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Physiological and Pathological Aspects of Human NK Cells

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1. Introduction

Natural Killer (NK) cells were first defined, more than 30 years ago, on the basis of their unique capability of killing spontaneously different targets including tumor and virus-infected cells. Thus, since their first discovery, NK cells came across as a potential attractive tool for the implementation of immuno-therapeutic strategies for different diseases. Accordingly, besides the plethora of studies aimed at investigating their functional and phenotypic properties, considerable efforts have also been spent in the past several years to understand how these cells could be generated and how their functions could be regulated and/or manipulated. At the same time further attention has been paid on their alteration in vivo and their potential role in the occurrence of NK cell-based hematological malignancies.

2. NK cells: From a function to a multifaceted population

2.1 The discovery of NK cells

NK cells were originally identified on a functional basis. In the middle '70s, it was discovered that healthy individuals could display selective cytotoxic activity against tumor or virally infected cells and that this activity was hidden within the circulating lymphocyte population (West et al., 1977; Santoli et al., 1978). This implied the presence in the Peripheral Blood (PB) of a lymphocyte subset capable of killing different targets without previous sensitization. These cells, that for their functional properties were termed 'Natural Killer', were then characterized morphologically as Large Granular Lymphocytes (LGL) by virtue of their size and cytolytic granule content. Finally, in the late '80s, NK cells were more precisely defined with the $CD3^-CD56^+CD16^+$ surface immuno-phenotype. This also allowed the identification of the CD16/FcγRIII as the receptor responsible for the Antibody-Dependent Cell Cytotoxicity (ADCC) function shown by NK cells (Lanier et al., 1986; Trinchieri, 1989). In the same period, the circulating NK cell population, which represented approximately 10-15% of PB

lymphocytes, was further split into $CD56^{bright}CD16^{dim/neg}$ and $CD56^{dim}CD16^{bright}$ cell subsets, expressing respectively low and high cytolytic properties. In the following 10 years great efforts were done to widen the list of NK cell markers and to define the surface receptors responsible for the regulation of NK cell cytotoxicity. Finally, in the last decade it was discovered that NK cells could exert different regulatory functions, and many studies were oriented to the definition of novel NK cell subsets involved in such unexpected functional features.

2.2 The regulation of NK cell function: Activating and inhibitory receptors

Once NK cells could be physically identified and isolated, the goal was to understand how these cells could work; and the first questions were: "How can a single NK cell kill different targets?" and "how do NK cells recognize and spare self normal cells?" The answer to these questions was indicated by Karre in his "missing self hypothesis". He postulated that NK cells could sense the absence (or reduction) of self MHC class I molecules (MHC-I) during cell-to-cell interaction: the missing recognition of self would allow NK cells to kill the targets, while recognition of self MHC-I would inhibit their cytolytic activity (Ljunggren & Karre, 1990). This would explain how NK cells could kill tumor or virus infected cells, which frequently undergo surface MHC-I down-regulation, and spare self non-pathogenic cells, equipped with appropriate levels MHC-I. The hypothesis was then confirmed, and the inhibitory MHC-I-specific receptors responsible for the recognition of Self were identified. In humans, major HLA-I-specific inhibitory receptors are represented by the C-type lectin CD94/NKG2A dimer that recognizes the non-classical HLA-E alleles, and the Killer Ig-like Receptors (KIR). The CD94/NKG2A encoding genes are located in the NK Receptor complex on chromosome 12, while the KIR locus is located in the Leukocyte Receptor Complex in Chromosome 19. The KIRs constitute a family of strictly homologous proteins characterized by the presence of two or three extracytoplasmic Ig-like domains (KIR2D or KIR3D) and a long cytoplasmic tail (KIR2DL or KIR3DL) containing Immuno Tyrosine-based Inhibitory Motifs (ITIMs) for the inhibitory signal transduction. Each KIR recognizes a specific epitope common to a defined group of HLA-I alleles. In particular, KIR2DL1 recognizes the Lys80 containing C2 epitope, that is shared by a group of HLA-C alleles; KIR2DL2/L3 recognize the Asn80 containing C1 epitope, that is shared by the remaining alleles of the locus HLA-C; KIR3DL1 recognizes the Ile/Thr80 containing HLA-Bw4 epitope, that is common to certain HLA-A and HLA-B alleles, while KIR3DL2 recognizes certain HLA-A alleles (Biassoni et al., 2001; Parham, 2005). Interestingly, KIR3DL2 has also been recently shown to bind microbial nucleic acids at the cell surface and to shuttle them to TLR9 in the endosome, suggesting for certain KIR a novel (or rather an ancient) function as pathogen sensors (Sivori et al., 2010). The HLA-I-specific inhibitory receptors are clonally distributed within the NK cell population of each individual. Most NK cells express one or more receptors and at least one that recognize an autologous HLA-I allele. Recently, a $KIR^{-}NKG2A^{-}$ NK cell subset has been described in different donors. These cells, however, appeared to be poorly functional. This suggested that the expression and the engagement of inhibitory receptors during maturation of NK cells could dictate the acquisition of their cytolytic potential (Licensing theory) (Anfossi et al., 2006). The KIR family also includes activating KIRs. These are homologous to their inhibitory counterparts, but display a short cytoplasmic tail (KIR2DS or KIR3DS), lack ITIMs and associate an ITAM-bearing molecule (DAP12) to transduce activating signals. Similarly, also for NKG2A, does exist a short-tailed activating counterpart, termed NKG2C. The meaning of these receptors is poorly known. It

has been hypothesized that they may recognize HLA-I molecules loaded with viral or tumor peptides, or, with low affinity, normal HLA-I molecules. In this latter case, when target cells selectively down-regulate HLA alleles, recognized by inhibitory KIR, the engagement of activating KIR may result in NK cell activation. The simultaneous expression of activating and inhibitory KIRs may therefore represent a strategy whereby NK cells can recognize and kill pathologic cells uniquely by detecting changes in their HLA alleles repertoire. At present, however, binding to HLA-I molecules has been formally demonstrated only for KIR2DS1 and NKG2C.

NK cells are also equipped with a large array of non-HLA-specific triggering receptors and co-receptors, whose engagement by specific Ligands expressed on target cells induces NK cell cytotoxicity and cytokine release (Moretta A. et al., 2001; Vivier et al., 2011). The group of NK-triggering receptors is largely heterogeneous: it encompasses molecules either structurally unrelated or belonging to different molecular families. At variance with KIRs, triggering receptors are expressed by virtually all NK cells and, in most cases, by different lymphocyte subsets or monocytes. The activating receptor Ligands till now identified, appear to be over-expressed in one or more of the following cases: tumor transformation, viral infection, cell stress or activation. Consistently, most of the activating receptors have been shown to be variably involved in the recognition and killing of virus infected and/or tumor cells. Two members of CD2 family: 2B4 (CD244) and NTBA, which recognize CD48 and NTBA respectively, have been involved in the clearance of EBV-infected cells. NKp80 and NKG2D, two Killer cell Lectin Receptors (KLR) encoded in the NK gene Complex on human chromosome 12, recognize molecules that can be expressed on tumor and/or virus infected cells. NKp80 recognizes AICL (encoded in the same NK gene complex) that is expressed on activated monocytes, but it can be also expressed on malignant myeloid cells (Welte et al., 2006). NKG2D recognizes MHC-I-related stress-inducible molecules of the MIC and ULBP families, that can be up-regulated on virus infected or tumor cells. Three receptors, that are expressed on cytotoxic lymphocytes (both NK and T cells), DNAM-1 (CD226), TACTILE (CD96) and CRTAM, recognize members of the Nectin/Nectin-Like (NecL) family (Fuchs & Colonna, 2006). In particular DNAM-1, that is the most highly espressed on NK cells, recognizes Nectin2 (CD112) and NecL5 (CD155 - poliovirus receptor) (Bottino et al., 2003). Nectin/NecL molecules are involved in the formation of various types of cell-to-cell junctions, especially in epithelial cells, neurons or fibroblasts. Nectins 1 and 2, and NecL5 can also serve as viral entry receptors. These molecules, however, are frequently up-regulated in tumor cells of different histotypes. In addition, CMV infection can alter NecL5-Nectin3 intercellular interaction: in this case NecL5 would be exposed outside the cellular junction and allow DNAM-1 recognition.

While all the above-mentioned activating receptors are expressed by different leukocyte subsets, NKp30, NKp46 and NKp44 triggering receptors are restricted to NK cells. NKp44 and NKp46 are involved in the recognition of both viral antigens (as the influenza virus HA) (Mandelboim et al., 2001) and ligands expressed by tumor cells. NKp30 recognizes B7H6 and BAT-3 (Brandt et al., 2009; Pogge von Strandmann et al., 2007). B7H6 is a member of the B7 family (which includes ligands for stimulatory/inhibitory T cell co-receptors CD28/CTLA4). B7H6 is poorly expressed on normal cells, while it is up-regulated in different tumor cell lines. BAT-3 is a nuclear factor released in exosomes by tumor cells and Dendritic Cells (DC) in response to stress/activation stimuli. The tumor ligands for NKp46

and NKp44 remain still undefined. The NKp30, NKp46 and NKp44 receptors are structurally and genetically unrelated. NKp46 encoding gene is located within the Leukocyte Receptor Complex in Chromosome 19, while the NKp30 and NKp44 encoding genes are located in separated regions in chromosome 6. However, for their expression pattern, restricted to NK cells, and their impressive capability of triggering NK cell cytotoxicity against an extremely wide range of tumor cell lines, they were grouped and collectively termed Natural Cytotoxicity Receptors (NCRs).

