as supported by previous experiments by (Addison, North et al. 2005). The main NK cell–mediated killing mechanisms is via perforin and Granzyme but there are no significances among $CD8^+$ and $CD8^-$ NK cells as well as with, no differences were

found among the two subpopulations and the same behaviour is detected for IFNɣ secretion.

It has been shown that resting umbilical cord blood mononuclear cells have less cytotoxicity (Tanaka, Kai et al. 2003) than PBMCs but upon stimulation they are able to trigger similar responses as equally treated PBMCs (Nomura, Takada et al. 2001).

In a more effective way than peripheral blood NK cells, that only show overall differences on the expression of CD8 when stimulated (Figure 11), umbilical cord blood NK cells respond more promptly showing differences among the different stimuli (Figure 30) presumably by a more rapid response to stimuli than peripheral blood NK cells.

An *in vitro* maturation of umbilical cord blood NK cells may be achieved according to data shown in Figure 32 (A), which combined with the increase of CD8 expression and relating these results with others discussed above, $CD8^+$ cord blood NK cells may have a more cytotoxic function, similarly to their equivalents in peripheral blood but with a greater response in what regards the time needed for that response to occur.

Umbilical cord blood cells are thought to have a low cytokine secretion profile but it is believed that upon stimulation they are able to rapidly respond (Kaminski, Kadereit et al. 2003), although, no significant changes were achieved in regard to IL-10 production among the two new NK cells subpopulations proposed in this work (Figure 33). Further investigation on cytotoxic granules and cytokine profile is needed.

Alterations among cell populations within disease are common. Few changes in cell number in CML and lung cancer patients (Figure 34 and Figure 38) and cell phenotype for lung cancer patients (Figure 39) were identified. For instance, in lung

cancer patients an exacerbated number of NK cells were found and the discovery of an abnormal maturity state of these cells may explain the theory of a constant activation of NK cells, due to the presence of tumour cells, leads to a push toward maturation which is possibly a more cytotoxic state. In the case of CML patients, no differences were identified either in the expression of neither CD8 nor CD57 (Figure 35 and 37), thus besides and increase in number, these cells appear to be normal. This fact can be explained by a disease control with tyrosine-kinase inhibitors. The only phenotypic alteration in these patients was related to an improved expression of CD7 by $CD8^+$ NK cell subpopulation. As discussed above, CD7 is believed to be related to an increased cytotoxicity revealing that in this pathology, $CD8^+$ NK cells may play an even more cytotoxic effect than the same subset in healthy individuals.

iNKT cells have been suggested to play an important role in immunoregulation (Bendelac, Rivera et al. 1997). In fact, NK properties and cytokine-profile production has been demonstrated for iNKT cells (Kitamura, Iwakabe et al. 1999).

Among the samples studied, none of them show significant differences in regard to the percentage of iNKT cells when compared with healthy individuals or either in the expression of CD8 by this cell type (Figure 43 and 44).

CD69 has been demonstrated to be rapidly induced in the surface of activated lymphocytes (Risso, Smilovich et al. 1991). Regarding the data obtained (Figure 45), it is possible to postulate that $CD8^+$ iNKT cells are the subpopulation that appears to be more efficiently activated since a higher expression of CD69 is recorded. The expression of this early activator marker was increased in CML patients in comparison to healthy individuals, whereas no differences were found for lung cancer patients. CD69 was found to be up regulated by the expression of the fusion gene BCR-ABL and

down-regulated by the suppression of BCR-ABL in CML (Hantschel, Gstoettenbauer et al. 2008). Thus the increased expression of this marker in CML comparatively to healthy individuals and lung cancer presented in this work are in accordance with the expectations. It has been shown that the activation of iNKT cells leads to the activation of both innate and acquired immune cells (Nishimura, Kitamura et al. 2000). In this way, it is possible to say that the communication between innate and acquired immune system, *via* iNKT cells is not affected in lung cancer patients and it might be over-activated in CML patients.

Recent evidences suggest that TLRs are expressed by γδ T cells (Wesch, Beetz et al. 2006). In fact, stimuli with Poly (I:C), an agonist of TLR3, has revealed itself as powerful as a cocktail of cytokines in regard to IFN-γ production in comparison with non-stimulated cells (Figure 48). Still, no significant differences were found in response to LPS. The present data suggest that some TLR are expressed by γδ T cells.

IFN-γ affects the proliferation of hematopoietic stem cells and affects colony formation. In addition, IFN-γ genotype has been reported to be related with the response to treatment in CML. The presence of mutations in IFNG gene affecting its pathway, showed to be the most significant alteration in the prediction of cytogenetic remission (Kim, Kong et al. 2010). Data shown in Figure 49 identify a clear difference in IFN-γ production by γδ T cells from CML patients relatively to healthy individuals. Further studies having in view the IFNG genetic profile may clarify the reason for such a high production of IFN-γ.

A more detailed study of iNKT and γδ T cells would be of great value, increasing the understanding of their role in NK cells function as well as possible advantages in the conjugation of these different cell types.

Conclusions and future perspectives

The present work aimed to understand how the presence or absence of CD8 in NK cells may influence their functions and how these differentiated functions can be useful for immunotherapy.

Hence, we demonstrated that $CD8^+$ NK cells may be an innately a more mature and cytotoxic subset than their counterparts (showed by the higher expression of CD57 and CD7) and that $CD8^-$ NK cells seem to be more of a cytokine secretor subset (IL-10 and TGFβ). We also demonstrate that upon cultivation with target cells, both subpopulations are able to produce similar amounts of IFN-ɣ, Granzyme B and Perforin, were only minor differences can be envisioned. Since $CD8^-$ NK cells do not acquire CD57 or CD7 upon stimulation, a different mechanism by which this subpopulation acquire cytotoxicity when co-cultured with target cells may be involved. The expression CD137 by NK cells was successfully modulated by the stimuli used in this work.

As $CD8^+$ NK cells appear to be more innately cytotoxic than their counterparts, a similar role for CD8 in NK cells and T cells may be proposed. We suggest that this adhesion molecule play an important role in NK cell ligation to target cell, activation and cytotoxicity by enhancing cell to cell contact.

Umbilical cord blood $CD8^+$ NK cells respond to stimuli prompter than their equivalents in peripheral blood. $CD8^+$ iNK T cells are more rapidly activated both in lung cancer and in healthy individuals than $CD8^-$ cells. In addition, iNKT cells in lung cancer show an increased early activation profile than healthy individuals.

In lung cancer patients, NK cells were characterized by an augmented expression of CD57. It is possible to assume that an excessive activation due to the presence of tumour cells may lead to a push toward maturation of NK cells.

Natural killer cells from Chronic Myeloid Leukaemia patients do not show differences in the expression of CD8 nor CD57. The only change detected was related to an increased expression of CD7 by the $CD8^+$ subpopulations of NK cells.

The expression of CD8 by iNKT cells in CML and lung cancer patients was similar to healthy individuals. Both subpopulations of NK reveal increased activation when compared to healthy individuals.

γδ T cells were effectively stimulated by Poly (I:C) in order to produce IFN-γ in healthy individuals. The production of this cytokine by non-stimulated γδ T cells from CML patients showed to be augmented.

In order to evaluate the exact function of CD8 in NK cells, we expect, in a first approach, to block CD8 receptor on NK cells and assess the functional properties of this cells in comparison with $CD8^-$ NK cells. Additionally, we intend to transduce CD8 into $CD8^-$ NK cells and evaluate the acquisition or not of $CD8^+$ cells functions. A ^{51}Cr release assay, were CD8 subpopulations of NK would be assessed separately may highlight their distinctive functions.

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