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Developmental characterization of Group 1 Innate Lymphoid Cells: from mouse to human

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Abstract

Natural Killer (NK) cells are a heterogeneous population of cytotoxic cells that can be grouped in phenotypically and functionally different subsets. Among them, human $CD56^{bright}$ and $CD56^{dim}$ NK cells show important differences in their cytotoxic activity, cytokine production, and responses to cytokine activation. Moreover, $CD56^{bright}$ NK cells differ from $CD56^{dim}$ ones for the phenotypic expression of CD117, CD16 and the HLA class I inhibitory receptors (CD94/NKG2A and KIRs). $CD56^{bright}$ NK cells have been proposed to represent either a mature NK cell subpopulation or an immature stage of the $CD56^{dim}$ NK subset. Considered that $CD56^{bright}/CD16^{dim/neg}$ NK cells are virtually all licensed by CD94-NKG2A expression, it is not clear which subset represents the real immature stage of the licensed $CD56^{dim}CD16^{bright}$ NK cells.

Human $CD56^{bright}$ NK cells are thought to be the counterpart of mouse thymic NK (tNK) cells, because they share some characteristics like the requirement for GATA3 and the dependence on IL-7, but it is not completely clear weather they are NK cells or a different subset of Group 1 Innate Lymphoid Cells (Group 1 ILCs); in fact tNK cells have been described with hybrid features of immature NK cells and ILC1.

We have investigated the mechanisms governing tNK cell functions, demonstrating that tNK cells express the transcription factor EOMES and that they developed independent of the essential ILC1 factor TBET, confirming their placement within the NK lineage. Moreover, tNK cells developed independent of the E protein transcription factor inhibitor ID2 and their numbers were only mildly affected by the loss of ETS1.

Our data revealed that in the thymus of mice there is an absence of ILC1, setting the stage for deeper studies of the relationship between murine tNK cells and human $CD56^{bright}$ NK cells.

In the first part of this project, using culture systems capable of generating $CD56^{bright}$ and $CD56^{dim}$ NK cells from the human hematopoietic progenitors CD34+ circulating in the peripheral blood through the administration of appropriate cytokine combinations, we have been able to characterize the differentiating NK cells. Thus, we indicate that $CD56^{dim}$ and $CD56^{bright}$ NK cells, would originate from distinct progenitors, which, along with their differentiation into mature cells, would generate two distinct cell NK subsets with convergent phenotypes and functions. Moreover, during their development $CD56^{dim}$ and $CD56^{bright}$ NK cells would exploit different mechanisms to prevent cytotoxicity against healthy cells.

Introduction

1. Innate Lymphoid Cells

NK cells represent a homogeneous group of IFN-γ producing cells, which express characteristic markers (NK1.1 in mouse and CD56 in human, and NKp46 in both species) and depend on the transcription factor T-bet for their development. In 2008 a second subset of cells, involved in the mechanisms of the innate immunity, has been discovered. These cells share many attributes with conventional NK (cNK) cells, but they differ in several aspects like their transcriptional requirement and their localization, suggesting that they differ for some aspect in their role in immune response (Diefenbach et al., 2014). All of these cells, unlike adaptive immune cells, develop in the absence of recombination-activating gene 1 (Rag1) or 2 (Rag2), they require few hours after their activation to respond, and they all develop from the common lymphoid progenitor (CLP); moreover, they all have a lymphoid morphology and they lack myeloid and dendritic antigens (Spits & Cupedo 2012). These cells, called ILCs (Innate-like Lymphoid Cells), have been discovered and divided into three main groups (Annunziato et al., 2015) based on their cytokine and transcription factor expression (Figure 1): The ILC1 family is composed of the T-bet expressing cells and includes NK cells and ILC1

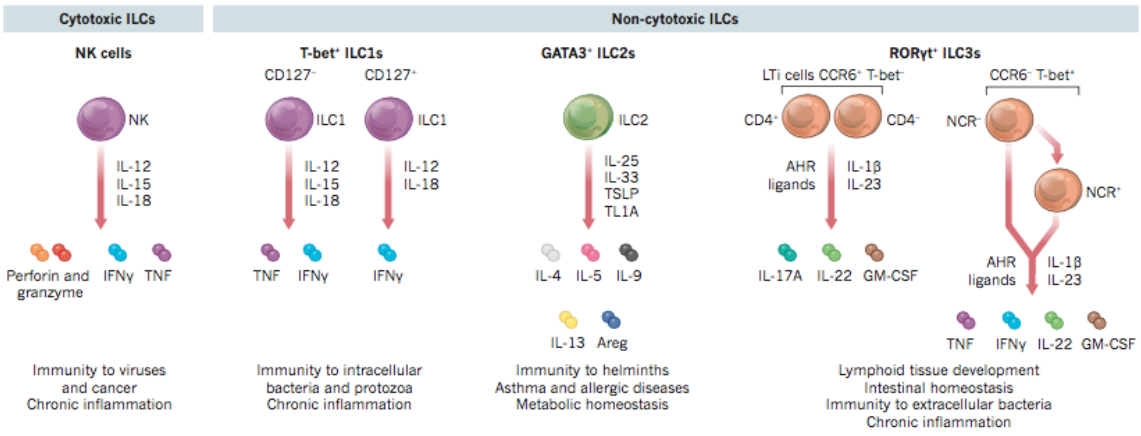

Figure 1: Main subsets of the Innate Lymphoid Cell family. [From: Artis & Spits, 2015]

cells; ILC2 are Gata-3-expressing cells (also known nuocytes) originally discovered in lung, skin, or fat tissue (Price et al., 2010; Neill et al., 2010); and ILC3 that produce IL-17 and/or IL-22 and express the transcription factor Rorγt. All of the three populations are made up of several subsets, but among them Group 1 ILC is the most heterogeneous and confused subset. In fact, NK cells have been considered the prototype of Group 1 ILCs for several years, but more recently it has become evident that distinct populations within this group exist and their diversity seems to be very important for immune protection.

1.1 Group 1 Innate Lymphoid Cells: NK and ILC1 cells

NK cells where identified for their ability to spontaneously lyse tumor cell lines in vitro, but more recently other recombinant activating gene (RAG)-independent ILC populations have been discovered. The ILC1 family, better known as Group 1 ILCs, is mainly composed of T-bet expressing cells: NK cells and ILC1 cells. Group 1 ILC is a group of non-cytotoxic lineage negative (Lin-) cells which can produce IFN-γ and TNF-α and are involved in immunity to intracellular bacteria and parasites (Fuchs et al., 2013; Klose et al., 2014). To date the ILC1 lineage is not well defined but it's known that it requires TBET for lineage specification and function and produce "type 1" cytokines such as IFN-γ (Spits et al., 2013). NK cells have been considered the prototype member of this group, but they are not the only one. Another subset of group 1 ILCs that produces IFN-γ but not Th2 cell- or Th17 cell-associated cytokines, and that is distinct from NK cells, has been identified in mice and humans (Vonarbourg et al., 2010). In humans, the ILC1 subset lacks expression of c-kit (also known as CD117) and expresses high levels of TBET and low levels of RORγt. ILC1 have been identified in a variety of different tissue locations resulting in the identification of distinct populations.
In the adult liver NK cells coexist with ILC1. These ILC1 are distinct from cNK cells because they do not circulate throughout the body and they remain at their steady state only in the liver (Peng et al., 2013). Liver ILC1 are $CD49a^{+}Trail^{+}$ (Takeda et al., 2005), and Trail seems to be a marker to distinguish these two different lineages. Moreover, these two populations have different transcription factor requirement and a distinct gene profile (Daussy et al., 2014). Liver ILC1 share the expression of some antigens like NKp46, CD122 and NK1.1 with cNK cells, but they also express a pool of antigens not common to that expressed by cNK cells like some chemokine receptor and adhesion molecules (CXCR6, CXCR3), cytokines and cytokine receptors. A functional profile of liver NK cells has been outlined, and it correspond to that of a cell population that surprisingly kill target cells with mechanisms that differ from NK cells, and that could be involved in regulatory roles either directly or indirectly via interactions with T cells.
In the intestine it has been identified at least two populations of ILC1. Fuchs and colleagues characterized a human ILC1 subset that produces INF-γ in response to IL-12 and IL-15 and which has a unique phenotypic profile. This population of cells have been found in human tonsils and express markers such as CD160, CD49a, CXCR6 and CD39 (Fuchs et al., 2013); it has been proposed that because of sharing some features with tissue-resident memory $CD8^{+}$ T cells intraepithelial ILC1 may be their innate counterparts; with an activated-memory phenotype and the secretion of INF-γ and other lytic mediators, these subsets of ILC1 could have a pro-inflammatory function. The murine counterpart of human intraepithelial ILC1 has been identified in the $CD160^{+}$ $NKp46^{+}$ $NK1.1^{+}$ INF-γ producing cells in response to stimulation with IL-12 and IL-15

(Fuchs et al., 2013) and they are largely independent of IL-15Rα, corroborating that these ILCs are distinct from conventional NK cells, which require IL-15Rα for development.

Another study revealed that in inflamed intestine from individuals with Crohn's disease there is an accumulation of INF-γ producing ILC1 (Bernink et al., 2013), and this subset of cells is the most represented ILC subset. Even if at least a portion of these cells differentiated from ILC3 cells under the influence of IL-12, ILC1 develop after colonization of the gut with commensals and this population may be involved in the early innate immune response against certain bacteria. Moreover, the adoptive transfer of human fetal hematopoietic stem cells (HSC) into transgenic mice lacking lymphocytes, NK cells, and ILCs, demonstrated that human ILC could reconstitute the intestinal ILC compartment in mice (Bernink et al., 2013).

In mouse salivary glands (SG) another population of ILC1 has recently been identified. This population of cells express both TBET and EOMES, but are poor producers of IFN-γ and opposite to cNK cells they do not depend on NFIL3 to develop (Cortez et al. 2014). How this ILC1 group fits into the innate landscape is not completely clear because these SG ILC1 have some confusing characteristics: they have a unique integrin pattern of expression (e.g.: CD103, VLA1) similar to that of intestinal intraepithelial ILC1s, which however are largely NFIL3 dependent and produce IFN-γ. Moreover, SG ILC1 express EOMES, while liver $VLA1^+$ NK cells do not.

1.2 ILC1 and NK cells homeostasis

The immune system has evolved the ability to have a broad reactivity but also a high specificity to protein antigens thanks to the continuous expression or recombination activation genes (*Rag1/2*) by B and T cells; few selected B and T cells can recognize an antigen and clonally expand to produce a long-lived memory effector. In contrast, a heterogeneous pool of short-lived NK cells mediates the inflammatory response by secreting pro-inflammatory cytokines and cytotoxic granules. The current understanding of lymphocyte homeostasis is that when a system experiences a deficiency in a cell type due to infection/chemotherapy/irradiation, the biological system induces a replacement of these cells from progenitors or residual cells. For that reason, the homeostasis and the activation of NK cells is tightly regulated in an antigen-independent manner, both with extrinsic and intrinsic factors.

Intrinsic factors

Among the intrinsic factors involved in NK and ILC1 homeostasis, an important role is made by the protein tyrosine phosphatase CD45 (encoded by *Ptprc*), which is a key

negative regulator of both NK cell and liver ILC1 homeostasis (Huntington et al., 2005); in fact, hepatic ILC1 and cNK cell numbers are significantly elevated, correlating with an increased proliferation. Nevertheless, ILC1 and NK cells develop differently and they also have different transcription factor requirements, so the exact mechanism of how CD45 negatively regulates NK cell and ILC1 homeostasis is not clear.

In the differentiation of peripheral NK cell subsets and their functional diversification, an important role is acted by the Ikaros family of zinc finger proteins Aiolos (encoded by *Ikzf3*). It is early expressed during haematopoiesis and it regulates several aspects of lymphoid lineage development (Morgan et al., 1997), and mice lacking this gene display an unusual arrest in NK cell differentiation (Holmes et al., 2014). This *Ikzf3*$^{-/-}$ NK cells produce less IFN-γ even though they show a higher in vivo killing potential, and they still have normally expressed transcription factors known to regulate NK cell development. The arrested NK cell differentiation phenotype of *Ikzf3*$^{-/-}$ mice resembles that of B-lymphocyte-induced maturation protein-1 (Blimp-1, encoded by *Prdm1*) deficiency (Holmes et al. 2014). Even though Blimp-1 plays a key role in the terminal B- and T- cells differentiation, its expression increases during NK cell differentiation and is rapidly up regulated upon IL-12 and IL-21 stimulation.

Also Forkhead box protein O1 (*Foxo1*) is a transcription factor that has recently been identified as a negative regulator of NK cell differentiation. The homeostatic cytokines IL-2 and IL-15 induced the phosphorylation of *Foxo1*, preventing it from binding to its target gene, like TBET, which is essential for NK cell differentiation and *Tbx21*$^{-/-}$ mice do not develop mature NK cells and have significantly fewer total NK cells (Daussy et al., 2014; Gordon et al,. 2012). The expression of TBET and Foxo1 during NK cell ontogeny is inversed with TBET increasing and Foxo1 decreasing during differentiation. Foxo1 was found to bind Tbx21 at the proximal promoter region and Tbx21 expression was significantly elevated in Foxo1-null NK cells indicating that Foxo1 acts as a repressor of TBET expression to limit NK cell differentiation in vivo (Deng et al., 2015).

Extrinsic factors

The principle factor known to regulate NK cell homeostasis is IL-15 (Huntington 2014) but IL-15 is also necessary for the development and maintenance of other lymphocyte subsets. Also, IL-15 is important for driving NK cell maturation, in particular it is responsible of the up regulation of KLRG1 (Huntington et al., 2007). IL-15 is critical for the enhanced homeostatic proliferation and accumulation of KLRG1^{+} NK cells in *Rag1*$^{-/-}$ mice: in fact there is a dose-dependent reduction of KLRG1^{+} NK cells with the deletion of one or two copies of IL15. Also commensal bacteria could have a role in the

homeostatic expansion of NK cells, probably linked to IL-15 production via myeloid and non-hematopoietic cells as a result of NOD signalling. This competition between NK cells and T cells for IL-15 and commensal bacteria have important consequences for immune responses. This competition between NK cells and T cells for IL-15 and commensal bacteria have important consequences for immune responses. In response to MCMV, $KLRG1^{-}$ NK cells are functionally superior to $KLRG1^{+}$ NK cells and experience a significantly greater Ly49H-m157 expansion 7 days of post-infection (Kamimura & Lanier, 2015).

IL-2 is another member of common γ cytokine family, like IL-15, but even if they have sometimes overlapping pathways and overlapping functions, $IL\text{-}2^{-/-}$ and $IL\text{-}15^{-/-}$ mice present very different phenotypes suggesting unique roles for these cytokines in NK cell homeostasis (Ring et al., 2012); in fact both cytokines require the heterodimeric IL-2R β/γ complex for their signalling, whereas IL-15Rα is required to trans-present IL-15 to IL-2Rβ/γ expressing cells but does not intrinsically alter IL-15 signalling (Lodolce et al. 1998).

Transforming growth factor beta (TGF-β) is another potent immune-regulatory cytokines (Laouar et al., 2005); and it has been proposed that dendritic cells are a possible source of TGF- β1 for NK cells during immune response, and they can suppress and/or alter NK cell activity by altering TGF-β1 and IL-12 levels (Sarhan et al., 2015).

2. Transcriptional regulation of innate lymphoid cell fate and generation of different ILC1 and NK cell subsets

In the hematopoietic system, the hematopoietic stem cell (HSC) is the multipotent and self-renewing cell which gives rise to the generation of all the hematopoietic lineages. Haematopoiesis is a multistep process, during which HSC progressively lose the cell-fate potential, and the main step in lymphopoiesis is the generation of the common lymphoid progenitor (CLP).

NK cell is considered the founding member of the ILC family, one of the three lineages of lymphocytes to originate from the CLP, along with T and B cells, and several transcription factors and growth factors are known to be involved in CLP development into the downstream precursor. In these years, rare Lineage negative cells have been identified in the fetal liver, fetal gut and adult bone marrow; these cells express CD127 and the α4β7 integrin, and have also lost B and T cell potential even if they can still generate NK cells, dendritic cells and LTi cells (Yoshida et al., 2001). Recently, a committed ILC precursor (ILCp) has been identified within the IL-7Rα+α4β7+ population in bone marrow and fetal liver (Constantinides et al., 2014). This precursor expresses high levels of the transcription factor pro-myelocytic leukemia zinc finger protein (PLZF, encoded by Zbtb16) and it is required for ILC development. Several transcription factors have been identified to drive the generation of the ILCs subsets, which act in different ways and in the different lineages (Figure 2).

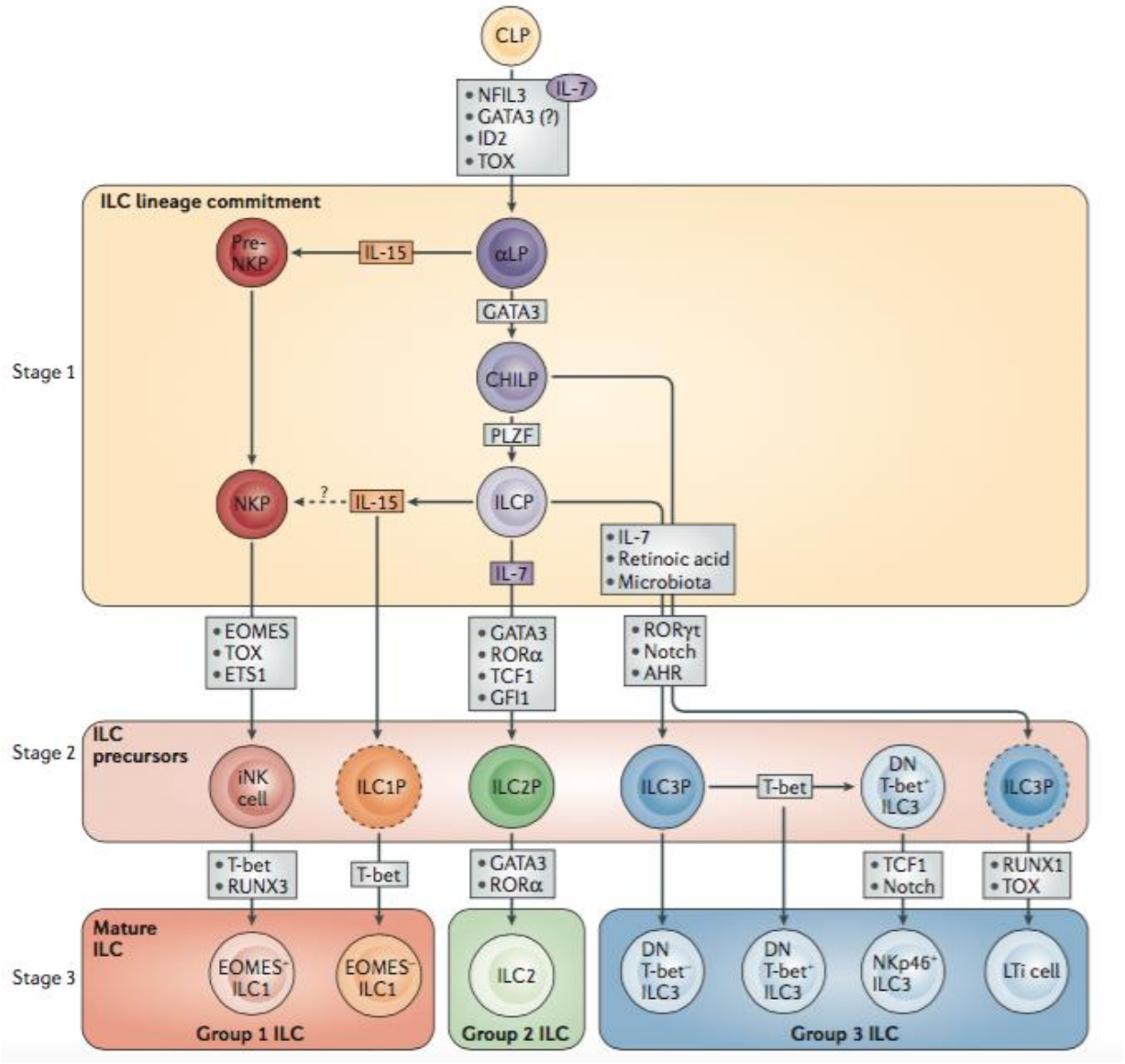

Figure 2: General model for (mouse) ILC development. Innate lymphoid cells (ILCs) differentiate from haematopoietic stem cells via a common lymphoid progenitor (CLP). Interleukin-7 (IL-7) and the transcription factors ID2 and NFIL3 promote the differentiation of potential common precursors of ILC-restricted progenitors. Immature NK (iNK) cells appear after expression of EOMES. ILC1s may derive from NKPs and/or CHILPs in response to an IL-15-induced transcriptional programme that involves transcription factors such as TBET, EOMES, GATA3 and/or NFIL3. I [From: Serafini et al., 2015]

2.1 The Common Lymphoid progenitor (CLP)

ID2

ID2 is a member of the ID family of transcriptional repressor; proteins of this family lack the DNA-binding domain and share the highly conserved helix-loop-helix domain (Sun et al., 1991). ID2 regulates the differentiation and the development of a lot of lineages, and its ablation has dramatic effects on the differentiation of myeloid and

lymphoid lineages (Verykokakis et al., 2014). Members of this family bind and functionally inactivate a set of transcription factors known as E-proteins, like E2A, E2-2 and HEB within the hematopoietic system.
ID2 deficient mice have been reported to have a selective loss of NK cells and lymphoid tissue, whereas B- and T-cell development is substantially the same (Boos et al., 2007). Moreover, overexpression of ID proteins inhibits B cell and T cell development, while it strongly promotes ILC generation, in particular NK cells (Heemskerk et al., 1997).
All CLPs express little or no ID2, and high levels of ID2 would essentially restrict the lymphoid precursor to the ILC lineage (Klose et al., 2014). Moreover, some ILCs can develop in the absence of ID2 if also E protein are ablated (Boos et al., 2007), indicating how ILCs development might represent a default pathway for CLPs: ID2 induction appears to be one of the first molecular steps in the induction of the ILC lineage; however, the mechanism of Id2 up-regulation in ILC progenitors remains unclear.

NFIL3

NFIL3 (nuclear factor interleukin-3; also known as E4-binding protein 4, or E4BP4) has been first described as a critical transcriptional regulator for NK cell development affecting mature NK cells (Gascoyne et al., 2009; Kamizono et al., 2009) and thymic NK cells (Seillet et al., 2014a). It is broadly expressed in different tissue and it is involved in several developmental and biological processes. Within lymphocytes, NFIL3 ablation has a dramatic effect on NK cell development in particular at the pre-NKP stage, but it seems to be not so fundamental in mature $NKp4^+$ cells (Gascoyne et al., 2009). NFIL3-deficient mice also display a broad loss of ILC populations including ILC1, 2, and 3 together with LTi cells. This loss appears to stem from inhibition of the development of the bone marrow $\alpha4\beta7^+$ lymphoid progenitors and ILCPs (Seillet et al., 2014b) and thus the development of a common innate lymphoid progenitor, prior to PLZF up-regulation.
CLPs express little or any NFIL3, but it can be regulated by cytokines and IL-7 can strongly induces NFIL3 expression in CLP (Ikushima et al., 1997). NFIL3 is not required for all ILC and discrimination between different peripheral subsets has been linked to the induction of EOMES. Indeed, all EOMES-expressing NK cells, including conventional medullary and thymic NK cells, are absent in absence of NFIL3, whereas $TRAIL^+$ NK cells that do not express EOMES appear unaffected by its loss (Seillet et al., 2014a). Even if the strength importance for NFIL3 in the NK cell development, how its expression is regulated is not completely clear.

GATA3

The transcription factor GATA3 has many roles in T cell and ILC2 differentiation (Tindemans et al., 2014). In 2006 for the first time a role for GATA3 has been appreciated for thymic NK (tNK) cells, a subset of NK cell different from that of cNK cells (Vosshenrich et al., 2006); since then it has been shown that deletion of GATA3 in all hematopoietic cells impaired the development of all IL-7Rα$^+$ ILC subsets but did not interfere with the development of cNK cells (Yagi et al., 2014), showing that in the absence of GATA3, Lin$^-$ CD127$^+$ cells fail to develop in the fetal liver and adult bone marrow.
In general, GATA3 has a broad role in ILCs development, and it has been proposed that it is involved in the segregation of "helper" from "killer" ILC lineages, because the development of splenic CD127$^-$ EOMES$^+$ NK cells still occur also in its absence (Samson et al., 2003).

