LiceNsed to Kill

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Thesis:

Licensed to kill: towards Natural Killer cell immunotherapy

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Licensed to kill

towards Natural Killer cell immunotherapy

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Aan mijn ouders, jullie zijn mijn basis.
Aan Peter, bij jou vind ik mijn thuis.

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1. General introduction

Hematological malignancies, such as leukemia, are life-threatening diseases that find their origin in a derailment of the hematopoietic system. Allogeneic stem cell transplantation (SCT) is often the final treatment modality in which the patient is transplanted with hematopoietic stem cells (HSCs) from an allogeneic donor that give rise to a new and healthy hematopoietic system. After SCT, residing tumor cells are eradicated by donor-derived T cells (GVL reactivity). However, these alloreactive T cells may also provoke strong graft-versus-host reactivity against healthy host tissues. This often leads to graft-versus-host disease (GVHD), which can result in mortality. Although HLA-matching between donor and recipient has strongly decreased the occurrence of GVHD, other immunogenic factors may still trigger donor T cell alloreactivity. Therefore, immunotherapeutic strategies augmenting GVL reactivity while reducing GVHD are urgently needed. Recently, natural killer (NK) cells have been described to provoke efficient GVL reactivity without the induction of GVH responses. This makes NK cells important candidates to exploit for adoptive immunotherapeutic strategies in leukemia treatment, which is the focus of this thesis.

2. The hematopoietic system

2.1 Hematopoiesis

The development of the human blood compartment, termed hematopoiesis, is a highly coordinated process by which pluripotent hematopoietic stem cells (HSCs) give rise to the functional mature hematopoietic system consisting of red blood cells (i.e. erythrocytes), white blood cells (i.e. lymphocytes, granulocytes, monocytes) and platelets. As mature hematopoietic cells have a limited life-span, hematopoiesis is a continuous process throughout life.[1] Besides giving rise to mature functional hematopoietic cells, HSCs are capable of self-renewal to maintain the pool of pluripotent progenitors.

Hematopoiesis can be described as a hierarchical process that is tightly regulated and starts in the bone marrow (Figure 1). The HSC population consists of long-term HSCs (LT-HSCs) which give rise to short-term HSCs (ST-HSCs).[2] Both populations are capable of self-renewal. ST-HSCs differentiate into a multi-potent progenitor (MPP) population that has no capacity of self-renewal. MPPs further develop into two early committed progenitor lineages; the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The CLP lineage gives rise to the mature lymphocyte population consisting of NK, T and B cells.[3] The CMP lineage further differentiates into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs).[4] However, the hierarchical relationship between MEP and other progenitors is now under debate as it has recently been suggested that MEPs may directly arise from MPPs.[5] MEPs further differentiate into erythrocytes and megakaryocytes, the latter giving rise to platelets. The GMP lineage gives rise to mature monocytes, macrophages, dendritic cells

and granulocytes (i.e. neutrophils, eosinophils and basophils). The hematopoietic progenitor for dendritic cells, however, remains controversial as these cells can arise from both myeloid and lymphoid progenitors.[6;7]

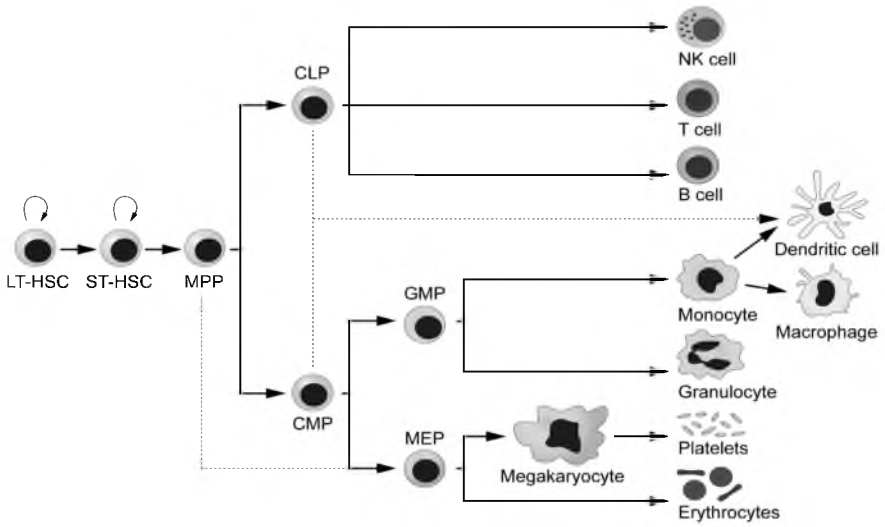

Figure 1. Human hematopoiesis. The hematopoietic system originates from hematopoietic stem cells (HSCs) that reside in bone marrow. Long-term HSCs (LT-HSC) and short-term HSCs (ST-HSC) either undergo self-renewal or differentiate into multi-potent progenitors (MPP) that give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). CLPs differentiate into mature NK, T and B cells. CMPs further differentiate into multi-lineage progenitors giving rise to granulocytes and macrophages (GMP), and megakaryocytes and erythrocytes (MEP).

During hematopoiesis, the proliferation potential and life-span of cells decreases. The functional mature white blood cells collectively function as the immune system that provides protection and defense against pathogens and malignant-transformed cells.

2.2 Leukemia

The balance between HSC self-renewal and differentiation towards the various hematopoietic cell lineages is considered to be critical to maintain hematopoietic homeostasis.[8-10] Fate decisions of HSCs on life and death, self-renewal and differentiation are important processes that play a crucial role in the regulation of HSC numbers and their lifespan. Defects in these processes can contribute to hematopoietic insufficiencies and the development of hematological malignancies, such as leukemia.

Leukemia is a type of blood cancer that starts in the bone marrow and is characterized by an abnormal accumulation of malignant hematopoietic cells (leukemic cells). These leukemic cells are arrested in their hematopoietic development and proliferate faster than normal hematopoietic cells. As a result, leukemic cells colonize the available space in bone marrow and hamper

normal hematopoiesis resulting in reduced numbers of healthy progenitor cells. If untreated, leukemic cells overwhelm the bone marrow, enter the blood stream, and eventually invade other compartments of the human body, such as lymph nodes, spleen, liver, and the central nervous system. The most common types of leukemia are acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL).[11] Acute leukemias are characterized by a rapid progression and accumulation of immature malignant-transformed hematopoietic cells (myeloblasts or lymphoblasts). Chronic leukemias usually progress slowly as compared with acute leukemias and the malignant clone originates from a more mature hematopoietic cell population. Treatment modalities often include chemotherapy, radiation, and/or allogeneic stem cell transplantation.

3.Allogeneic stem cell transplantation

3.1 General procedure

Allogeneic stem cell transplantation (SCT) is an intensive but often final treatment modality for patients with leukemia or other life-threatening hematological malignancies.[12;13] For allogeneic SCT, bone marrow has been the traditional source of donor-derived hematopoietic stem cells (donor HSCs).[13;14] However, donor HSCs are now usually mobilized into the peripheral blood using hematopoietic growth factors like G-CSF, followed by leukapheresis.[15] The use of these mobilized donor peripheral blood stem cells (donor PSCs) is associated with better engraftment and hematological recovery after SCT without increased risk of graft-versus-host disease (GVHD).[16;17] Umbilical cord blood harvested at the time of delivery is also a source for donor HSCs.[18-20]

Before transplantation, patients receive a severe myeloablative conditioning regimen with high dose chemo-radiotherapy, often combined with total body radiation (TBI), in order to eradicate all non-malignant patient-derived hematopoietic cells and the majority of tumor cells to ensure proper engraftment of the donor HSCs and to decrease the tumor burden in the patient. After conditioning, donor HSCs are transfused into the patient to give rise to a new hematological and immunological system resulting in complete donor chimerism and the induction of an immune mediated graft-versus-leukemia (GVL) response by donor T cells against residing tumor cells. As an alternative to the severe myeloablative conditioning regimen, a reduced intensity nonmyeloablative conditioning (RIC) regimen can be used prior to transplantation followed by a donor lymphocyte infusion (DLI) after SCT.[21-23] Following transplantation, patients are strictly monitored and treated with immunosuppressive drugs to prevent GVHD.[24-28] Once engraftment has been secured and no severe GVH reactions have occurred within the first months after SCT, immunosuppressive treatment can be tapered. Nevertheless, patients remain vulnerable for microbial and viral infections, and the development of chronic GVHD. Besides these complications, patients may still risk relapse of their underlying hematological malignancy. The basis for transplant related complications, such as GVHD, lies within the tissue compatibility between donor and recipient.

3.2 Immunobiology

3.2.1 Allorecognition

Allorecognition is an immune process that is orchestrated by human leukocyte antigens (HLA). They provide the human immune system the ability to discriminate between "self" and "non-self", and regulate immune responsiveness to foreign invaders (e.g. bacteria, viruses, fungi) and malignant -transformed cells. HLA molecules are highly polymorphic proteins and are encoded by the HLA complex.