Among the triggering receptors, NKp30, NKp46, NKp44, NKG2D and DNAM-1 are indispensable for the NK-mediated recognition and killing of tumor cells. Different studies have demonstrated how their blockade, impairment of function, or expression failure, heavily compromise the efficacy of NK cells in killing tumor cells in vitro, or in containing tumor growth in animal models (Bottino et al., 2004; Iguchi-Manaka et al., 2008; Guerra et al., 2008; Halfteck et al., 2009). Notably, NK cells can significantly improve their functional capabilities in response to various cytokines including type I IFN, IL-2, IL-15, IL-12 and IL-18. In this context, exposure to IL-2, IL-15 and IL-12 can indeed induce up-regulation of NCRs, NKG2D and DNAM-1. In particular major effect is exerted on NKp44 that is not expressed at all on resting NK cells (Biron et al., 1999; Moretta A. et al., 2001; Della Chiesa et al., 2006; Balsamo et al., 2009; Vivier et al., 2011).

Thus, NK cells by their inhibitory and activating receptors can sense the altered expression of both protective self HLA-I molecules and a large array of pathogenic markers, and on this basis can discriminate which cells have to be killed.

The fact that, at variance with activating receptors, the inhibitory HLA-specific receptors are clonally distributed, allows the generation of a repertoire of NK cell subsets capable of sensing alteration of even single HLA-alleles. Importantly, this phenomenon is the basis for the exploitation of alloreactive NK cell subsets in aplo-identical Hematopoietic Stem Cell Transplanation (HSCT) in hematological malignancies (see section 2.4).

2.3 Multiple NK cell subsets and functions

Besides their involvement in direct pathogen clearance, NK cells are also implied in the regulation of the immune responses. Indeed they can produce and respond to various cytokines, functionally interact with different immune cell types and participate at regulation of T cell functions, in particular at Th1/Th2 polarization (Cooper et al., 2001; Moretta A. et al., 2005; Scordamaglia et al., 2008).

Up today several NK cell types, with distinct phenotype, function and anatomical location have been described (Table 1).

The $CD56^{bright}CD16^{dim/neg}KIR\text{-}NKG2A^{+}$ NK cells are characterized by low granule content and low cytolytic activity but produce large amounts of cytokines, in particular IFN-γ. These cells cover 5-10% of circulating NK cells but represent the large majority of NK cells in Lymph Nodes (L.N.). At these sites, upon interaction with maturing DC, $CD56^{bright}$ NK cells can proliferate and produce IFN-γ, thus favoring Th1 response (Fehniger et al., 2003; Ferlazzo & Munz 2004).

The classical $CD56^{dim}CD16^{bright}$ NK cells (approx. 90% of circulating NK cells) are highly cytotoxic, and also produce cytokines, in particular IFN-γ and TNF-α and, to a lesser extent,

chemokines, such as MIP-1β. These cells may migrate to peripheral tissues driven by chemokines and type I IFNs. Indeed, $CD56^{dim}CD16^{bright}$ NK cells express receptors (CXCR1 ChemR23 and CX_3CR1) specific for chemokines (CXCL8, Chemerin and CX_3CL1 respectively) that are usually produced during inflammatory events by Macrophages, Neutrophils, DC and Endothelial cells (Moretta A. et al., 2005; Della Chiesa et al., 2006b; Parolini et al., 2007). In addition they also express GPR56, a molecule that would function as receptor for Extra Cellular Matrix components (Della Chiesa et al., 2010). In inflamed tissues, different viral or bacterial products (i.e. PAMPs - Pathogen Associated Molecular Patterns) may either directly activate NK cells (which express TLR6 and TLR9 PAMP receptors) or induce DC, plasmacytoid DC (pDC), M1 type Macrophages, to produce cytokines capable of activating NK cells. At these sites, $CD56^{dim}CD16^{bright}$ NK cells can physically interact with immature DC (iDC) and, by the engagement of NKp30 and DNAM-1, they can release TNF-α and HMGB1 and promote DC maturation. Once activated, NK cells, by using the same receptors (NKp30 and DNAM-1), can also kill iDCs (and spare mature DC - mDC). As compared to mDC, iDC express lower HLA-I levels, and are not "protected" from activated NK cells. This phenomenon may represent either a mechanism to eliminate those DC that have not properly undergone maturation or a signal to terminate the response and avoid chronic inflammation (Zitvogel et al., 2002; Moretta A. et al., 2005).

Recently, a novel $CD56^{+}NKp44^{+}$ NK cell type was described in MALT (tonsils and Peyer patches), and was called NK22, by virtue of its ability to produce IL-22 in response to IL-23 (Colonna, 2009). IL-22 is a IL-10 family cytokine with anti-bacterial effects, as it maintains epithelial-cell barrier function in the gut thus contrasting bacterial dissemination. The meaning of NK22 cells, as well as their ontogenesis, are not fully understood, nevertheless these cells may play an important role in constraining inflammation and in defense against bacterial infection in the mucosa.

A unique NK cell subset expressing the phenotype $CD56^{bright}CD16^{dim/neg}KIR^{+}NKG2A^{+}$, populates decidua in the first trimester of pregnancy (Moffet-King, 2002; Hanna et al., 2006). These cells display peculiar functional features: they express the NCRs but are poorly cytolytic; rather, the engagement of NKp30 and NKp44 would induce them to produce a defined pattern of chemokines and pro-angiogenic factors (see Table 1). These factors would favor trophoblast migration and decidua vascularization, ensuring an appropriate placenta and fetus development. Decidual NK (dNK) cells may also have a role in the induction of tolerance at the maternal/fetal interface. dNK cells, by producing IFN-γ, would induce expression of IDO in decidual myelomonocytic cells. In turn IDO, together with TGF-β, would favor induction and proliferation of Tregs. Interestingly, unlike PB NK cells (see section 2.4), dNK cells are resistant to the inhibition of the IDO-induced Trp-catabolite, L-kynurenine. This resistance implies that dNK cells can maintain production of IFN-γ and sustain the tolerogenic pathway over time (Vacca et al., 2010).

It has been recently proposed that the $CD56^{bright}CD16^{dim/neg}KIR^{-}NKG2A^{++}$ cells could undergo further differentiation through different stages (Bjorkstrom et al., 2010; Lopez-Verges et al., 2010). The relatively immature and poorly cytotoxic $CD56^{bright}CD16^{dim/neg}KIR^{-}NKG2A^{++}$ cells, would progressively increase the expression of cytolytic granules, CD16, KIRs and CD57, down-regulate NKG2A, and modify the kinetics of IFN-γ release. In line with this hypothesis, four phenotypically distinct NK cell populations, possibly representing sequential stages of this differentiation process, have been identified within circulating pool:

$CD56^{bright}CD16^{dim/neg}CD57^{-}KIR^{-}NKG2A^{++}perforin^{+/-}$, $CD56^{dim}CD16^{dim}CD57^{-}KIR^{-}NKG2A^{++}perforin^{+}$, $CD56^{dim}CD16^{bright}CD57^{+/-}KIR^{+}NKG2A^{+}perforin^{++}$, $CD56^{dim}CD16^{bright}CD57^{+}KIR^{+}NKG2A^{-}perforin^{+++}$.

Interestingly, along these putative differentiation steps NK cells would also progressively lose CCR7 and CD62L expression and acquire the fraktalkine receptor CX_3CR1, thus skewing their initial homing capabilities to L.N., towards inflamed Peripheral Tissues (Juelke et al., 2010; Hamann et al., 2011). This latter point should not be disregarded, especially in view of the future perspective of selecting appropriate NK cell subsets for cancer immunotherapy.

NK cell subpopulation (phenotype)	Anatomical/ tissue localization	Functional features
$CD3^{-}$ $CD56^{bright}CD16^{dim/neg}$ $KIR^{-}NKG2A^{++}$ $CCR7^{++}CD62L^{++}$ $CD57^{-}$	Lymph Nodes 5-10% PB NK cells	Differentiation towards KIR+ cells Cytokines production (IFN-γ TNF-α) mDC-induced prolif. and IFN-γ prod. (promote Th1 polarization)
$CD3^{-}CD56^{dim}CD16^{bright}$ KIR^{+}/KIR^{-} $NKG2A^{+}/NKG2A^{-}$ $CCR7^{-}CD62L^{+/-}$ $ChemR23^{+}CX3CR3^{+}$ $GPR56^{+}$	90% PB NK cells inflamed periph. tissues?	Cytotoxicity Cytokines production (IFN-γ TNF-α) Induction of DC maturation
$CD56^{-}CD161^{+}$	5-10% PB NK cells	Produce type2 cytokines (IL-5 IL-13) Low cytotox. Expanded in HIV patients
$CD3^{-}CD56^{bright}CD16^{dim}$ KIR^{+}	Decidua (1^{st}. trimester pregnancy)	Low cytotox. $IFN\text{-}\gamma^{+}$ $IL\text{-}8^{++}$ $IP\text{-}10^{+}$ $VEGF^{+}$ Regulatory interactions with Trophoblasts (Placenta development) Decidua vascularization Induction of Tregs
$CD3^{-}CD56^{+}NKp44^{+}$	MALT	$IL\text{-}22^{+}$ / mucosal immunity
$CD3^{-}CD56^{+}CD16^{+/-}$ KIR^{+}	Liver	Cytotoxicity

Table 1. Surface phenotype, anatomical localization and function of different NK cell subsets

2.4 Role of NK cells in the control of tumors

The impressive advances obtained in the last 15-20 years in the knowledge of the mechanism of action of NK cells and of their regulation, have been fuelled by the evident therapeutic potential that these cells have shown since their first discovery. However, although the efficacy of NK cells in containing tumors in vivo has been demonstrated in

different animal models (Kim et al., 2000; Smyth et al., 2000; Guerra et al., 2008; Halftech et al., 2009), the way to develop effective NK-based immunotherapy has been elusive for many years and only very recently it yielded promising results.