PLZF

The transcription factor PLZF has a very important role in the differentiation of T cell subsets, but only recently its role in the differentiation of ILCs has been demonstrated. Fetal liver and bone marrow PLZF$^+$ precursor include committed ILC precursor; however, PLZF deficient mice have only partial defect in ILC1 and ILC2 development, thus, even if its molecular targets are not totally known, it seems to be important for the generation of CHILP from CLP. Fate mapping experiments in mice have shown that PLZF expression promotes commitment to the ILC lineages, but not LTI or NK cells (Constantinides et al., 2015).

2.2 Differentiation of NK cells and ILC1

Transcription factors govern the development of NK cells, starting from the earliest progenitor. Although many of these DNA-binding and chromatin-modifying proteins are shared with other cells of the immune system or even with non-hematopoietic cells, some are unique to the NK cell lineage. Already mentioned transcription factors ID2, E proteins, PLZF and Nfil3 are among proteins known to drive early stages of NK cell development. Additional transcription factors, including TBET, EOMES, and ETS1 play specific roles at distinct stages of NK cell development and maturation.

TBET and EOMES

TBET is the transcription factor encoded by Tbx21, and it is the signature transcription factor for mature Group 1 ILC that produce IFN-γ; it binds to the *Ifng* locus to activate

it via chromatin remodelling that allows other binding of factors that improve *Ifng* expression (Djuretic et al., 2007). Both ILC1 and NK cells express TBET, but they depend on it in a different manner, while only NK cells express EOMES so it can be used to discriminate between these two subsets.

In NK cells, TBET is required altogether with EOMES to promote their maturation and function (Gordon et al., 2012). These two transcription factors act in a sequential manner, but TBET directs the development of iNK cells and stabilizes the immature phenotype while EOMES induces the expression of a diverse repertoire of Ly49 receptors in NK cells and acts to maintain the mature phenotype (Gordon et al. 2012). EOMES seems to be a downstream target of NFIL3 in NK precursor (NKP) (Male et al., 2011) which drives early stage differentiation, and this is an explanation for the severe NK cell deficiency in NFIL3 deficient mice. Indeed, these mice have other severe defects also in the other ILCs lineages, suggesting that EOMES is not the only downstream target of NFIL3 (Seillet et al., 2014a). Moreover, Analyses of EOMES reporter mice showed that at steady-state $TBET^{+}$ $EOMES^{-}$ cells appear to be a stable population that do not subsequently give rise to $TBET^{+}$ $EOMES^{+}$ cells, suggesting that these cells are not immature, but represent a distinct population (Daussy et al., 2014). Daussy and colleagues also demonstrated that TBET expression is repressed in the bone marrow allowing the development of $EOMES^{+}$ NK cells, and $EOMES^{-}$ NK cells are not precursors of $EOMES^{+}$ NK cells in homeostatic conditions and rather correspond to a distinct lineage of ILCs (Daussy et al., 2014). It was also demonstrated that TBET and EOMES cooperate to induce high expression of CD122, the β chain that binds IL-15 (Intlekofer et al., 2005)

ETS1

ETS1 is a member of the large family of ETS transcription factors, it is a proto-oncogene expressed in all lymphoid lineages, with widespread roles in development (Sharrocks, 2001). ETS1 is required for the optimal development of mature NK cells (Barton et al., 1998), and NKP are significantly reduced in the absence of ETS1, in fact transcriptional analysis have revealed that ETS1 sustains ID2 and TBET expression in this progenitors (Ramirez et al., 2012).

3. Natural Killer cells

Natural Killer (NK) cells were first discovered in the mouse at the beginning of 1970s as a population of cells able to spontaneously kill cancer cells (Kiessling et al., 1975), and few years later they were found also in human (Pross & Jondal, 1975; Jondal & Pross, 1975). This name is due to their capacity of spontaneous cytotoxicity (natural killing) that was different to that one mediated by cytotoxic T lymphocytes; in fact T cell cytotoxicity is dependent on the expression of Major Histocompatibility Complex (MHC), while NK cells were able to kill also tumor cells without the expression of MHC antigens on their surface (Trinchieri & Santoli, 1978). Even if they were first considered as a "background noise" in T cells cytolytic assays, about ten years later NK cells were characterized as a distinct type of mononuclear cells in the peripheral blood (Perussia et al., 1983; Lanier et al., 1986b); they were described as Large Granular Lymphocytes (LGL) because of their homogeneous morphology with granules inside the cytoplasm (Grossi et al., 1982), and they were present in both lymphoid organs and non-lymphoid peripheral tissues. Nevertheless, because NK cells share the LGL morphology with some other subsets of T cells and dendritic cells, they can't be described only on the basis of their morphology.

NK cells are included as part of the innate immune system, with monocytes and granulocytes (Scott & Trinchieri, 1995), because they rapidly respond to infectious agents without prior sensitization, and they are highly cytotoxic versus infected cells and tumor cells when these cells do not express class I MHC molecules (MHC-I) (Zamai et al., 2007). NK cells represent about the 10-15% of all the circulating cells in the peripheral blood, but they can be found also in other lymphoid tissues, spleen, lymph nodes and bone marrow (Ferlazzo et al., 2004), and in case of necessity they can also migrate towards sites of inflammation.

Even if T and NK cells share some functional and phonotypical characteristics they have complementary action, because T cells have the requirement that MHC-I is expressed on the surface of pathogen-infected cells in order to recognize them, while NK cells can lyse target cells without prior sensitization resulting powerful effector when B and T cells are not enough and for that reason they have been considered among the main components of the innate immunity. Moreover, as opposed to T cell and B cell responses that are dictated by unique, somatically recombined, and clonally distributed antigen receptors, NK cell responses are controlled by a more limited repertoire of germ line-encoded receptors (Vivier et al., 2011). The main feature that distinguishes NK cells from other lymphoid populations is the lack of specific receptors for foreign antigens, in particular the absence of the surface receptor CD3, the T Cell receptor

(TCR) typical of T lymphocytes or surface Immunoglobulins (sIg or B Cell Receptor, BCR) characteristics of B lymphocytes (Ritz et al., 1985; Lanier et al., 1986a).
NK cells play a pivotal role in defending our body, with an intense cytolytic activity against tumor cells, cells infected by viruses or bacteria, and allogeneic cells (Moretta, et al., 2001) being able to lyse them through the activation of cytolytic pathway in the absence of a previous stimulation, and being active against cells that do not express MHC class I molecules (Lanier et al., 1985).
The most important aspect in defining this population able to mediate the spontaneous cytotoxicity has been to unequivocally identify it through the presence of surface markers in the absence of CD3/TCR or sIg. Although NK cells do not express rearranged gene products TCR, adult NK cells expressing the CD3 ζ chain; moreover, NK fetal constitutively present in the cytoplasm the γ, δ, and ε chains of CD3 molecule (Lanier et al., 1992). Activated NK cells in the peripheral blood of adult individuals express the ε chain of the CD3 intracytoplasmic (cytCD3ε), but not the other chains of this receptor with the exception of the ζ chain (that is bound to CD16). Some evidence suggests that the initial expression of cytCD3ε can identify a common T/NK progenitor present in the fetal thymus (Miller et al., 1994; Spits et al., 1995) but no functional role has been found in NK cells.
The lack of CD3ε chain in other hematopoietic lines makes this molecule potentially important in discriminating NK cells and immature T cells, when these lack other markers or phenotypic functional characteristics. However, human NK cells exhibit numerous other proteins on the cytoplasmic membrane that can be used as phenotypic markers exclusive for easily distinguish them from other lymphocyte populations.
NK cells can be divided into functionally distinct populations, which seem to be different in humans and mice: human NK cells can be mainly classified based on the expression levels of CD56 and CD16, while mouse NK cells share many characteristics with human NK cells, but the lack of CD56 expression and other surface markers makes it difficult to identify functionally comparable NK cell subsets in mice.

3.1 Natural Killer cell markers and receptors

CD56

CD56 is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily (Cunningham et al., 1987) and it has a molecular weight between 175 and 220 kDa, it is characterized by the presence of an immunoglobulin domain in the extra-cellular portion. The CD56 molecule is homologous to the neural cell adhesion molecule N-CAM, but it is not able to transduce an activator signal and it is not directly involved in

the activation of NK cytolytic machinery. CD56 is involved into an increased adhesion between the NK cell and its target (Lanier et al., 1991), and through homophilic binding it plays an important role in the process of target cell killing by an apoptosis mechanism (Takasaki et al., 2000). NCAM in the peripheral blood is prematurely expressed on NK cell surface and it is considered an important marker for the identification of NK cell subsets in the human.

CD16

One of the most studied activating receptor expressed on the NK cell surface, is the low affinity receptor for the crystallisable fragment (Fc) of IgG, CD16 (FcγRIIIa).

CD16 is a transmembrane glycoprotein of 50-80 kDa and it is expressed by most human NK cells, activated monocytes and a subpopulation of T lymphocytes (Lanier et al., 1985). This receptor is important because it is a signal-transducing molecule that after the binding with its ligand, it induces (also in combination with other stimuli) the transcription of genes encoding for other surface activation molecules or cytokines important in enhancing NK cells activation (Anegòn et al., 1988).

CD16 has low affinity for the crystallisable fragment (Fc) of G Immunoglobulins (Ravetch & Perussia, 1989), it binds opsonized targets cells and it activates the antibody-dependent cell cytotoxicity (antibody dependent cell-mediated cytotoxicity, ADCC) mechanism, through the association with γ chain of the high affinity receptor for IgE (FcεRI) and the ζ chain of the CD3 that contains an activator immune-receptor (Immune Tyrosine-based activating Motif, ITAM) (O 'Shea, et al., 1991).

The cross-linking of CD16 with its ligand leads to a rapid activation of the tyrosine-kinases protein (PTK), which follows the phosphorylation of a tyrosine residue of the ITAM sequence of the intracytoplasmic tails of FcεRIγ and CD3ζ chains. Subsequently, they are recruited on this phosphorylated PTK site belonging to the Syk/ZAP70 family (Brumbaugh et al., 1997) and the Src family (Azzoni et al., 1992) and they are able to phosphorylate both the adapter protein LAT (Jevremovic et al., 1999) and the phosphatidylinositol-3-kinase (PI3K) (Weiss & Littman, 1993). At this point distinct metabolic events can occur: on one side there are activated phospholipase C-γ1 and C-γ2 which, in turn, lead to the activation of protein kinase C (PKC), by the production of diacylglycerol (DAG) as a second messenger, and to a transient increase in the intracellular concentration of Ca2 +, due to the inositol 1,4,5-triphosphate (IP3) (Einspahr et al., 1991; Ting et al., 1992). Another way involves the Vav-Rac1 protein (Galandrini, et al., 1999), while a different pathway involves the activation of phosphatidylinositol-3-kinase (PI3K), which plays a major role only in the ADCC mechanism. The phosphorylation of the ITAM sequences allows, besides the generation of second messengers, also the translocation into the nucleus of transcription factors such as NFATp and NFATc.

After stimulation by CD16, NK cells mediate antibody-dependent cell-mediated cytotoxicity (ADCC), and they secrete a variety of cytokines such as IFN-γ, tumor necrosis factor (TNF)-α, and some colony stimulating factors such as IL-3 and granulocyte monocyte colony stimulating factor, GM-CSF.
Because of the low affinity for immunoglobulin G (IgG1 and IgG3 specifically recognizes) CD16 binds only to IgG coated cells or immune complexes and not to free circulating IgG in the blood; so that the free IgG in the plasma do not interfere with the recognition by the CD16 cell covered by Ig, allowing the accomplishment of the cytotoxic action versus target cells. In this process the IgG have the dual role of specific recognition structures of the target cell that needs to be eliminated and mediator of NK cell binding of the target cell, ensuring the specificity effector.
The CD16 antigen is not an exclusive molecule of NK cells, it is also present on the surface of granulocytes, where, however, it does not induce the ADCC mechanism, in fact the main function of CD16 on granulocytes is to enable the anchoring of the latter to opsonized cell, thus allowing the process of phagocytosis. This is due to the fact that CD16 on granulocytes is not an integral membrane protein, and, unlike the NK cells, it has no got the transmembrane and intracytoplasmic domains able to transduce the signal inside the cell, being CD16 simply anchored to the surface cell by means of a phosphatidylinositol.

CD27

CD27 is a member of the tumor necrosis factor receptor superfamily, known to play a very important role in cell growth and differentiation, as well as apoptosis or programmed cell death (Prasad et al., 1997). It binds to ligand CD70, and it is one of the most important markers for the characterization on NK cells in mice: altogether with the gradual decrease of CD11b expression, there is the gradual up-regulation of CD27, which became a marker of the latest stages of differentiation of NK cells (Chiossone et al., 2009).

NK inhibitory and activating receptors

NK cells mediate spontaneous immune response against a variety of cells including, in some conditions, the autologous cells. The study of the regulatory mechanisms of this response has involved many researchers and has led to the definition of a new mechanism of lymphocyte regulation mediated by surface receptors, both activators and inhibitors, expressed by NK cells (Moretta &Moretta, 2004).
A key role in the recognition of appropriate target cells is played by the ubiquitously expressed major histocompatibility complex (MHC) class I molecule, a ligand for which NK cells generally have multiple receptors. NK receptor gene complexes are

intimately associated from a genetic and functional point of view with MHC recognition, and the interaction of various combinations of NK cells receptors (NKRs) and MHC class I molecules may have contribute also to human survival in the presence of epidemic infections (Parham, 2005). In the absence of inhibitory signals, the activating receptors induce biological responses and this is the reason why NK cells spontaneously lyse tumor cell lines which do not express HLA class I antigens (Moretta et al., 2001). Healthy cells are protected from lysis mediated by NK cells by the expression of the major histocompatibility complex class I (MHC-I) complex that acts as a ligand for the inhibitory receptors of NK cells (Lanier, 2005).

NK cell receptors should be distinguished according to their function. Most of the molecules expressed on the NK cell membrane is expressed by other hematopoietic cells, such as T-lymphocytes, myeloid cells and monocytes. Moreover, many of these receptors are not expressed by all NK cells, indicating the existence of phenotypic and functional heterogeneity within this cell population. Receptors involved in inhibitory functions (or those whose activation induces the inhibition of the cytotoxic activity and cytokine production), are important because they represent a sort of safety-check in preventing attacks by NK cells against normal autologous cells and they are represented by killer immunoglobulin receptors (Killer Immunoglobulin (Ig) -like receptor, KIR), which recognize different allelic groups of HLA-A -B, -C molecules; by the complex CD94 / NKG2A that recognizes HLA-E molecules, and to a lesser extent by the LIR. A common feature of inhibitory receptors is the presence in their intracytoplasmic chain of a tyrosine immune-receptor with inhibitory function (immune-receptor Tyrosine-based Inhibitory Motif, ITIM) which, following the binding with the appropriate MHC, activates the phosphatase SHP-1 and SHP-2. These phosphatases in turn inhibit the cascade of signals induced by activating receptors. There are also several receptors involved in the activation of NK cells, like the natural cytotoxicity receptors (Natural Cytotoxicity Receptors, NCRs) NKp46, NKp44, NKp30. These receptors are very important not only for their functions but also because they are expressed almost exclusively by NK cells, so they can be very important for their identification. In the NK immune response there are also numerous surface molecules that perform specific functions such as co-activators (CD2, 2B4, DNAM- 1) and adhesion molecules (such as CD56 and LFA-1).

Inhibitory NK receptors

Inhibitory receptors are randomly distributed on NK cells surface and they distinguish different subsets NK. In the early '90s, basing on some experimental evidences showing that NK cells killed preferentially cancer cells, which don’t express MHC-I molecules, the hypothesis of an immune surveillance mechanism which eliminate cells with deterioration in the expression of MHC molecules was formulated. In particular, the

cytolysis is inhibited when the appropriate molecules HLA (the major histocompatibility system of the human) of class I are expressed by target cells, but it is implemented when these molecules, which identify its own cells as "self", are missing, and there is the situation known as "missing self" (Ljunggren & Kärre, 1990). The “missing self” hypothesis suggests that NK cell could attack only target cells with a reduced or aberrant MHC or HLA-I (like cancer cells or virus-infected cells), become unable to send inhibitory signals to NK cells and, therefore, they become susceptible to NK lysis. Thus, when MHC-I is expressed (normally by healthy tissues) on target cells, the activation of NK cells in inhibited (Kärre, 2008). Almost all NK cells express at least one specific inhibitory receptor for MHC I "self-molecules" and NK cells that do not express them are hypo-functional (Anfossi et al., 2006), in this way the autologous cells that express MHC I antigens are protected from NK lysis. This inhibition is essential for the role of NK cells: because of the abundant expression of MHC-I on many cells, NK cells remain non-responsive to healthy tissue (Figure 3).

In the last decade there was the molecular characterization of human NK inhibitory receptors, which allowed their division in different classes, among these there are structurally distinct families of receptors that are sensitive to the expression of MHC class I molecules I: the family of the KIRs (Killer immunoglobulin-like receptors) and LIRs (Leukocyte immunoglobulin-like receptor-1) belonging to the immunoglobulin superfamily and the receptors belonging to the family of C-type lectins (CD94/NKG2A-C) (Moretta et al, 1996). The expression of KIR and CD94/NKG2 receptors is not restricted to NK cells, because they are expressed also by a subpopulation of T lymphocytes, which inhibits both the cytotoxicity and the production of cytokines induced by TCR. Inhibitory receptors in NK cells determine the inhibition of signals derived from activating receptors, including cytotoxicity signals and the production of cytokines (Augugliaro et al., 2003).

Humans and primates have evolved different families of receptors when compared to mice; but even if they have different developmental origins and structures there are some evidences of their convergent evolution, such as the ITIM (immunoreceptor Tyrosine-based Inhibitory Motifs) and DAP-12 ITAM (immunoreceptor tyrosine-based activating motifs) signalling, the recognition of MHC-I and the presence of both inhibitory and activating receptors (Barten et al., 2001)

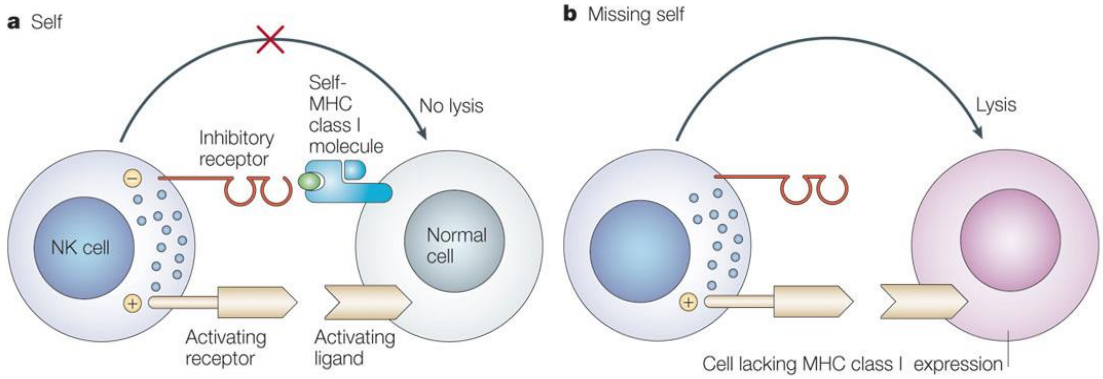

Figure 3: Schematic representation of the "missing-self" hypothesis. **a**. NK cell interacting with a normal autologous target cell which expresses the appropriate MHC-I so that inhibitory signals block the lysis. **b**. NK cell is activated by the missing of expression of MHC-I on the surface of the target cell; it does not receive inhibitory signals and therefore lyses the target cell [Adapted from: Kumar & McNerney, 2005].

KIRs

KIR (Killer Immunoglobulin-like Receptors) are integral membrane glycoproteins expressed on the surface of human NK cells and also T cell, in which an extracellular, a transmembrane and an intracytoplasmic portion can be distinguished; they have evolved from the Ig-superfamily and they have two or three Ig-like domains with short or long cytoplasmic tail that are able to specifically recognize different types of HLA (Colonna & Samaridis, 1995; Wagtmann et al., 1995). Some members of the KIR family specifically bind certain alleles of HLA class I, and the decision of which KIRs are expressed on each NK cells is randomly regulated by the methylation of *KIR* gene loci (Chan et al., 2003).

KIR receptors specifically bind HLA-A, -B and –C molecules, but each individual has a different KIR haplotype, that is the expression of a particular repertoire of *KIR* genes; but three KIR genes are common to all haplotypes: KIR3DL3, KIR2DL4, KIR3DL2. Based on the number of Ig domains present in the extracellular portion there are two subfamilies KIR: KIR2D (which has two Ig domains: D1 and D2) and KIR3D (with three Ig domains: D0, D1, D2). The members of each members of KIR subfamily differ also for the length of the intracytoplasmic chain, those long-chain (long, L), which has two ITIM motifs, induce a signal type inhibitory (KIR2DL and KIR3DL); in contrast, KIR with short tail (KIR2DS and KIR3DS) have the ITAMs sequences that generate activation signals upon interaction with their respective ligands.

KIRs inhibitory receptors are:

- KIR2DL: it is also called p58 (Moretta et al., 1993) and it has two extracellular Ig domains (D1 and D2) and an intracytoplasmic chain with 76-84 amino acidic

residues. The recognition of KIR2D depends mainly on the nature of the MHC-I amino acid present at position 80; in this family we can find two main subsets:

- KIR2DL1, also called CD158a, which is a protein of 58kDa that binds HLA-C2 molecules and it induces an inhibitory signal after the contact of NK cells with their target (Melero & Salmeròn, 1994).
- KIR2DL2, also called CD158b, which is a glycoprotein of 58kDa that binds HLA-C1 molecules, and it leads to the inhibition of NK cell-mediated cytotoxicity.

- KIR3DL: it is also called p70 (Litwin et al., 1994) and it has three extracellular Ig domains (D0, D1, D2) and an intracytoplasmic chain with 84-95 amino acidic residues. Also this family have different members, but the main can be considered KIR3DL1 (also called CD158e1 or NKB1). It is a glycoprotein of 70kDa and it binds some HLA-A and HLA-B alleles of the serologic group Bw4.

The biochemical mechanisms that mediate this inhibition are known only in part and it was only recently discovered that inhibition of NK function involves recruitment and activation of the tyrosine phosphatase SHP-1. The signal transduction cascade of KIR inhibitory receptor is triggered at the level of the ITIM amino acid sequence, present on the cytoplasmic tail of these receptors. When this structural motif containing tyrosine residues recognizes its specific ligand, there is the phosphorylation of a tyrosine residue, the recruitment and activation of a phosphatase (SHP-1) thus determining the inhibition of activating signal which, otherwise, would lead to the lysis of the target cell (Burshtyn, et al., 1996).

There are also KIR activators which have an extracellular portion similar to that of inhibitors, but a short intracytoplasmic portion, in particular:

- KIR2DS, also called p50 (Bottino, et al., 2000), has two extracellular Ig domains (D1 and D2) and an intracytoplasmic chain with 39 amino acid residues. In this family, it is possible to distinguish subgroups:
  - KIR2DS1 (also called CD158h) is a glycoprotein of 50 kDa which upon interaction with a group of HLA-C alleles HLA-denominated C2, it produces a activating signal.
  - KIR2DS2 (also called CD158j) is a 50 kDa glycoprotein which results in a signal that activates the cytotoxicity mediated by NK cells, but its ligand is still not well known.
- KIR3DS1 (also called CD158e2) has three immunoglobulin extracellular domains (D0, D1, D2) and it is able to transduce activating signals when it encounters its ligand. It seems that it can bind the HLA-alleles Bw4, but in a manner dependent on the assembled peptide.

- The KIR2DL4 is actually a receptor with activating function, despite the long cytoplasmic tails, and that recognizes the HLA-G molecule expressed exclusively in the fetus.

The cytotoxic effect of NK cells toward target cells is a balance between inhibitory KIR and stimulating KIR: the former can inhibit the cytotoxic activity mediated by KIR activators if the target or antigen presenting cell also expresses the ligand for the inhibitory KIR (Moretta &Moretta, 2004). While recognizing the same HLA-I molecules, the latest evidence suggests that the KIR activators have greater affinity for HLA-I associated peptides of viral antigens; while the KIR inhibitors have greater affinity for HLA-I associated peptides self-antigens.

KIRs have a role in the induction of NK cell tolerance of self-tissue, preventing the activation of NK cells against normal healthy tissues. It's important to notice that KIR and HLA segregate independently and the expression of KIRs is not driven by HLA (Gumperz et al., 1996).