3.2.2 The HLA complex

The HLA complex, also known as the major histocompatibility complex (MHC) in mice, is a gene cluster located at chromosome 6 and is the most polymorphic region in the human genome. HLA molecules are encoded in two regions of the HLA complex: the class I and class II region (Figure 2). The HLA class I region encodes the classical HLA-A, -B, -C molecules (class Ia) and the nonclassical HLA-E, -F, -G molecules (class Ib). They contain a highly polymorphic heavy chain (45 kDa), consisting of three α subunits, which is noncovalently associated to the nonpolymorphic β2-microglobulin (β2m) chain (12 kDa).[29] The β2m chain is encoded by a gene located on chromosome 15 outside the HLA complex. HLA class I molecules present endogenously-derived antigenic peptides to cytolytic $CD8^+$ T cells. The classical HLA-A, -B, and -C antigens are expressed on the cell surface of all nucleated cells and are therefore considered important for transplantation immunobiology, whereas the nonclassical HLA-E, -F, and -G antigens show a more limited tissue distribution and their significance for transplantation immunobiology is still unclear. The genes of the HLA class II region encode HLA-DR, -DQ, and -DP molecules consisting of two highly polymorphic chains (α and β) that each contain 2 subunits. Both HLA class II chains are encoded within the HLA complex. The HLA class II molecules are mainly expressed by antigen-presenting cells (APCs), such as monocytes, dendritic cells and B cells. These cells take up foreign antigens from the extracellular fluid. After processing, exogenous-derived antigenic peptides are loaded on HLA class II molecules and presented to helper $CD4^+$ T cells.

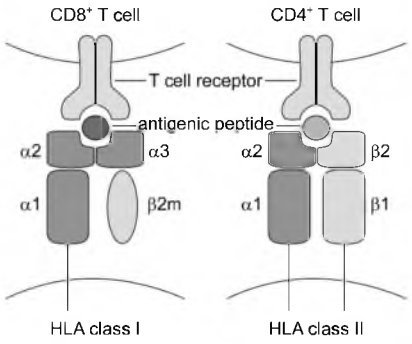

Figure 2. HLA class I and class II molecules. HLA molecules are important for allorecognition and discrimination between "self" and "non-self". HLA class I molecules each consist of a highly polymorphic heavy chain (45 kDa), which is noncovalently associated with β2m. They present endogenous-derived antigenic peptides to $CD8^+$ T cells. HLA class II molecules each consist of two highly polymorphic heavy chains and present exogenous-derived antigenic peptides to $CD4^+$ T cells.

3.2.3 Development of GVHD

In allogeneic SCT, the immune system derived from the donor can recognize healthy host cells as "non-self", resulting in GVH reactions that contribute to GVHD. The development of GVHD can be described in three sequential phases: (1) induction of "danger" signals; (2) activation, expansion, and differentiation of donor T cells; and (3) migration of donor T cells and recruitment of other effector cells followed by the destruction of host tissues (Figure 3).

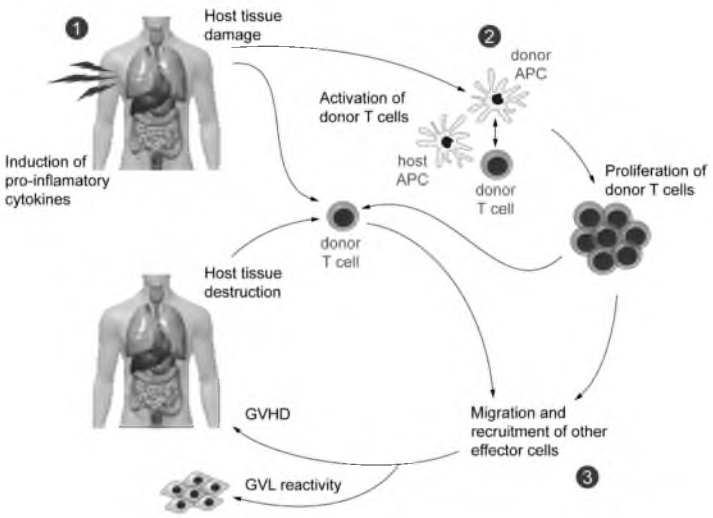

Figure 3. Development of graft-versus-host disease (GVHD). GVHD development can be described in three sequential phases: (1) induction of "danger" signals due to host tissue damage by the conditioning regimen prior to allogeneic SCT and the activation of host APCs; (2) activation, expansion, and differentiation of alloreactive donor T cells; and (3) migration of alloreactive donor T cells and recruitment of other effector cells followed by the destruction of host tissues (GVHD) and graft-versus-leukemia (GVL) reactivity.

In phase 1, due to the toxic conditioning regimen prior to allogeneic SCT, damaged host tissues respond by producing "danger" signals, including proinflammatory cytokines (e.g. TNF-α, IL-1, IL-6) and chemokines, leading to increased expression of allogeneic HLA molecules, co-stimulatory molecules, and adhesion molecules, and activation of host APCs.[30;31] Phase 2 starts with the activation of donor T cells by host APCs expressing allogeneic HLA molecules and co-stimulatory molecules.[32;33] Donor $CD4^+$ T cells recognize allogeneic HLA class II molecules presented by host APCs and are activated through co-stimulatory B7/CD28 interactions. Activation of these cells leads to the production of proliferative cytokines (e.g. IL-2) resulting in clonal expansion and differentiation into Th1 or Th2 cells (defined by the cytokines that they produce). These cells have been associated with differences in GVHD severity.[34;35] Donor $CD8^+$ T cells directly recognize allogeneic HLA class I molecules and become activated through co-stimulatory CD40/CD40L interactions. For proper activation and expansion, donor $CD8^+$ T cells require allorecognition of host APCs and cytokine stimulation by activated donor $CD4^+$ T cells.[33] Allorecognition of host APCs by donor T cells is critical for GVHD initiation and occurs despite the early disappearance of host APCs post-SCT.[32;33] Donor-derived APCs are then able to further augment $CD8^+$ T cell-mediated GVHD, presumably by acquiring and presenting host antigens (cross-priming).[36] Phase 3 is characterized by the migration of the activated donor T cells towards GVHD target tissues (gut, skin, liver, and lung), followed by the recruitment of other

donor effector cells (e.g. monocytes, B cells, NK cells), resulting in subsequent tissue injury.[37] During this phase GVL reactivity also takes place. Target tissue destruction and GVL reactivity are mediated through cell surface and soluble effector molecules (i.e. FasL, TNF-α, TRAIL, perforin, granzymes, IFN-γ). Subsequently, tissue damage leads to increased production of proinflammatory cytokines inducing GVHD continuation and further aggravation of the disease counterbalancing successful outcome of GVL reactivity.

3.3 HLA-matching to limit GVHD

As the incidence of acute GVHD is directly associated with the degree of mismatch between HLA molecules, accurate matching of HLA alleles between donor and recipient is essential. Development of high-resolution screening techniques have allowed transplantation centers to enhance the level of HLA-matching for HLA-A, -B, -C, -DR, and DQ, which contributes to improved engraftment and better overall survival after allogeneic SCT.[38-40] In some cases, an isolated HLA-C mismatch between donor and recipient may be allowed, depending on the transplantation setting and the type and stage of the malignancy.[41-43] The relevance of additional HLA-DP matching on clinical outcome is still under debate, but is currently not used as a matching criterion for donor selection.[44-47] Although the golden standard is to aim for full HLA compatibility between donor and recipient, development of functional cellular tests have demonstrated that certain HLA mismatches may be functionally less immunogenic and do not necessarily result in poor clinical outcome (referred to as "permissible mismatches").[42;48] Given the increasing number of identified HLA alleles, this extends the pool of potential donors for patients that lack a fully HLA-identical sibling donor.

3.4 Balancing between GVHD and GVL

Despite HLA compatibility between donor and recipient, substantial numbers of patients still develop GVHD, due to genetic differences that lie outside the HLA complex, known as minor histocompatibility antigens (mHAgs).[49-51] Minor HAgs are immunogenic peptides encoded by polymorphic genes and are presented by HLA class I and II molecules on the cell surface where they can be recognized by donor T cells.[52-55] Removal of donor T cells from the graft may prevent mHAg-induced GVHD. However, radical T cell depletion increases the risk of graft failure and delayed immunological recovery.[56;57] The use of partially or repleted T cell grafts in combination with RIC regimens has resulted in durable engraftment in sibling and voluntary unrelated donor (VUD) transplantations while significantly reducing the risk for GVHD. The reduced antitumor activity related to such protocols necessitates the use of pre-emptive DLI infusions, consisting of donor T cells, to promote GVL reactivity.[23;58-60] The efficacy and curative effect of DLI has been demonstrated in a variety of hematopoietic malignancies.[61] Nevertheless, relapse may still occur depending on the sensitivity of the malignancy to the effects of DLI. Furthermore, escalating doses of DLI to boost GVL reactivity increase the risk for GVHD. Therefore, strategies for adoptive immunotherapy augmenting GVL reactivity and reducing GVHD are urgently needed. There is

increasing evidence that donor T cells that mediate GVHD are not the only effectors of GVL and that GVHD and GVL may be separable. NK cells are one of the potential candidates for immunotherapeutic use and are currently exploited for NK cell-based immunotherapy, which is the focus of this thesis.