After the initial attempts by Rosenberg's group (Rosenberg et al., 1985), several trials had been done to set protocols for the adoptive transfer of ex-vivo activated autologous NK cell populations or for the use of different cytokines to elicit NK cell responses in vivo (Sutlu & Alici, 2009). The advantages of these approaches, however, appeared minimal: both because of the technical limits in expanding ex vivo large bulk populations (as repeated infusions were necessary to overcome the short life-span of transferred mature NK cells), and because of the putative adverse effects induced by IL-2 in vivo (such as the possible expansion of $CD25^+$ Tregs or the induction the AICD) (Ghiringhelli et al., 2005; Rodella et al., 2001). In addition, another possible limitation could be that tumor cells could retain sufficient HLA-I expression to protect themselves from autologous NK cell attack. A turning point in the definition of efficient NK-based immunotherapeutic strategies against cancer, was represented by the study by Velardi and co-workers, primarily aimed at the evaluation of the clinical outcome of aplo-identical HSCT in Acute Myeloid Leukemia (AML) (Ruggeri et al., 2002). In this study the authors suggested that, in the presence of HLA/KIR mismatch (i.e. recipient and donor expressing different HLA-I alleles that are recognized by different KIRs), NK cells that developed from donor's progenitors could play an active role in reducing both the risk of leukemia relapse and even the Graft versus Host Disease (GvHD). This implied that heterologous NK cells could develop in the conditioned host, and kill Leukemic blasts more efficiently than autologous NK cells could do. Further studies by different groups have led to the current hypothesis that in the host, heterologous NK cell population expressing KIRs specific for donor's and not for recipient's HLA-I alleles can develop and be educated (licensed) by the donor's bone marrow cells, thus acquiring efficient killing capabilities towards host (allogeneic) malignant cells or activated leukocytes (Pende et al., 2009; Cooley et al., 2010; Haas et al., 2011; L. Moretta et al., 2011). The functional capabilities of allogeneic NK cells would also explain the reduction of GvHD observed in patients undergoing HLA/KIR mismatched HSCT, as, besides the elimination of heterologous Leukemic blasts (Graft-versus-Leukemia - GvL - effect), donor's NK cells would also be involved in the killing of those host's DC capable of priming donor's T cells. Interestingly, this alloreactive NK cell population may persist (and exert the its beneficial effect) for years (Pende et al., 2009; Haas et al., 2011) without causing apparent detrimental clinical signs ascribable to NK cell activity.

The above studies open new perspectives for the exploitation of NK cells in immunotherapy. In this context, different trials have been designed for the treatment of hematological malignancies, and in some cases also for solid tumors (Miller et al., 2005; Terme et al., 2008; Sutlu & Alici, 2009). Several problems, however, still remain to be solved. For example the fact that immature, not fully competent, NK cells may predominate (and persist) in circulation during (and after) the recipient immunological reconstitution (Nguyen et al., 2005) with negative effects on the anti-tumor activity and surveillance against viruses. Another problem regards a general negative effect that malignancies may exert on NK cell functions. In this context, different studies have reported a down-regulation of activating receptors in circulating NK cells from tumor patients (Sanchez et al., 2010; Le Maux-Chansac et al., 2005; Fregni et al., 2011). Moreover , in solid tumors, the tumor microenvironment

may play an additional negative role by contrasting both the responses and the infiltration capabilities of the immune effector cells (Albertsson et al., 2003). In this context, different studies have indicated that in some tumors, infiltrating NK cells may be rare or poorly functional (Albertsson et al., 2003; Carrega et al., 2009; Platonova et al., 2011). At the tumor site several stromal components, induced by aberrant tumor-driven inflammation, can contribute to NK cell down-regulation (Mantovani et al., 2008). Tregs, by the release of TGF-β, may suppress NK cell functions (Zimmer et al., 2008). The induction of M2 macrophages may reduce the macrophage-mediated NK cell activation (as this effect is prominently sustained by M1 macrophages) (Bellora et al., 2010). Also the Tumor Associated Fibroblasts (TAF) may contrast NK cells in their anti-tumor activity. Indeed, fibroblasts derived from melanoma lesions were found to inhibit, by mean of cell-to-cell contact and PGE_2 release, the IL-2-driven up-regulation of NKp44, DNAM-1 and NKp30 on NK cells (Balsamo et al., 2009). Finally, even the tumor cells can induce down-modulation of activating NK receptors. Several tumor cell lines constitutively express IDO, an enzyme involved in the Trp catabolism. The IDO-induced Trp catabolite Kynurenine has been shown to down-regulate NKp46 and NKG2D (Della Chiesa et al., 2006). In addition, tumor cells can also induce down-regulation of NKG2D or DNAM-1 on NK cells by the release of soluble NKG2D-Ligands (Doubrovina et al., 2003) or by the prolonged engagement of DNAM-1 in cell-to-cell contact (Carlsten et al., 2009)

3. Human Natural Killer cell development

3.1 In vitro NK cell development

3.1.1 Acquisition of NK cell receptors and function

Most of information available on human NK cell development came from in vitro studies of NK cell differentiation from $CD34^+$ Hematopoietic Stem Cells (HSC) or $CD34^{+/-}CD45RA^+$ early lymphoid precursors. These cells can be isolated from different sources such as fetal liver, Bone Marrow (BM), Thymus, PB and Umbilical Cord Blood (UCB) and stimulated with IL-2 or IL-15, in the presence or in the absence of feeder cells, to obtain NK cell differentiation (Freud & Caligiuri , 2006). In the 90s' it was shown that it was possible to obtain $CD3^-CD56^+$ CD94/NKG2A$^+$ cytolytic NK cells from either $CD34^-CD45RA^+CD7^+CD1a^-$ early thymic precursors (Mingari et al., a1991,b1997; Lanier et al., 1992; Sanchez et al., 1994) or $CD34^+$HSC isolated from BM or UCB (Miller et al., 1994; Lotzova et al., 1993).

Currently, optimal culturing conditions require the simultaneous presence of different cytokines: Stem Cell Factor (SCF), FMS-Like Tyrosine Kynase Ligand (Flt3-L), IL-7, IL-15 and IL-21.

During in vitro differentiation the development of NK cells proceeds through a step-by-step process (figure 1) (Freud& Caligiuri, 2006). Freshly isolated HSC precursors already express CD117 and Flt3, which are the SCF and Flt3-L receptors, respectively, and play an important role for their proliferation and survival before any cell lineage commitment takes place . The first cell markers that would suggest a NK-lymphoid commitment are the IL-2/IL-15 receptors CD122, CD132 and the IL-7 α-chain receptor (CD127). These receptors play an important role in transducing proliferation and differentiation signals upon interaction with the appropriate cytokines (IL-15 and IL-7 respectively). However, in humans, these markers are rarely detectable on ex-vivo isolated $CD34^+CD45RA^+$ precursors, while they can be detected on small fractions of precursor after first days of culture (Freud& Caligiuri, 2006).

The first surface cell marker that clearly identify NK cell precursors is CD161 (Bennet et al., 1996). It can be detected on small percentages of $CD3^{-}CD56^{-}CD117^{+}$cells, which already express CD244 co-receptor: the acquisition of NKp44 activating receptor and CD56 represents the following differentiation step. Next, the expression of the NKp46, NKG2D and DNAM-1 activating receptors and of CD94/NKG2A inhibitory receptor occurs. The acquisition of CD16 and KIRs can be hardly observed in vitro (Mingari et al., 1997; Miller et al., 2001; Sivori et al., 2002). These receptors are typical of functionally differentiated circulating NK cells: their acquisition by small percentages of cell precursors undergoing differentiation, appears after long term cultures in the presence of IL-21, a pro-inflammatory cytokine (Sivori et al., 2003). On the other hand, the early expression of NKp44 may be related to the fact that recombinant IL-15, present in the culture, rapidly activates NKp44 gene transcription. Hence, NKp44 transcription could be differently regulated from that of the other activating receptors.

This sequential process of cell marker acquisition identifies NK cell precursor intermediates with different functional properties (Freud & Caligiuri, 2006; Grzywacz et al., 2006). Early NK cell precursors $CD117^{+}CD161^{+}CD244^{+}CD56^{+/-}$ $NKp44^{+/-}$ are not cytolytic but can secrete cytokines such as GM-CSF and IL-13. These cells lack the expression of all the other activating receptors and inhibitory receptors. The next stage of in vitro NK cell differentiation correlates with the definitive acquisition of CD56 and of NKp44 ($CD117^{+}CD161^{+}$ $CD244^{+}CD56^{+}NKp44^{+}$): these precursors can be defined as immature NK cells (iNK). Immature NK cells secrete large amounts of CXCL8 and low amounts of IL-22 while they do not produce IFN-γ (Freud & Caligiuri, 2006; Vitale et al., 2008; Tang et al., 2011). The production of CXCL8 could have a role in the modulation of hematopoietic cell lineage commitment. In particular, CXCL8 inhibits myelopoiesis (Youn et al., 2000) and, thus, might favour NK cell differentiation. On the other hand, iNK cells are not cytotoxic. Indeed they have not acquired yet cytolytic granules in their cytoplasm, important NK activating receptors (i.e. NKp46), and the adhesion molecule LFA-1, that strongly contribute to the activation of cytolytic machinery (Bryceson et al., 2006).

The acquisition of a weak cytolytic activity correlates with the expression of NKp46 activating receptor and LFA-1; however, at this stage of development, the NKp46-mediated cytotoxicity against susceptible cell targets may be inhibited by the CD244 co-receptor, that acts as inhibitory receptor on these iNK cells. CD244 works as an activating co-receptor on mature NK cells thanks to the recruitment of an intra-cytoplasmic adaptor molecule SAP. However, on $CD117^{+/-}CD161^{+}$ $CD244^{+}CD56^{+}NKp46^{+/-}CD94/NKG2A^{-}LFA\text{-}1^{+/-}$ iNK cells SAP it is not synthetized yet, leading to inhibitory rather than activating function of CD244 (Moretta et al., 2001; Sivori et al., a2002, b2003; Vitale et al., 2008).