Ly49 receptors

Instead of having polygenic and polymorphic KIRs, rodents have expanded their Ly49 genes, resulting in a remarkable diversity across different inbred mouse strains (Kirkham & Carlyle 2014). This complex in the mouse comprises about 20 genes and pseudo genes similar to KIRs in humans from a functional point of view, because both receptors families have inhibiting and activating members.

Ly49 receptors are type II integral membrane proteins that form disulphide-linked homodimers on the cell surface, and they composed of a carboxy-terminal lectin domain, also known as NK domain (NKD), which gives specificity for distinct allotypic groups of MHC-I and MHC-I like molecules (Karlhofer et al., 1992). The NKD is bind to the cell membrane by an extended stalk region, about 70 amino acidic residues in length. Within this lectin domain, like in the KIRs family, there is genetic variation both in which Ly49 genes are present and in the sequence of individual genes across different mouse strains (Carlyle et al., 2008).

More than 20 Ly49 genes have been identified, the majority of which encode for inhibitory receptors, whose prototypes are Ly49A and Ly49C. Both of them have the ITIM motif in their cytoplasmic regions that, after the binding of the molecule in the target cell, recruit and activate phosphatases such as SHP-1, to inhibit NK cell activation (Nakamura et al., 1997). Within the Ly49 family, there are some members that have been evolved more recently, like Ly49D and Ly49H, that have gained the capacity to interact with the small disulphide bonded homodimers such as DAP12 or DAP10 (Smith et al., 1998).

The pathway of MHC-I recognition by Ly49 receptors requires the presence of a peptide bound in the furrow of the MHC molecule, and each Ly49 receptor has a

different specificity for this peptide binding, because they exhibited different binding proprieties (Hanke et al., 1999). It has been found that Ly49 molecules, such as Ly49A, not only bind its ligand on potential target cells (*trans*), but also it is constitutively associate on the same cell (*cis*); thus lowering the threshold at which NK cell activation exceeds NK cell inhibition, cis interaction allows optimal discrimination of normal and abnormal host cells (Doucey et al., 2004). Moreover, the nature of Ly49 receptors (*cis* or *trans*) can affect the signalling outcome.

C-type lectin family of receptors

The receptors CD94/NKG2 are heterodimers composed of a common chain, CD94, associated with one of the products of genes NKG2. The most representative of the inhibitory receptor superfamily of C-type lectins consists of the CD94 glycoprotein subunits and NKG2A tied in a heterodimeric complex. These receptors react with a non-classical MHC-I on the surface of target cells, and they seem to be crucial for the prevention of inappropriate NK cell activation (Borrego et al., 1998); in particular their ligand is the product of the HLA-E gene, which expression depends on the presence of MHC-I molecules and it is expressed in most normal autologous cells (Braud et al., 1998). The interaction of the receptor with the HLA-E therefore allows to prevent "self-reactivity" against normal cells. In humans the expression of these receptors seems to be related to *KIR* gene expression, as suggested in an important study where they demonstrate that NK cell clones lacking the expression of an inhibitory KIR, expressed an inhibitory CD94/NKG2 heterodimer (Valiante et al., 1997).

The CD94 is the invariable component of the receptor, and it is a type II integral membrane protein, encoded by a single gene with no apparent polymorphism (Chang, et al., 1995), and which has a very short intracytoplasmic chain that alone it is not capable of inducing a signal transduction after the bounding with the specific ligand; for this reason to be functional, CD94 need to be bounded through a disulphide bond with a member of the NKG2 family receptors.

NKG2 is a multigene family expressed either on the cell surface of NK cells on $CD8^+$ T lymphocytes (Ykoyama & Seaman, 1993), consisting of five different proteins (NKG2A, NKG2B, NKG2C, NKG2D/F, NKG2E). Like KIRs, some members have inhibitory function (NKG2A and B) and other have activating functions (NKG2C, NKG2D/F, NKG2E).

Despite the structural heterogeneity of the various inhibitory receptors that interact with MHC I molecules, the mechanism responsible for their inhibitory activity is common. In fact in the intracytoplasmic tail of an inhibitory receptor, both KIR of the CD94/NKG2 family (-A / -B), there are one or two ITIM sequences, containing tyrosine residues, which upon interaction with the ligand MHC I are phosphorylated. The phosphorylated ITIM domain is therefore responsible for the recruitment of tyrosine

phosphatases, and in particular SHP-1, responsible for the propagation of the negative signal and then by blocking the activation of NK cell (LeDrean, et al., 1998). The other members of the NKG2 family instead transduce activating signals, because they are associated with trans-membrane proteins such as DAP10 and DAP12 that contain ITAM sequences. It is important to underline the fact that each inhibitory receptor bound can only inhibit the activators of receptors signals that are around it (Kaplan, et al., 2011).

Another member belonging to the superfamily of C-type lectins is the NKR-P1 receptor (or CD161), expressed in dimeric form on most NK cells and on a subpopulation of T lymphocytes (Lanier et al., 1994). The genes coding for NKR-P1 were identified both in humans and in rodents and are located in a chromosomal region called "NK complex". Unlike the mouse, in humans there is only one family member, NKR-P1A, while in the mouse NKR-P1 receptors can be both activating and inhibitory, and five different receptors have been identified (Plougastel et al., 2001). The physiological ligand of NKR-P1 seems to be the lectin-like transcript-1, LLT1. The interaction between NKRP1A on NK and LLT1 on target cells inhibits both the cytotoxic activity that the secretion of cytokines (Rosen et al., 2008). In humans, some evidence suggests that it can work as a receptor both activating and inhibitory, depending on the cell type.

LIRs

The family of the LIRs (Leukocyte Inhibitory Receptors) is quite wide and also distributed in other cell types, including B cells, dendritic cells and certain T lymphocytes (Fanger et al., 1999). These receptors such as KIRs, are inhibitory receptors able to recognize a wide variety of MHC-I molecules presenting a very similar structure characterized by the inhibitory ITIM sequences and thereby acting with the same mechanisms.

The function of LIR in the regulation of NK cell activation is not completely clear, but it has been found a specific LIR which binds UL18, a protein encoded by human cytomegalovirus, with greater affinity than for HLA-I (Chapman et al., 1999).

NK activating receptors

According to the hypothesis of the missing-self, in the absence of inhibitory signals due to a failure or reduced expression of HLA class I on the target cell membrane, the activating receptors lead to the activation of NK cell that is now free to perform its cytotoxic activity and to produce cytokines (Moretta et al., 2001). NK cells have many activating and co-activating receptors. In addition to activators members of KIRs and NKG2 family (especially NKG2D), among the activating receptors members of the

NCR (Natural Cytotoxicity Receptors) family are very important, and they are represented by NKp46, NKp44 and NKp30.

NCRs

NK cells express three different receptors that mediate directly the natural cytotoxicity, called NCR, which are NKp30, NKp44 and NKp46; they were discovered in the late 1990s as being express on human NK cells. Even if they share some functional skills, the do not share many similarities either in their amino-acid sequence or in their structure (Joyce & Sun, 2011).

The NCRs belong to the Ig superfamily (Ig-SF), and they are integral membrane proteins in which an extracellular portion with one or two Ig domains, a transmembrane portion and an intracellular portion can be identified. The intracellular portion is associated through a disulphide bridge to molecules presenting ITAM sequences and that transduce activation signals of the natural cytotoxicity mediated by NK cells when they are engaged with their specific ligands (Figure 4).

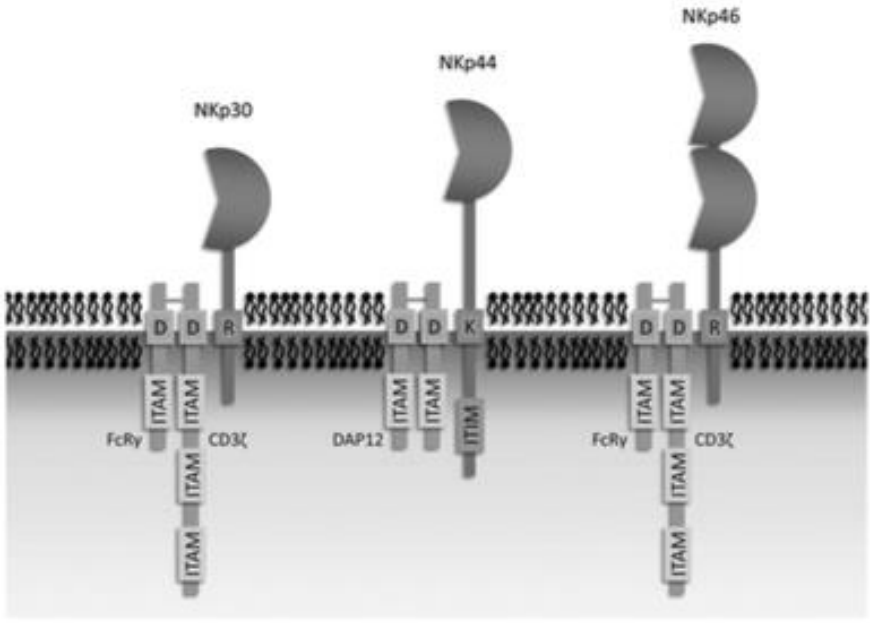

Figure 4: Schematic representation of the NCRs molecules. All NCRs have a positively charged amino acid in the transmembrane domain but NKp30 and NKp44 possess only one Ig-like domain, whereas NKp46 has two. [From: Kruse et al., 2014].

The **NKp30** receptor is a glycoprotein of 30 kDa that is expressed in all mature NK cells including those immature that are produced in vitro from $CD34^+$ cells and it cooperates with NKp46 and NKp44 in the induction of cytotoxicity against several tumor targets (Pende et al., 1999), moreover it can be expressed also on other type of cells, such as cord blood T cells after IL-15 exposure (Tang et al., 2008). NKp30 is a

molecule composed of one extracellular Ig domain, the transmembrane domain has a charged arginine associated with the negatively charged present in the transmembrane portion of the CD3ζ molecule, and the intracellular domain has no additional signalling domain. It is associated to the ζ chain of the CD3 complex and both the CD3ζ homodimers and the CD3ζ/FcRg heterodimers can bind to the charged arginine in the transmembrane domain. There are six different splicing variants of NKp30 known to be expressed on the cell surface: three of them encoding for a molecule with an extracellular V-type domain, and the other three encoding for a C-type Ig domain (Neville & Campbell, 1999). NKp30 plays an important role in the selection (editing) of dendritic cells induced by NK cells, the latter are in fact able to recognize and eliminate immature dendritic cells that would not do properly the work of antigen presentation (Della Chiesa et al., 2003). The cross-linking mediated by the specific monoclonal antibody (mAb) on NKp30, induces cellular responses identical to those induced by NKp46: flow of Ca^{2+} ions, cytotoxicity, and production of cytokines. Recently it was shown that TGF-β1 (Transforming Growth Factor β1) influences the expression of NKp30 and in part also that of NKG2D to the cell surface. TGF-β1, which is issued by various tumor such as melanoma, neuroblastoma, cancer and leukemia, is proficient in inducing a negative regulation of the expression of the receptor-inducing cytotoxic activity, as if cancer cells have found a mechanism escape surveillance by NK cells (Romero et al., 2001). Among the cellular ligands that NKp30 is able to recognize we find B7-H6 (Pogge von Strandmann, et al., 2007), BAT3 (Brandt, et al., 2009), the pp65 protein of HCMV (Arnon, et al., 2005), and heparan sulphate (HS) as a co-ligand (Hecht, et al., 2009).

The **NKp44** receptor is another member of the NCR family, with a molecular weight of 44KDa, which induces NK-mediated cytotoxicity as a result of cross-linking with specific antibodies. There is no coding gene for NKp44 in the mouse, but the gene has been found in other primates; in particular in humans it is expressed on the surface of activated cells (Vitale et al., 1998). NKp44 is composed of a single extracellular V-type Ig domain, a single transmembrane domain rich in lysine amino-acidic residues, and a short cytoplasmic domain containing a sequence with a not functional ITIM motif (Campbell et al., 2004); only recently it has been demonstrated that it can be functional depending on the ligand (Rosental et al., 2011). The transmembrane portion is responsible for associating this molecule with the KARAP/DAP12 complex, which in turn has only one ITAM region (Lanier et al., 1998). Although the NCR ligands are still poorly understood, it has been recently seen that the protein E of the flavivirus can be linked to the NKp44 receptor (Hershkovitz, et al., 2009).

NKp46 was the first NCR to be identified; it is the only NCR conserved in human and mice and it is expressed on NK cells regardless of whether they are resting or activated (Sivori et al., 1997). It is a transmembrane glycoprotein of 46 kDa characterized by two extracellular domains of the Ig-like C2 type, followed by a sequence of amino-acidic residues that connects them with the transmembrane and cytoplasmic portion (Pessino et al., 1998). The intracytoplasmic portion does not contain the ITAM domains necessary to promote activating signals, but the signal mediated by NKp46 depends on its association with the adapting molecules CD3ζ and FCεRIγ, containing the ITAM sequences that, after their phosphorylation at the level of the tyrosine residue, transduce the activation signal. It is seen that the polypeptides CD3ζ and FcεRIγ are also involved in other intracellular signal transduction, for example via CD16. Following the assignment of this receptor with its ligand there is a mobilization of Ca^{2+} ions from intracellular stores and the release of lytic granules, which determine the lysis of target cells and release of cytokines. NKp46 also has an important role in the regulation of NK cell function, in fact in a mutant mouse in which NKp46 was not stably expressed at the cell surface, NK cells were hyper responsive due to an overexpression of the Helios transcription factor (Narni-Mancinelli et al., 2012). A possible ligand of NKp46 is represented by the hemagglutinin (HA) of influenza virus (Mandelboim et al., 2001). The interaction between the HA protein and the receptor provides a mechanism by which NK cells can specifically recognize and eliminate virus-infected cells. In vitro studies have shown that after an initial up-regulation of NKp46 in response to the virus, occurs a subsequent down-modulation of the receptor probably induced by chronic stimulation produced virus (Jost et al., 2011). A recent study by Jaron-Mendelson has demonstrated that a dimerization (between two molecules NKp46) needs to happen to let NKp46 perform its task because it influences the binding of the receptor to the target cells via an allosteric effect (Jaron-Mendelson et al., 2012). It is unclear whether the dimerization is contingent upon ligand binding and allosteric change produces the signal transduction or whether the dimerization affects ligand binding.

NKG2D

Recently, some studies have shown that clones of NCR^{bright} NK cells could kill some tumor cell lines through an NCR-dependent mechanism, while the killing of other cellular targets requires coordinated action by both the NCR and NKG2D. Thus, NKG2D plays an important role in activating NK cells, and its activity is complementary to that of NCR (Pende et al., 2001); in particular, this receptor has been shown to be important in the NK cell-mediated control of some cancers (Guerra et al., 2008).

NKG2D is only related to the NKG2 family, and it does not form a heterodimer with CD94, but it is expressed as a homodimers, and its signalling works by recruiting DAP-

10 or DAP-12 molecules; in fact, to perform its cytolytic function, it needs the association with adapter proteins that transduce the signal such as DAP10 and DAP12. These proteins contain ITAM motifs in their intracytoplasmic portion and are able to activate the enzyme PI 3-Kinase (phosphatidyl-inositol-3 kinase), after the phosphorylation of a tyrosine residue present in the intracytoplasmic chain ITAM (Wu et al., 1999). In the mouse there are two isoforms of the NKG2D molecule, a longer isoform and a shorter one, and after their stimulation they signal through DAP-12 resulting in both cytokine secretion and cytotoxicity, and through DAP-10 they stimulate a strong cytotoxic response (Gilfillan et al., 2002). In human, NK cells only express the long isoform of NKG2D, which associates with DAP-10 to induce both cytotoxic and cytokine-mediated response.

NKG2D recognizes different ligands, including MHC-I related proteins whose expression is regulated by a DNA damage and heat shock response pathways. In humans these ligands are represented by surface molecules induced by stress such as MHC-like protein, and MIC-B MIC-A (MHC class I-related chain A and B) and UL16-binding proteins called proteins, ULBPs (Groh et al., 1999; Sutherland et al., 2001). The MIC-A and MIC-B molecules are transmembrane molecules normally expressed gastro-intestinal epithelium but also on other epithelia such as the lung, breast, kidney, ovary, prostate and some pathologies such as colon cancer and melanoma. The expression of these molecules is increased in response to cellular stress and following the infection by pathogens. Recently it has been discovered another ligand for NKG2D known as ULBP-16, produced by the human cytomegalovirus. In the mouse, NKG2D binds to retinoic acid early transcript-I molecules (α, β, γ, δ and ε), as well as mouse UL16-binding-like transcript-I and H60 molecules (Carayannopoulos et al., 2002).

NKG2D has been shown to have a role in the immune response to certain immunogenic tumors, which have been reported to secrete NKG2D ligands, such as MIC-A, which can serve as a decoy to NK cells (Groh et al., 2002). Tumor cells use different strategies to escape NKG2D mechanism, like the secretion of transforming growth factor-β1, which can lead to down regulation of expression of NKG2D on NK cells (Castriconi et al., 2003).

Co-activating receptors

Other surface molecules are expressed on NK cells (but also on other lymphoid cells), and they seem to have a co-receptor function. In fact, their ability to activate NK cells depends on the simultaneous activation of other activating receptors (Moretta et al., 2001) and they are able to amplify the cytotoxic effect when co-stimulated with activating receptors (NCR and NKG2D CD16), acting as co-activators. Some of these proteins are: 2B4, CD2 and DNAM-1 (Bryceson et al., 2006)

2B4 (CD244)

The receptor 2B4, also known as CD244, is a glycoprotein of 70 kDa expressed on all human and mouse NK cells, and on $CD8^+$ cytotoxic T lymphocytes, TCRγδ, on monocytes and basophils (Nakajima et al., 1999). The ligand of 2B4 is represented by the CD48 molecule, expressed by all the hematopoietic cells, and in both human and mice there are contrasts regarding the outcome of stimulation through this receptor, in fact activation or inhibition could result from the signalling induced by the recruited adapter proteins.

2B4 could be a multifunctional receptor, and it has been postulated that the result of the triggering may be dependent on the stage of NK cell maturation (Lanier, 2005). 2B4 is characterized by the presence of four tyrosine motif in its cytoplasmic tails which, following the tyrosine phosphorylation, may be associated with a small cytoplasmic protein of 14 kDa said Src homology 2 domain-containing protein (SH2D1A, also called SLAM-associated protein, SAP) (Poy et al., 1999). This molecular association is fundamental for the triggering of signals that led to the activation of NK cell. However, the activation of 2B4 can also produce the recruitment of a phosphatase containing SH2 domains (SHP). Thus, the activation depends on the competition of these two molecules in binding 2B4. So when SH2D1A joins 2B4, is also prevented the generation of inhibitory signals mediated by the tyrosine phosphatase, SHP (Lewis et al., 2001). This effect is due to the binding of the phosphatase SHP-1 with the cytoplasmic domain of 2B4. The two isoforms present in mice have different cytoplasmic domains, signalling either activation or inhibition (Schatzle et al., 1999).

Studying the in vitro differentiation of human NK cells from $CD34^+$ progenitors in the cord blood, it has been revealed that 2B4 seems to appear early during the NK differentiation and this molecule in the early stages of NK differentiation seems to have an inhibitory function. This function serves to improve tolerance to "self" NK differentiating cells which express the NCR receptor, in particular NKp46 and NKp30, and do not exhibit inhibitory receptors that bind HLA-Specific (Sivori et al., 2002).

Moreover, experiments on *resting* NK cells showed that 2B4 synergizes with activating receptors NKp46, NKG2D and DNAM-1 (Bryceson et al., 2006). CD16 also very well cooperates with 2B4, in fact it has been seen that the synergy with NKp46 appears to be significantly lower than that with CD16. While the cross-linking of the 2B4 monoclonal antibody with NKG2D or DNAM-1 has only caused a small but reproducible increase in intracellular Ca^{2+}, with CD2 the elevations of Ca^{2+} levels were lower and CD56 there have been at all (Bryceson et al., 2005). The 2B4 behaves as a co-receptor with NKp46, but studies of NK cells with low levels of NKp46 expression have confirmed the hypothesis that 2B4 may also increase the NK cell activation induced by other activating receptors such as NKp44 or CD16 (Sivori et al., 2000).

DNAM-1 (CD226)

DNAM-1, also known as CD226, is a transmembrane glycoprotein of 65 kDa of 318 amino acids and it is involved in adherence and in the transduction of lymphocyte activation signal. It seems to have a synergistic action only with NKp46 and 2B4 (Bryceson et al., 2006). It is constitutively expressed upon 50% of NK cells, but it is also found in monocytes, lymphocytes T and in a small subset of B lymphocytes (Bottino et al., 2003). This receptor is a member of the Ig superfamily, it has two extracellular Ig-like domains of the V type and is associated with PVR molecules and nectin-2 (CD155 and CD112) playing a key role in cell adhesion, in the activation of cytolysis mediated by T and NK cells and the secretion of cytokines (Shibuya et al., 1999). These ligands can be up regulated on some tumor cells, implicating DNAM-1 in some NK-cell mediated anti-tumor responses (Masson et al., 2001). Moreover DNAM-1 is involved in the lysis of tumor cells that do not express ligands for NK cell-activating receptors, suggesting that this receptor is more than just co-stimulatory (Gilfillan et al., 2008).

DNAM-1 is involved in a variety of cellular functions that include both innate immunity that adaptive: it intervenes for example in the activation of co-stimulatory signals mediated by LFA-1 on the proliferation and differentiation of $CD4^+$ T cells into Th1 cells (Shibuya et al., 2003), in the activation of macrophages and platelets, in adherence to the vascular endothelial cells (Shibuya et al., 2005). In fact DNAM-1 is physically associated to the LFA-1 adhesion molecule, influencing its function (Shibuya et al., 1999), so DNAM-1 may be involved in the building of stable interactions between NK and target cells.

CD2

CD2 is a glycoprotein of approximately 50 kDa belonging to the Ig super-family, it is expressed by T lymphocytes and NK cells. Specific antibodies directed against some of the CD2 epitopes are capable of promoting the adhesion between the effector cell and target cell, and then to activate the NK cytotoxicity. The main ligand of CD2 is another member of the Ig superfamily membrane protein, CD58 (also known as LFA-3) (Selvaraj et al., 1987). The expression of CD58 only on the target cell is not sufficient to trigger the natural cytotoxicity, suggesting that the CD2 act as a co stimulatory receptor that increases, but does not induce the NK lysis. In particular it has been seen that CD2 presents a unique synergy for receptors associated with ITAM sequences as NKp46 (Bryceson et al., 2006).

Integrins

Another category of NK cell receptor is represented by integrins. They are heterodimeric integral membrane glycoproteins composed of a distinct alpha chain and a common beta chain. They have been found on various cell type, including NK cells and NKT cells and they are involved in cell adhesion and cell-surface mediate signalling.

One of the most important alpha chains is CD11. The CD11a (α_L of 180 KDa) corresponds to the antigen α_1 of the chain associated to the lymphocyte function (lymphocyte function-associated antigens 1, LFA-1), whose ligands are molecules like ICAM-1, ICAM-2 and ICAM -3. Other α subunits that are associated with β2 chain forming CD18 adhesion molecules expressed by the NK cells are CD11b (α_m) and CD11c (α_x). The CD11b (160 KDa), corresponds to the complement receptor 3 or CR3, as well as also is expressed by NK cells by monocytes, a subpopulation of T lymphocytes and granulocytes. It has been seen that CD11b is also the receptor for two clotting factors (factor X, and fibrinogen). The antigen CD11c (150 kDa) is expressed both by NK cells and monocytes.

LFA-1

The LFA-1 (Lymphocyte Function Associated Antigen-1) dimer, composed by CD11a/CD18 molecules, also said $\alpha_L\beta_2$, is one of the most important integrins that binds to intercellular adhesion molecules such as ICAM-1 (or CD54) (Wang & Springer, 1998), and is capable of promoting both the natural cytotoxicity and the ADCC mechanism. The β2 integrin, CD18 is a transmembrane glycoprotein of 678 amino acid residues with 6 sites for N-glycosylation. It has a cytoplasmic region of 46 amino acids, highly conserved, which contains various amino acids (tyrosine, serine and threonine different) that can be phosphorylated following stimulation; the extracellular portion is arranged in a region rich in cysteine highly conserved consists of 4 "tandem repeats" each and containing 8 cysteines in an highly conserved N-terminal region which is essential for the interaction with the ligand and for heterodimer formation.