4. Natural Killer cells

4.1 General

NK cells are members of the innate immune system. They are important in the initial phase of defense against infections and play an important role in tumor surveillance. Their name was based on their ability to kill target cells without prior sensitization.[62;63] As they are morphologically recognized as relatively large lymphocytes containing azurophilic granules they have also been known as large granular lymphocytes (LGL).[64] NK cells comprise approximately 5-15% of the peripheral blood lymphocyte (PBL) population and are also found in lymph nodes, spleen, bone marrow, lung, liver, intestine, omentum and placenta.[65] NK cells are believed to originate from the same common lymphoid progenitor lineage as T and B cells in bone marrow.[66] However, they do not rearrange T cell receptor genes or immunoglobulin (Ig) like, respectively, T and B cells do. Resting NK cells can be recognized based on their expression of CD56 (neural cell adhesion molecule; NCAM). Since CD56 is also expressed by other immune cells, NK cells are identified by the expression of CD56 combined with the lack of CD3, which are both present on NK-T cells. NK cells can be further characterized based on their expression of CD56 in combination with CD16, a low affinity Fc receptor (Figure 4).[67] In peripheral blood, approximately 90% of NK cells shows low expression of CD56 and high expression of CD16 on the cell surface. These cells are collectively referred to as the $CD56^{dim}CD16^{+}$ or $CD56^{dim}$ NK cell subset. The other 10% of NK cells shows a high level of CD56 expression and almost no expression of CD16 on the cell surface. Together, these cells are known as the $CD56^{bright}CD16^{+/-}$ or $CD56^{bright}$ NK cell subset. The $CD56^{dim}$ NK cell subset is characterized by a highly cytolytic behavior towards target cells, whereas $CD56^{bright}$ NK cells abundantly produce cytokines, such as IFN-γ and TNF-α, upon activation. The production of cytokines by NK cells influences the Th1/Th2 bias of the adaptive immune response by activating Th1 cells. Thereby, NK cells form a bridge between innate and adaptive immunity.[68]

NK cells lyse susceptible target cells (e.g. virus-infected cells, malignant-transformed cells) by one of two mechanisms: “natural killing” (no prior sensitization) or antibody dependent cellular cytotoxicity (ADCC). Natural killing is initiated by activating signals from a variety of stimulatory receptors that can be inhibited by a variety of inhibitory receptors. In ADCC, the activating receptor FcRγIII (CD16) binds to the Fc piece of antibodies bound to target cells. In both mechanisms, target cells are lysed by the release of cytolytic proteins (i.e. granzymes and perforines) or by the induction of apoptosis.[69]

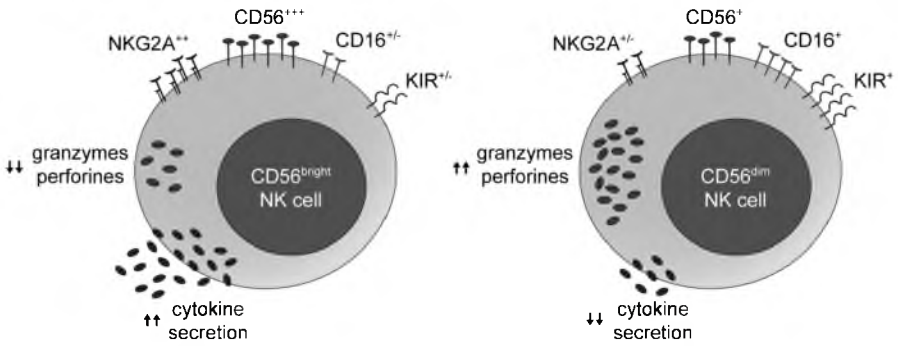

Figure 4. Natural killer cell subsets. NK cells can be divided in two major subsets based on their expression of CD56 and CD16. $CD56^{bright}$ NK cells have a high CD56 and low CD16 expression profile, and are specialized in cytokine secretion (e.g. TNF-α, IFN-γ). In addition, they highly express inhibitory receptor NKG2A. $CD56^{dim}$ NK cells have a low CD56 and high CD16 expression profile, and are highly cytolytic. Their function is predominantly inhibited through KIR.

4.2 NK cell receptors

The NK cell receptor repertoire forms the basis for NK cell immune surveillance and NK cell activity. NK cell immune surveillance is regulated through the recognition of HLA class I molecules by inhibitory receptors. Subsequent activation of NK cells is triggered through the recognition of activating ligands by stimulatory receptors.

4.2.1 Inhibitory receptors

NK cells survey potential target cells for the absence or loss of expression of classical HLA class I molecules or non-classical HLA class I specific signals through inhibitory killer immunoglobulin-like receptors (KIRs) and lectin-like receptors.[70;71] The cytoplasmic domains of all inhibitory NK cell receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM).[72-74] These domains recruit intracellular tyrosine phosphatases SHP-1 or SHP-2 that mediate the inhibition of cytotoxicity and cytokine release.[72;73;75;76]

The lectin-like receptor complex CD94/NKG2A forms an inhibitory receptor that recognizes non-classical HLA-E molecules.[77] As the expression of HLA-E is promoted by the binding of signal sequence-derived peptides from HLA class I molecules, it is thought that HLA-E expression serves as a barometer of classical HLA class I expression.[78] The purpose of the inhibitory CD94/NKG2A receptor complex may, therefore, be to monitor the overall HLA class I expression. KIRs, on the other hand, allow for a more subtle immune surveillance as these receptors scan the presence of specific classical HLA class I molecules.

KIRs are encoded by a family of polymorphic and highly homologous genes, and recognize polymorphic epitopes present on HLA-A, -B, or -C molecules; HLA-A3 and -A11 are recognized by KIR3DL2, HLA-Bw4 is recognized by KIR3DL1 and receptors KIR2DL1, KIR2DL2, and KIR2DL3 are able to distinguish HLA-C into HLA group C1 and HLA group C2 molecules.[79] The

various KIRs are classified by the number of immunoglobulin-like (Ig) extracellular domains as 2-domain (2D) or 3-domain (3D). They are further subdivided on the basis of the length of their cytoplasmic tail in L (long) or S (short).[80] Different KIRs sharing the same number of Ig domains and length of the cytoplasmic tail are distinguished by number at the end of their name, e.g. KIR2DL2 or KIR2DL3. KIRs are clonally distributed among NK cells within each individual, which creates a complex combinatorial repertoire of NK cell specificities for HLA class I molecules.[80]

4.2.2 Stimulatory receptors

Some members of the CD94/NKG2 receptor and KIR family have stimulatory properties. NKG2C is a stimulatory member of the CD94/NKG2 family and competes with CD94/NKG2A for the recognition of HLA-E.[77;81] KIR2DS and KIR3DS are stimulatory members of the KIR family.[82-84] Instead of ITIM, these stimulatory receptors contain a positively charged amino acid residue in their transmembrane domain that associates with the negatively charged DAP-12 molecule. DAP-12 contains an immunoreceptor tyrosine-based activating motif (ITAM).[85] There is evidence that the stimulatory KIRs bind self-HLA class I molecules with lower affinity as compared with the inhibitory receptors.[86;87] Thus autoimmunity can be prevented by a balance towards negative NK cell regulation. Similar to the inhibitory receptors, the HLA class I-specific stimulatory receptors are expressed in a variegated and predominantly stochastic fashion by NK cells.[88]

Besides stimulatory members of the CD94/NKG2 receptor and KIR family, NK cells also express a variety of other stimulatory receptors. The biological roles of many of these receptors are not well understood, primarily because the ligands for these receptors have not been fully identified. The main triggering receptors for NK cell activity are the natural cytotoxicity receptors (NCR) and NKG2D.[89-92] Their stimulation causes direct killing of target cells and their stimulatory signals can even override the inhibition of NK cells.

NCR consist of three members; NKp30, NKp44, and NKp46. NCR belong to the immunoglobulin superfamily and molecular cloning of NCR confirmed that they are structurally distinct.[93;94] The ligands for NCR remain controversial. Some groups have proposed viral antigens as being the ligands for NCR based on their role in the lysis of virus-infected cells.[91;92;95] NCR have also been shown to mediate lysis of tumor cells and that NK cells with a NCR^{dull} phenotype are unable to kill tumor cells, suggesting that their ligands may be upregulated or induced upon malignant transformation of cells.[96-98] NKp30 and NKp46 are both uniquely expressed on resting and activated NK cells, whereas NKp44 is only present on IL-2 activated NK cells.[99]

Unlike NKG2A, NKG2D is not associated with CD94, but is a homodimer that needs association to the adaptor molecule DAP10 for stable cell surface expression.[100] NKG2D recognizes HLA class I-like molecules, such as MIC A and MIC B. It has been shown that the expression of NKG2D ligands, MIC A/B, ULPB1, ULPB2, and ULPB3 are upregulated by cells in times of stress, virus infection, and malignant transformation.[90;101;102] NKG2D is constitutively expressed on all human NK cells and can be upregulated through stimulation by IL-15, IL-12 and IFN-α.[103;104] Stimulation of NKG2D complements NCR activation in mediating NK cell lysis of tumor cells.[105] Similarly, cooperation between NKG2D and stimulatory KIRs has been shown for both cytolytic activity and

IFN-γ secretion.[106] Therefore, it is possible that NKG2D may serve both as a primary stimulatory receptor, whose engagement triggers cytotoxicity, and also as a co-stimulatory receptor, which cooperates with other activating receptors (e.g. activating KIR or NCR) for cytokine secretion. A similar phenomenon is seen on cytomegalovirus-specific T cells, where NKG2D acts as a co-stimulatory receptor for TCR-dependent signals.[107-109]

Other stimulatory receptors that are involved in NK cell activation are co-stimulatory receptors NKp80 and 2B4 (CD244).[110] NKp80 and 2B4 both function synergistically with NCR.[111;112] In addition, CD16, CD69 and DNAM-1 have been shown to trigger NK cell-mediated lysis in redirected cytotoxicity assays.[113-115]

4.3 NK cell allorecognition

4.3.1 The "missing self" hypothesis

In 1976, Snell *et al.*[116] observed a correlation between the susceptibility of target cells to NK cell lysis and the absence, or low, expression of HLA class I molecules on the target cells. Absent or low expression of HLA class I molecules is common in virus-infected and malignant-transformed cells, which are the usual targets for NK cell lysis. Therefore, they proposed that NK cell receptors may not only interact with HLA class I molecules, but that these receptors are also able to detect a decrease in HLA class I expression.