The full acquisition of cell functions typical of mature NK cells, such as IFN-γ secretion and cytolytic activity, correlates with the surface bright expression of NKp46, the expression of NKG2D and DNAM-1 activating receptors, CD94/NKG2A inhibitory receptor and of LFA-1 molecule. At this stage, SAP starts to be synthesized, and CD244 acquires a co-stimulatory activity. These cells are defined as $CD56^{bright}$ NK cells since they are very similar to $CD56^{bright}$ $CD94/NKG2A^{+}CD16^{-}KIR^{-}$ NK cells present in the PB. Notably, differentiation is a continuous process and cells may not be synchronized in their maturation status: hence, a certain heterogeneity of the different cell subsets may be observed.

Altogether these experimental evidences indicate that NK cell development is tightly regulated and that evolution had particularly tuned the acquisition of cytolytic activity. NK cells, to become cytotoxic, must express at least the NKp46 activating receptor and the LFA-1 adhesion molecule. Simultaneously, they have to loose the inhibitory activity of CD244 co-receptor. The ability to control NK cytotoxicity by inhibitory CD244 could be a "safe" mechanism, important in the interactions between NK cell precursors and other hematopoietic cells. CD244 ligand is the CD48 molecule, which is expressed by different types of leucocytes and hematopoietic precursors (Cannons et al., 2011). On the other hand, iNK cells secrete a peculiar pattern of cytokines, such as IL-22 and CXCL8, involved in inflammatory process, defence against bacteria, neo-angiogenesis and modulation of haematopoiesis. Hence, iNK cells might exert important crosstalk with other cells present in microenvironment where their differentiation takes place.

3.2 Factors that modulate NK cell differentiation

Hematopoietic cell lineage commitment depends on a wide variety of factors. Genetic components dictate the initial commitment but the milieu that surrounds lineage precursors may have a key role in the fate of these cells. The balance between specific transcription factors and the appropriate cytokines and hormones can change the commitment of intermediate lymphoid precursors, inducing their switch towards alternative lineages (Laiosa et al., 2006; Doulatov et al., 2010). In this context, in vitro analysis have shown that NK cells can share intermediate precursors not only among lymphoid cells but also with dendritic cells and myeloid cells (Miller et al., 1999; Marquez et al., 1998; Perez et al., 2003; Vitale et al., 2008; Grzywacz et al., 2011).

3.2.1 Cytokines

Cytokines are important factors that modulate NK cell differentiation. SCF and Flt3-L have a role in the first days of in vitro development because they induce HSC to enter into the cell cycle, and sustain precursor proliferation and survival (Yu et al., 1998). Then, IL-7 supports the lymphoid lineage commitment, since this cytokine is involved in T cell homeostasis and lymphoid differentiation (Ma A. et al., 2006). However, it is IL-15 that plays a key role in NK cell differentiation, through the interaction with IL-2/IL-15 common β and γ chains receptors (CD122 and CD132, respectively). Indeed, IL-15 has been shown to be critical in the terminal differentiation of $CD117^{+/-}CD161^{+}CD56^{+}$ iNK towards mature NK cells (Mrozek et al., 1995; Freud & Caligiuri, 2006). In murine models, NK cell deficiencies are more pronounced in mice lacking IL-15 or its receptors, than in mice lacking IL-2 or IL-7 related products (Di Santo et al., 1990; Giliani et al., 2005; Kennedy et al., 2000). In vitro assays revealed that high dose soluble IL-15 binds to its receptors and promote NK cell differentiation but, in vivo, IL-15 is primarily detectable complexed to its IL-15 Receptor α (IL15-Rα) present on accessory (stromal) cells (Dubois et al., 2002). IL-15/IL15-Rα complex would then present the cytokine *in trans* to $CD122^{+}CD132^{+}$ cells, meaning that also stromal cells could exert an important role in the terminal differentiation of iNK cells (Miller et al., 1994; Briard et al., 2002; Vacca et al., 2011). Finally, IL-21 have been shown to increase the proportions of $CD56^{+}KIR^{+}$ cells undergoing in vitro differentiation (Sivori et al., 2003).

3.2.2 Transcription factors

In vitro and in vivo studies using mice with loss-of-function mutation helped to clarify the role of many transcription factors (TFs) involved in NK cell commitment, proliferation and maturation. Some of them, like the E proteins, orchestrate a lymphoid-biased cellular context versus myeloid compartment (de Pooter et al., 2010) but must be down-regulated to allow NK cell differentiation (Boos et al., 2007). Other TFs are important both in the NK cell and T cell commitment, such as Notch-1 or Id2 (Benne et al., 2009; Boos et al., 2007). Id2 is an helix loop helix TF, able to modulate the activation the E protein E2A, and have been shown to exert a prominent role in NK cell commitment. Recently, other two TF have been suggested to induce definitive early NK cell commitment: a High Mobility Group protein, called TOX, and a basic leucin zipper, E4PB4 (also known as NFIL3)(Aliahmad et al., 2010; Gascoyne et al., 2009; Kamizono et al., 2009). It has been shown that IL-15 activates E4PB4 that, in turn, would activate Id2 transcription, leading to a definitive NK cell commitment and expansion of NK cell precursors. In humans, E4BP4 and Id2 expression can be observed either in ex-vivo-isolated early committed $CD34^{+/-}CD122^{+}CD127^{+}$ NK cell precursors either in in vitro-derived $CD117^{+/-}CD161^{+}CD56^{+}LFA\text{-}1^{-}NKG2A^{-}$ iNK cells (Hughes et al., 2010; Vacca at al., 2011). On the other hand, TOX would influence the activation of T-bet, a TF that correlates with the acquisition of cytolytic activity and the ability to produce IFN-γ by more differentiated $CD161^{+}CD56^{+}LFA\text{-}1^{+}$ $NKp46^{+}$ $CD94/NKG2A^{+}$ NK cells (Yun et al., 2011).

Expression of others TFs appears to correlate with the different stages of NK cell differentiation. RORC correlates with the secretion of IL-22 by iNK cells (Tang et al., 2011) while the expression of EOMES (a T-box TF), similar to T-bet, correlates with IFN-γ production by more differentiated NK cells (Glimcher et al., 2004).

3.2.3 Corticosteroids

In the last years the studies on in vitro NK cell development offered new important clues on NK cell lineage commitment and on the factors that may modulate this process.

The attempt to improve models of in vitro NK cell differentiation, induced many groups to test additional factors, besides cytokines, that could favour in vitro NK cell differentiation: in particular, stromal cells and/or corticosteroids. These new protocols revealed an unexpected function for glucocorticoids, which were already known to exert a modulatory effect on T cell development (Jondal et al., 2004). Studies with Hydrocortisone (HC) showed that it was possible to obtain NK cells from the in vitro culture of $CD33^{+}CD14^{+/-}$ myeloid progenitors isolated from UCB (Perez et al., 2003). These results suggested that, in cord blood, it was possible to switch the differentiation of monocyte precursors towards NK cells. We obtained similar results with Methylprednisolone (MePDN). This corticosteroid is commonly used as first line of treatment for acute GvHD after allogeneic HSCT. Our hypothesis was that MePDN could inhibit not only mature NK cell proliferation and functions but also the in vitro NK cell differentiation. Surprisingly, our results showed that pharmacological concentrations of MePDN accelerated NK cell differentiation and were able to induce myeloid precursors to switch their differentiation towards $CD161^{+}CD56^{+}NKp44^{+}$ iNK cells (Vitale et al., 2008). More recently, Grwaycz et al., with an elegant in vitro experiment, provided evidence that HC, in combination with a stromal cell line, induce differentiation of $CD33^{+}CD13^{+}CD115^{+/-}$ myelomonocytic precursors into cytolytic $CD56^{+}NKp46^{+}CD94/NKG2A^{+}KIR^{+/-}CD16^{+/-}$ NK cells (Grwaycz et al., 2011).

These results offer important clues to better clarify some major issues that have been discussed in the last years.

The first one is related to the hematopoietic cell lineage commitment. Current models of haematopoiesis suggest that HSC may commit early to the erythroid/platelet lineages or to leukocyte lineages. However, once committed to the leukocyte lineage, hematopoietic precursors would retain a high plasticity. Thus, the choice to terminally differentiate towards myeloid or lymphoid lineages would then depend on the role of lineage-specific transcription factors and on a permissive milieu (Doulatov et al., 2010). This would be of particular interest for NK cells, which are the only lymphocytes assigned to natural immunity compartment and that appear to be the connection ring with acquired immunity.