It has been shown that LFA-1 (CD11a/CD18) is involved in the action NK cytotoxic against target cells (Nakamura et al., 1990). It has been hypothesized that the ability of NK cells to recognize target cells can be the consequence of multiple products at the same time ties by adhesion molecules and activating receptors, rather than by a single receptor for the recognition of the target cell. The adhesion molecules would act primarily by allowing the initial binding to the target cell while activating receptors, modulated by those inhibitors, would trigger the cytotoxicity mediated by NK cells. The adhesion activity, mediated by integrins, may also be modulated by signals from other receptors. In fact, it has been seen that the engagement of activating receptors by their respective ligands on target cells plays an important role in adherence of NK cell mediated by LFA-1. On the contrary, when the inhibitory receptors recognize HLA on

the target cells, they may interfere in this process blocking the adhesion of NK cells from the beginning of contact between cells (Burshtyn et al., 2000). the engagement of the receptor co-activating 2B4 with its ligand CD48, induces a rapid accession dependent on LFA-1 of the NK cells to tumor cells, thus revealing that the 2B4 significantly increases the ability of LFA-1 to interact with its ligand ICAM-1 (Hoffmann, et al., 2011). Other studies had shown that LFA-1 is associated physically with DNAM-1 receptor in both NK lymphocytes in T cells stimulated with anti-CD3 antibodies (Shibuya et al., 1999).

Mac-1

CD11b/CD18 (also referred as to Mac-1 and CR3 [complement receptor 3]), is expressed on human and mouse NK cells, and in particular the CD11b integrin has been defined as a major marker of NK-cell maturation (Hayakawa & Smyth, 2006). In the bone marrow and lymph nodes $CD11b^{low}$ NK cells are the more abundant, and they develop into $CD11b^{high}$ NK cells, acquiring all the features of mature NK cells.

The heterodimer $\alpha_M\beta_2$ is expressed on the surface of many leukocytes including monocytes, granulocytes, macrophages, and natural killer cells. The integrin Mac-1 is expressed by peripheral NK cells, and although the majority of NK cells in spleen, peripheral blood and lung are $Mac\text{-}1^{hi}$, portions of NK cells in the bone marrow and liver are $Mac\text{-}1^{low}$, and its expression correlates with the capacity of NK cells to produce cytokines, in particular IFN-γ (Kim et al., 2002).

Mac-1 is highly expressed on NK cells with the functional capacity to produce cytokines and show cytotoxicity. It mediates inflammation by regulating leukocyte adhesion and migration and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation (Solovjov et al., 2005).

VLA-1

VLA-1 is another receptor that binds extracellular matrix proteins such as collagen and laminin, and the α1 integrin subunit of this receptor is CD49a, both in human and mouse. CD49a is expressed on various cells, including T and immature NKT cells, and it has been associated with inflammation (De Fougerolles et al. 2000).

Moreover, CD49a is a specific collagen IV receptor in VLA-1-high τδ and CD8+ αβ cells and can transmit signals to these lymphocytes to spread and express IL-2R, and it is known to be an important marker in defining a population of tissue memory $CD4^+$ T cells thet acts as a rapid effectors upon reinfection (Chapman & Topham, 2010).

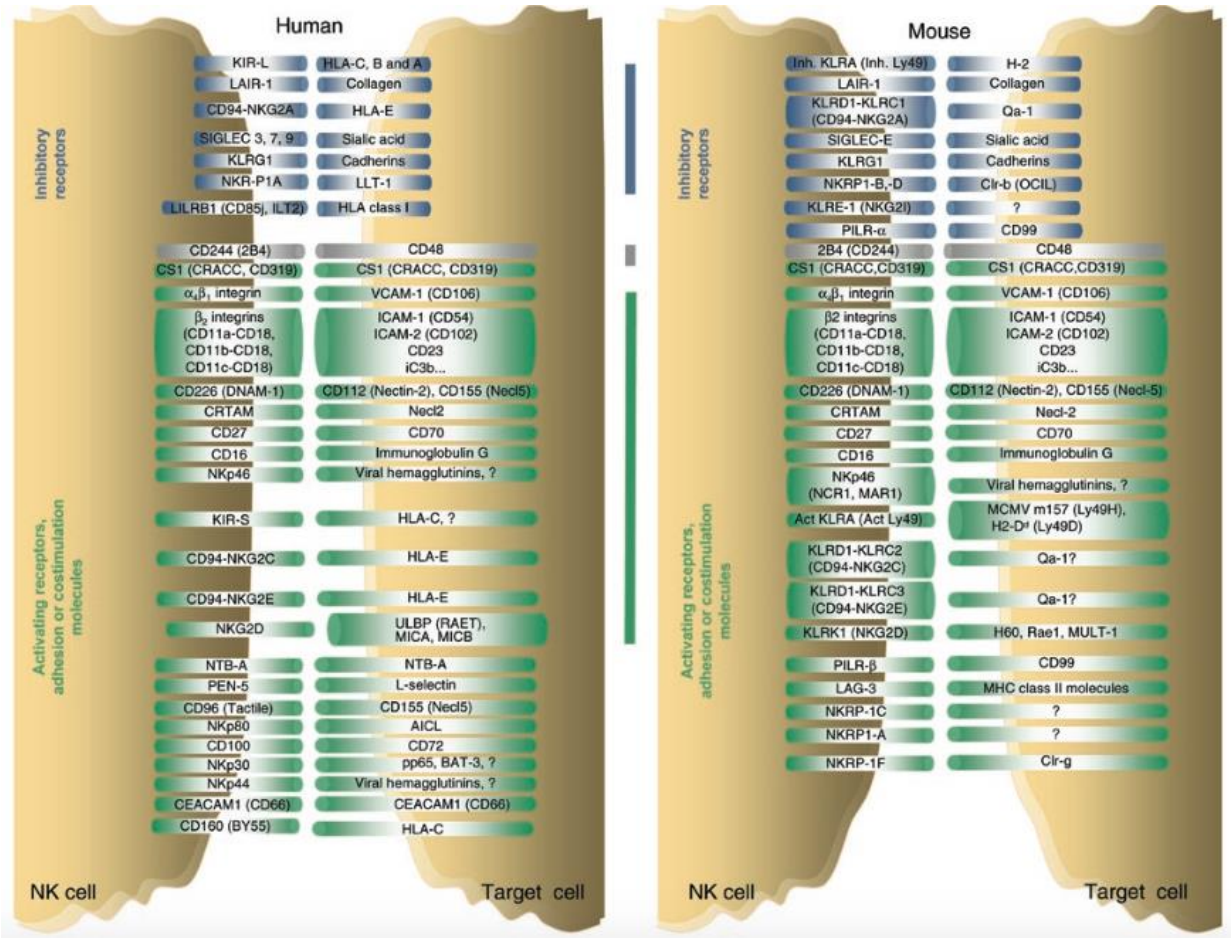

Figure 5: Human and Mouse schematic representation of NK cell molecules interaction with different signals of the target cell. [From: Vivier et al., 2008].

4. Natural Killer cell functions

4.1 Cytotoxic activity

The majority of human NK cells express CD16 receptor, so they can lyse the target cells through the ADCC mechanism. However, the CD16 receptor is not responsible for the spontaneous cytotoxicity of NK cells, demonstrated by the fact that blocking CD16 with monoclonal antibodies inhibits the ADCC activity without changing the "Natural Killing" activity. Thus NK cells have at least two distinct recognition structures, thanks to which they can attack the target cell: one is represented by CD16 and the other is constituted by a family of receptors, characteristic of NK cells, responsible for the recognition not mediated by antibodies of the target cell.

Around 1990, parallel studies in humans and mice, have discovered that NK cells recognize MHC class I molecules, thanks to the presence of receptors on their cell surface, the activation of which can lead to an inhibition or an activation of the spontaneous cytotoxic activity (Moretta and Moretta, 2004). Contrarily to the non-activated T cells, which must first proliferate and then differentiate to develop the cytotoxic ability characteristic of cytotoxic T lymphocytes (CTL) (Schwartz, 1992), NK cells can lyse target cells without a pre-sensitization. In a few minutes, after binding to the target cells lacking MHC class I molecules (such as erythroleukemic K562 line in human), NK cells (especially $CD56^{dim}$) orient cytoplasmic granules containing the lytic proteins embedded in a proteoglycan matrix (Lowin et al., 1995) towards the region of cell contact and release their contents, causing the death of the target cells. This is due to the destruction of the cell membrane by perforin and DNA fragmentation due to the serine esterase, also known as granzyme (Young, 1989).

Perforin is a polypeptide of 550 amino acids with regions of homology with other members of C6-C9 complement; it polymerizes in the presence of calcium and causes transmembrane pores on the surface of the target cell. The homologous sequences of perforin with complement proteins explain how it acts. In fact, similarly to the activated complement proteins, perforin polymerizes by forming pores on the membranes of target cells in a Ca^{2+} -dependent way (Liu et al., 1995) thus allowing the entry of Na^{+} ions within the cell then the swelling due to H_2O entry, and lysis (Duke, et al., 1989). Perforin is a protein that is synthesized as an inactive 70 kDa precursor (Lichtenheld et al., 1988) which is activated, during its biosynthesis, by proteolytic cleavage within an acid compartment (Uellner et al., 1997). The presence of proteoglycans within the lytic granules keeps perforin in an inactive state, with the secretion of cytotoxic proteins, as a result of increased concentrations of Ca^{2+} and pH, proteoglycans free perforin that can fit into plasma membrane of the target cell (Masson et al., 1990) where the phosphor-

choline, polar head of the phosphatidyl-choline, acts as a specific receptor Ca^{+2}-dependent for the perforin molecules (Tschopp et al., 1989). After the insertion, the polymerization of individual molecules of perforin occurs, with the consequent formation of variable-diameter pores (10-20 nm), which allows the entry of Na^{+} and H_20 determining the lysis of the target cell.

Granzymes (A-H) are a family of serine-neutral esterase, they are not equipped with a real cytotoxic activity, but are able to induce rapid DNA fragmentation of the target cell in the presence of "sub-lytic" doses of perforin. Among them, granzymes A and B are the most abundant in the NK cell and best characterized. For the release of granzyme, a first model proposed that these enzymes were entering through the pores created by perforin transmembrane, but a newer model suggests that granzymes enter into the target cell by endocytic vesicles and not directly through the channels formed by perforin. In fact the membrane damage caused by perforin on the target cell would activate a repair process causing endocytosis of the damaged cell membrane with granzyme and perforin, which are located near the damaged area, and which would thus be introduced into the target cell. According to this new model perforin, besides damaging the plasma membrane, allows the release of granzyme in the cytosol of the target cell. Once in the cytosol of the target cell, granzymes and in particular granzyme B is able to activate different caspases and to initiate the apoptotic program (Shresta et al., 1998). The cell-mediated cytotoxic action therefore takes place, through the combined action of perforin and apoptotic proteins present in the granules.

NK cells can induce the death of the target cell using at least three distinct mechanisms:

1. Ca^{2+} -dependent spontaneous cytotoxicity via NCR/NKG2D activation and release of lytic granules;
2. Ca^{2+} -dependent antibody-dependent cell-mediated cytotoxicity (ADCC) via activation of CD16 and release of lytic granules;
3. Ca^{2+}-independent spontaneous cytotoxicity mediated by members of the TNF family (tumor necrosis factor) such as Fas-ligand (Fas-L), TNF-α, in the free or membrane-bound form cell, CD40-ligand and TRAIL.

Mechanisms mediated by the exocytosis Ca^{2+}-dependent of lytic granules lead to the death (necrosis or apoptosis) of the target cells in a few minutes (20-60 minutes), while the Ca^{2+}-independent cytotoxicity determines the apoptosis of target cells, which requires more time (3 hours or more).

Ca2 + -dependent: Spontaneous cytotoxicity and ADCC

This process is regulated by the balance between the stimulation of activating receptors (NCRs, NKG2D) and co-activators (DNAM-1, 2B4) and inhibitory (KIR, CD94-NKG2A). This eventually results in the recognition of the target as harmful or normal.

In the case in which prevail the NK cell activators stimuli, the content of lytic granules become free determining the death of the target cell. In the dependent cytotoxicity process by the release of the granules (both spontaneous and ADCC) it can be distinguished five different phases:

1. Adhesion between effector and target through the adhesion molecules (LFA-1, CD2);
2. Recognition by activating receptors (NCRs, NKG2D, CD16) or coactivators (2B4, DNAM-1) and inhibitory (KIR, CD94-NKG2A);
3. Polarization of the granules to the point of contact between effector and target;
4. Degranulation at the level of immunological synapses;
5. Death of the target cell.

The ADCC mechanism (Antibody Dependent Cell-mediated Cytotoxicity) is implemented through the interaction of the surface receptor CD16 or FcRgammaIIIA with crystallizable fragment (Fc) of IgG (Ravetch & Perussia, 1989; Ravetch & Kinet, 1991) and it is an important link between specific immunity mechanisms and the innate immunity. The ADCC has been studied mainly in NK cells, which have the ability to kill cancer cells without phagocytosis or involvement of MHC molecules. The events that characterize the ADCC and the CD16-mediated lytic mechanisms can be summarized as follows:

1- Binding portion of the F (ab ') 2 IGG (which contains the hypervariable regions) to an antigen on the target cell membrane;
2- Fc fragment of intact IGG recognition by the CD16 receptor on NK cells;
3- Induction of the activating signal cascade in the NK cell, which results in the exocytosis of lytic granules and release of their contents in the contact area between effector cell and target cell (Henkart & Yue, 1988);
4- Death of the target cell as a result of the lysis by the perforin or to the activation of apoptosis by granzyme (Trapani & Smyth, 2002).

Spontaneous Ca^{2+}-independent cytotoxicity

The identification of cytotoxic activities Ca^{2+}- and perforin-independent CTL-mediated cytotoxicity led to the discovery of the action mediated by the interaction of Fas ligand present on the NK cell membrane with the Fas receptor expressed on the target cell membrane (Young et al., 1987; Kagi et al., 1994). Ca^{2+}-independent cytotoxicity is mediated by the TNF receptor family members, also known as the family of death receptors as ligands that belong to this family (Fas Ligand or Fas-L, TRAIL and TNF - α) (Henkart & Yue, 1988) bind receptor (Fas, TRAIL-R1, TRAIL-R1 and TNF-R2) containing the intracytoplasmic region of a particular amino acid sequence known as the death domain. These proteins are secreted or expressed on the surface of NK cells and

bind with their receptors on target cells leading to the trimerization of the receptor resulting in the transduction of an apoptotic signal through the activation of the executive "caspase" pathway. Unlike the lysis, the cell into apoptosis has, at least initially, only a partial alteration of cell membrane permeability, which only subsequently disintegrates into the process that takes the name of "secondary necrosis."
The expression of Fas-L on the surface of NK cells (or T cells) is regulated by various cytokines and can be induced by stimulation of the CD16 receptor. Fas-L may be present in both a membrane-associated form and in a soluble form. Through recombinant DNA techniques, by analogy with the structure of the Fas-L, it has been cloned another molecule of the TNF family, called TRAIL (TNF-Related Apoptosis-Inducing Ligand) capable of inducing apoptosis through specific death receptors, defined TRAIL - receptors 1 and 2, or DR4 and DR5 (Wiley et al., 1995; MacFarlane et al., 1997).
Until now, they have been identified 4 distinct membrane receptors for TRAIL, called TRAIL-R1, -R2, -R3 and -R4. TRAIL-R1 and R2 are both transmembrane receptors type I and possess a cell death domain that transduces an apoptotic signal as a result of their trimerization induced by TRAIL. TRAIL-R3 and R4 differ to R1 and R2, because TRAIL-R3 is a molecule anchored to the glycol-phospholypidi without transmembrane and intracytoplasmic component, while TRAIL-R4 despite being a type I transmembrane protein, presents an incomplete cytoplasmic death domain. Since R3 and R4 compete with R1 and R2 to the binding capacity of TRAIL without inducing apoptosis, it has been proposed that TRAIL-R3 and R4 are able to protect normal cells by the induction of death by TRAIL (Ashkenazi, 2002). It has been shown that NK cells, both mature and immature, use TRAIL or Fas-L in a differentiated way to kill susceptible cells (Zamai et al., 1998). In particular, TRAIL is expressed earlier of Fas-L during differentiation NK (Zamai et al., 1998).
Another lytic mechanism used by NK cells is cell-mediated cytotoxicity dependent by the interaction CD40/CD40-Ligand (Carbone et al., 1997). CD40 is a membrane glycoprotein of 50 kDa, expressed on the cell surface of B lymphocytes, dendritic cells and monocytes. This molecule is a member of the TNF/NGF receptor family, which also include the antigens CD27, CD30 and Fas (CD95). The CD40-L is a membrane glycoprotein having a molecular weight of 39 kDa, present on the cell surface of activated T and NK lymphocytes. The CD40/CD40-L interaction is required for the activation of B cells dependent on T and NK cells and to kill the target cell, which express CD40.

4.2 Cytokines production

NK cells have been first identified for their cytotoxic activity, but a further characteristic is their ability to produce cytokines in response to various stimuli, in particular in early viral infections. Cytokines are soluble molecules that mediate and regulate the immune response, inflammation and haematopoiesis, also influencing the differentiation and the production of other cytokines in various cell types. NK cells produce cytokines such as IFN-γ, TNF-α (Anegon et al., 1988), lymphotoxin (LT), but also the Colony Stimulating Factors (CSFs) such as GM-CSF, M-CSF and IL-3 (Cuturi et al., 1989), as well as IL-5 (Warren et al., 1995), IL-8, IL-10 and IL-13.

Among cytokines produced by NK cells, one of the most important and abundant is IFN-γ. It is also called type II interferon, and it is an homodimeric protein made by two subunit of 21-14 kDa, with a critical role for innate and adaptive immunity against viral and bacterial infections (Schoenborn & Wilson 2007). The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immune stimulatory and immune-modulatory effects. IFN-γ promotes an immune response by acting directly on infected monocytes (activating their anti-microbial mechanisms endogenous), and promoting the differentiation of $CD4^+$ T helper type 1 (Th-1) cells (which direct the immune response towards a T cell-mediated response type). In the inflamed lymph nodes, NK cells are able to promote the priming of Th-1 cells by secreting IFN-γ, which is necessary for Th-1 polarization (Martín-Fontecha et al., 2004). In vitro studies have demonstrated the importance for DC-derived IL-12 in the induction of IFN-γ production by NK cells in different systems, both in humans and mice (Gerosa et al., 2002; Dalod et al., 2003; Kikuchi et al., 2004). Following an infection due to intracellular pathogens the main stimulus that induces the secretion of IFN-γ by NK cells appears to be the release of IL-12 by infected monocytes (Scharton & Scott, 1993). Also, in a sort of loop, NK cells by means of IFN-γ can promote the maturation of dendritic cells (DC), which in turn activate NK cells by means of IL-12, (Walzer et al., 2005). NK cells have also a complex role in the control of some major life-threatening infections like the case of *Plasmodium falciparum* infection, where the early production of IFN-γ through the cooperation between monocytes and NK cells seems to be very important in the promotion of protective immunity (Roetynck et al., 2006).

After the cell target recognition by NK cell activating receptor, there is the production also of TNF-α, which, altogether with IFN-γ, is essential in viral and tumor clearance (Balkwill et al., 2009); in its soluble form it is a protein of 17 kDa, and it is involved in systemic inflammation. The production of both IFN-γ and TNF-α by NK cells is linked to their cytolytic activities: they make target cells sensitive to NK cytotoxicity. In particular, cancer cells sensitized by these two cytokines express inducible levels of

ICAM-1, and this up-regulation is involved in the induction of the cytolysis of target cells, suggesting a role for IFN-γ and TNF-α in synergistically enhance the cytolytic function of NK cells (Wang et al., 2012).

INF-γ and TNF-α, as well as promoting the development of an inflammatory response after an immunological insult, possess also a potent inhibitory action on haematopoiesis (Broxmeyer et al., 1986). In vitro studies have demonstrated that NK cells are able to inhibit the development of myeloid cells from stem hematopoietic progenitors (Bellone et al., 1993); this effect could be almost in part, a consequence of the release of these cytokines, but it's not totally clear whether the production of these cytokines by NK cells has as its physiological target mature or immature hematopoietic cells.

5. Natural Killer cell subsets

5.1 Mouse NK cells subsets

Although NK1.1 is currently the most specific serologic marker on $CD3^-$ NK cells in C57BL/6 (B6) mice (Kim et al., 2000), early NK precursors do not yet express this marker. In addition, these precursors do not express DX5 —a pan–NK cell marker that was identified as the integrin α2 subunit associated with β1—which suggests that the $CD122_+$ $NK1.1^-$ $DX5^-$ population contains the earliest NK-committed precursor (Ikawa et al., 1999). The development of NK cells occurs in the bone marrow, where cells committed to the NK cell lineage undergo a series of developmental stages characterized by the acquisition or the loss of some surface markers (Yokoyama et al., 2004). In the periphery, mature splenic NK cells express several integrins, including CD11b, and other members of the β2 integrins family such as DX5, suggesting that the integrins expression during the differentiation is strictly regulated.

Splenic NK cells

Splenic NK cells are the conventional NK cells found in mouse. In knockout mice lacking IL-15 or any other chain of its receptor (α, β, γ), splenic NK cells are absent. In physiological conditions, splenic NK cells development occur largely in the bone marrow where they acquire the whole set of receptors and surface markers; out in the periphery mature splenic NK cells can be further distinguished by differential expression of CD11b and CD27 (Kim et al., 2002). Thus, conventional splenic NK cells display developmental markers associated with maturation.
NFIL3 has a clear role in splenic NK cells development: mice lacking NFIL3 have no splenic NK cells (Gascoyne et al., 2009; Kamizono et al., 2009), whereas the related T-box transcription factors, TBET and EOMES, play a much more complex roles in NK cell development (Townsend et al., 2004; Gordon et al., 2012), with splenic NK cells showing a less mature phenotype in the absence of TBET.

Tissue resident NK cells

In the mouse adult liver, at least two distinct populations of NK cells, distinguished by mutually exclusive expression of CD49a and DX5 (Peng et al., 2013). These two populations have distinct gene profile (Daussy et al., 2014), and detailed phenotypic analysis revealed that DX5 and CD49a are mutually exclusively expressed on liver NK cells. Even if both populations express NKp46, CD122, and NK1.1, only liver NK cells express CX3CR1, CD62L, S1PR1, and S1PR5 which are not found in the ILC1 (Seillet et al., 2014a). Liver NK cells phenotypically are pretty similar to conventional splenic

NK cells, but they are two different subsets, in fact studies developed with parabiont mice have revealed that the CD49a^{+} DX5^{-} cells are tissue resident NK cells, with low levels of CD11b and high levels of TRAIL (Kim et al., 2002). These liver tissue resident NK cells are distinct from immature cNK cells found in the bone marrow.
Also in mouse the presence of uterine tissue resident NK cells has been evaluated. It's well known that NK cells are normally present in the non-pregnant uterus (Yadi et al., 2008), but they expand at the site of embryo implantation during pregnancy (Hatta et al., 2012). Like cNK cells, uterine NK cells require IL-15 for development (Ashkar et al., 2003), they are cytotoxic as they express perforin and granzymes, and they produce IFN-γ (Ashkar et al., 2000). Interestingly, however, uterine NK cells appear relatively normal in TBET-deficient mice (Tayade et al., 2005) and recent studies suggest that a subset of uterine NK cells can be distinguished from cNK cells (Yadi et al., 2008).

Thymic NK cells

Among the lymphoid tissues, the thymus has NK cells with surface marker phenotypes resembling those of immature cNK cells (Vossenrich et al., 2006). Di Santo and colleagues found that when compared to splenic NK cells, thymic NK cells show a low expression of Ly49 and CD11b, similarly to immature NK cells in the bone marrow do. These thymic NK (tNK) cells are absent in athymic nude mice, indicating that a functional thymus is required for their development. Thymic NK cells have a unique requirement for the transcription factor GATA3 and they all express CD127 (IL-7Rα), in fact they repopulated peripheral lymphoid organs, and their homeostasis is strictly dependent on GATA3 and IL-7. Even if they are poor cytolytic effectors, tNK cells have the ability to produce large amounts of IFN-γ (Vossenrich et al., 2006). Moreover, peripheral thymic NK cells require a thymus for development and can develop in vivo and in vitro from double negative (CD4^{-} CD8^{-}) 1 (DN1) subset of immature thymocytes (Vargas et al., 2011), indicating that they do not develop directly in the BM, unlike cNK cells.
Of note, for the first time since the development of NK cells, a comparison between human and mouse NK cells has been possible: CD56^{bright} NK cell in human peripheral blood are CD127^{+} and they also express more GATA3 than CD56^{dim} ones. Thus, the two human and two mouse NK cells subsets may have similar developmental and functional properties.