It was not until 10 years later that Ljunggren and Kärre demonstrated the regulation of NK cell activity. They showed that murine lymphoma cells with low, or absent, MHC class I expression were less malignant than wild-type cells after low dose inoculation in syngeneic mice, and that the rejection of these cells was regulated through innate immunity, preferably through NK cell-mediated lysis.[117] Resistance to NK cell-mediated lysis of tumors with low MHC class I expression could be restored by reintroduction of MHC class I molecules.[118;119] Based on these data, they proposed the "missing self" hypothesis, which is nowadays still appreciated as the basic model for NK cell activation (Figure 5).[120]

4.3.2 Licensed to kill

As the KIR repertoire of NK cells is encoded by a set of highly polymorphic genes and segregates independently from HLA class I genes during NK cell development, it is essential that the KIR repertoire of NK cells properly corresponds with the HLA environment to provide self-tolerance and prevent autoimmunity. There have been several hypotheses on the acquisition of self-tolerance by NK cells. Raulet *et al.*[88] proposed that an individual NK cell can simultaneously express multiple inhibitory KIRs in a stochastic fashion. The only rule appears to be that every NK cell has at least one inhibitory KIR specific for self-HLA class I in order to avoid autoreactivity. This is referred to as the "at least one receptor" model.[88] Others have suggested a "receptor calibration" model in which the acquisition of the KIR repertoire may be related to changes in the HLA class I environment and is dependent on the HLA class I haplotype.[121-123] However, these studies involved *in vitro* cultures that could alter the intrinsic features of NK cells that may be different from the *in vivo* situation. Recently, the acquisition of self-tolerance was demonstrated

in an *in vivo* murine study. Kim *et al.*[124] showed that NK cells from MHC-deficient mice were functionally immature as they were defective in cytokine secretion upon *ex vivo* stimulation as compared with wild type NK cells, indicating that MHC-specific receptors are involved in the acquisition of functional competence. They also found a correlation between the expression of an inhibitory receptor for self-MHC class I and the capacity of an individual naive NK cell to be activated to produce cytokines and lyse susceptible target cells. Based on their findings they proposed the "licensing" model, in which NK cells acquire functional competence through "licensing" by self-HLA class I molecules, resulting in two types of self-tolerant NK cells: licensed or unlicensed. This model was confirmed by others, both in mice and human, demonstrating that NK cells without expression of known self-receptors were found to be hyporesponsive.[125;126] Thus, in order for NK cells to get their "license to kill", they need to fulfill the requirement of HLA class I-specific receptor engagement by self-HLA.

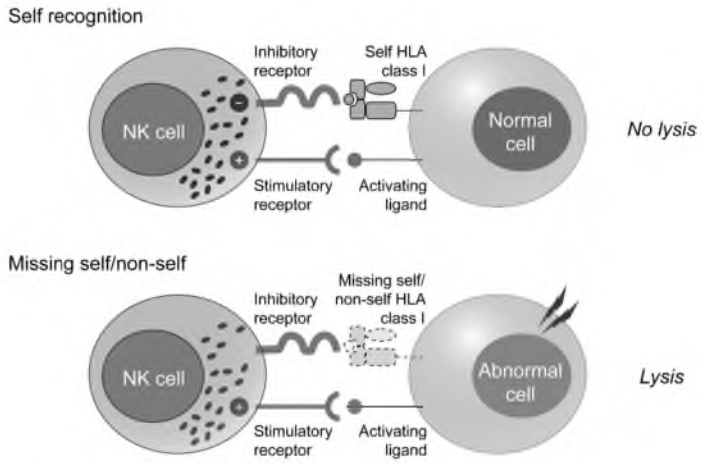

Figure 5. "Missing self" hypothesis. NK cell activity depends on a balance between inhibitory (i.e. KIR, CD94/NKG2A) and stimulatory (e.g. NCR, NKG2D) signals. In steady state, NK cells are inhibited from activation by the recognition of self-HLA class I molecules, which overrules potential stimulatory signals (self recognition). In case of virus infection or malignant transformation, cells may downregulate self-HLA class I molecules, while upregulating activating ligands that trigger NK cells to respond resulting in lysis of the infected/transformed cells (missing self). After allogeneic SCT, donor NK cells may be triggered by host leukemic cells due to reduced HLA-matched class I molecules (HLA-matched SCT), or the presence of non-self HLA class I molecules (HLA-mismatched SCT), combined with strong stimulation by upregulated activating ligands.

5. NK cells and their therapeutic role in SCT

5.1 Evidence for GVL

Deficient HLA class I expression has been described for leukemic cells making them susceptible targets for NK cell-mediated lysis. However, this phenomenon was not ubiquitously observed in the autologous setting for patients with different forms of leukemia. In CML, NK cell numbers and NK cell function have been shown to decrease progressively during the spontaneous course of the disease, but could be recovered upon IFN-α treatment.[127;128] Moreover, activated autologous NK cells were shown to suppress the growth of primitive CML progenitors in long-term *in vitro* cultures.[129] In AML, however, autologous NK cells were demonstrated to be impaired in their cytolytic function, which correlated with a low NCR cell surface density (NCR^{dull}).[98] Moreover, these NK cells were impaired in regulating DC physiology (killing the surplus of immature DCs), which could lead to specific T cell tolerization by expanded immature DCs expressing leukemia-derived antigens.[97] Allogeneic SCT may overcome the impairment of NK cell-mediated lysis.
In different SCT settings, NK cells have shown to play an important role in the anti-tumor response within the first months after transplantation.[130-133] In haploidentical SCT, Ruggeri *et al.*[134] showed that donor alloreactive NK cells isolated from peripheral blood of the recipient were able to lyse tumor cells derived from the recipient, implying that within one month after SCT, NK cells may be able to provide some degree of immune reactivity by targeting residual tumor cells still present in the recipient. Moreover, fast recovery of NK cells and predicted GVL reactivity towards host tumor cells has been associated with decreased relapse rates and better overall survival of the patient.[135-137] Altogether these data suggest that NK cells play an important role in the control and clearance of leukemic cells after allogeneic SCT.

5.2 Evidence for the prevention of GVHD

The haploidentical transplantations performed by Ruggeri *et al.*[132] additionally suggested that NK cells may prevent the development of GVHD. For patients, the prevalence of GVHD was significantly lower using grafts with potential NK cell alloreactivity in the GVL direction as compared with grafts without potential NK cell alloreactivity. In a murine model, they demonstrated that mice transplanted with non-T cell depleted grafts could be rescued from GVHD upon infusion of alloreactive NK cells. Mice infused with non-alloreactive NK cells died as they were not protected from GVHD. They also demonstrated that alloreactive NK cells are able to lyse recipient APCs, thereby preventing interaction with donor T cells which otherwise would initiate GVHD. Recently, a novel mechanism for NK cell-mediated GVHD reduction was demonstrated, whereby alloreactive donor NK cells were able to inhibit and lyse alloreactive donor T cells during the initiation of GVHD.[138] Overall, these studies demonstrate that alloreactive NK cells may, directly or indirectly, reduce or prevent the occurrence of GVHD while retaining GVL reactivity.

5.3 Exploitation of NK cell alloreactivity

The ability to induce GVL reactivity while reducing or preventing GVHD makes alloreactive NK cells important candidates for immunotherapeutic strategies in leukemia treatment. Since 2005, several studies have explored the safety of NK cell-based immunotherapy within various settings. Koehl *et al.*[139] demonstrated the feasibility of good manufacturing practice (GMP)-compliance NK cell isolation and expansions for clinical applications. In this study, IL-2 activated NK cells were used for repeated transfusions in three pediatric patients with multiple relapsed ALL or AML. Unfortunately, all three patients died of relapse or disease-related complications. The feasibility of NK-DLI was tested by Passweg *et al.*[140] in patients after haploidentical SCT, demonstrating that NK cell infusions after SCT were well tolerated without the induction of GVHD. The feasibility of NK cell infusions was further assessed by Miller *et al.*[141] in a non-transplantation setting and demonstrated successful adoptive transfer and *in vivo* expansion of haploidentical NK cells in patients with poor-prognosis AML. However, only 5 out of 19 patients showed complete hematological remission. Shi *et al.*[142] later showed that infusion of haploidentical KIR-ligand mismatched NK cells proved safe and feasible for advanced multiple myeloma patients in the setting of autologous SCT. In this study, 50% of the patients achieved (near) complete remission. Overall, these studies indicate that NK cell-based immunotherapies may be safe, well tolerated, and do not induce GVHD. Nevertheless, results also demonstrate that the success of NK-cell based immunotherapies may depend on the type of malignant disease, stage, and the overall health of the patient.

6. Scope and outline of this thesis

The development of NK cell-based immunotherapies for cancer treatment is still in its infancy. So far, the exploitation of NK cell alloreactivity for immunotherapeutic purposes has only been pursued in the HLA-haploidentical setting. However, for the treatment of hematological malignancies, HLA-matching between donor and recipient is the preferred setting for allogeneic SCT and immunotherapeutic DLI (**chapter 1**). Therefore, the main focus of this present thesis was to explore the facilitation of alloreactive donor NK cells in HLA-matched allogeneic SCT. For this purpose, we studied the effects of two different graft types, the feasibility of skewing NK cell alloreactivity towards specific malignant cells, and the potential side-effects of immunosuppressive regimens.