The second issue regards the role of NK cells after HSCT and their use in immunotherapy to obtain GvL reaction. The possibility that myeloid precursors, in the presence of corticosteroids, could generate more rapidly functional NK cells offers important clues also in the clinical settings (Vitale et al., 2008; Grwaycz et al., 2011). It is conceivable that NK

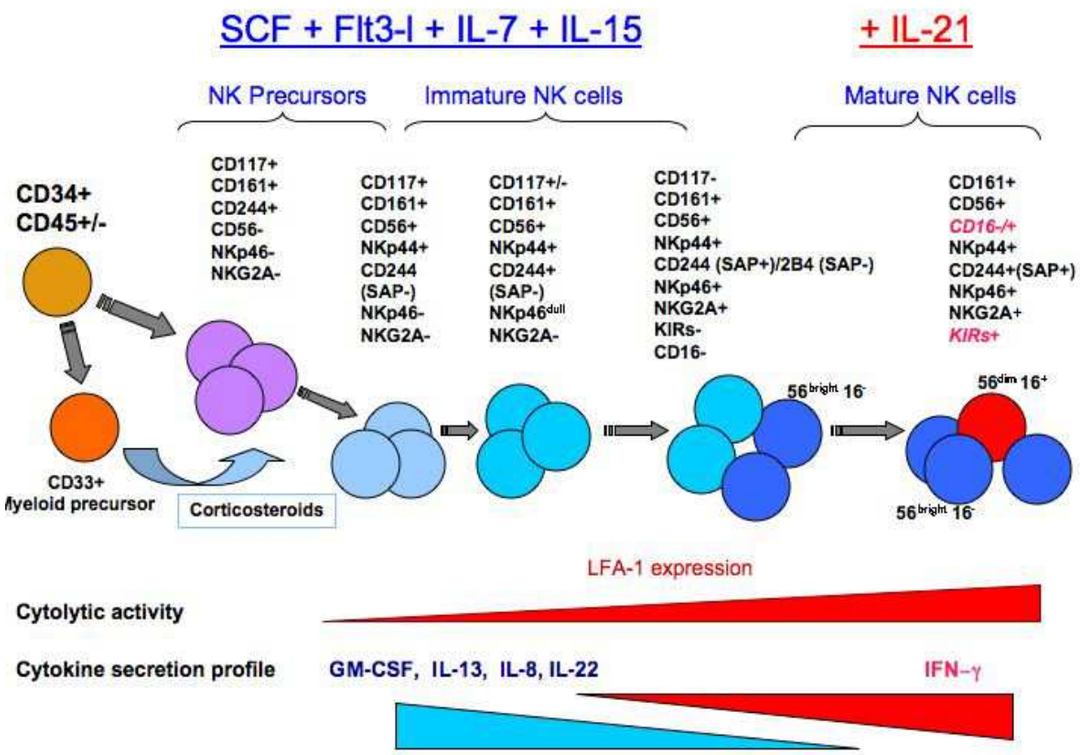


Fig. 1. Acquisition kinetic of receptors and functions by NK cells undergoing in vitro cell development.
Three main precursor subsets may be identified along different time intervals of culture: NK cell precursors, immature NK cells and more differentiated CD56^{bright} NK cells. The appearance of CD56^{dim} CD16^{+}KIR^{+} NK cells (red cell) is a late and rare event and is more achievable in the presence of IL-21. Stages of differentiation are endowed with a peculiar surface phenotype and functions. In particular the acquisition of cytolytic activity correlates with the expression of LFA-1, activating receptors and the production of IFN-γ, while high secretion of IL-8 correlates with the immature NK cell stage.

cells, present in high percentages in peripheral blood of patients at the earliest time intervals after HCST, may derive, at least in part, from myeloid precursors. Importantly, this maturation could not occur wholly in BM but also in PB and other sites. This is the third important issue that have been matter of debate of the last years: the site of in vivo human NK cell development and maturation.

3.3 In vivo NK cell development and maturation

NK cells were generally believed to differentiate into the BM from $CD34^+$ hematopoietic stem cells (Freud & Caligiuri, 2006). However, NK cell developmental intermediates were detectable in vivo neither in the BM nor in thymus. The possibility to observe in vivo any different NK cell developmental stages detectable in vitro, came from analysis of lymphocyte recovery in patients undergoing allogeneic HSCT. Analysis of PB of these patients revealed that, in some of them, the first waves of lymphocytes recovering at early time after transplant (2-3 weeks) are mostly NK cells. These lymphocytes are characterized by the $CD56^{bright}$ $CD16^-$ $CD94/NKG2A^+KIR^-$ phenotype and a dull expression of NCRs, while $CD56^{dim}CD16^+CD94/NKG2A^+KIR^+$ NK cells can be detected later, (4-8 weeks after transplant). (Vitale et al., 2000; Shilling et al., 2003; Vitale et al., 2004).

3.3.1 Sites of in vivo NK cell development: Stages of NK cell differentiation

The finding that in vitro models of NK cell differentiation led to the generation of $CD56^{bright}$ $CD16^-$ NK cells, supported the hypothesis that, in vivo, this subset could be a precursor reservoir, able to promptly differentiate towards more cytotoxic $CD56^{dim}CD16^+$ upon specific stimuli. However, experimental evidences that support this hypothesis have been acquired only recently. Freud and co-workers discovered that $CD45RA^+CD117^-$ and $CD34^+CD45^+CD117^+CD161^{+/-}$ cells were enriched in Secondary Lymphoid Tissue (SLT), particularly in lymphnodes and tonsils. Importantly, these precursors were able to generate selectively NK cells upon culture in the presence of appropriate cytokines. They defined these cells as pro-NK and pre-NK cells respectively. Further analysis revealed that in SLT, it was possible to detect and isolate four different subsets of NK cell precursors representing different stages of NK cell differentiation (Freud et al., a2005, b2006).

Stage 1: $CD34^+CD45RA^+CD117^-CD161^-$ pro-NK cells; stage 2: $CD34^+CD45RA^+CD117^+CD161^{+/-}$ pre-NK cells; stage 3: $CD34^-CD117^+CD161^+CD56^{+/-}$ $NKp46^-CD94/NKG2A^-KIR^-CD16^-$ immature NK cells; stage 4: $CD117^{+/-}$ $CD161^+CD56^+NKp46^+CD94/NKG2A^+KIR^{+/-}CD16^-$, defined as $CD56^{bright}$ NK cells. Further analysis suggested that also the expression of CD122 and CD127 could help to identify stage 1/2 of pre-NK cell precursors. These discoveries confirmed for the first time that in vitro models of NK cell differentiation may have in vivo similar counterpart. Indeed, the above stages 2, 3 and 4 remind the differentiation steps observed in vitro (Freud & Caligiuri et al., 2006; Caligiuri, 2008). This discovery suggests that hematopoietic precursors could migrate from BM to SLT and generate NK cells in organs far from the BM. It is possible that at least a fraction of $CD56^{bright}$ NK cells present in SLT could differentiate from these precursors upon interaction with DC and other cells capable of presenting membrane-bound IL-15.

As mentioned above a particular subset of NK cells has been found to be enriched in tonsils and gut-mucosa associated tissue: these cells are $CD56^{+/-}NKp46^{+/-}NKp44^+NKG2A^-$ and

produce IL-22. Their development appears to be independent from IL-15. However, whether these cells may represent a new subset of NK cells (called NK22 cells) or simply immature NK cells with peculiar in vivo functions is still a matter of debate (Colonna, 2009).

In conclusion, BM microenvironment may provide a fundamental support for the early stages of NK cell differentiation but peripheral tissues, in particular SLT, could provide a unique cell-to-cell interactions and cytokines to induce the terminal NK cell differentiation.

Surface markers	STAGE 1: Pro-NK	STAGE 2: Pre-NK	STAGE 3: iNK	STAGE 4: CD56 ^{bright}	STAGE 5: CD56^{dim} CD16^{+}
CD34	+	+	-	-	-
CD45RA	+/-	+/-	(+)/-	+/(-)	+/(-)
CD117	-	+	+	+/-	-
CD122	-	-	-	+/-	+
CD127	dull	dull	+	+/-	-
CD161	-	+/-	+	+	+
CD244	+	+	+	+	+
CD56	-	(+)/-	+/-	+	+
NKp44	-	-	+/(-)	(+)/-	(+)/-
NKp46	-	-	-	+	+
NKG2A	-	-	-	+	+/-
KIR	-	-	-	(+)/-	+/-
CD16	-	-	-	-	-

Table 2. Expression of surface markers by NK cell precursor intermediates isolated in SLT, according to model proposed by Freud and co. Legend: +/- variable expression; (+)/- majority of cells negative; +/(-) majority of cells positive; dull= weak expression

3.3.2 Other sites of in vivo NK cell development

The next step was to verify the possibility that NK-committed cell precursors could be isolated in other peripheral tissues, where peculiar NK cell subsets were enriched. Indeed NK–committed precursors were found in gut, endometrium and placenta (Chinen at al., 2007; Male et al., 2010; Vacca et al., 2011). In all these districts there is a high concentration of CD56^{bright} NK cells characterized by immune-regulatory activity. In particular, NK cells present in human decidua (dNK) during the first trimester of pregnancy display a peculiar phenotype (CD56^{bright}CD16^{-}NKG2A^{+}KIR$^{-/+}$) and exert peculiar functions. For long time dNK cells were supposed to derive from PB NK cells undergone phenotypic and functional modifications upon interaction with decidual microenvironment. Recently, different experimental evidences suggested that dNK cells could derive from NK precursors already present in endometrium or in decidua, able to promptly differentiate upon stimuli given by the onset of pregnancy (Male et al., 2010; Vacca et al., 2011). In particular, it has been shown that NK cell lineage-committed CD34^{+}CD127^{+}CD122^{+} cells expressing E4BP4 and Id2 TFs are present in human decidua (dCD34^{+}) during the first trimester of pregnancy. They can undergo in vitro differentiation into functional CD56^{bright}CD16^{-} NK cells in the presence of suitable cytokines. More importantly, they could also differentiate without exogenous cytokines when co-cultured with decidual stromal cells (dSC), able to express endogenous

membrane-bound IL-15. These results suggest that interaction between $CD34^+$ cell and decidual stromal cells would be sufficient to promote in situ NK cell differentiation.

3.3.3 The stage five of human NK cell differentiation: $CD56^{dim}$ $CD16^+CD94/NKG2A^{+/-}$ KIR^+ NK cells

As already discussed, several evidences suggest that $CD56^{dim}$ NK cells may derive from $CD56^{bright}$ NK cells. After HSCT the first wave of NK cells is represented by $CD56^{bright}CD16^-CD94/NKG2A^+KIR^-$ cells while $CD56^{dim}CD16^+CD94/NKG2A^{+/-}KIR^+$ cells appear later. Importantly, $CD56^{bright}$ display longer telomeres than $CD56^{dim}$ NK cells. Moreover, different phenotypically defined NK cell subsets have been recently proposed as functional intermediates between the $CD56^{bright}$ and $CD56^{dim}$ cell types (see section 2.3) (Romagnani et al., 2007; Juelke et al., 2010).