5.2 Human NK cell subsets

At least in peripheral blood, CD56 and CD16 markers largely discriminate cytotoxic NK cells from other perforin-expressing lymphocyte subsets. Moreover, NKp46 has also been employed as a singular marker of NK cells, but this receptor, which is involved in triggering of natural cytotoxicity, can also be expressed by ILC3 subsets (Reynders et al. 2011). Thus, according to the density of expression of CD56 and CD16 molecules, we can recognize two major subpopulations of NK cells, both phenotypically and functionally distinct: the $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim/neg}$ (Cooper et al., 2001).

The different expression of CD16 on the cell surface lead to functional consequences for the ADCC mechanism in the two main NK cells subsets: NK CD56dim cells have high levels of CD16 expression, thus, a higher antibody-dependent cell-mediated cytotoxicity (ADCC) compared to $CD56^{bright}$ NK cells (Leibson, 1997). In addition, $CD56^{dim}$ resting cells in general (i.e.: those present in the circulation and not yet activated), naturally have a more cytotoxic activity than resting $CD56^{bright}$ ones.

$CD56^{dim}$ NK cells

$CD56^{dim}$ NK cells, so called because of their low-density surface expression of CD56 and high expression of CD16 and KIR, represent approximately 90% of NK cells in peripheral blood. They represent a subset specialized to immune-surveillance; they have many cytolytic granules that give them a strong cytotoxic activity and are able to produce cytokines (mainly, IFN-g and TNF-a) upon target cell recognition. $CD56^{dim}$ undergo an educational process called "licensing". According to this mechanism, only the NK cells that express at least one inhibitory receptor for self-MHC-I molecules (such as a KIR or NKG2A-CD94), are capable of triggering spontaneous cytotoxic activity against target cells with reduced levels or lack of HLA-I molecules (Anfossi et al., 2006). This process involves the formation of two kind of NK cells: those functionally competent, known as "licensed," authorized to kill in a missing-self fashon, whose effector responses are inhibited by self HLA-I molecules through the same receptors by mean of which they have earned the licensing, and those functionally incompetent, called "unlicensed".

$CD56^{bright}$ NK cells

Most of the human $CD56^{bright}$ NK cells (about 50-70%) does not express CD16 (for this are indicated as $CD56^{bright}/CD16^{neg}$) while the remaining percentage shows a low density of expression of the FcγRIII and are indicated as $CD56^{bright}/CD16^{dim}$ NK cells. This subset is mostly present in the lymph nodes and tonsils (Ferlazzo et al., 2004).

There are evidences that precursors of $CD56^{bright}$ NK cells leave the bone marrow, go through the peripheral blood and come in the lymph-nodes, where they differentiate into $CD56^{bright}$ NK cells under the influence of cytokines locally produced by stromal cells and dendritic cells of the lymph-nodes (Caligiuri, 2008).
This subset express very low levels of perforin and granzymes when compared to canonical $CD56^{dim}$ NK cells and cytotoxic effector T cells: tenfold and threefold lower expression levels of perforin, respectively (Chiang et al., 2013). Resting $CD56^{bright}$ NK cells possesses a relatively low cytotoxicity but under the influences of the cytokine milieu produce high amounts of pro-inflammatory cytokines, such as interferon IFN-γ, tumor necrosis factor TNF-α, IL-5, IL-10 and IL-13, playing essentially a type immune-regulatory function (Cooper et al., 2001; Jacobs et al., 2001; Martin-Fontecha et al., 2004).
Low levels of CD16, as well as NKG2C and activating KIR expression, implies that circulating $CD56^{bright}$ NK cells do not significantly contribute to ADCC or other forms of cytotoxic immune-surveillance. Of note, target cell recognition by circulating $CD56^{bright}$ NK cells induces very little IFN-γ and TNF–α, likely reflecting inaccessibility of the *Ifng* locus to transcription factors induced by co-activating receptors.

Tissue resident NK cells

NK cells are widespread throughout lymphoid and no-lymphoid tissues, so they have been found in other tissues besides peripheral blood. Studies of this so called “tissue-resident” NK cells have revealed distinct features in different tissues. They should therefore be viewed as a spectrum of cells uniquely influenced by their microenvironments. With respect to cytotoxic function, tissue-resident NK cells express comparatively low levels of perforin, and they are generally known to be poor cytotoxic mediators, as is the case for uterine and liver-derived NK cells (Burt et al. 2009; Kopcow et al. 2005). Most NK cells in lymph nodes and tonsils often express high levels of CD56, and lack perforin, being more similar to $CD56^{bright}$ (Ferlazzo & Münz, 2004). These cells promptly produce INF-γ in response to IL-12, IL-15 and IL-18 (Cooper et al., 2001). Relative to other lymphocyte subsets, they are particularly abundant in the liver and female reproductive tract. In these organs, human NK cells display distinctive phenotypes (Burrows et al., 1993; Burt et al., 2009; Koopman et al., 2003).
Liver NK cells express high levels of Trail and can thereby induce apoptosis of hepatocytes (Dunn et al., 2007), and lack expression of CD62L and express low levels of EOMES and TBET (Burt et al., 2009; Marquardt et al., 2015).

Uterine NK cells still express EOMES, but with low levels of TBET (Tayade et al., 2005). Moreover, uterine NK cells produce vascular and endothelial growth factor (VEGF) as well as placental growth factor (PLGF), supporting their role in vascular remodelling (Hanna et al., 2006).
Similar to circulating $CD56^{bright}$ NK cells, tissue-resident $CD56^{bright}$ NK cells also lack expression of CD16. Thus, they cannot mediate ADCC, and they may thus vary widely in their phenotype for adhesion receptors and transcription factors.

Subsets relationships

Basing on these observations, in recent years a discussion has been opened about the inter-relationship between the two main subsets. Some researchers suggest that NK cells can be divided into two functionally distinct subpopulations (similar to CD4 and CD8), while others think that they belong to different stages of the same NK lineage differentiation NK. Fauriat and colleagues (Fauriat et al., 2010) have shown that the $CD56^{bright}$ propensity to produce a large quantity of cytokines compared to $CD56^{dim}$ NK cells depends on stimulation with exogenous cytokines. $CD56^{dim}$ cells, however, produce a greater quantity of cytokines and chemokines after stimulation mediated by contact with the target cells. These results demonstrate that NK secretory function has two distinct pathways of activation in the two subsets. The $CD56^{bright}$ NK cells would be more susceptible to stimuli mediated by soluble receptor ligands, while $CD56^{dim}$ ones respond to stimuli related to the contact and the recognition of target cells, which involve the classic NK activating receptors.
These results would suggest that the two subsets represent two distinct cell populations with a converging phenotype and cytotoxic and secretory functions (Campbell et al., 2001; Fauriat et al., 2010). It has also been proposed that the maturation of NK cells would be characterized by the down-regulation of CD56 and the acquisition of CD16 and KIR. According to this line of thought, $CD56^{bright}/CD16^{dim}$ thus constitute an intermediate stage between the most immature $CD56^{bright}/CD16^{-}$ and more mature $CD56^{dim}/CD16^{bright}$ (Nagler et al., 1989). NK cells are thought to primarily develop in the bone marrow. However, fetal thymus and liver contain bipotent T/NK progenitor cells that possess the ability to develop into NK cells (Carlyle et al., 1997; Spits et al., 1998). Similar to T and B cells, NK cells require the common gamma chain of the IL-2 receptor complex for their development. The lack of the common gamma chain (γc) results in a near complete loss of NK cells under steady-state conditions (Di Santo 2006; Ma et al., 2006). Among the cytokines that need of γc to transduce their signals, IL-15 is thought to be required during the entire life span of NK cells (Di Santo 2006; Yokoyama et al., 2004).

IL-2, IL-12, IL-15 have shown to be able to increase the cytotoxicity of NK cells, but only IL-2 and IL-15 are able to promote their proliferation (Gately et al., 1998). All NK cells express the surface heterodimeric receptor with intermediate affinity interleukin-2 (IL-2) IL-2Rβγ (CD122, CD132), and in 1990 some studies have shown that $CD56^{bright}$ NK cells also express the high affinity heterotrimeric receptor for IL-2: IL-2Rαβγ (CD25, CD122, CD132) in fact these cells are able to proliferate both in vitro and in vivo in response to low doses (picomoles) of IL- 2 (Caligiuri et al., 1993). $CD56^{bright}$ NK cells have another receptor, the tyrosine kinase c-kit (CD117), whose ligand, Kit ligand (KL) also called stem cell factor (SCF), increases the proliferation induced by IL-2 and it promotes the survival of these cells up-regulating Bcl-2 expression (Carson, et al., 1994). On the contrary, the resting $CD56^{dim}$ NK cells only express the heterodimeric receptor with intermediate affinity for the cytokine IL-2, they are c-kit negative, and weakly proliferate in vitro when subjected to high doses of IL-2, even if these doses of IL-2 on these cells induce the expression of the heterotrimeric high affinity receptor for IL-2 (Baume et al., 1992).

$CD56^{dim}$ NK cells have high levels of expression of KIR unlike $CD56^{bright}$ NK cells that have a low or even absent expression of these molecules, instead they express the inhibitory receptor CD94/NKG2A. The $CD56^{dim}$ constitutively express NKp46 and NKp30, and NKp44 is present only on the surface of activated NK cells; the $CD56^{bright}$ instead express all three NCR. The NKG2D receptor is present in both cell subsets. CD2 has a higher density of expression on $CD56^{bright}$ compared to $CD56^{dim}$ NK cells (Lima et al., 2001), moreover a CD2neg population has been identified (about 23%) in CD56dim NK cells. Based on these observations, NK cell differentiation studies aimed to clarify whether $CD56^{dim}$ and $CD56^{bright}$ subsets represent two different NK lineages or belong to two steps of the same maturational pathway were performed (Cooper et al., 2001; Nagler et al., 1989; Zamai et al., 2012; Ferlazzo et al., 2004).

Aim of the work

The recent discovery of a number of other lymphoid subsets involved in the innate immune response, has questioned part of the notions already acquired. Although until a few years ago it was believed that NK cells are the main effector of innate immunity, with the discover of the ILC family it has become evident that there are many actors with important roles in the immune defence, inflammation, tissue remodelling and cancer.

Group 1 ILCs, which includes already-known NK cells and ILC1, is the most controversial group among all the ILC groups, and growing evidence are bringing to light the existence of different subset of cells belonging to this group. Moreover, the fact that NK cell and ILC1 have phenotypic and transcriptional characteristics sometimes divergent, sometimes overlapping, makes the clarification even more difficult. Also, the fact that there is a substantial heterogeneity within mouse and human NK cells, and despite of effort in clarifying the evolutionary relationship between these two models, a lot of uncertainties are still in this area.

The purpose of this work was to investigate the differentiation and maturation step of NK cells in humans, trying to bring to light evidences on the relationship between the two main NK cell subsets. To do this, I have used culture systems capable of generating $CD56^{bright}$ and $CD56^{dim}$ from the human hematopoietic progenitors $CD34^{+}$ circulating in the peripheral blood, through the administration of appropriate cytokine combinations. To characterize the differentiating NK cells, I have performed a series of flow cytometric investigations to assess their phenotypic and functional features.

Given that improved understanding of mouse thymic NK cell development should aim knowledge of human $CD56^{bright}$ NK cells which resemble murine thymic NK cells, in the second part of my project I have investigated possible relationships between human NK cells and murine NK cells. To do that I have studied the transcriptional factor requirement of thymus in mice lacking some genes known to be involved in the development and differentiation of NK cells and ILC1.

Materials and Methods

1. Mouse model

All mice used in the present study were on a C57BL/6 background, one of the most used inbred strain of laboratory mouse (Mekada et al., 2009), and all mouse lines were housed at the University of Chicago Animal Resource Center in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Conventional knockout Mice

C57BL/6 *Rag1*$^{-/-}$ mice were purchased through The Jackson Laboratory (Bar Harbor, ME USA) and housed at the University of Chicago for less than 2 weeks, and some were inbred with *Il7Ra*^{Cre}*Ets1*$^{f/f}$, *Gzmb*^{Cre}*Id2*$^{f/f}$ and Cre^{+} littermate (LMC) controls. Mice homozygous for the *Rag1*^{tm1Mom} mutation produce no mature T cells or B cells, and the thymus of the mutant mice contains 15 to 130 times fewer cells than heterozygous or wild-type siblings.
Tbx21$^{-/-}$ mice and their controls were purchased from The Jackson Laboratory (Bar Harbor, ME USA) and housed at the University of Chicago for less than 2 weeks. These mice were homozygous for the target mutation and they were knocked-out for the T-box transcription factor TBX21.
Nfil3$^{-/-}$mice (Geiger et al., 2014) and LMC were provided by Dr. Joe Sun (Memorial Sloan Kettering Cancer Center, New York) and were housed at MSKCC according to the guidelines of their Institutional Animal Care and Use Committee.

Conditional knockout mice

To analyse some transcription factors, two conditional knockout mice have been used in this study. Conditional gene knockout is a technique used to eliminate a specific gene in a certain tissue (Orban et al., 1992); thus, is possible to avoid any lethal or incompatible situations with the life of the animal (Gu et al., 1994). The most common technique is the Cre-lox recombination system (Figure 6).

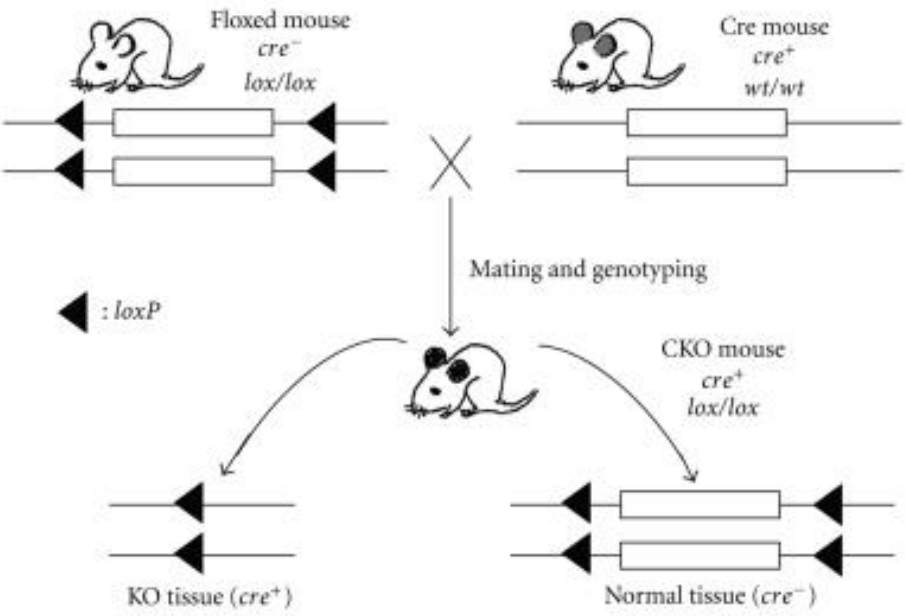

Figure 6: Schematic representation of the conditional gene knockout system. [Adapted from: Le, 2011].

The Cre recombinase enzyme is a 38 kDa bacteriophage P1 protein that catalyses the recombination between two 34 bp loxP (locus of X-over of P1) recognition sites without the need for any cofactor.

This enzyme, specifically recognizes two lox (loci of recombination) sites within DNA and causes recombination between them. The recombination occurs through DNA inversion, excision/integration, and translocation (Friedel et al., 2011), but the excision of loxP-flanked ("floxed") DNA segments is the most widely used for in vivo genome modification.

In these protocols standard techniques have been used to produce mice in which the functional region of the gene of interest is floxed, so that such mice with the conditional allele, when crossed with an effector mouse line expressing Cre in a tissue-specific manner, give a progeny in which the conditional allele is inactivated only in Cre expressing cells.

$Ets1^{f/f}$ mice and $Id2^{f/f}$ mice were generated in the University of Chicago Transgenic Core Facility using 129/SvJ embryonic stem cells. The offspring were backcrossed onto the C57BL/6 background for >12 generations.

A targeting vector containing the floxed sites along with exons 8 and 9 containing the ETS1 binding domain was generated and introduced to germline DNA through homologous recombination. After backcrossing, the $Ets1^{f/f}$ mice were crossed to $Il7ra^{Cre}$ mice, for >12 generations (Schlenner et al., 2010; Zook et al., 2016). The same has been done crossing $Id2^{f/f}$ mice with $Gzmb^{Cre}$ mice. $Rag1^{-/-}Gzmb^{Cre}Id2^{f/f}$ mice have deleted the Id2 gene in all hematopoietic cells (Xu et al., 2015).

WT and $Rag1^{-/-}$ mice as well as $Rag1^{-/-}Il7Ra^{Cre}Ets1^{f/f}$ ($Ets1^{-/-}$), $Rag1^{-/-}Gzmb^{Cre}Id2^{f/f}$ mice, and their $Rag1^{-/-}Cre^{+}$ littermate controls (LMC) were maintained in a specific pathogen

free facility at the University of Chicago according to the guidelines of the University of Chicago Institutional Animal Care and Use Committee (IACUC). At an age between 7 and 10 weeks, conditional and conventional knockout mice and their LMC have been sacrificed using CO_2 asphyxiation followed by cervical dislocation.

Cell preparation

Thymus (and spleen as internal control) have been isolated from mice and maintained in FACS buffer for all the time of sample processing. Thymus have been mechanically crushed and mushed in Petri sterile dishes to obtain single cell suspensions, which have been maintained on ice. CD8 lineage depletion was performed by staining thymocytes with CD8-biotin followed by streptavidin-magnetic beads (Miltenyi Biotech) and then the cells were passed over LS magnetic columns (Miltenyi Biotech) to obtain a flow through that was depleted for CD8+ cells.
Cells suspensions were incubated for 15 minutes on ice with an unlabelled purified CD16/32 (2.4G2.1) blocking antibody before the addition of any biotinylated or flourochrome-conjugated antibodies (FITC, PE, APC, PECY7, Percp-cy5.5, AF780, and Brilliant violet 421). Single cell suspensions were stained for flow cytometry for 20 minutes on ice using standard procedures. The antibodies TCRβ, TCRγδ, CD3e, CD4, CD8a, NK1.1, DX5, CD49a, CD103, CD27, CD11b, CD127, TRAIL, CD69, CD244, NKp46 and Ly49G2 were purchased from eBiosciences, BioLegend, or BD Pharmingen.
PBS57 loaded and unloaded CD1d tetramers were obtained from the NIH Tetramer Facility (Atlanta, GA).
The Foxp3 Transcription Factor Staining Buffer Set (eBioscience) was used for the intracellular staining with the EOMES and TBET antibodies (eBiosciences).

Flow cytometry

Flow cytometry was performed on a BD LSRIII Fortessa, and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Paired and unpaired Student t-test were calculated using Prism 6 (GraphPad Software); p-values <0.05 have been considered significant.

2. Human model

Blood sample

Peripheral blood leukocytes (PB) or buffy coat have been obtained from healthy donors and provided to us by the Transfusion centre of Urbino Hospital (Urbino, Italy).
Blood has been slowly layered in a 50 ml test tube over a volume of 15ml of Ficoll (Ficoll/Histopaque- 1077; Sigma-Aldrich, St. Louis, MO, USA), a poli-sucrose and sodium diatrizoate based solution with a density (1.077) equal to that of the mononuclear cells alive and centrifuged at 2800 rpm for 30 minutes. In this way the erythrocytes and granulocytes sedimented on the bottom of the tube, while mononuclear cells remained at the interface between this and the plasma. Mononuclear cells were then harvested and subjected to a series of washings of 10-15 minutes at a decreasing speed to also remove most of the remaining red blood cells and platelets.
Finally, a count of mononuclear cells has been done by counting them on a Neubauer chamber after dilution of an aliquot of cells with the Trypan blue dye.
A small cell aliquot was labelled with directly conjugated antibodies to define the phenotype of cells obtained prior to isolation of $CD34^+$ hematopoietic stem cells.

$CD34^+$ isolation

DNase I at a concentration of 10 μg/mL at room temperature (15-25°C) for at least 15 minutes has been used to avoid cell aggregation prior to labelling and separation.
$CD34^+$ hematopoietic progenitors were isolated from peripheral blood lymphocytes due to positive immune-magnetic separation technique, using the "Vario MACS magnetic cell sorting program"(Miltenyi Biotec, Auburn, CA), a magnetic method for sorting cells and the $CD34^+$ insulation kit in accordance with the guidelines provided by the company (Figure 7).
To obtain the $CD34^+$ hematopoietic progenitor, cells have been incubated with a colloidal paramagnetic solution of microbeads (microbeads) conjugated with an anti-human CD34 monoclonal antibody (isotype IgG1; QBEND/10 clone) and passed through a column equipped with a metal matrix formed by small spheres; this column, during the separation of $CD34^+$ cells, is placed within a magnetic field that allows to block the microbeads conjugated to the antibody. It follows that cells presenting the CD34 antigen are retained within the matrix of the column while the mature lymphocytes are eliminated in the eluate. To allow the collection of $CD34^+$ cells, the column is removed from the magnetic field to leave the microbeads from their magnetic bond. The collection rate is the fraction of $CD34^+$ progenitor cells (Figure 7).

To prevent that the above-mentioned antibody, and the magnetic beads conjugated to it, to bind in a non-specific way or to interact with the Fc fragments of immunoglobulins present on the surface of CD34 negative cells, a solution called "Blocking Solution" it was used. The Blocking Solution, added in the cell suspension before the marbles, helps to ensure the specificity of the link between magnetic microbeads and $CD34^{+}$ cells.

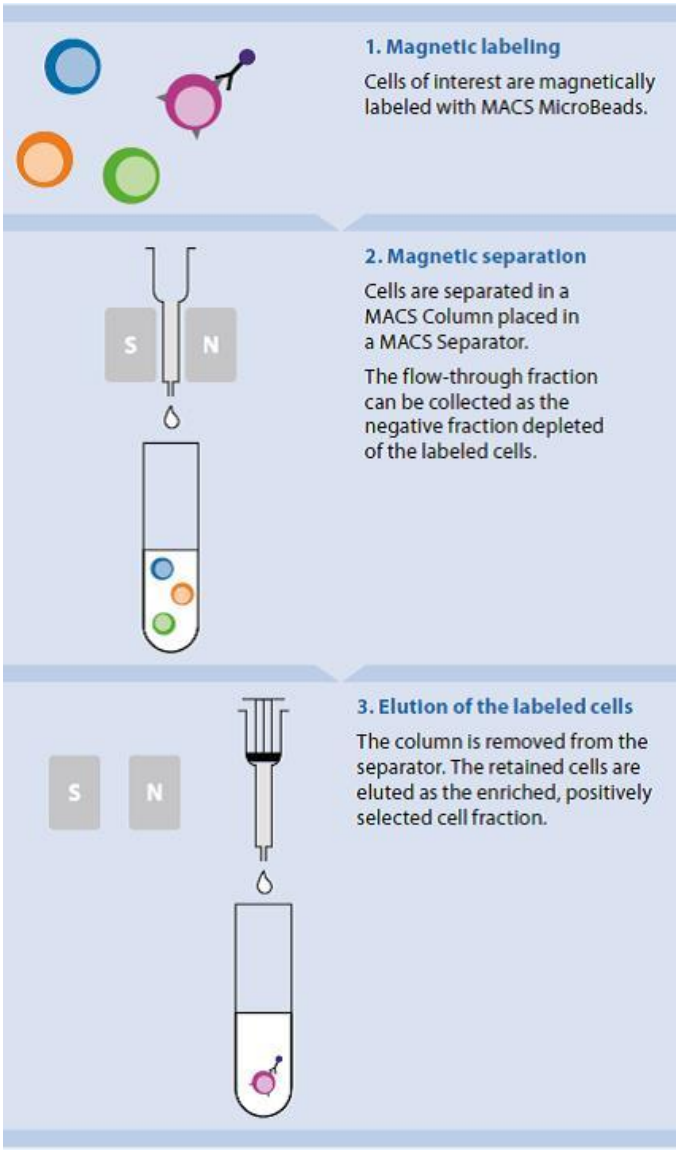

Figure 7: Schematic representation of the Vario MACS magnetic cell sorting programto isolate CD34+ hematopoietic progenitors

Cell cultures

The purity of $CD34^+$ cells selected was determined by flow cytometry with FACS Calibur (Becton-Dickinson BD Bioscences, San Jose, CA) cytometer, using a monoclonal antibody (mAb) that recognizes a class III epitope of the CD34 molecule (HPCA-2, BD Biosciences Pharmingen), directly conjugated to Phycoerythrin (PE). Only samples with a purity of 80-95% of $CD34^+$ cells has been used.