In **chapter 2**, we studied the alloreactive potential of mature donor-derived NK cells when given as part of the graft in HLA-matched allogeneic SCT. The results of this study elegantly demonstrate that the use of $CD3^+/CD19^+$ depleted grafts facilitates strong NK cell cytolytic responses directly after SCT, and the rapid emergence of a NK cell receptor phenotype that is more prone to activation as compared with the use of conventional $CD34^+$ selected grafts, which are devoid of donor NK cells. In **chapter 3,** we examined the plasticity of the KIR repertoire and the cytolytic response of mature human peripheral blood NK cells in the presence of specific KIR receptor-

ligand (i.e. HLA-C) mismatches. Here, we show that HLA class I can skew the mature NK cell phenotype and enrich the alloreactive NK cell population towards leukemic target cells. This may be important for future clinical applications of NK cell-based immunotherapies using mature NK cells in transplantation settings. As immunosuppressive drugs (ISDs) are frequently used for GVHD prophylaxis after allogeneic SCT or DLI, we further explored the effect of commonly used ISDs (i.e. cyclosporin A (CsA), mycophenolic acid (MPA), and rapamycin (Rapa)) on human peripheral blood NK cells *in vitro* (**chapter 4**). Our results showed that CsA, MPA and Rapa each have distinct effects on NK cell phenotype and function, which may have important implications for NK cell function *in vivo* after transplantation.

As NK cell alloreactivity may also be exploited in non-transplant settings (i.e. adoptive cancer immunotherapy), we further contributed to the development of a clinical grade NK cell product and established an extremely efficient cytokine-based culture system for *ex vivo* expansion of NK cells from hematopoietic stem and progenitor cells from umbilical cord blood (UCB) (**chapter 5**). The *ex vivo*-generated NK cells efficiently lysed myeloid leukemia and melanoma tumor cell lines, and mediated cytolysis of primary leukemia cells at low NK:target ratios. This culture system exemplifies a major breakthrough in producing pure NK cell products from limited numbers of $CD34^+$ cells for NK cell-based immunotherapy.

As thorough knowledge of NK cell development is essential for the development of NK cell-based immunotherapies, we finally studied NK cell development in various human tissues using 10-color flow cytometry (FCM) (**chapter 6**). Using newly developed 10-color FCM panels, we were able to identify seven NK cell developmental stages in bone marrow and found phenotypical evidence that NK cell development is divided over multiple compartments of the human body (i.e. bone marrow, lymph nodes, spleen, peripheral blood) combined with differential *in situ* maturation of the NK cell receptor repertoire.

Finally, **chapter 7** summarizes this thesis and the applicability of NK cell-based immunotherapies in combination with allogeneic SCT or as a standalone immunotherapy in the treatment of leukemia is put in perspective.

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Chapter 2

$CD3^+/CD19^+$ depleted grafts in HLA-matched allogeneic peripheral blood stem cell transplantation lead to early NK cell cytolytic responses and reduced inhibitory activity of NKG2A

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Abstract

Natural killer (NK) cells play an important role in the anti-tumor response early after stem cell transplantation (SCT). As part of a prospective randomized phase III study, directly comparing the use of $CD3^+/CD19^+$ depleted peripheral blood stem cell (PBSC) harvests with $CD34^+$ selected PBSC harvests in allogeneic HLA-matched SCT, we here show that, the use of $CD3^+/CD19^+$ cell depleted PBSC grafts leads to early NK cell repopulation and reconstitution of the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, with concomitant high cytolytic capacity. In the CD34 group this process took significantly longer. Also, in the CD3/19 group after reconstitution,
a higher percentage of killer immunoglobulin-like receptor (KIR) positive NK cells was found. Although similar percentages of CD94 positive NK cells were found in both groups, in the CD34 group, almost all expressed the inhibitory CD94/NKG2A complex, whereas in the CD3/19 group, the inhibitory CD94/NKG2A and the activating CD94/NKG2C complex were equally distributed. This preferential development of NKG2C expressing NK cells in the CD3/19 group was paralleled by a loss of NKG2A-mediated inhibition of NK cell degranulation. These results show that the use of $CD3^+/CD19^+$ depleted grafts facilitates strong NK cell cytolytic responses directly after SCT, and the rapid emergence of a NK cell receptor phenotype that is more prone to activation.

Introduction

Natural killer (NK) cells are potent immune effector cells involved in the clearance of virus-infected and tumor cells. They are able to lyse infected or abnormal cells without prior sensitization or preactivation by antigen-presenting cells. NK cells survey potential target cells through a range of inhibitory and stimulatory receptors that detect the loss (or reduction) of expression of human leukocyte antigen (HLA) class I molecules or other nonclassical HLA class I specific signals.[1,2] The balance between the inhibitory and stimulatory signals triggers and modulates the NK cell effector function and the response of NK cells towards other cells.

The cytolytic function of NK cells is regulated by killer immunoglobulin-like receptors (KIR) including both inhibitory receptors (KIR-DL) and stimulatory receptors (KIR-DS). Whereas the ligands for the activating receptors are not yet unequivocally established, the ligands of KIR-DL receptors are specific for HLA class I molecules. KIR receptors are clonally distributed among NK cells within each individual.[3] Next to the KIR, another important receptor in modulating NK cell responses is the CD94/NKG2 heterodimeric complex. This receptor is part of the C-type lectin family and its ligand is the HLA-E class I molecule.[4] The CD94/NKG2 heterodimer can either have an inhibitory (NKG2A) or a stimulatory (NKG2C) function. Other NK cell receptors include the natural cytotoxicity receptors (NCR; NKp30, NKp44 and NKp46) and NKG2D (a C-type lectin homodimer). These receptors are capable of activating NK cells, whereby inducing NK cell-mediated cytolytic responses.[5]

As NK cells are efficient effectors in eradicating tumor cells, they play an important role in the anti-tumor response after allogeneic stem cell transplantation (SCT).[6-9] In HLA-haploidentical SCT, Ruggeri *et al.*[10] showed that donor alloreactive NK cells isolated from peripheral blood of the recipient were able to lyse tumor cells derived from the recipient, implying that within one month after SCT, NK cells may be able to provide some degree of immune reactivity by targeting residual tumor cells still present in the recipient. Indeed, fast recovery of NK cells and predicted alloreactivity towards host tumor cells have been associated with decreased relapse rates and better overall survival of the patient.[11-13] Recently, however, Nguyen *et al.*[14] showed that early-reconstituting NK cells have an immature phenotype and that NK cell alloreactivity is impaired at an early stage after haploidentical SCT. The results of this study emphasize that proper reconstitution of the NK cell receptor repertoire is probably more important for the induction of alloreactive NK cell responses than just fast NK cell recovery. One way to overcome delayed immune reconstitution of NK cells following SCT is to change the selection procedure of the graft. Recent data have shown that graft selection for haploidentical SCT by depletion of $CD3^+$ and $CD19^+$ cells, instead of positive $CD34^+$ selection, leads to faster engraftment and better overall immune reconstitution.[15]

In our centre, a phase III clinical study started in January 2006, directly comparing the use of $CD3^+/CD19^+$ depleted peripheral blood stem cell (PBSC) harvests with commonly applied $CD34^+$ selected PBSC harvests in allogeneic HLA-matched SCT. We investigated the immunological recovery and function of the NK cells after allogeneic SCT for a follow-up period of one year and compared the results between patients either having received a $CD3^+/CD19^+$ cell depleted graft or a $CD34^+$ cell selected graft.

Materials and methods

Study population

In a single-centre randomized phase III clinical study at the Radboud University Nijmegen Medical Centre (RUNMC), patients with AML in first complete remission, ALL in first complete remission or high risk MDS (RAEB, RAEB-t) in complete remission, eligible for allogeneic peripheral blood stem cell transplantation (PSCT) received G-CSF-mobilized peripheral blood stem cells of an HLA-identical sibling donor (SIB) or an HLA-A, -B, -C, -DR, -DQ-matched voluntary unrelated donor (VUD). In this study, patients were randomized to receive a peripheral blood stem cell graft processed either by positive $CD34^+$ immunomagnetic selection or T ($CD3^+$) and B cell ($CD19^+$) depletion by negative immunomagnetic selection using a CliniMACS cell selector device (Miltenyi Biotec, Bergisch Gladbach, Germany). All transplants contained at least $3x10^6$ $CD34^+$ cells and 0.5 $x10^6$ T cells/kg body weight. The amount of T cells was achieved by adding back cells from the harvest. Between January 2006 and November 2007, 23 patients were included in this study. This study was approved by the medical ethics committee of the RUNMC and written informed consent was provided by patients and donors according to the Declaration of Helsinki. Table 1 outlines the demographics of the patients selected for this study. This study is registered at http://clinicaltrials.gov as "T-Cell and B-Cell Depletion in Allogeneic Peripheral Blood Stem Cell Transplantation" (registration number: NCT00306332).

Table 1. Patient characteristics.

	Patient groups	
	$CD34^+$ selection	$CD3^+/19^+$ depletion
N	14	9
Median age of recipient, years (range)	46 (22-61)	47 (25-60)
Median age of donor, years (range)	46 (25-65)	42 (22-72)
Donor/recipient relation		
SIB	6	5
VUD	8	4
Diagnosis		
AML	8	4
ALL	3	2
MPD (low risk)	0	1
MDS	2	2
NHL	1	0

Abbreviations: SIB, sibling donor; VUD, voluntary unrelated donor; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; MPD, myeloproliferative disorder; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma.