However, in in vitro assay, it is almost impossible to observe significant expansions of KIR^+ NK cells from any type $CD34^+$ cell precursors. Similar problems must be related to our inability to fully recreate the in vivo milieu in in vitro assay. $CD56^{dim}$ may acquire their surface phenotype and functional properties upon peripheral tissues/blood microenvironment stimuli. However, signals that drive the differentiation of $CD56^{dim}$ NK cells, both during normal homeostasis and infections, remain still elusive. Recent studies provided evidence that $CD56^{dim}$ cells change their phenotypic properties and continue to differentiate throughout their lifespan. The loss of expression of NKG2A, the acquisition of KIRs and CD57 would allow the identification of sequential steps of cell maturation accompanied by a progressive decline of cell proliferation and of responsiveness to cytokine stimulation. In particular, CD16 and KIR would be acquired at late stages of peripheral blood NK cell maturation (Björkström et al., 2010; Lopez-Vergès et al., 2010). Since the acquisition of KIR repertoire in each single NK cell is a stochastic process, related to the KIR genotype and polymorphism, it is important to understand how NK cells may be educated to avoid auto-reactivity. They should acquire appropriate KIR able to prevent them from killing healthy self-cells. However, in PB of normal donors, it is possible to detect NK cells expressing KIR mismatched for self HLA-I ligands or not expressing any HLA-I inhibitory receptor at all. These cells could represent a danger as they would not recognize self HLA-I on self normal cells. As above mentioned, however, it has been recently proposed that during their development only those NK cells expressing inhibitory receptors specific for self HLA-I ligands would acquire full functional competence, while cells that fail to express such receptors (i.e. potentially autoreactive NK cells) would retain a state of hypo-responsiveness. It has to be noted, however, that the question on how such licensing/educational process could actually occur in vivo is still matter of debate (Parham, 2006; Vivier et al., 2011).

The interest for NK cells and their clinical application for the control of leukemic relapse after allogeneic HSCT is enormously increased in the last years: hence, it is mandatory trying to clarify the epigenetic factors that regulate KIR acquisition and functions, because their expression pattern on allogeneic donor NK cells play a crucial role in the eradication of recipient's leukemia (Moretta et al., 2011).

4. Classification of NK cell disorders

The World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues encompasses four distinct entities, two of which are provisional: 1) NK

cell lymphoblastic leukemia/lymphoma (*provisional*) (Borowitz et al., 2008); 2) Chronic Lymphoproliferative Disorder of NK cells (*provisional*) (Villamor et al., 2008); 3) Aggressive NK cell leukemia (Chan et al., 2008a); and 4) Extranodal NK/T-cell lymphoma, nasal type (Chan et al., 2008b). In addition, on the basis of morphology, immuno-phenotype, functional NK cell activity, and expression of cytotoxic molecules, NK cell neoplasms can be divided into immature and mature categories (Jaffe, 1996).

In recent years rare cases of lymphoblastic lymphomas/leukemia arising from immature NK cells has been reported, although the lack of suitable markers for immature NK cells mentioned above makes it difficult to distinguish NK-cell lymphoblastic lymphoma (LBL) from precursor T-cell LBL. It is also worth mentioning that the plasticity of hematopoietic-cell lineage seems greater than previously thought, and relations between phenotypically dissimilar neoplastic disorders are being reassessed. In contrast, it is believed that chronic lymphoproliferative disorder of NK cells, aggressive NK cell leukemia and extranodal NK cell lymphoma, nasal type, originate from mature NK cells (Chan et al., 2008a and b). Relevant biological features of NK cell neoplasms are summarized in Table 3.

	NK cell lymphoblastic leukemia/ lymphoma	Extranodal NK/T-cell lymphoma, nasal type	Aggressive NK cell leukemia	Chronic Lymphoproliferative Disorder of NK cells
Age	Pediatric	Middle-aged adult	Young to Middle aged adult	Adults (6th decade)
Geographic distribution	Worldwide	Asia	Asia	Worldwide
Cell origin	Immature NK cell	Mature NK cell	Mature NK cell	Mature NK cell
Relevant phenotype	sCD3-CD4-CD13-CD33- CD16-CD7+ CD2+CD56+CD3e+	CD2+sCD3-cCD3e+CD56+CD16- CD57-Granzyme B+ Perforin+TIA1+	sCD3-cCD3e±CD56+CD16± CD57-, CD94+	CD3-CD8±CD16+CD56+CD57+ KIRs±CD94/NKG2A± Granzyme B+Perforin+
Relevant cytogenetic aberrations	Complex karyotype	Deletion 6q	Deletion 6q Complex Karyotype	Usually Normal Karyotype
EBV association	No	Yes	Yes	No
Clinical course	Aggressive	Aggressive	Very Aggressive	Indolent
Prognosis	Poor	Poor	Poor	Good

Table 3. Clinical and Biological features of NK cell neoplasms

4.1 Immature NK cell neoplasms

4.1.1 NK cell lymphoblastic leukemia/lymphoma, provisional

A considerable confusion has been generated in the literature concerning this type of disorder, mostly due to the definition of NK cell leukemia on the basis of expression of CD56 antigen. This is indeed the most important and sensitive NK marker; unfortunately, CD56 is not specific for NK cells and can be also expressed in AML and ALL and blastic plasmacytoid dendritic cell neoplasms (BPDCN). On the other hand it may not be expressed by immature NK cells. NK cell lymphoblastic leukemia/lymphoma can be considered in cases showing blastic morphology, expressing CD56, TdT, immature T associated markers such as CD7 and CD2, and cytoplasmic CD3ε, but lacking the expression of surface CD3, CD19, CD20, CD13, CD33, and MPO (Liang & Graam, 2008). These patients frequently presented with leukemia and lymphadenopathy without skin involvement and were negative for EBV. TCR and/or Ig genes were in germline configuration in all cases in which the tests were performed. Outcomes of these patients were absolutely unfavourable. The immature morphology with NK cell-associated phenotype and genotype suggests that these tumours represent the true precursor NK cell neoplasms.

Some well characterized cases of NK precursor tumours with lymphomatous presentation that expressed CD94 1A transcripts have been reported (Lin et al., 2005). CD94 1A, a distal promoter of the CD94 molecule, is activated only by IL-15 (Lopez-Botet et al., 1997). Lin et al., recently reported that CD94 1A is the predominant form found in immature NK cells and it is expressed in TCR^- lymphoblastic leukemia (NK lineage LBL) but not in TCR^+ LBL (T lineage LBL) (Lin et al., 2005). By studying 21 patients with LBL and, on the basis of the expression of CD94 1A transcripts and the lack of TCR, the above investigators identified 7 patients with LBL of immature NK cell origin ($CD94\ 1A^+$, TCR^-). It is noteworthy that those NK-LBLs occurred in young patients and had better outcomes as compared with patients who had T-LBL ($CD94\ 1A^-$, TCR^+); none of the tumours was positive for CD56 (Lin et al., 2005). Thus, the use of CD94 1A associated with TCR appears to be more suitable than CD56 for identifying an immature NK cell neoplasm.

A standard treatment protocol for immature NK cell neoplasms has not been established mainly because of the rarity of these cases. Current chemotherapy strategies for non-Hodgkin's lymphoma or acute lymphoblastic leukemia (ALL) were the most commonly used. However, the overall outcomes were dismal and HSCT represents the only effective therapy to achieve a complete remission (Lin et al., 2005).

4.2 Mature NK cell neoplasms

4.2.1 Extranodal NK/T cell lymphoma, nasal type

The WHO classification encompasses both nasal NK/T cell lymphoma and extra-nasal NK/T cell lymphoma in the same category (Chan et al., 2008a). They share the same histology, even though these lymphomas may have different clinical manifestations, treatment approaches, and prognoses (Oshimi, 2007). Although most cases are genuine NK cell neoplasms, the term "NK/T" rather than "NK" is used because this entity also includes cytotoxic T cell neoplasms (Sozumiya et al., 1994). Nasal and extra-nasal NK/T cell lymphoma are invariably associated with EBV and have a ethnic predisposition, being more

prevalent in Asia, Mexico, and Central and South America (Chan et al., 2008b; Kwong, 2005) and rare in Western Countries, the Middle East and Africa.

Nasal NK/T cell lymphomas refer to tumours that occur in the nose and the upper aero digestive tract (Oshimi, 2007; Cheung et al., 1998). They are the most common type among primary lymphomas of the nasal cavity (Cheung et al., 1998). The site of disease is primarily in the midline and includes the nasal cavity in more than 80% of cases. The tumour is locally invasive and might infiltrate surrounding tissues and organs, such as oropharynx, palate, orbits, till the appearance of the characteristic mid-facial destructive lesions, the so called "lethal midline granuloma" (Cheung et al., 1998). Common symptoms include nasal discharge, nasal obstruction, purulent rhinorrhea, epitasis and local swelling of the nasal bridge. The tumours may be destructive, leading to the highly characteristic midline perforation.