$CD34^+$ purified cells were suspended in RPMI medium (Sigma- Aldrich, USA) supplemented with 1% antibiotics, 1% L-glutamine (Sigma Chemicals, St Louis, MO) and 10% fetal bovine serum (FBS) decomplemented (at 56°C for 45 minutes).After being arranged at an optimal concentration of $1x10^5$ cells per mL, $CD34^+$ were cultured at 37°C, 5% CO2, in the presence of Flt3-L (20 ng/mL) and IL-15 (20 ng/mL) (PeproTech, London, UK), with or without IL-21 (20 ng/mL) to induce NK cell differentiation.

Every 4 days about half of the volume in each well has been removed, and it was replaced with fresh medium supplemented with serum, antibodies and the respective cytokines. To obtain a high percentage of NK cells (> 90%), Flt3- ligand was added only during the first 15 days of culture. These culture conditions refer to the "primary culture".

$CD56^{bright}$ NK cells (> 95%) generated from $CD34^+$ hematopoietic progenitor cells, after 30 days of primary culture, were put into secondary culture for further 15 days in the presence of IL-15 or IL-15 + IL-21. In some experiments, after the successful removal of $CD34^+$ cells, mature NK cells were isolated by negative selection from eluted lymphocytes (T cells, B, and NK) using the Vario-MACS (Miltenyi Biotec) and the NK Isolation II Kit . Eluted $CD56^+$ NK cells (purity> 95%) were then grown for analysis and comparison with NK cells generated from $CD34^+$ progenitors. The purity of NK cells was determined for each isolation by flow cytometry using the combination of anti-CD5FITC, anti-CD16PE and an anti-CD56PECy5.

The purified NK cells were cultured for 15-30 days in RPMI with 10% FBS and treated with 20 ng/mL of IL-15. NK cells generated in culture were analysed by flow cytometry to identify phenotypic characteristics of subset-specific, as well as their cytotoxic capacity.

To detect granzyme B and perforin, cells were stained for NK cell surface markers, and then treated with the fixation kit/permeabilization Fix / Perm kit (Caltag Laboratories, Burlingame, CA) prior to detection of intracellular proteins by appropriate monoclonal anti-granzyme B-PE and anti-Perforin-FITC.

Intracellular cytokines detention

Cells were incubated ($5x10^6$/mL, 6 hours, 37°C) in medium with or without Phorbol Myristate Acetate (PMA) (10^{-9}M) and Ca^{2+} ionophore (A23187, 0.1 µg/mL) (all reagents from Sigma Chemical). Brefeldin A (10 µg/mL) was added during the last 3 hours. Cells have been harvested and a Fix/Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA) was used to fix and permeabilize cells for intracellular cytokine detection combined with surface phenotyping.

Cytotoxic activity

The study of the functional response of NK cells could not be exempt from the real analysis of NK cells cytotoxicity against target populations. It is well known that members of the TNF family dispatch the cytotoxic activity of NK cells through both the lytic mechanism through the combined action of perforin/granzyme that through the apoptotic induction mediated. We have developed a detection method to discriminate between to lytic-induced and apoptotic-induced death by exploiting the use of propidium iodide (PI) at high concentration (50mg/ml) (Zamai et al., 1996) and the marking of the target cells in green with DIOC18 dye.

Target cells were incubated over night with 5µM of DIOC18 (green fluorescent dye, D-275, Molecular Probes). After washing, target cells were placed in contact with the effector cells according to certain effector cell ratios: target (E: T), then incubated for a time of 2 hours to evaluate the Ca^{2+}-dependent lytic mechanism and for 6 hours in presence of 1mM EGTA and 2 mM $MgCl_2$ to assess the Ca^{2+}-independent lytic mechanism.

During the last 30 minutes PI was added at a concentration of 50 µg/ml. The lytic activity was found to be visible after 1 hour of incubation on both K562 target cells and Jurkat cells at different effector/target ratios (E:T). With this staining technique it is possible to discriminate the death of the target cells by exploiting the different modifications of the permeability of the plasma membrane that occur during the necrotic and apoptotic processes. Partial modifications during the early stages of apoptosis allow the PI to penetrate only partially (PI^{dim}), while PI is free to enter in secondary necrosis or necrotic cells (PI^{bright}). Target cells lives remain rather negative to PI (PI-).

NK-sensitive lines K562 (Fas-/CD48-) and Jurkat (Fas^+/TRAIL-$R2^+$/$CD48^+$) (Sivori, et al., 2002) (Bennett et al., 1996) were grown in RPMI 1640 with 10 % FBS at 37°C and in an atmosphere of 5% CO2. These tumor cell lines were used as targets to assess both cytotoxic activity of NK cells: calcium-dependent and calcium-independent.

Flow cytometric analysis allowed to calculate the percentage of specific death, using the following formula:

$$\frac{\text{\% of target cells DIOC18 or PI after incubation with effectors - \% spontaneous cell death}}{\text{100- spontaneous cell death}} \times 100$$

Flow cytometry

Conjugated monoclonal antibodies with different fluorochromes: FITC (fluorescein isothiocyanate), PE (Phycoerythrin), PerCP (Peridinin-clorofilproteina), APC (Allophycocyanin), PerCpCy5.5 (tandem Peridinin-clorofilproteina and cyanine 5.5), PECy7 (tandem Phycoerythrin and cyanine 7) or PECy5 (tandem Phycoerythrin and cyanine 5) were purchased from different companies. In particular: anti-CD16, -CD56, -CD158a/h, -CD158b/j, -CD158e, -NKG2A, -CD107a, -CD3, -CD5, -NKG2D, - CD94, -NKp30, - NKp44, -NKp46, -CD11a, -CD11c, -CD18, -CD117, -2B4 (CD244), -CD34, -CD117 (c-kit) -Granzyme-B, -Fas (CD95), -FasL (CD95L) -CD40L and anti-perforin. The phenotype of NK differentiating cells selected was determined by flow cytometry with FACSCalibur (Becton-Dickinson BD Bioscences, San Jose, CA) cytometer.

Results

4.1 Mouse model

Characterization of Innate-like lymphoid cells in wild-type mice

Vossenrich and colleagues first described a population of NK cell in the thymus of the mouse different form other cNK cells yet known (Vossenrich et al., 2006). These cells, in addition of having a low cytolytic potential, have a unique requirement for GATA3 transcription factor and they are dependent on IL-7. In order to better define a presumable comparison between these cells and human $CD56^{bright}$ NK cells, we first characterize the phenotype of these tNK cells in the thymus of mice.

The population of interest was made by the Lineage negative (TCRβ, TCRγ, CD3ε, CD4, CD8 / Lin^-) $CD122^+/NK1.1^+$ innate-like cells in the thymus. Our findings were consistent to what was previously known about heterogeneity of these ILC-like cells in the expression of DX5 (Vossenrich et al., 2006), with about the 20% cells expressing DX5 on the surface (Figure 8).

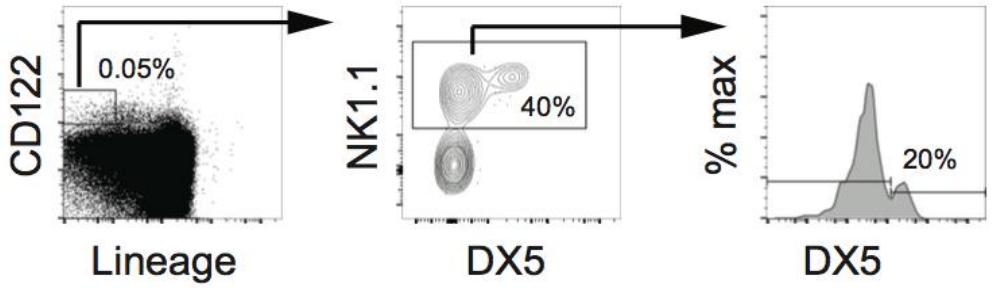

Figure 8: Representative flow cytometry analysis for innate-like lymphoid cells (ILC-like), defined as Lin^-CD122^+ $NK1.1^+$ cells. Lineage cocktail included TCRβ, TCRγ, CD3ε, CD4, CD8. DX5 expression on ILC-like cells is also shown in the histogram

This population of ILC-like cells showed heterogeneity also in other characteristic surface markers. The majority of these cells were DX5- with high levels of expression of CD127 and CD49a, a marker usually associated with ILC1 and tissue resident cells (Sojka et al., 2014). Also, many of the $DX5^-$ cells expressed high levels of CD103, the αE integrin that is also associated with tissue resident T cells (Woodberry et al., 2005). It is important to note that the $DX5^+$ thymic NK cells lack all this markers of tissue residency. Moreover $DX5^+$ ILC-like cells highly expressed GATA3 and EOMES, whereas the $DX5^-$ population expressed GATA3 but low levels of EOMES (Figure 9).

Because NK cells can be distinguished from ILC1 by their expression of the transcription factor EOMES and their ability to develop in the absence of TBET, which is required for development of ILC1, the fact that $DX5^+$ cells expressed both GATA3

and EOMES, confirmed the fact that the minor population of tNK cells in wild-type mice had a phenotype of "immature" tNK cells ($CD122^+/NK1.1^+$ $/CD127^+$ $/DX5^+$ $/CD11b^{lo}$), whereas the majority of Lin^- $/CD122^+$ $/NK1.1^+$ cells were $DX5^-$ cells with a an innate-like or ILC1 phenotype ($CD122^+$ $/NK1.1^+$ $/CD127^+$ $/CD49a^+$ $/CD103^+$).

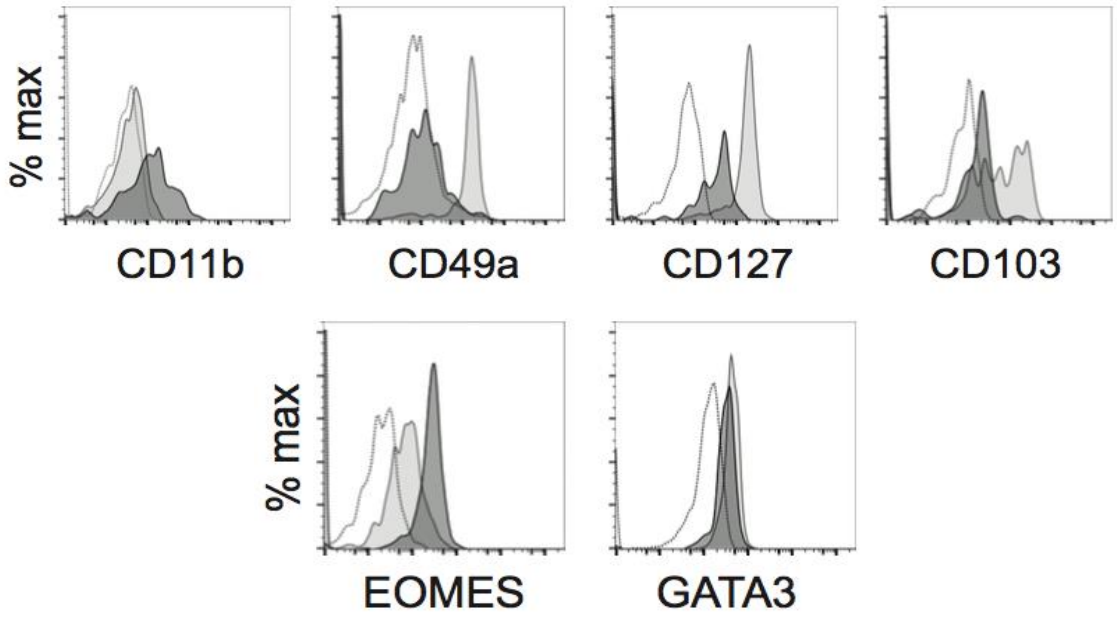

Figure 9: surface expression of CD11b, CD49a, CD127 and CD103, and intracellular expression of EOMES and GATA3, for $DX5^+$ (dark grey) and $DX5^-$ (light grey) ILC-like cells. The unshaded profile is the FMO. All data are representative of at least 7 independent experiments.

To further address the designation of these subsets of cell in the thymus of the wild-type mice, we analysed *Tbx21*$^{-/-}$ mice (TBET- deficient), where we saw an approximate 50% decrease in the number of Lin^- $/CD122^+$ $/NK1.1^+$ thymocytes, but nearly all of the remaining cells (>90%) were $DX5^+$. In the absence of TBET there was a specific loss of $CD49a^+$, $CD127^{hi}$, $CD103^+$, and $DX5^-$ cells. Therefore, tNK cells developed in TBET-deficient mice but the Lin^- $/CD122^+$ $/NK1.1^+$ $/DX5^-$ population of ILC-like cells were TBET-dependent (Figure 10).

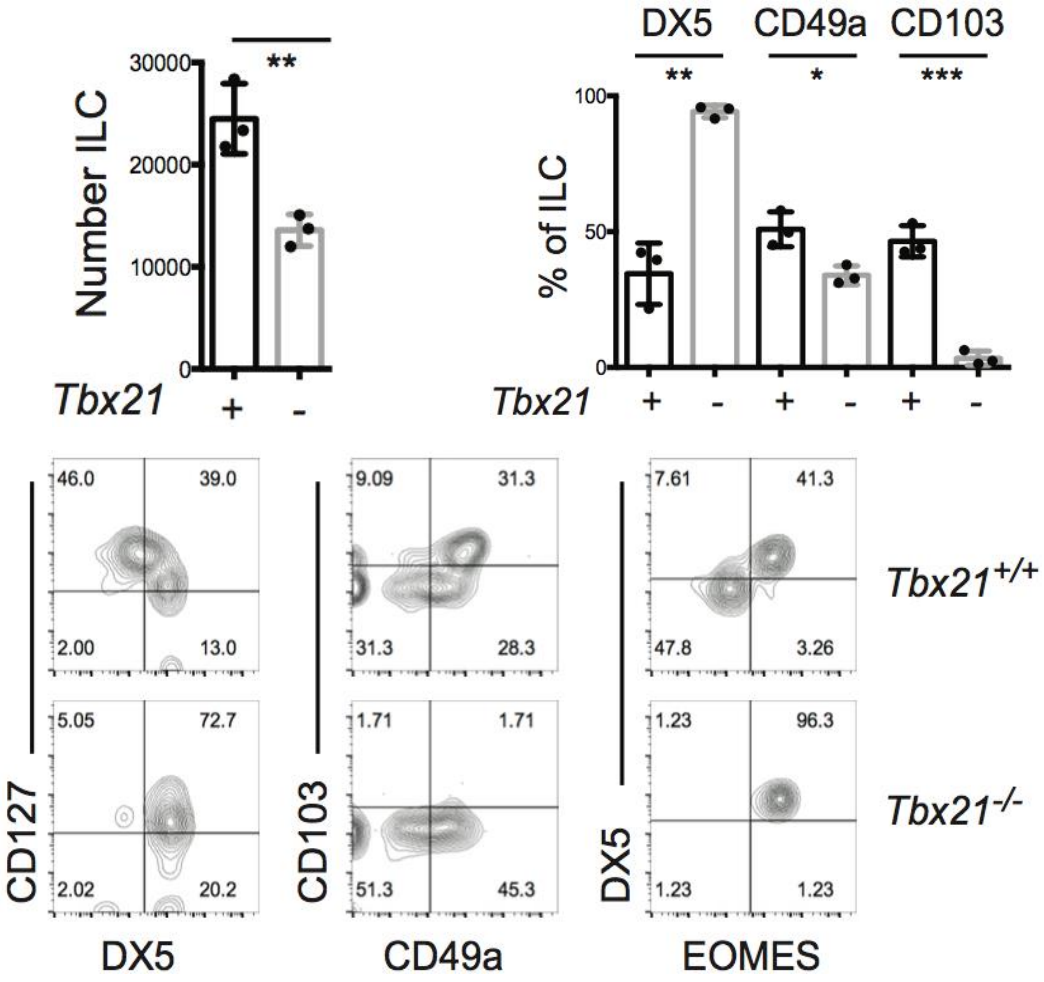

Figure 10: Number of thymic ILC-like cells in *Tbx21*$^{+/+}$ and *Tbx21*$^{-/-}$ mice. Summary of the percent of *Tbx21*$^{+/+}$ (+, black) and *Tbx21*$^{-/-}$ (-, grey) thymic ILC-like cells expressing DX5, CD49a and CD103. Each dot represents one mouse. Error bars are SEM. * p<0.05, **p<0.01, ***p<0.001On the bottom: flow cytometry analysis expression of CD127 versus DX5, CD103 versus CD49a, and DX5 versus EOMES on thymic ILC-like cells in *Tbx21*$^{+/+}$ and *Tbx21*$^{-/-}$ mice. Gates were set using negative controls.

We tested another transcription factor involved and essential for the development of cNK cells and some subsets of ILC1, but it is known to be not required for innate-like T cell development (Gascoyne et al. 2009). Thus, to confirm that the DX5^{+} tNK cells were related to NK cells, we tested whether these cells develop in the absence of NFIL3.
In *Nfil3*$^{-/-}$ mice there was a near complete loss of DX5^{+} tNK cells, as it can be seen in the two histograms below. However, total Lin^{-} /CD122^{+} /NK1.1^{+} cell numbers were not significantly altered by the loss of NFIL3 despite the reduced frequency of DX5^{+} cells, an observation suggesting that the other ILC-like cells may have expanded in the absence of NFIL3 (Figure 11).

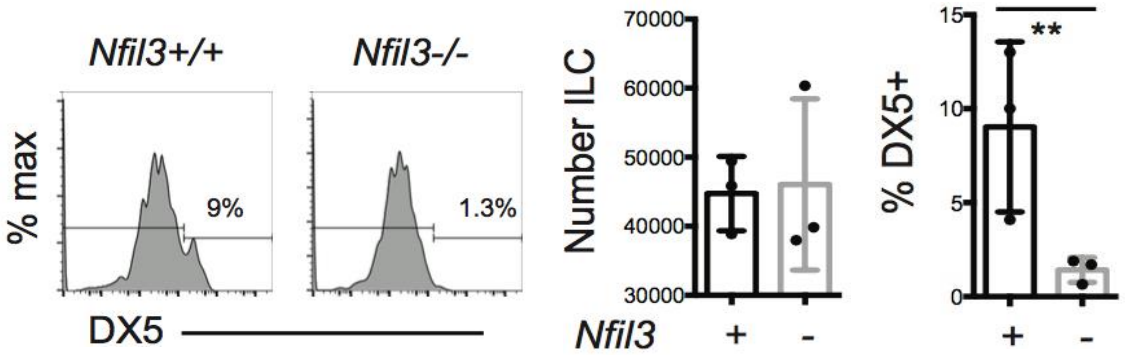

Figure 11: histograms showing DX5 expression on thymic ILC-like cells from Nfil3+/+ and Nfil3-/- mice. In the middle: thymic ILC-like numbers and, on the right, the percent of DX5+ in Nfil3+/+ and Nfil3-/- mice. Each dot represents one mouse. Error bars are SEM. **p<0.01.

In this first set of experiment we have demonstrated that Lin^- /$CD122^+$ /$NK1.1^+$ thymocytes are heterogeneous and include tNK cells and other innate-like lymphoid cells. Thymic NK cells are $CD127^+$ /$GATA3^+$/ $EOMES^+$ cells that require NFIL3 but not TBET for their development, consistent with their designation as NK cells rather than ILC1, and consistent with previous studies that showed a loss of NK cells in the thymus of *Nfil3*$^{-/-}$ mice (Seillet et al., 2014). Moreover, our data suggest that the Lin^- $CD122^+$ /$NK1.1^+$ /$DX5^-$ subset of thymocytes are TBET-dependent, NFIL3-independent, innate-like lymphocytes.

Thymic NK cells in *Rag1*$^{-/-}$ mice acquire markers of tissue residency

To date, it was believed that tNK cells were the majority of NK cells in the thymus, and that they had a signature phenotype and transcription factor requirement different to that of cNK cells. The fact that the majority of the Lin^- /$CD122^+$ /$NK1.1^+$ cells in WT mice were DX5-, let us think if they could be ILC1 with features of tissue residency or an innate-like T cell subset with very low expression of TCRβ that was not depleted by our Lineage cocktail.

We note that also increasing the stringency of the Lin- gate the frequency of these cells did not change. Thus, to further investigate the identity of this unexpected population of thymic cells, we examined Lin^- /$CD122^+$ /$NK1.1^+$ cells in *Rag1*$^{-/-}$ mice, which lack adaptive lymphoid cells.

Rag deficient mice showed a complete loss of the $DX5^-$ population of cells (97% of Lin^- /$CD122^+$ /$NK1.1^+$ cells expressed DX5), suggesting that the major population of $DX5^-$ cells in WT mice were T lymphocytes (Figure 12).

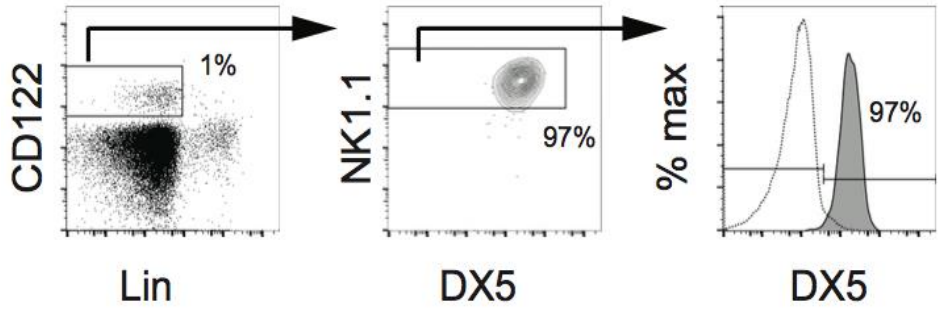

Figure 12: Representative flow cytometry analysis for ILC-like cells in Rag1-/- mice and their expression of DX5.

To better identify this population we used CD1d tetramers, and we found that the majority of thymic NK1.1^{+} cells were invariant NKT cells that stained positively with PBS57-loaded CD1d tetramers; these cells also expressed CD49a and about 50% of them were positive for CD103, consistent with the phenotype of thymic Lin^{-} /CD122^{+} /NK1.1^{+} /DX5^{-} cells. However, there were NK1.1^{+} cells that stained for CD49a and CD103 that were not detected by CD1d-tetramers suggesting that there may be additional innate-like T lymphocyte subsets that express these markers (Figure 13).

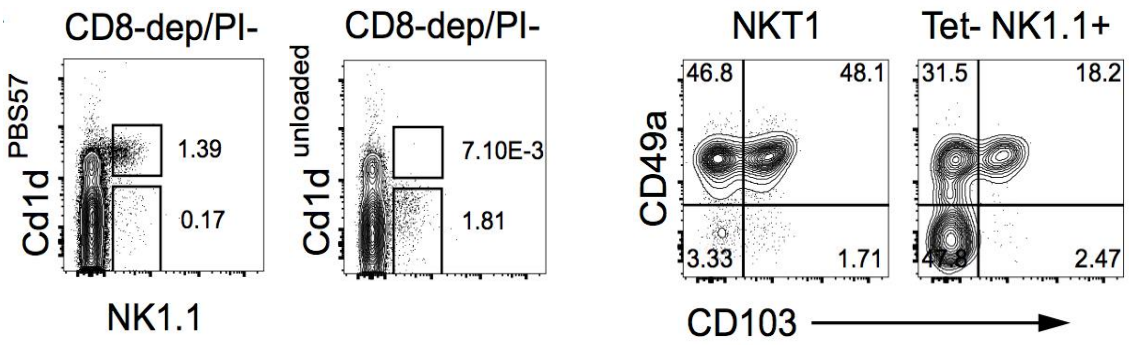

Figure 13: CD8-depleted thymocytes were stained with fluorescently labeled NK1.1 and PBS57 loaded CD1d tetramers or unloaded CD1d tetramers. On the right: the population NK1.1 and CD1dPBS57 tetramer (NKT1) or lacking CD1dPBS57 tetramer staining (Tet-NK1.1+) were examined for expression

Also in *Rag1*$^{-/-}$ mice the population of DX5^{+} tNK cells expressed CD127 and GATA3, with low levels of CD11b, highlighting the fact they were the same of their counterpart in WT mice. However, these cells also expressed CD103 and CD49a, typical markers of tissue residency, but the fact that they clearly expressed EOMES, confirmed the fact that they were tNK cells and not ILC1 with an acquired DX5 expression (Figure 14).