Patient treatment procedures

All patients transplanted with a graft from a sibling donor were conditioned with idarubicine (total dose 42 mg/m^2) i.v.[16], cyclophosphamide (120 mg/kg body weight) i.v., and fractionated total body irradiation (TBI) given in two equal fractions on days -2 and -1 to a total dose of 9 Gy. In 2 patients TBI was replaced by busulphan (3.2 mg/kg body weight) intravenously. In patients transplanted with a graft from a voluntary unrelated donor (VUD) the conditioning regimen consisted of cyclophosphamide (120 mg/kg body weight), anti-thymocyte globulin (ATG) (2 mg/kg body weight) every 24 hours at four consecutive days (day -7 to -3) i.v and fractionated TBI given in two equal fractions on days -2 and -1 to a total dose of 9 Gy. The stem cells were infused 24 hours after completion of TBI or 72 hours after the last dose of busulphan. All patients were treated with cyclosporine A (CsA) 3 mg/kg/d i.v. from day -1 to +14, followed by 2 mg/kg/d i.v. infusion until oral administration of CsA (6 mg/kg/d) was possible. CsA was gradually tapered off and discontinued 12 weeks post-grafting. Patients who did not develop acute GVHD > grade 2 and/or chronic GVHD, according to the criteria described by Glucksberg and Shulman, received pre-emptive donor lymphocyte infusion (DLI) at a dose of 0.1 x 10^8 $CD3^+$ cells/kg body weight. All patients received oral selective gut decontamination (Ciprofloxacin), as well as co-trimoxazole for Pneumocystis carinii prophylaxis and oral acyclovir for prophylaxis of herpes infections.

Collection and preparation of samples

Blood samples (50 ml) were taken from patients before and at 0.5, 1, 2, 3, 6, 9, and 12 months after SCT. When possible, blood samples were collected from each donor before SCT. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Roskilde, Denmark). A fraction of the isolated PBMC was used to analyze the phenotype of the NK cells in detail. The remaining cells were cryopreserved in liquid nitrogen until further use. To evaluate immunological recovery, immunophenotyping was performed on fresh blood samples from patients.

Phenotypic analysis

The phenotype of NK cells was analyzed by four-color flow cytometry measured on a Coulter Epics XL (Beckman Coulter, Miami, FL, USA). For cell surface staining, the following conjugated mAbs were used: CD45-PE, CD16-FITC (Dako, Glostrup, Denmark), CD3-ECD, CD56-PCy5, CD158a,h-PE, CD158b_1/b_2,j-PE, NKG2A-PE, NKp30-PE, NKp44-PE, NK46-PE (Beckman Coulter, Miami, FL, USA), NKB1-PE (BD Biosciences, Erembodegem, Belgium), CD94-PE (Immunotech, Marseille, France), NKG2C-PE and NKG2D-PE (R&D Systems, Minneapolis, CA, USA). Isotype controls were used for marker settings. Analysis was performed using CXP analysis software (Beckman Coulter). All flow cytometric analyses were performed with a combination of CD3, CD16 and CD56 mAbs combined to a fourth specificity to gate specifically

on $CD3^{-}CD56^{dim}CD16^{bright}$ and $CD3^{-}CD56^{bright}CD16^{low/neg}$ NK cell populations and to distinguish them from NK-T and T cells. A dual platform technique was used to evaluate the immunological recovery of NK cells. With this technique, the absolute numbers of NK cells were calculated based on the flow cytometrically assessed percentage of $CD45^{+}CD56^{+}CD16^{+/-}CD3^{-}$ cells and the white blood cell count from a hematology cell analyzer (ADVIA120 Hematology System, Bayer Diagnostics, Leverkusen, Germany).

Functional analysis

For functional studies, cryopreserved PBMC were thawed and cultured overnight in the presence or absence of rhIL-2 (100 U/ml; Chiron, Amsterdam, the Netherlands) and rhIL-15 (10 ng/ml; BioSource International, Camarillo, CA, USA) in culture medium consisting of RPMI 1640 supplemented with pyruvate (0.02 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% human pooled serum (HPS), in a 37 °C, 95% humidity, 5% CO_2 incubator.

^{51}Chromium-release assay

Pre-stimulated or unstimulated PBMC were used to examine the NK cell cytolytic activity using the major histocompatibility complex (MHC) class I-deficient human erythroleukemia K562 cell line (ATCC#; CCL-243) as target cells at PBMC:target ratios ranging from 80:1 to 0.625:1 in a standard 4-h ^{51}Cr-release assay. All experiments were performed in triplicates and for each patient all samples were run together in one assay in order to avoid inter-assay variation. The percentage specific lysis was calculated as follows: % specific lysis = ((experimental release - spontaneous release)/(maximum release - spontaneous release))x100%. In order to express NK cell cytolytic activity independent of an arbitrary selection of E:T ratio and to allow reliable comparisons between samples of patients tested at different moments in time, the specific lysis was converted into Lytic Units (LU). LU are defined as the number of effector cells contained in 10^6 effector cells required to lyse 20% of the target cells.[17]

Redirection assays

Redirection assays were performed using the P815 cell line (ATCC#; TIB-64) as target cells. P815 cells were incubated in culture medium with pre-stimulated PBMC at an E:T ratio of 1:2 in the presence of mAbs (10 µg/ml) specific for NKG2C, NKG2A and NKp46 (R&D Systems, Minneapolis, CA, USA) or with IgG1, IgG2a and IgG2b isotype controls (R&D Systems, Minneapolis, CA, USA) at 37 °C, 95% humidity and 5% CO_2. E:T ratio's were based on the amount of live NK cells present in the PBMC fractions after overnight culture. To measure NK cell degranulation, cells were washed after two hours of incubation, stained on ice with fluorochrome-conjugated anti-CD107a (BD Biosciences, Erembodegem, Belgium), anti-CD56 and anti-CD3 mAbs and analyzed by flow cytometry. To calculate the specific degranulation, non-specific degranulation induced by isotype controls was deducted from the degranulation given by specific triggering. IFN-γ produced by NK cells was analyzed by intracellular staining after 5 hours of incubation with P815 target cells at

37 °C with 5 ng/µl of Brefeldin A (Sigma-Aldrich) for the last 4 hours. First, a surface staining was performed using anti-CD56 and anti-CD3 mAbs. For intracellular staining, Fix and Fix/Perm buffer (eBioscience, San Diego, CA, USA) were used according to the manufacturer's instructions in combination with IFN-γ-PeCy7 mAb (eBioscience, San Diego, CA, USA).

Statistical analysis

Nonparametric tests were used to compare continuous variables between two groups. To test differences between both patient groups or within one group between donors and recipients after SCT, a Mann-Whitney U test was performed. A Wilcoxon signed rank test was used to compare differences within each group between recipients before and after SCT. *P*-values <.05 were considered statistically significant.

Results

Patients

Patients eligible for inclusion in the study were randomized on a 1:1 basis for treatment with either a positive $CD34^+$ selected graft (CD34 group) or a T and B cell-depleted graft ($CD3^+$/$CD19^+$ depletion; CD3/19 group). Between January 2006 and November 2007, 14 patients were included in the CD34 group and 9 patients were included in the CD3/19 group. Patient and donor characteristics are depicted in Table 1. The composition of the grafts selected by $CD34^+$ selection and $CD3^+$/$CD19^+$ depletion are summarized in Table 2.

Table 2. Graft composition.

		Patient groups	
		$CD34^+$ selection	$CD3^+/19^+$ depletion
Nuclear cells	($x10^8$/kg)	7 ± 3	634 ± 156
$CD3^+$ cell	($x10^6$/kg)	0.43 ± 0.1	0.58 ± 0.1
Log T cell depletion		*2.9*	*2.8*
NK cells ($CD56^+CD3^-$)	($x10^6$/kg)	0.03 ± 0.02	26.6 ± 5.2
$CD19^+$ cell	($x10^6$/kg)	0.08 ± 0.06	0.40 ± 0.20
Log B cell depletion		*3.0*	*2.4*
$CD34^+$ cell	($x10^6$/kg)	4.6 ± 1.6	8.8 ± 3.9
CFU-GM	($x10^6$/kg)	53 ± 47	113 ± 31
BFU-E	($x10^6$/kg)	30 ± 19	207 ± 67

Abbreviations: CFU-GM, colony-forming units-granulocytes and macrophages; BFU-E, burst-forming units-erythrocytes.

After SCT, patients of both groups showed similar engraftment kinetics and there were no remarkable differences in the immunosuppressive regimens between the two groups (Table 3). There were no major differences in the occurrence of graft versus host disease (GVHD), fungal infections and relapse rate. The incidence of bacterial infections was slightly higher in the CD34 group, possibly due to the fact that the $CD34^+$ selected grafts contained less monocytes and granulocytes. In case of viral infections, we saw a tendency towards a higher infection rate in the CD3/19 group. This is due to a higher number of CMV reactivations that occurred within the CD3/19 group (4 patients vs. 1 patient in the CD34 group).

Table 3. Clinical follow-up.

	Patient groups	
	$CD34^+$ selection	$CD3^+/19^+$ depletion
N	14	9
Median follow-up; months (range)	12 (1-12)	6 (2-12)
Engraftment [a]		
$>0.5x10^6$/ml neutrophils	11 (10-15)	11 (10-16)
$>20x10^6$/ml platelets	11 (9-21)	11 (8-21)
Immunosuppression [b]		
CsA	82 (23-570)	85 (55-570)
Additional steroids	3	1
Acute GVHD		
No	8	5
Grade I	2	3
Grade II	2	1
Grade III	2	0
Grade IV	0	0
Chronic GVHD [c]		
No	6	4
Limited	4	2
Extensive	2	2
Infections		
Viral	4	5
Bacterial	10	5
Fungal	3	2
Relapse		
No	10	6
Yes	4	3

[a];The hematological recovery for each group is given as median days with ranges. [b];The immunosuppression for each group is given in median days after transplantation with ranges (CsA) and the number of patients receiving additional steroids. [c];Within 3 months after SCT, 2 patients died within the CD34 group and 1 patient died within the CD3/19 group.