Extra-nasal NK/T cell lymphomas represent the counterpart of nasal NK/T cell lymphomas and involve any other part of the body. Males are predominantly affected, and the median age of presentation is the fifth decade. Primary sites of involvement include the skin, gastrointestinal tract, salivary glands, spleen, and testis (Chan et al., 1997). Patients with extra-nasal NK/T cell lymphoma more likely exhibit an advanced stage of disease with significantly higher general involvement, high levels of lactate dehydrogenise and a significantly decrease of haemoglobin and platelet count as compared with patients who have nasal NK/T cell lymphoma (Chan et al., 1997). The histological features are similar, regardless of the involved sites. Mucosal sites often show ulceration. A diffuse infiltrate of lymphoid cells is found in association with tissue necrosis and coagulation, although in some cases infiltrating cells lack atypical morphology, resulting in misdiagnosis as chronic inflammation. An angiocentric and angiodestructive growth pattern with associated fibrinoid changes in the blood vessels is frequently observed. In most patients, the neoplastic cells are characterized by the $CD45^{+}$ surface (s)$CD3^{-}$ cytoplasm (c)$CD3\varepsilon^{+}$ $CD56^{+}$ phenotype and lack myeloid and B lymphoid markers. Proliferating cells are also positive for cytotoxic granules, granzyme B, perforin, TIA. Rarely cells express CD30 and CD7, while CD56 negative cases have also been reported. Association with EBV can be demonstrated in nearly all patients (Harabuchi et al., 1990). Using *in situ* hybridization technique, EBV-encoded RNA can be found in neoplastic cells and Southern Blot analysis can detect monoclonal proliferation of EBV. Analyses of the terminal repeat region of the EBV genome indicates that the virus is in a clonal episomal form. Other than providing an indirect proof of the clonal nature of the lymphoid proliferation, this finding suggests that the EBV might play an etiologic role in mature NK cell neoplasms (Minarovitz, 1994). A defect in immune surveillance for EBV infection is demonstrated by the high frequency of 30-base pair deletions of the LMP1 gene in EBV-infected Asians. In addition, amino acid changes in the sequence coding HLA-A2- restricted CTL epitopes of the LMP1 and LMP2 genes, and low frequency of HLA-A*0201 in NK/T cell lymphoma patients have been reported (Kanno et al., 2000; Harabuchi et al., 2009). Genetic alterations have been detected in the tumour suppression genes and several oncogenes enabling tumour cells to proliferate and resist apoptosis. Mutations of *p53* have been demonstrated, with variable frequencies (24 to 60%). Mutation of *c-kit* can also be frequently demonstrated, as far as *Fas* gene mutations (Hoshida et al., 2003; Shen et al., 2002). The most common cytogenetic aberration is deletion of 6q. A recent microarray study showed that several genes associated with vascular biology, EBV induced genes, and *PDGFRa* gene are over-expressed, pointing to the deregulation of the

tumor suppressor HACE1 in the frequently deleted 6q21 region (Huang et al., 2010). Moreover, in NK/T cell lymphoma, gene signatures related to angiogenesis, genotoxic stress and proliferation, and signaling pathways (TGF-β, Notch and Wnt), were significantly enriched as compared to IL2-activated normal NK cells. Interestingly, NK/T lymphoma cells of NK lineage have a very similar molecular profile to that of NK/T-cell or peripheral T-cell lymphoma of γδ-T cell lineage (Iqbal et al., 2010).

The clinical outcome of patients with nasal NK cell lymphoma is variable. Most observational studies have consistently demonstrated that radiotherapy is superior to chemotherapy alone in patients with stage I/II disease (Sakata et al., 1997). Some patients with early-stage disease are cured by radiation therapy. It has been demonstrated that radiotherapy, either as initial treatment or as part of the chemotherapy regimen, is the single most important key to a successful outcome (Ribrag et al., 2001). However, some patients with early-stage disease have early local or systemic recurrences and die of disease. For patients with stage III/IV disease, chemotherapy is the treatment of choice (Kwong, 2005). In several published series, the median survival of patients with advanced-stage disease was approximately 12 months. Extra-nasal NK cell lymphomas are clinically aggressive, the response to therapy is poor, and most patients die within 6 months after diagnosis. The long-term remission rate with allogeneic HSCT is less than 10% (Cheung et al., 1998).

4.2.2 Aggressive NK cell leukemia

First described by Fernandez et al., (Fernandez et al., 1986), aggressive NK cell leukemia (ANKL) is a systemic disease, more common in Asians than in Caucasians (Chan et al., 2008a), which is characterized by the presence of neoplastic NK cells in the peripheral blood, bone marrow, liver and spleen and by a rapidly progressive clinical course with poor prognosis. There is an equal sex incidence in men and women. The disease typically affects young to middle-aged adults with a median age in the third decade. At presentation, patients usually are very compromised with systemic symptoms, liver dysfunction, and hepato-splenomegaly sometimes accompanied by systemic lymphoadenopathy. In contrast to extra-nodal NK cell lymphoma, skin lesions are uncommon. The clinical progression is devastating despite treatment, and most patients survive for only days to weeks. Disseminated intravascular coagulation and hemophagocytic syndrome are often seen during the course of disease (Oshimi, 2007). In tissue sections, the neoplastic infiltrate is diffuse and destructive with lymphoid cell population usually appearing monomorphous (Siu, Chan & Kwong, 2002). Morphologically, leukemic cells are slightly larger than normal LGLs (Oshimi, 2007). There is an ample amount of pale or slightly basophilic cytoplasm that contains fine or coarse azurophilic granules. These cells are $sCD3^{-}cCD3\varepsilon^{+}CD56^{+}CD16^{+}$ (75% of cases), $CD57^{-}$ $CD94^{+}$ with a germ line configuration of β and γ genes of TCR. The chemokine system plays a critical role in the tumor cell diffusion, leading to the fulminant clinical courses. Serum levels of soluble FasL, IL-8, MIP-1α, and MIP-1β are significantly elevated in ANKL patients and proliferating cells are highly positive for Fas, IL-8, RANTES, MIP-1α, and MIP-1β (Makishima et al., 2007). Although clonal EBV is found in tumour cells in most patients and EBV is considered to be the etiological agent (Oshimi, 2007), little is known about the mechanisms through which EBV infection triggers clonal proliferation of NK cells. A defective T cell and NK cell response to EBV infection may play a role in the development of this disorder. However up to 10% of cases have been shown to be EBV-negative (Suzuki et al., 2004).

Several chromosomal abnormalities have been reported. In particular, the finding of abnormalities involving del(6q) in aggressive NK cell leukemia and in extranodal NK/T lymphoma provides a biological link between these two diseases (Wong, Chan & Kwong, 1997). A recent array-based comparative genomic hybridization study on 27 NK cell lymphoma/leukemia cases, classified into two disease groups based on the World Health Organization Classification (10 ANKL cases and 17 extranodal NK/T lymphomas, nasal type), showed recurrent gain of 1q and loss of 7p15.1–p22.3 and 17p13.1 in ANKL (Nakashima et al., 2005). The same study also demonstrated clear genetic differences between aggressive NK cell leukemia and extranodal NK/T cell lymphoma, suggesting that these are two separate entities (Nakashima et al., 2005).

Aggressive NK cell leukemia is a catastrophic disease with an almost uniform mortality. A few patients have a clinical response with conventional chemotherapy (Kwong, 2005), although the response is typically transient and survival is measured in days to weeks. Allogeneic HSCT results in short-term remission in a few patients (Kwong, 2005). Taking together, big efforts for the recognition of new therapeutical targets and for development of new experimental protocols are urgently required to address the issue of ANKL therapy. As a matter of fact, recent data in a murine model have reported impressive response by targeting of survivin by nanoliposomal ceramide (Liu et al., 2010).

4.2.3 Chronic lymphoproliferative disorder of NK cells (provisional)

The chronic lymphoproliferative disorders of NK cells (CLPD-NK) are included among the novelties of the current WHO classification (Villamor et al., 2008). These rare and heterogeneous disorders are characterized by a chronic expansion of mature appearing NK cells (usually more than 2,000/μl) in peripheral blood for more than 6 months (Loughran, 1993; Semenzato et al., 1987; Tefferi et al., 1994; Semenzato et al., 1997; Oshimi, 1996), without a clearly identified cause (Figure 2). Patients are usually adults with a mean age of 60 years without gender and racial predisposition (Pandolfi et al., 1990). In recent years several studies have been published focusing on the pathogenetic mechanisms of this disease (Zambello & Semenzato, 2009; Loughran et al., 1997; Zambello et al., 2003; Hodge et al., 2009; Gattazzo et al., 2010; Epling-Burnette et al., 2008). NK cell activation in response to an unknown stimulus, likely of viral origin, is postulated to play a role in the initial steps of CLPD-NK by selecting NK clones (Zambello & Semenzato, 2009). No prototypical HTLV infection was demonstrated in these patients. However, the evidence that sera from a series of patients from Europe and USA reacted with the recombinant HTLV env protein p21E, suggests that exposure to a protein containing homology to BA21 may be important in the pathogenesis of this lymphoproliferative disorder (Loughran et al., 1997). In contrast with other mature NK cell neoplasms, EBV is not usually detected within affected lymphocytes (Zambello & Semenzato, 2009). It is believed that BM, which is frequently involved in CLPD-NK patients, represents the setting where the putative inciting antigen could reside. In this compartment, DCs may represent the target of infection (Zambello et al., 2005). Bone marrow biopsies demonstrated a topographic distribution of DCs and NK cells that indicates a close contact between the two cell types (Zambello et al., 2005). Patients' NK cells also showed a reduced capability of promoting Mo-DC maturation and of killing iDC (Balsamo et al., 2009). These findings could be explained, at least in part, by the low expression levels of NKp30 activating receptor, usually involved in the molecular

interactions occurring between NK cells and DC. It is suggested that impairment of DC killing capabilities detected in patients' NK-GLs may allow an accumulation of DC that, at certain sites, may sustain the chronic proliferation of NK cells themselves. DCs are also likely to represent the source of IL-15 that is crucial in the mechanisms sustaining the maintenance of NK proliferation. IL-15 has been found to mediate its activity by interfering with Bcl-2 family members, and more specifically by modulating Bid expression (Hodge et al., 2009). Hodge et al., demonstrated that CLPD-NK cells express low levels of Bid, that are reversed by blockade of IL-15 signaling (Hodge et al., 2009). Bid is also increased following bortezomib (Velcade/PS341) treatment and this effect is coordinate with increased susceptibility to Fas- or TRAIL- independent apoptosis. In fact, bortezomib increased cell surface expression of DR4, a TRAIL death receptor decoy. The inability of death receptors to account for the apoptosis may be explained by the putative role of Bid in DNA damage and repair. It is possible that elevation of Bid expression in CLPD-NK cells promotes S phase cell cycle block and death (Hodge et al., 2009).