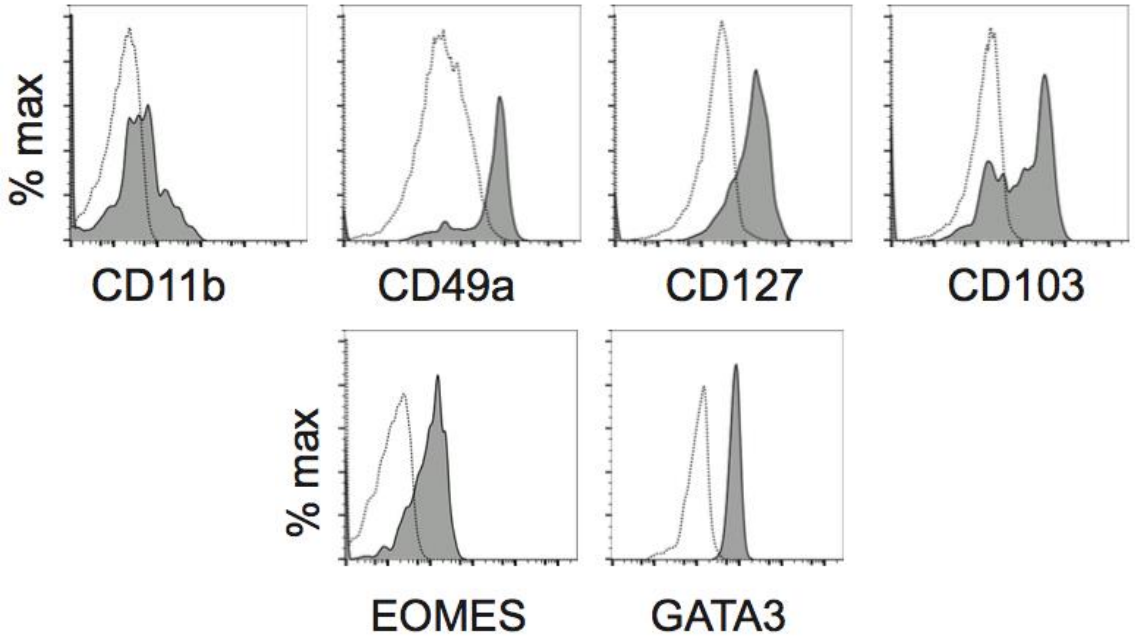

Figure 14: On the top: Surface expression of CD11b, CD49a, CD127 and CD103 for DX5+ tNK cells. On the bottom: intracellular expression of EOMES and GATA3, is shown for DX5+ tNK cells. The unshaded profile is the FMO. Data are representative of 4-7 independent experiments.

A recent report showed that salivary gland ILCs are strongly impacted by TGFβ signalling for the acquisition of tissue residency markers (Cortez et al., 2016). In a similar way the fact that *Rag1*$^{-/-}$ tNK cells expressed CD49a and CD103 (Figure 15), suggested that in the absence of T cells tNK cells could be impacted by factors that can drive the expression of these proteins such as TGFβ or related factors.

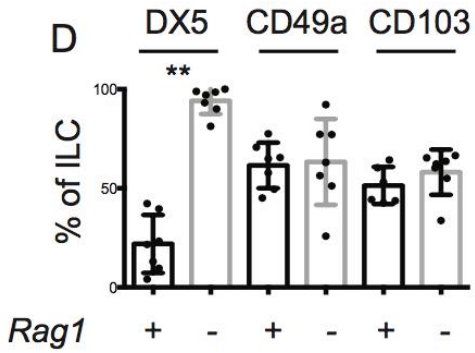

Figure 15: Summary of the percent of thymic ILC-like cells that expressed each of the indicated markers in *Rag1*$^{+/+}$ (+, black) or *Rag1*$^{-/-}$ (-, grey) mice. Each dot represents one mouse. Error bars are SEM. * $p<0.05$, ** $p<0.01$.

We note that despite the loss of Lin^{-} /CD122^{+} /NK1.1^{+} /DX5^{-} cells in the of *Tbx21*$^{-/-}$ mice, tNK cells did not acquire expression of CD103. Therefore, the loss of this "innate-like" T cells population may not be sufficient to expose tNK cells to the factors

necessary to induce CD103, although some of these cells do express CD49a. Alternatively, TBET may be required for expression of CD103 on these cells.

Thymic NK cells are ID2-independent

There has been substantial controversy over whether NK cells in the thymus require ID2. In the fetal thymus, $CD3^-$ /$NK1.1^+$ "NK cells" are ID2-dependent whereas in the adult mouse NK cells were reported to be present in the thymus (Ikawa et al., 2002; Boos et al., 2007). However, in the adult, the $Id2^{-/-}$ genotype is lethal on the C57BL/6 background so the adult $Id2^{-/-}$ mice were examined on a 129/J x FVB/NJ background, where the critical NK cell marker NK1.1 is not expressed (Mesci et al., 2006).
We decided to use a conditional knockout for ID2, where the gene is deleted in all hematopoietic cells (Xu et al., 2015), so we examined tNK cell numbers in $Rag1^{-/-}$ $GzmbCre^{+}Id2^{f/f}$ mice on a C57Bl/6 background (hereafter designated $Id2^{-/-}$). In these mice, tNK cell numbers were indistinguishable from ID2-sufficient littermate control mice (LMC) and these cells expressed DX5, CD127, CD103 and CD49a indicating that tNK cell development was ID2- independent (Figure 16).

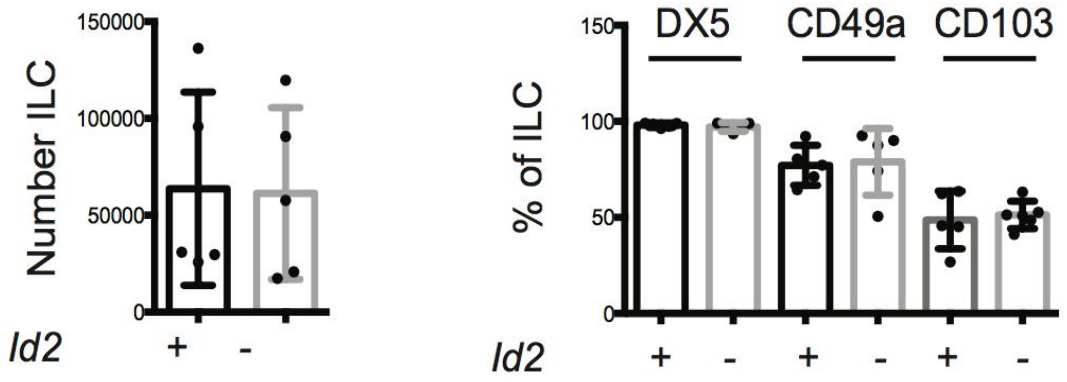

Figure 16: On the left: number of thymic ILC-like cells in $Id2^{+/+}$ and $Id2^{-/-}$ mice. Each dot is one mouse. On the right: percent % of $Id2^{+/+}$ (+, black) or $Id2^{-/-}$ (-, grey) thymic ILC expressing DX5, CD49a or CD103. Data are representative of 4-7 independent experiments. Error bars are SEM. * $p<0.05$, ** $p<0.01$.

Interestingly, ID2-deficient tNK cells failed to express any detectable CD11b and had heightened expression of CD27 (Figure 17), indicating that they may be even more immature than their LMC counterparts. To check the authenticity of this immature phenotype, we analysed tNK cells from $Il7ra^{Cre}Id2^{f/f}$ mice, which lack ID2 in all lymphoid cells and have an intact Rag1 gene, and a similar immature phenotype, with lower expression of CD11b and a higher expression of CD27, was observed (data not shown).

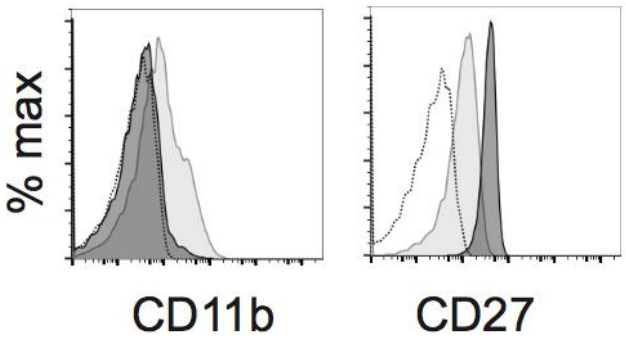

Figure 17: Flow cytometry analysis for CD11b and CD27 on *Id2*$^{+/+}$ (light grey) and *Id2*$^{-/-}$ (dark grey) tNK cells. Unshaded histogram is FMO control. Data are representative of 4-7 independent experiments.

ETS1 prevents tNK cell maturation

It has been previously shown that ETS1 is a crucial transcription factor strictly necessary for the optimal development of NK cells (Barton et al., 1998). Thus, to further address the transcription factor requirement of tNK cells, we examined *Rag1*$^{-/-}$ *Il7ra*^{Cre}*Ets1*$^{f/f}$ mice (Zook et al., 2016), and in contrast to what seen in *Id2*$^{-/-}$ mice, tNK cell numbers were decreased in the absence of ETS1 to approximately 50% of LMC. However, all of the Lin^{-}/CD122^{+}/NK1.1^{+} remained cells were tNK cells as indicated by expression of DX5 and EOMES (data not shown). In the absence of ETS1 the frequency of CD103^{+} tNK cells was substantially reduced whereas the frequency of CD49a^{+} cells was similar to LMC. However, even if not statistically significant, the intensity of CD49a expression was decreased in the absence of ETS1 (Figure 18).

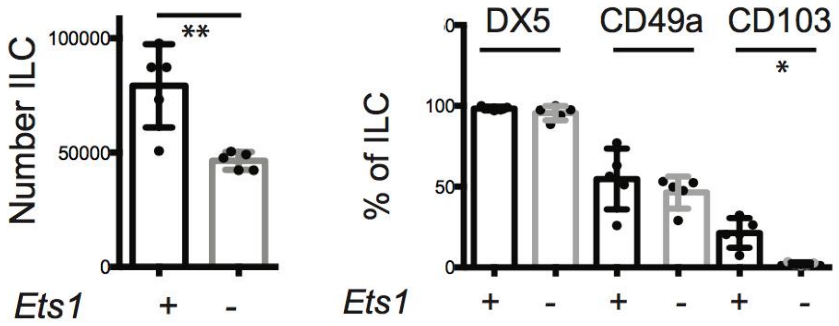

Figure 18:Number of thymic ILC-like cells in *Ets1*$^{+/+}$ and *Ets1*$^{-/-}$ mice. Percent of *Ets1*$^{+/+}$ (+, black) or *Ets1*$^{-/-}$ (-, grey) thymic ILC expressing DX5, CD49a or CD103. Each dot is one mouse. Data are representative of 5-7 independent experiments. Each dot represents one mouse. Error bars are SEM. * $p<0.05$, ** $p<0.01$.

Importantly, *Ets1*$^{-/-}$ tNK cells continue to express CD127 indicating that these tNK cells are not peripheral cNK cells that gained access to the thymus. Moreover, expression of CD127 on these cells indicates that ETS1 does not regulate Cd127 gene transcription in tNK cells, as it does in conventional T cells (Grenningloh et al., 2011). We also observed that *Ets1*$^{-/-}$ tNK cells had increased expression of CD11b and decreased CD27 suggesting that these cells undergo maturation in the absence of ETS1 (Figure 19).

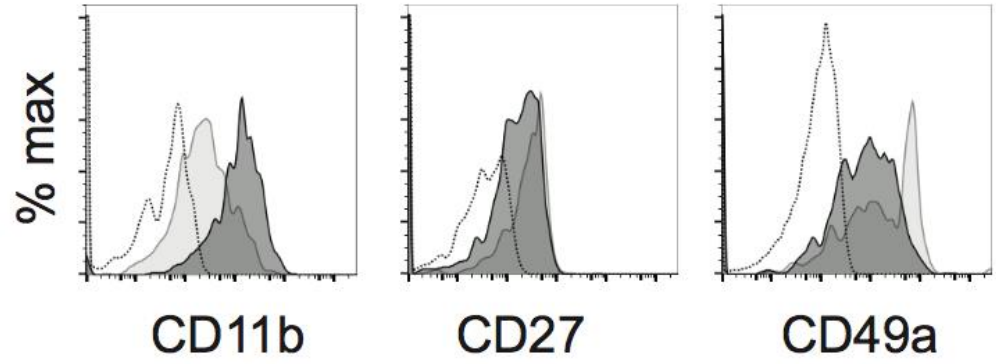

Figure 19: Flow cytometry analysis for CD11b, CD27, and CD49a on *Ets1*$^{+/+}$ (light grey) and *Ets1*$^{-/-}$ (dark grey) tNK cells. Unshaded histogram is FMO control. Data are representative of 5-7 independent experiments.

Our data indicate that ETS1 is not absolutely required for the development of tNK cells but it regulates their maturation.

4.2 Human model

Early differentiation of CD56^{dim} and CD56^{bright} NK cells from CD34+ cells

To better understand the already known differences between the two populations of NK cells, in vitro differentiation systems starting from CD34^{+} hematopoietic progenitors were used. It is known that the differentiation with Flt3-L + IL-15 induces the generation of CD56^{bright} NK cells, while the addition of IL-21 promotes the expansion of the CD56^{dim} population with strong cytotoxic activity (Parrish-Novak, et al., 2000), thus, in a first set of experiments we compared the two systems of NK cell differentiation using Flt3-L+IL-15 with and without IL-21. After 15 days of culture with Flt3-L and IL-15 a small percentage of cells was clearly characterized by relatively large dimensions (high forward and side scatter characteristics) and by CD56 at high-density of expression, the CD56^{bright} population. Instead, after 15 days of culture with IL-15, Flt3-L and IL-21, the CD34^{+} hematopoietic progenitors differentiated into both CD56^{dim} and CD56^{bright} NK populations. In fact, a CD56^{bright} NK population (similar to that in the culture without IL-21) and another one (the majority of cells) characterized by low scatter characteristics and CD56 expression, were clearly distinguishable.
As for cultures without IL-21, the small percentage of CD56^{bright} cells can be further divided into CD18 positive and negative, differently all CD56^{dim} cells express CD18.
CD16 receptor, mostly absent on CD56^{bright} cells, is expressed at medium-low density on most (but not all) CD56^{dim} cells, suggesting that this receptor is up regulated slightly later than the CD18 molecule. By focusing on CD56$^{dim/neg}$ cells with low forward scatter characteristic (Region 1, R1), we evaluated the expression of different NK receptors distinguish between CD16^{+} cells (more mature) and negative (immature stages of NK CD56^{dim} subset). Some CD56^{dim}/CD16^{neg} NK cells expressed CD244, CD94, NKG2A, NKG2D and the KIRs, but not NCRs. The majority of CD56^{dim}/CD16^{+} cells did not express NKp44, suggesting that they were in a not activated state. The fact that NCRs appear slightly later than NKG2D antigen suggests the existence of an immature NCR^{neg}/NKG2D^{+} stage during the differentiation of the CD56^{dim} NK cell lineage, a phenotype which reminds that described in several chronic viral diseases (Bjorkstrom et al., 2010). Of note, the majority of CD56^{bright} NK cells gated R1, express NKp44 antigen, while for CD56^{dim}/CD16^{+} cells the opposite was true (Figure 20).

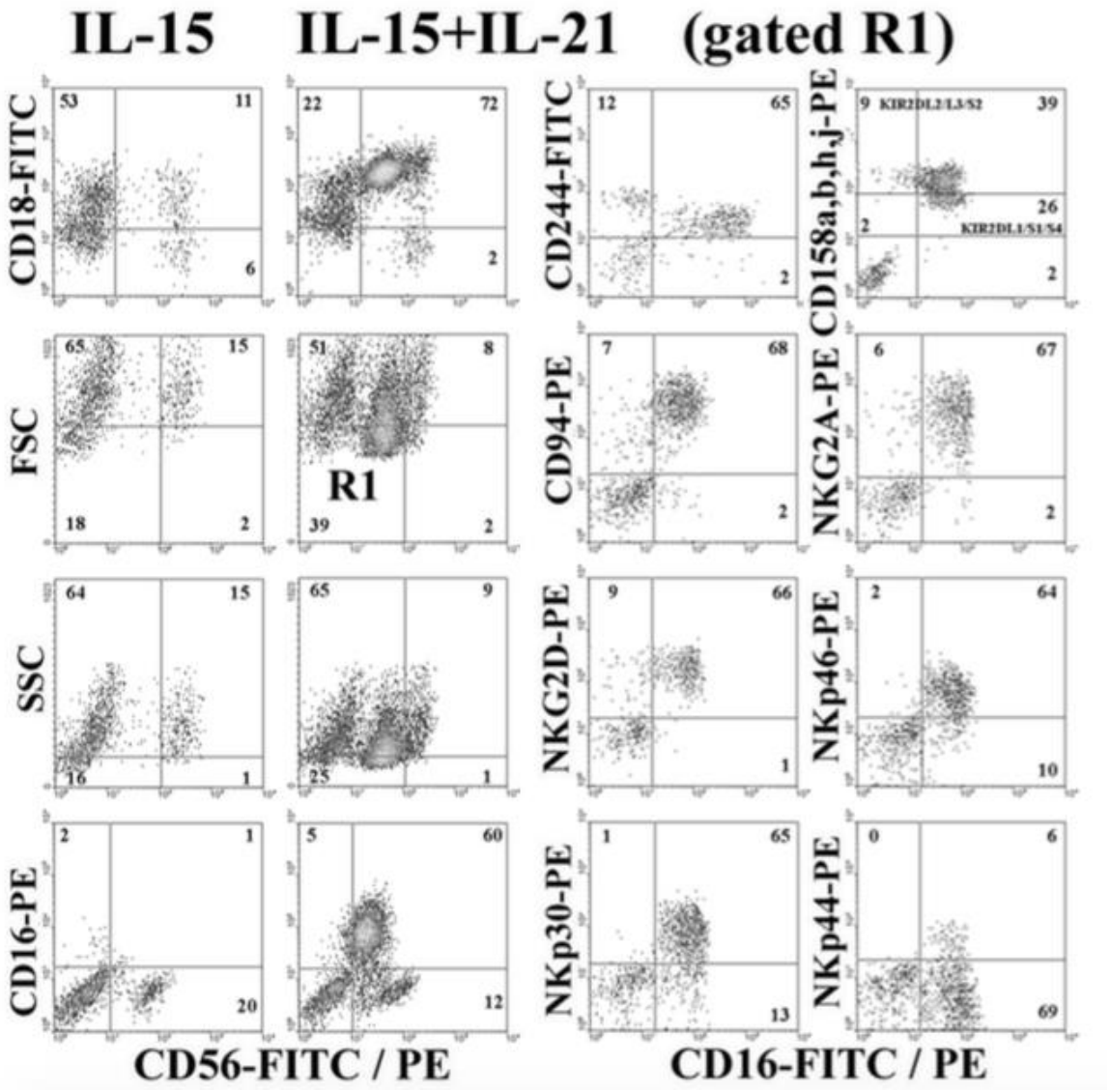

Figure 20: Surface expression of molecules and scatter characteristic of the in vitro generated cells after 15 days of culture of human CD34$^+$ cells with Flt3-L and IL-15 with or without IL-21. The two columns on the right side of the panel display the antigen expression of the cells gated in the region 1 (R1). The percentages of positive cells are indicated in each quadrant.

CD56^{dim} NK cells generated in vitro express cytotoxic effector molecules (Granzyme-B$^+$/Perforin$^+$/LFA-1$^+$) and are short life

After 15 days of culture with IL-15, Flt3-L and IL-21, CD56^{dim}/CD16$^+$ cells, generated from CD34$^+$ hematopoietic progenitor, expressed Perforin, Granzyme-B, LFA-1 and, typical of CD56^{dim} cells, not CD117 (Figure 21).

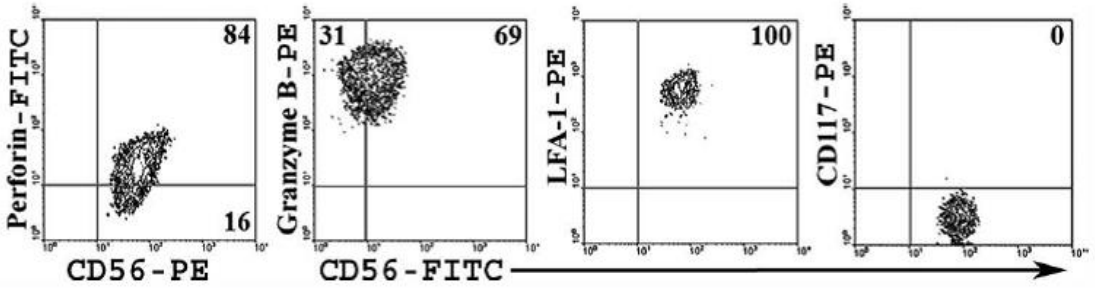

Figure 21: Surface and intracellular molecule expression of CD56^{dim}-gated NK cells generated in vitro after 15 days of CD34^{+} cell culture with Flt3-L, IL-15 and IL-21.

CD56^{dim} NK cells generated in vitro, early expressed both inhibitory receptors (CD94, NKG2A and KIRs) necessary to ensure self-tolerance, and molecules associated to the cytotoxic activity (LFA-1, NKG2D, Granzyme B and perforin) (Figure 20 and 21).
After 25 days of culture with IL-21, we observed a significant expansion of the CD56^{bright} NK cell subset, while CD56^{dim} NK cells generated in culture showed an increase in the density of CD56, CD16 and NKp44 (Figure 22) indicating their further maturation and activation. At this maturational stage was no longer possible to distinguish the two subgroups according to the CD18 vs. CD56 expression.

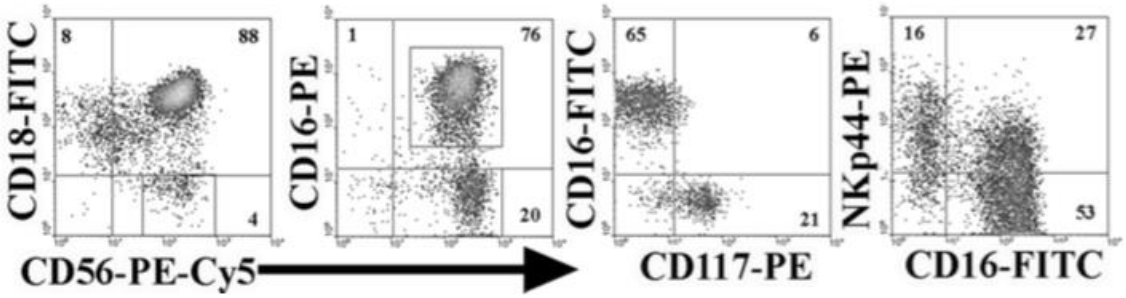

Figure 22:Surface expression of molecules expressed after 25 days of in vitro culture of human CD34^{+} cells with Flt3-L, IL-15 and IL-21.

Differently from CD56^{bright}, CD56^{dim} NK cells had a short life cycle and they quickly died, in fact, after 30 days of culture they were no longer detectable.

Expression of cytotoxic effector molecules in CD56^{bright} NK cells generated in vitro: identification of immature CD56^{bright}/Granzyme-B^{-}/Perforin^{-}/TRAIL^{+} NK cells

NK cells generated in vitro after 20-30 days of culture with Flt3-L + IL-15 (without IL-21), despite expressing the NCRs and NKG2D (but not CD16) activating receptors, mediated a relatively low cytotoxic activity compared to that of mature NK cells (Sivori et al., 2002; Zamai et al., 1998). It has been suggested that reduced cytotoxic activity of NK cells obtained in vitro is linked to an inhibitory function of the 2B4 molecule (CD244) (Sivori et al., 2002). However, this reduced activity is visible against both

K562, which does not express CD48 the ligand for 2B4, and against the $CD48^+$ Jurkat cell line (Zamai et al., 1998), suggesting a non-exclusive role in the inhibition of the cytotoxic activity by the binding CD48-2B4. For this purpose, the expression of cytotoxic effector molecules, such as Granzyme-B and Perforin, has been evaluated. It is interesting to note that the Granzyme B and Perforin were co-expressed on only a subset of $CD56^{bright}/NCR^+$ cells generated *in vitro*, and the expression increases with the progression of time in culture (Figure 23 A). Unlike intracellular proteins, the surface molecules belonging to members of the TNF family are present on $CD56^{bright}$ NK cells already after 20 days of culture. In particular, TRAIL is also uniformly expressed, even if at a low density, on the surface of most $CD56^{bright}$ NK cells after 30 days of culture. The presence of other ligands of the TNF family such as, CD95L and CD40L has not been found. As already described, the CD94 antigen is used as a marker to distinguish two stages of NK cell differentiation (Freud et al., 2006; Grywacz et al., 2006). Of note, the percentages of $CD56^{bright}$/Granzyme-B^-/$Perforine^-$ were similar to those of $CD56^{bright}$/$LFA\text{-}1^-$ and $CD56^{bright}$/$CD94^-$/$NKG2A^-$, for this reason the co-expression of CD94/NKG2A with LFA-1 or lytic proteins has been investigated (Figure 23 B).