Repopulation of NK cells after SCT

To study NK cell repopulation and reconstitution of the NK cell receptor repertoire, peripheral blood samples were taken from each patient prior to SCT and at 0.5, 1, 2, 3, 6, and 12 months after SCT and were analyzed for the presence of NK cells by flow cytometric analysis. At each time point, the absolute numbers of NK cells were analyzed within whole blood samples (Figure 1A). In the CD34 group, at day 14 after SCT only limited amounts of NK cells were present in the peripheral blood as compared to absolute NK cell numbers prior to SCT. The absolute numbers of NK cells reached pre-SCT levels one month after SCT. Thereafter, levels slowly increased and reached significant levels at 6 months after SCT ($P<.05$, compared to numbers prior to SCT). In the CD3/19 group, absolute numbers of NK cells in the peripheral blood reached pre-SCT level already at fourteen days after SCT. One month after SCT, the absolute NK cell numbers increased even further ($P<.05$). Comparison of the CD34 group with the CD3/19 group shows that at day 14 after SCT significantly higher numbers of NK cells were present in the CD3/19 group ($P<.05$).

The repopulation of the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets was analyzed within isolated PBMC fractions by gating on the lymphocytes by $CD45^{+}$ and a low side scatter and subsequently on $CD56^{+}$ and $CD3^{-}$ cells. In the CD34 group, the repopulation of the two NK cell subsets seemed to start with the development of the $CD56^{bright}$ NK cells. The balance between the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets was in favor of the $CD56^{bright}$ NK cells until 6 months after SCT (Figure 1B). This is comparable with the results described by previous studies in which the development of NK cells after SCT was studied.[18,19] In contrast, in the CD3/19 group, both NK cell subsets were already present at fourteen days after SCT (data not shown). Moreover, the outgrowth of the $CD56^{bright}$ NK cell subset was less profound as seen within the CD34 group and the balance between the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets was more comparable with the donor situation prior to SCT.

In contrast to the different reconstitution kinetics of the NK cells, the reconstitution of both T and B cells was comparable between the CD34 and CD3/19 group during the first three months after SCT (Figure S1).

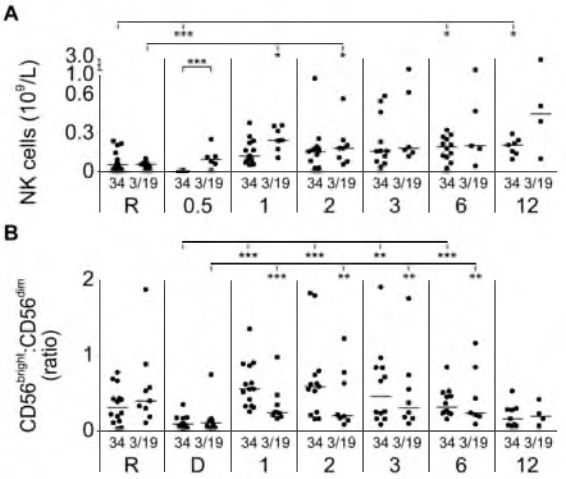

Figure 1. Recovery of NK cells after SCT. (A) The recovery of NK cells ($CD45^+CD56^+CD3^-$) after SCT within the CD34 group and the CD3/19 group. NK cell numbers were analyzed within whole blood samples of recipients before SCT (R) and at several time points after SCT using flow cytometry. Within each group, differences between recipients before SCT and after SCT were analyzed using the Wilcoxon signed rank test. The Mann-Whitney U test was used to test differences between both groups; **P*<.05, ****P*<.001. (B) Repopulation of NK cells and the different NK cell subsets, analyzed in freshly isolated PBMC fractions by flow cytometry, using CD3, CD45, CD16 and CD56 mAbs. Displayed are the ratios of the $CD56^{bright}$:$CD56^{dim}$ NK cell subsets before SCT and at different time points after SCT. R = recipients before SCT, D = donors. Within each group, differences between donors and recipients after SCT were analyzed using the Mann-Whitney U test; ***P*<.01, ****P*<.001.

Reconstitution of the NK cell receptor repertoire

As the repopulation of the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets was much faster in the CD3/19 group, suggesting an earlier presence of mature NK cells, we investigated whether this phenomenon was also reflected in the reconstitution of the NK cell receptor repertoire after SCT.

Comparison of KIR expression between the CD34 and the CD3/19 groups

In both groups, reconstitution of KIR2DL/S1, KIR2DL/S2/3 and KIR3DL1 expression was monitored in the total NK cell population ($CD45^+CD3^-CD56^+$) during the follow-up at different time points after SCT (Figure 2).

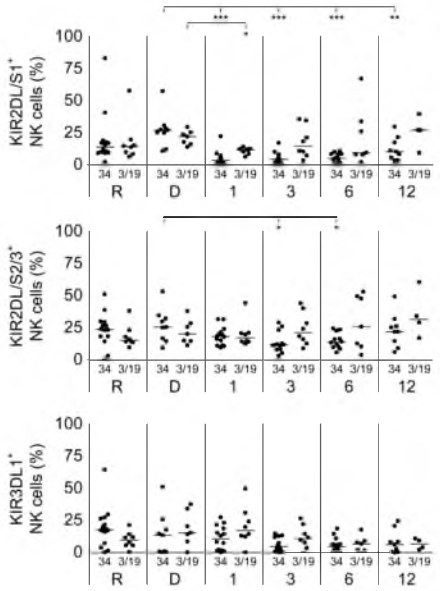

Figure 2. Reconstitution of KIR expression by NK cells. The reconstitution of KIR2DL/S1 (CD158a,h), KIR2DL/S2/3 (CD158b$_1$b$_{21}$j) and KIR3DL1 (NKB1) expression by CD45$^+$CD56$^+$CD3$^-$ NK cells was analyzed by flow cytometry in freshly isolated PBMC fractions, at different time points after SCT. Displayed are the percentage KIR positive cells in recipients before SCT (R), in donors (D) and in recipients at 1, 3, 6 and 12 months after SCT. Data are shown for the CD34 group and the CD3/19 group. Within each group, differences between donors and recipients after SCT were analyzed using the Mann-Whitney U test; **P*<.05, ***P*<.01, ****P*<.0001.

The reconstitution of KIR3DL1$^+$ NK cells already reached donor level within one month after SCT. Notably, during the remaining follow-up the percentage of KIR3DL1$^+$ NK cells was decreased and did not regain donor levels during the rest of the follow-up. Compared to KIR2DL/S, the reconstitution of KIR3DL1 seemed to show an opposite reconstitution pattern over time. This phenomenon has previously been described after HLA-mismatched/haploidentical SCT.[20]

In the CD3/19 group, KIR reconstitution was faster compared to the CD34 group (Figure 2). While in the CD34 group full reconstitution took a year, as has also been described by others[21], the NK cells in the CD3/19 group reached normal expression frequencies directly after SCT. Here, the amount of KIR2DL/S1$^+$ NK cells was only significantly decreased up to one month after SCT compared to donor level and within one year after SCT, expression levels were fully restored. In addition, the amount of KIR2DL/S2/3$^+$ and KIR3DL1$^+$ NK cells were already at donor level at 14 days after SCT (data not shown). Remarkably, during the remaining time of the follow-up the number of KIR2DL/S2/3$^+$ NK cells was enhanced from a median level of 19 percent at 2 months after SCT to 30 percent at one year after SCT. Similar to the CD34 group, reconstitution of the KIR3DL1$^+$ NK cells followed the same pattern over time and did not significantly decrease compared to donor level. Thus, as the reconstitution of the number of KIR2DL/S$^+$ NK cells was rather slow in the CD34 group and was significantly lower within the first months after

SCT, frequencies of KIR2DL/S expression in the CD3/19 group were not significantly different from donor level and reconstituted faster to full donor level. In summary, the NK cells quickly recovered in the CD3/CD19 group and maintained similar percentages of KIR bearing NK cells as compared to the donor before SCT.

Alternative reconstitution of CD94/NKG2A/C expression

During NK cell development, KIR expression is preceded by expression of CD94/NKG2A.[22] In the first months after SCT, NK cell subsets were shown to be characterized by a high frequency of CD94/NKG2A expression and low expression of KIR.[19-21] This suggests that at early stages after SCT, the function of NK cells is primarily regulated through the interaction of CD94/NKG2 heterodimeric complexes with HLA-E molecules. In both groups, the frequency of CD94 expressing NK cells was upregulated during the first 3 months after SCT, after which the percentage of CD94 expressing NK cells slowly decreased towards donor level (Figure 3).

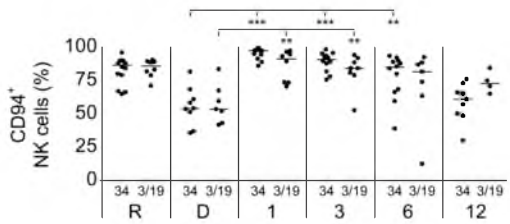

Figure 3. Reconstitution of CD94 expression by NK cells. CD94 expression by the $CD45^+CD56^+CD3^-$ NK cell population in freshly isolated PBMC fractions of donors (D) and of recipients before (R) and at 1, 3, 6 and 12 months after SCT, was analyzed using flow cytometry. Data are shown for the CD34 group and the CD3/19 group. Within each group, differences between donors and recipients after SCT were analyzed using the Mann-Whitney U test; $^{**}P<.01$, $^{***}P<.0001$.