A genetic susceptibility for this disease has been suggested and has been related to the detection of type B *KIR gene* repertoire which is characterized by a high number of activating genes (Zambello & Semenzato, 2009). In fact, a restricted pattern of KIR expression has usually been reported in these patients. A typical feature is the preferential expression of the KIR activating receptor isoforms and this pattern correlates with a reduced expression of other activating receptors, such as NCRs (Zambello et al., 2003). Together with a bias towards activating KIR expression, a deep silencing of inhibitory KIR through increased gene methylation has been recently demonstrated by our group (Gattazzo et al., 2010). More specifically, we showed the complete lack of KIR3DL1 expression in most analyzed patients, being the receptor expressed in 13% of patients as compared to 90% of controls ($p<0.01$). Interestingly, the results of methylation patterns of *KIR3DL1* promoter showed a significantly higher methylation status (0.76 ± 0.12 SD) in the patients with respect to the healthy subjects (0.49 ± 0.10 SD, $p<0.01$). These data suggest that together with the increased expression of activating receptors, the lack of the inhibitory signal could also play a role in the pathogenesis of disease (Gattazzo et al., 2010). Recent data on the pathogenesis of CLPD-NK are summarized in Figure 2.

Biochemical studies on the mechanisms sustaining the growth of NK cells in these patients have demonstrated a role of RAS farnesil transferase (Epling-Burnette et al., 2008), with clinical implications (see below). Pathological NK cells express CD16 and usually low levels of CD56 and CD57. As expected, cells express TIA1, granzyme and perforins, which correlate with the cytotoxic potential of these cells. CD94 is expressed at high density on patients' NK cells, frequently associated with the inhibitory subunit NKG2A, although, in a relevant number of cases, the dimer CD94/NKG2C has been reported (Zambello & Semenzato, 2003). Patients' NK cells express functional β and γ chains of IL-2/IL-15 receptor, which are strictly related to the role of these cytokines in the pathogenesis of disease (Zambello & Semenzato, 2009).

Most patients are asymptomatic, and the disease has a chronic indolent clinical course, similar to that reported for patients with T-LGL leukemia (Loughran, 1993; Semenzato et al., 1987; Tefferi et al., 1994; Semenzato et al., 1997; Oshimi 1996). In some cases this disorder is associated with other conditions, including pure red cell aplasia, vasculitic syndromes, solid and hematologic tumors, splenectomy, neuropathy and autoimmune disorders (Loughran,

1993; Semenzato et al., 1987; Tefferi et al., 1994; Semenzato et al., 1997; Oshimi, 1996). Recently, in patients with chronic myelogenous leukaemia, the association has been reported between treatment with dasatinib and the development of CLPD-NK. It has been suggested that the development of CLPD-NK might contribute on the control of Ph positive leukemic cells proliferation (Kim et al., 2009). Systemic symptoms, such as cytopenia (mostly neutropenia and anemia), are rare. Lymphoadenopathy, hepatomegaly, splenomegaly and cutaneous lesions are uncommon. Occasionally, patients present a slow progressive increase of peripheral blood NK cells and organ involvement. Several cases with a spontaneous complete remission have been reported (Zambello & Semenzato, 2009). Cytologically, the circulating cells show typical granular lymphocyte morphology, with moderate amount of pale cytoplasm that contains $\geq$ 3 azurophilic granules. Bone marrow biopsy is characterized by interstitial infiltration of cells with small nuclei and pale cytoplasm, which are difficult to recognize without the help of immunohistochemical techniques. Cytogenetic is normal in most cases (Zambello & Semenzato, 2009) and the germ line configuration of TCR is demonstrated, as expected for normal NK cells. Since clonality of proliferating cells is difficult to detect in these patients, the analysis of restriction fragment length polymorphism (RLFP) has been used as an indirect marker to demonstrate the clonality in some but not all patients. In rare case, in which EBV can be demonstrated in plasmid form within NK cells, the clonality of cells might be easily examined by Southern Blot analysis using probes recognizing the EBV terminal repeats (Kawa-Ha et al., 1989). Patients with CLPD-NK usually have an indolent clinical course and respond to immunosuppressive therapy with low doses of methotrexate (usually 10 mg/m^2/week) or of cyclophosphamide (50 or 100 mg/day) or cyclosporin (3-5 mg/kg/day) with or without inclusion of low doses of steroids (Lamy & Loughran, 2011).

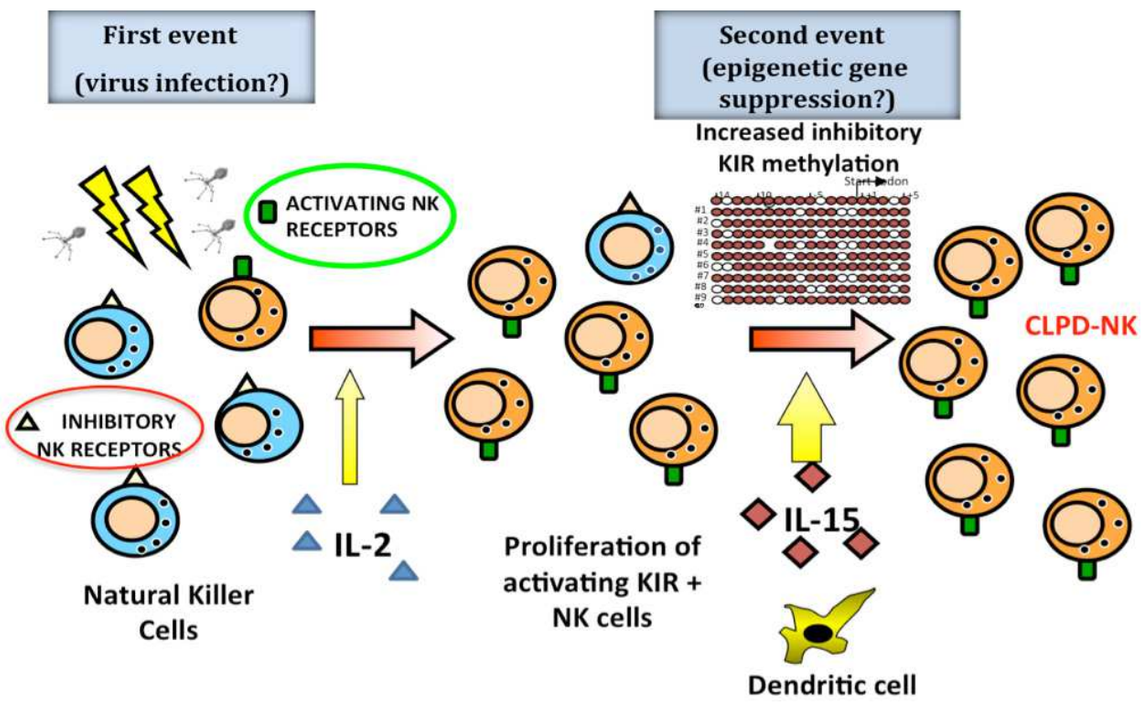


Fig. 2. Possible mechanisms of CLPD-NK pathogenesis.

5. Conclusions

NK cells are bone marrow–derived granular lymphocytes that have a key role in the recognition and in the killing of tumor- or virus-infected cells. The identification of a large array of surface NK receptors capable of transducing inhibitory or activating signals, let to explain how their effector functions could be regulated.

In recent years, it has also been shown that NK cells may play a role in the regulation of the immune response. This has been based on the finding that NK cells were able to functionally interact with different cells of the innate immune system including DCs, pDCs and macrophages. Besides the well known $CD56^{dim}CD16^{+}$ cytotoxic PB NK cells, different NK cell subsets located in specific tissue/organ compartments have been shown to exert various, alternative, regulatory functions. NK cells homing in SLT promote Th1 polarization. Conversely, decidual NK cells favour Treg expansion and play an important role in tissue remodelling and neo-angiogenesis process. In MALT, NK22 cells may support immune defence against bacteria invasion by their peculiar cytokine and chemokine secreting profile.

Whether the above described different NK cell subsets may derive from the same lineage or from different ones, is still a matter of debate. Studies on NK cell development may help to clarify the possible lineage relationship between the different NK cell subsets. In vitro models of NK cell development helped to define phenotypically and functionally different maturation stages that were, at least in part, confirmed by in vivo analysis. For example, the $CD56^{bright}CD16^{-}CD94/NKG2A^{+}KIR^{-}$ subset, that is largely represented at the end on in vitro development, represents the first wave of lymphocytes appearing in PB at early time intervals after HSCT. NK cells originate from $CD34^{+}$ hematopoietic precursors but several experimental evidences suggest that the NK cell development and terminal maturation does not occur wholly in the BM. Other secondary lymphoid organs, including L.N. and MALT, as well as decidua, may represent sites where NK cell development occurs. This suggest how defined subsets of NK cells may differentiate in peculiar tissues and point the attention on the role of the microenvironment in such a process. The full characterization of the genetic and epigenetic factors that may contribute to determine the type of NK cell that will develop is still in process and it will be important also to better define mechanisms leading to NK cell malignant transformation.

It is hopeful that investigation on NK cell neoplasm pathogenesis could lead to identify molecular targets and discovery of more efficient and less toxic treatments. The elucidation of pathways by which EBV transform NK cells might help to identify new molecular targets. Furthermore, the mechanisms of resistance to therapy, including alteration of apoptosis pathways should be deeply characterized. Thus, the definition the NK neoplasm patho-physiology and the identification of possible biological targets may help to improve therapeutical approaches.

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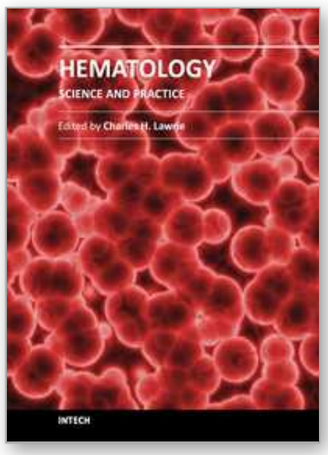
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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