Most $CD56^{bright}$ NK cells generated in vitro after 30 days of culture co-express Granzyme B with LFA-1 or CD94-NKG2A (whose distribution completely overlapped with CD159a antigen), suggesting that these molecules are expressed in a similar way during the differentiation of $CD56^{bright}$ NK cells. However, a small percentage of $LFA\text{-}1^-$ cells expressed the Granzyme B, suggesting that during differentiation intragranular cytotoxic molecules preceded the expression of LFA-1.

In contrast, TRAIL was expressed at low density on both immature $CD56^{bright}$/$LFA\text{-}1^-$ cells and more mature $CD56^{bright}$/LFA-1+, confirming that TRAIL is an activation marker expressed on the surface of immature NK stages (Zamai et al., 1998).

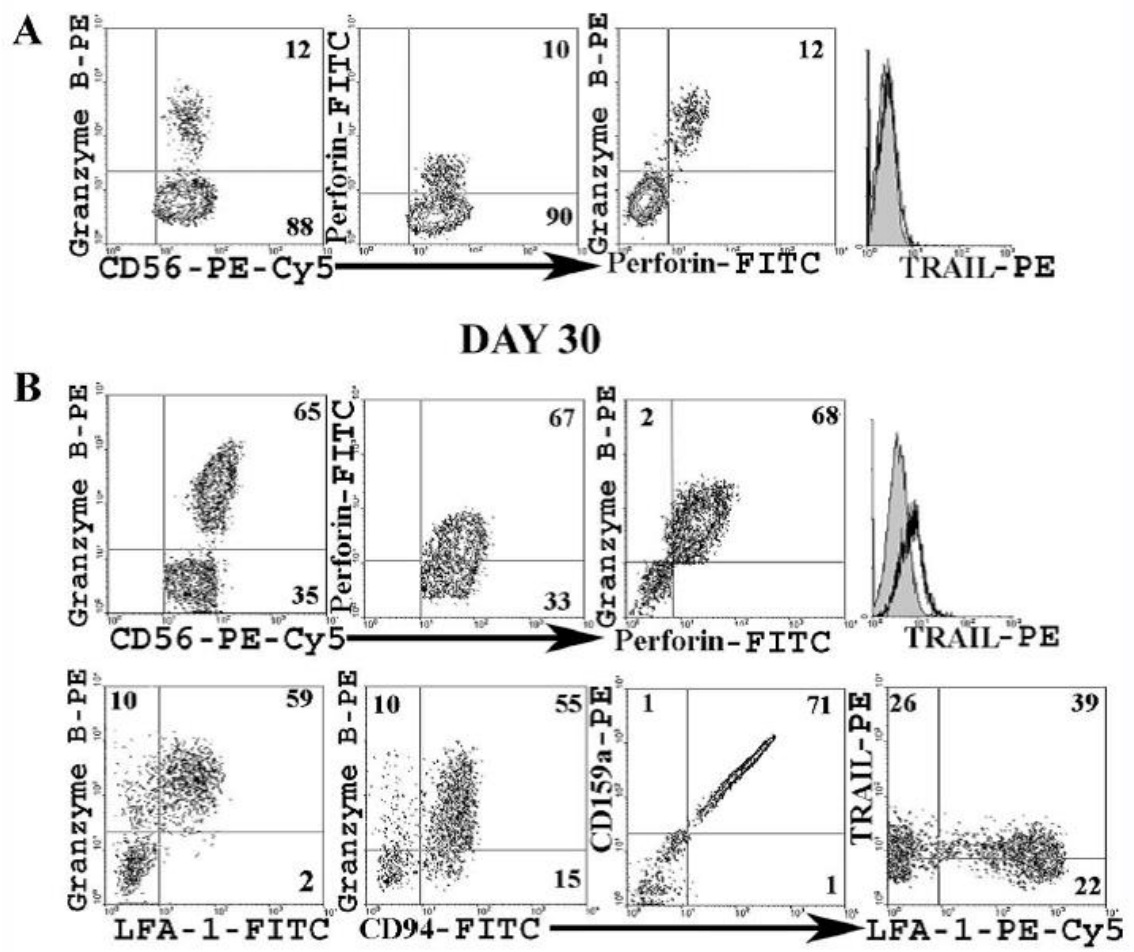

Figure 23: Surface expression of molecules at the CD56^{bright}/TRAIL^{+}/Granzyme B^{-}/ Perforin^{-} NK cell stage developed from CD34^{+} precursor in the presence of Flt3-L and IL-15. **A**- day 20 of primary culture. **B**- day 30 of primary culture.

Further differentiation of CD56^{bright} NK cells induced by IL-15 is inhibited by IL-21

It has been shown that the combination of IL-15 and IL-21 leads to the generation of the NK subpopulation CD56^{dim} (Parrish-Novak et al., 2000). In order to induce the differentiation and the subsequent generation of the CD56^{dim} subset from CD56^{bright} NK cells, NK cells obtained after 30 days of primary culture, that contain both immature and mature CD56^{bright}, were put in culture for further 15 days with IL-15 alone or in combination with IL-21.

IL-21 alone was not able to support the survival of cells in culture, while a cell viability of 95% was maintained in the cultures with only IL-15. Moreover, the proliferation induced by IL-15 alone, was inhibited by about 30% as a consequence of the addition of IL-21. In agreement with other work (Freud et al., 2006; Grzywacz et al., 2006; Barao et al., 20032001; Carayol et al., 1998), IL-15 promoted the subsequent differentiation of CD56^{bright}/CD117^{+} NK cells. In particular, IL-15 leads to an increase in the percentage of cells positive for LFA-1, CD94/NKG2A (Figure 24 A), CD11c and TRAIL-R4 (data

not shown), as well as for KIRs and CD16 (Figure 24 C). Moreover, after 15 days of secondary culture with IL-15, all NK cells were CD56^{bright} Granzyme-B^{+} (and Perforin^{+}) (Figure 24 A). In agreement with these observations, CD56^{bright} NK cells generated in vitro from CD34^{+} progenitors and peripheral blood NK cells cultured with IL-15 (obtained from the same donor), mediated a similar, high cytotoxic activity against Jurkat and K562 cells (Figure 24 B).

Interestingly, IL-21 did not induce the differentiation of the NK CD56^{dim}/CD16^{+} subset from CD56^{bright} NK cells (Figure 24 C), but this cytokine (IL-21) induced the differentiation of a low percentage of NK granzyme-B^{+} cells, LFA-1^{+}, CD16^{+} and KIR^{+}. These data show that the secondary cultures with IL-21 inhibit the differentiation of CD56^{bright} NK cells mediated by IL-15 (Figure 24 C). In agreement with these observations, CD56^{bright} NK cells in secondary culture with IL-15, mediated a cytotoxic activity against K562 cells higher than those cultured with IL-15 + IL-21 (Figure 24 D).

It is important to underline that CD16 and CD158a,b molecules were present only on CD56^{bright}/ LFA-1^{+} NK cells (Figure 24 C), further confirming the immaturity of CD56^{bright}/LFA-1^{-} NK cells.

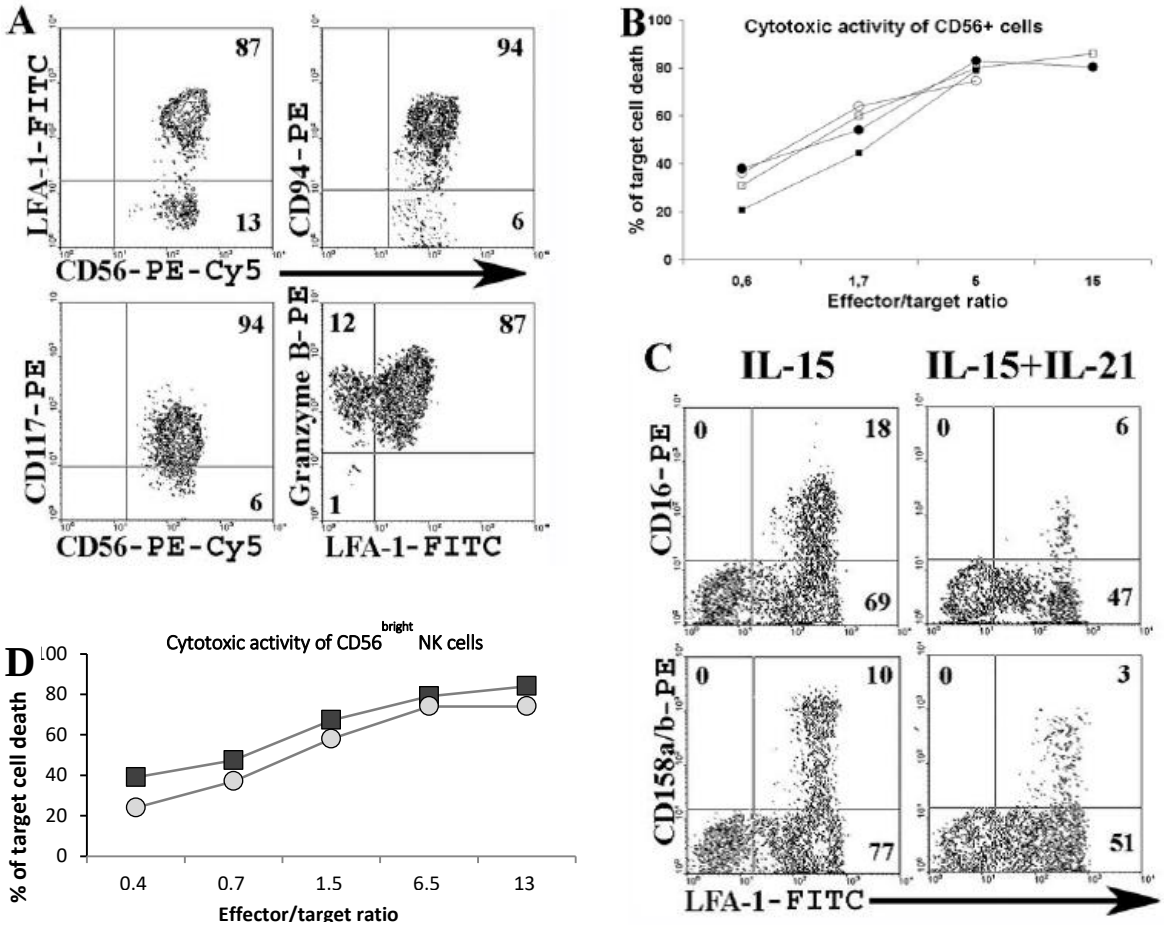

Figure 24: CD56^{bright} NK cell subset differentiation in the secondary culture with IL-15 is inhibit by IL-21. **A**- phenotype of CD56^{bright} NK cells after 15 days of secondary culture with IL-15. **B**- Cytotoxic activity against Jurkat (□, ●) and K562 (▪, ○) target cells of CD56^{bright} NK cells generated after 15 days of secondary culture with IL-15. **C**- phenotype of CD56^{bright} NK cells after 15 days of secondary culture in the presence of IL-15 with or without IL-21. **D**- cytotoxicity activity against K562 target cells of CD56^{bright} NK cells in the secondary culture with IL-15 (▪) or with IL-15 + IL-21 (○).

Discussion

NK cells are thought to primarily develop in the bone marrow. However, fetal thymus and liver contain bipotent T/NK progenitor cells that possess the ability to develop into NK cells (Carlyle et al., 1997; Sanchez et al. 1994; Spits et al., 1998). Similar to T and B cells, NK cells require the common gamma chain for their development Lack of common gamma chain, results in a near complete loss of NK cells (Di Santo 2006; Ma et al., 2006). IL-15 is thought to be required during the entire life span of NK cells (Di Santo 2006; Yokoyama et al., 2004). Although NK cells develop and mature in the bone marrow, they continue to mature in peripheral tissues and undergo "tuning" of functional competence dependent upon specific environmental cues, including MHC class I molecules (Orr and Lanier 2010). Other sites of development (liver, lymph node, thymus, and salivary glands) have also been proposed; however, whether the cells described at these sites represent unique NK cell subsets or distinct innate lymphoid cell (ILC) lineages remain to be determined. Lineage-tracing studies have suggested that NK cells and ILC1 originate from distinct precursors (Constantinides et al., 2014; Klose et al., 2014); however, recent data from the Immunological Genome Project found that these two populations possessed overlapping gene-expression patterns (Robinette et al., 2015). Thus, the distinction between NK cells and ILC1 remains controversial and complicated at the current time, and it is possible that ILC1 may represent a developmental stage of NK cells rather than a distinct lineage.

The purpose of the Ph.D project was to assess origin relationship between the two NK subpopulations, $CD56^{dim}$ and $CD56^{bright}$. In addition, we investigate the NK cells in the thymus of mice, which seems to be the murine counterpart of human $CD56^{bright}$ NK cells. Thymic NK cells (tNK) were first described by Vossenrich and colleagues (Vossenrich et al., 2006), before the discover of innate lymphoid cells (ILCs). For this reason, we wanted to better characterize these thymic cells to verify whether they represent a peculiar type of NK cells or they included also other lineage of ILCs.

We have demonstrated that the thymus contained a heterogeneous population of Lin^- $CD122^+/NK1.1^+$cells that included $DX5^-/CD49a^+/RAG1^-$ dependent innate-like T lymphocytes (ILT), a subset of which expressed CD103, and $DX5^+/CD27^+/CD11b^{lo}$ NK cells. The ILT cells were dependent on the transcription factor TBET but not NFIL3 whereas tNK cell development required NFIL3 but was independent of TBET, consistent with the classification of the latter as NK cells rather than ILC1 (Gascoyne et al., 2009; Kamizono et al., 2009; Robinette & Colonna, 2016). In contrast to cNK cells, tNK cell development was independent of ID2 although ID2-deficiency resulted in

increased expression of CD27 and a complete loss of CD11b on these cells. tNK cells also developed in mice lacking ETS1 and these cells were $CD127^+$ confirming that they were bone fide tNK cells. Interestingly, *Ets1*$^{-/-}$ tNK cells were primarily $CD27^-$ and $CD11b^+$ indicating that ETS1 was required to prevent tNK cell maturation (Chiossone et al., 2009). Our data demonstrate that tNK cells not ILC1 but have transcription factor requirements that only partially overlap with cNK cells.

Our observation that tNK cells arise independent of ID2 was surprising given that all ILCs and mature cNK cells have been reported to be ID2-dependent (Delconte et al., 2016; Satoh-Takayama et al., 2016). However, the $CD27^+CD11b^-$ NK cell subset is a very minor portion of bone marrow and peripheral cNK cells and this subset is present in $Id2^{-/-}$ mice. Therefore, tNK cells may be similar to cNK cells in their requirements for ID2 but this requirement was masked by the failure of tNK cells to mature into $CD11b^+$ cells. In contrast, ETS1 may play a unique role in tNK cells to prevent their maturation to a $CD27^-/CD11b^+$ stage.

ETS1 is a signal regulated transcription factor whose DNA binding activity can be regulated by Ca^{2+} signaling and its ability to activate transcription is regulated by the mitogen- activated protein kinase (Mapk) signalling pathway. Therefore, the immature phenotype of tNK cells could be a consequence of unique signals that prevent their maturation compared to the signals present in the bone marrow. Recent studies have indicated that $CD27^+/CD11b^-$ NK cells are naïve cells because they have not been activated by IL-15 or other cytokines that are induced in dendritic cells by microbial products (Kamimura et al., 2015). It is possible that the thymus lacks stroma or myeloid cells that can stimulate the maturation of tNK cells. Alternatively, tNK cells may be resistant to external maturation signals. tNK cells also express the alpha chain of the receptor for IL-7 (CD127), which shares the common γ chain with the IL-15/IL2 receptor alpha chain (CD25), and expression of two receptors that share a common component may diminish responsiveness to either cytokine if the common component is limiting (Cotari et al., 2013). Thus, the expression of CD127 on tNK cells may reduce their responsiveness to IL-15. This possibility is particularly intriguing given that tNK cells increase their expression of CD11b when ETS1 is deleted and ETS1 is known to limit the responsiveness of cNK cells to IL-15 (Ramirez et al., 2012). Therefore, the increased IL-15 responsiveness in $Ets1^{-/-}$ tNK cells may drive NK cell maturation and the expression of CD11b.

Whether the developmental program of tNK cells or their naïve state impacts their function also remains to be determined. Indeed, the function of tNK cells is currently not known. These cells could play a role in protecting the thymus from infection by thymic tropic viruses such the herpesvirus-related mouse thymic virus, which can infect neonatal mice and lead to immune suppression (Guignard et al., 1989). Similarly, these cells could function in the innate response to T cell leukemia (Dadi et al., 2016). tNK

cells can be found in peripheral lymphoid tissues and a relatively abundant in the lymph node (Vossenrich et al., 2006). These cells share many features with $CD56^{bright}$ NK cells in humans and may have similar function. $CD56^{bright}$ NK cells have been implicated in the regulation of dendritic cells and in tumor surveillance (Ferlazzo & Morandi, 2014). Whether peripheral tNK cells or $CD56^{bright}$ NK cells acquire tissue residency in any tissues is also not known but this is a possibility given our observation that CD49a and CD103 can be induced on tNK cells. A better understanding of the function of tNK cells will require model systems in which these cells can be selectively deleted altogether with some parabiosis experiment.

In our study, NK cells were differentiated from $CD34^{+}$ hematopoietic progenitors with Flt3-L, IL-15 and with or without IL-21. It is known that the combination of the cytokine Flt3-L and IL-15 is essential for generating $CD56^{bright}$ NK cells from hematopoietic progenitors (Yu et al., 1998, Loza et al., 2002). Co-administration of IL-21, IL-15 and Flt3-L instead determines the differentiation of progenitor cells in the $CD56^{dim}CD16^{+}$ subset (Parrish-Novak et al.,2000; Sivori et al., 2003; Zamai et al., 2012). $CD56^{bright}$ NK cells generated in vitro with the combination of cytokines in the absence of IL-21, have low levels of KIRs and CD16, while, typical of this population, they express high density of CD56 and CD117. These data indicate that these cells are the counterparts of the $CD56^{bright}$ NK subset present in the peripheral blood (Farag et al., 2006). Curiously, the $CD56^{bright}$ NK cells appear, both in vivo and in vitro after 3-4 weeks, unlike the $CD56^{dim}/CD16^{+}$ cytotoxic subset, which differentiate late *in vivo* or early *in vitro* (after 2-3 weeks of culture) if stimulated with IL -15, Flt3-L and IL-21 (Zamai et al., 2012; Parrish-Novak et al., 2000).

After 15 days of culture with IL-15, Flt3-L and IL-21, the $CD34^{+}$ hematopoietic progenitors primarily differentiate in cell with phenotype $CD56^{dim}/CD16^{+}$. The more immature $CD56^{dim}/CD16^{neg}$ NK cells express granzyme, perforin, LFA-1, 2B4 (CD244), CD94, NKG2A, NKG2D and the KIRs, while do not present NCRs. On the other hand, after 20-30 days of *in vitro* culture with FLT3- L and IL-15, a functionally immature stage of $CD56^{bright}$ NK cells has been identified, which does not perform the function mediated by the release of cytotoxic granules because they lack adhesion molecules and intragranular cytotoxic proteins. Nevertheless, these cells can potentially kill through a TRAIL-dependent mechanism.

The fact that, $CD56^{dim}$ NK cells generated in vitro from $CD34^{+}$ progenitors in the presence of IL-21, unlike the $CD56^{bright}$, express the LFA-1 adhesion molecule earlier than NCR activating receptors, suggests that the sequence of the antigen expression is different between the two subsets.

During normal *in vivo* differentiation, differentiating NK cells acquire the activating receptors, inhibitory receptors and cytotoxic function to prevent the self-aggression.

There are several hypotheses to reach the self-tolerance during NK differentiation (Raulet et al., 2006; Raulet et al., 2001). One possibility is that the expression of inhibitory receptors precedes that of the activating ones. Among the inhibitory receptors that bind MHC-I (necessary for the self-tolerance), the CD94-NKG2A heterodimer is one that appears early during the differentiation of NK cells (Raulet et al., 2001), and its inhibitory function has been demonstrated during *in vitro* differentiation (Carayol et al., 1998). Indeed, this mechanism of tolerance to "self" seems to intervene during differentiation of $CD56^{dim}$ NK cells.

However, as regards the differentiation of $CD56^{bright}$ cells, our data, clearly indicate that the activating molecules are expressed on immature NK cells, at higher percentages than MHC-I inhibitory receptors, suggesting that activating receptors precede the expression CD94-NKG2A heterodimer. Tolerance to "self" of NK cells could be due to the lack of ligands for activating receptors in the sites of NK differentiation. In this regard, it has been demonstrated that the immature myeloid cells, which are generated during the *in vitro* differentiation of NK cells (Bennett et al., 1996), express the ligands for NCRs (Nowbakht et al., 2005). Therefore it is not possible to exclude that some ligands for NK cell activating receptors are present during NK differentiation. Our data suggest that, in addition to the inhibitory function of the 2B4 (Sivori et al., 2002), the immature NK cell would not be lytic (and this would ensure the self-tolerance) as they do not express the adhesion molecules and intracellular lytic proteins. The early expression pattern of activating molecules is unclear, however there is some evidences to support the idea that the stimulatory signals are necessary to promote the expression of inhibitory receptors on differentiating NK cells (Raulet et al., 2001; Zamai et al., 2009).

Highly cytotoxic and fully functional $CD56^{bright}$ NK cells were obtained after secondary culture when the majority of NK cells also express inhibitory receptors that bind to MHC-I. However, to achieve the high cytotoxic activity that characterizes the $CD56^{bright}$ subset after 45 days of culture with IL-15, in addition to their full maturity in vitro it is also likely to occur an activation process. Indeed, typical of activated NK cells, is that they are characterized by an increased expression of the members of the TNF ligand family, molecules capable of increasing the cytotoxic NK function (Zamai et al., 2007). In particular, one of the members, the TRAIL surface molecule, occurs early during in vitro differentiation/activation of NK cells. Differently, FasL and CD40L appear late and are poorly detectable, probably because they are secreted rather than expressed on the cell surface (Zamai et al. 2012). The expression of TRAIL, similar to the intracytoplasmic production of TNF-α (Loza et al., 2002) was observed both on NK immature $CD56^{bright}/CD18^{-}$ cells than in mature ones $CD56^{bright}/CD18^{+}$, confirming that TRAIL is a marker of activation expressed at the level of immature stages of NK cells (Zamai et al., 1998).

Differently from cultures with $CD34^+$ progenitors, the presence of IL-21 did not induce the differentiation of $CD56^{dim}/CD16^+$ NK subset from $CD56^{bright}$ NK cells,and it rather inhibited their differentiation, downregulating IL-15-induced CD16 and KIR expression.

MHC-I inhibitory receptors would be expressed as early as NK cells, by upregulating molecules of the cytolytic machinery, become potentially cytotoxic. The reason of an early expression of activating molecules is not clear, anyway, some evidences support the idea that stimulatory signals are necessary to induce the expression of inhibitory receptors on developing NK cells (Raulet et al., 2006), finally leading to a functionally complete maturation. Moreover, the percentage of KIR^+ NK cells, which was usually higher than that of mature $CD56^{dim}$ peripheral blood ones (Sivori et al., 2012). To this regard, it is possible that the culture system, lacking cells able to present HLA-I to differentiating NK cells, may induce (as for MHC-I –deficient mice) (Raulet et al., 2001) high frequencies of HLA class-I specific receptors.

Altogether these data suggest that $CD56^{dim}$ and $CD56^{bright}$ NK cells, as for myeloid and lymphoid dendritic subsets, would originate from distinct progenitors, which, along with their differentiation into mature cells, would generate two distinct cell NK subsets with convergent phenotypes and functions. Moreover, during their development $CD56^{dim}$ and $CD56^{bright}$ NK cells would exploit different mechanisms to prevent cytotoxicity against healthy cells.

Indeed, the succession of surface markers, intracellular markers and cytotoxic functions acquired during differentiation (and activation) of NK cells is different between the 2 populations.

Of interest for cancer therapy, our data indicate that NK cells generated *in vitro* $CD56^{bright}$ after 45 days of culture with IL-15 acquire a phenotype and a function similar to that of cytotoxic $CD56^{bright}$ NK cells in peripheral blood, suggesting that the cytotoxic ability acquired during this culture time can be used against cancer cells.

A detailed knowledge of NK cell differentiation and acquisition of their of cytotoxic function is important to determine the culture conditions suitable for the generation of NK cells that can counteract the expansion of tumor cells or other diseases.

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