In the CD34 group, the amount of $CD94^+$ NK cells was higher during the first months after SCT. However, frequencies of CD94 expressing NK cells between the two groups were similar from 3 months to one year after SCT. Remarkably, the NKG2A expression levels in the CD3/19 group were significantly decreased from a median percentage of 76 percent at one month to 36 percent at one year after SCT ($P<.05$) while at the same time the frequency of NKG2C expressing NK cells was significantly increased from a median of 8 to 53 percent, respectively ($P<.01$) (Figure 4A). This phenomenon appeared to be specific for the CD3/19 group, as this was not seen within the CD34 group (Figure 4B).

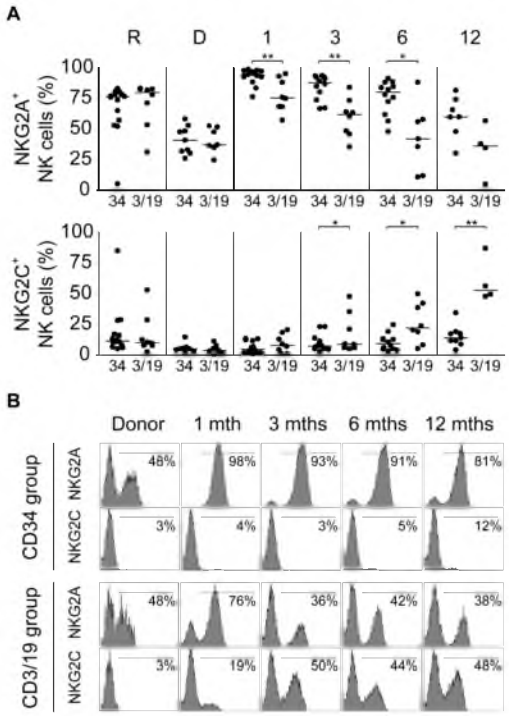

Figure 4. Reconstitution of NKG2A and NKG2C expression by NK cells after SCT. Expression of the inhibitory NKG2A and the activating NKG2C receptors by $CD45^+CD56^+CD3^-$ NK cells was analyzed by flow cytometry in freshly isolated PBMC fractions from recipients of both CD3/19 and CD34 groups. (A) Percentages of NKG2A and NKG2C expressing NK cells in recipients before SCT (R), in donors (D) and in recipients at 1, 3, 6 and 12 months after SCT in both groups. Differences between groups were analyzed using the Mann-Whitney U test; $^*P<.05$, $^{**}P<.01$. (B) A representative example of NKG2A and NKG2C expression on NK cells in individual recipients of either group over time. Histogram analysis shows the percentage of receptor expressing NK cells at different time points after SCT.

We observed no differences in the reconstitution of the other activating NK cell receptors (Figure 5). Although the frequencies of NKp30 expression seemed to drop slightly in the CD3/19 group as compared to the CD34 group, percentages of NKG2D and NCR expression were similar in both groups following SCT. In addition, frequencies of NKG2D, NKp30 and NKp44 expressing NK cells did not significantly change compared to donor levels. In both groups, the amount of NKp46 expression was significantly higher directly after SCT and remained above donor levels throughout the rest of the follow-up.

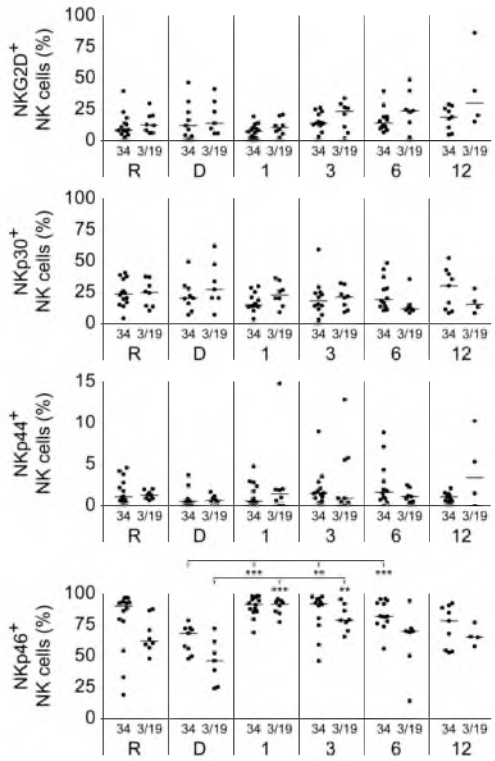

Figure 5. Reconstitution of activating NK cell receptors NKG2D, NKp30, NKp44 and NKp46. Percentages of NKG2D, NKp30, NKp44 and NKp46 expressing $CD45^+CD56^+CD3^-$ NK cells were analyzed in freshly isolated PBMC fractions for both the CD34 group and the CD3/19 group at 1, 3, 6 and 12 months after SCT. Within each group, differences between donors and recipients after SCT were analyzed using the Mann-Whitney U test; $^{**}P<.01$, $^{***}P<.0001$.

Cytolytic potential of repopulated NK cells

Cytolytic activity against HLA class I-negative target cells

We observed that, whereas in the CD34 group only few NK cells were present at day 14 after SCT and full repopulation in the CD34 group took place from one to two months after SCT, in the CD3/19 group the repopulation of NK cells had already taken place just after SCT. Also, we observed that the ratio of $CD56^{bright}$ versus $CD56^{dim}$ NK cells in the CD3/19 group after SCT was distinct from that found in the CD34 group. Thus, we set out to investigate whether the repopulation profiles and phenotypic differences were paralleled by a distinct functional activity of the cell populations. To assess the cytolytic potential of the repopulated NK cells after SCT, we performed cytolytic assays using the K562 cell line as a target. Prior to the assay, NK cells were incubated overnight in the absence or presence of IL-2 and IL-15 (Figure 6).

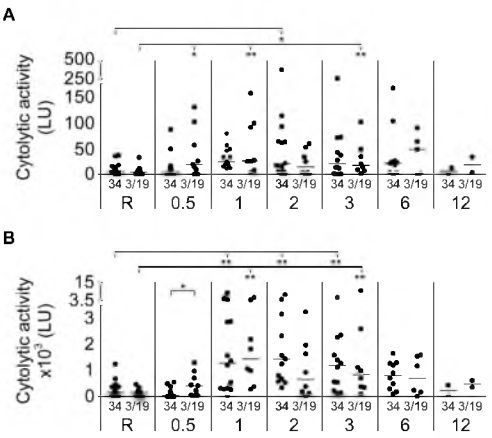

Figure 6. NK cell cytolytic activity against K562 target cells. Thawed PBMC fractions were cultured overnight in the absence (A) or presence (B) of 100 U/ml IL-2 and 10 ng/ml IL-15, and subsequently incubated with 51Cr-labeled K562 target cells in a standard 4-h ^{51}Cr release assay. Displayed are the Lytic Units (LU) within each PBMC fraction of the CD34 group and the CD3/19 group before SCT (R) and at 0.5, 1, 2, 3, 6 and 12 months after SCT. LU comparisons between recipients before SCT and after SCT were analyzed using the Wilcoxon signed rank test; **P*<.05, ***P*<.01. Differences between groups were analyzed using the Mann-Whitney U test; **P*<.05.

Unstimulated NK cells derived from the CD34 and CD3/19 group prior to SCT showed equal cytolytic activity (Figure 6A). But, after SCT, the NK cells in the CD3/19 group not only appeared to show a faster reconstitution of the different NK cell subsets and the NK cell receptor repertoire, at fourteen days after SCT they also showed stronger cytolytic capacity. Moreover, at this time point the cytolytic activity was even higher than before SCT (*P*<.05) and this was maintained throughout the rest of the follow-up. Parallel to the duration of NK cell repopulation, in the CD34 group enhanced cytolytic activity levels were only reached at one to two months after SCT. Although enhanced, the trend in cytolytic activity was similar for the pre-stimulated NK cells (Figure 6B). This implies that the use of $CD3^+/19^+$ depleted grafts for SCT may lead to an earlier and longer time frame of NK cell cytolytic activity after SCT.

Diminished NK cell inhibition through NKG2A triggering in the CD3/19 group

Previous studies showed that high expression levels of CD94/NKG2A after SCT may lead to impaired cytolytic NK cell function.[14] As the NKG2A:NKG2C ratio in the CD3/19 group was in favor of the stimulatory NKG2C receptor, we set out to investigate whether this distinct phenotype was associated with enhanced functional activity. To this end, we set up a redirection assay in which NK cells were triggered by P815 cells labeled with NKG2C and NKp46 specific antibodies in the absence or presence of NKG2A specific antibodies. Typically, triggering of NK cells with NKp46 and NKG2C antibodies leads to NK cell degranulation, whereas NKG2A triggering leads to inhibition of NK cell degranulation. At one month after SCT, the CD3/19 and CD34 groups showed similar NKG2A-mediated inhibition of NK cell degranulation (Figure 7), which is in line with the similar percentage of NKG2A expressing NK cells in both groups (Figure 5).

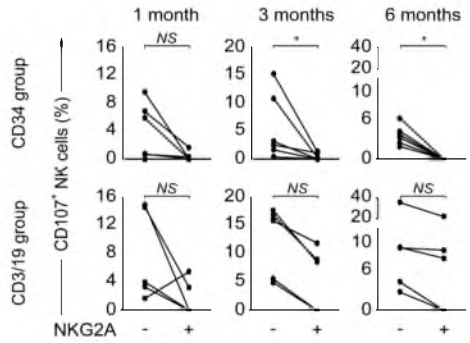

Figure 7. Redirected degranulation of NK cells by specific triggering of the activating NKG2C and NKp46 receptors in the presence or absence of NKG2A specific antibodies. PBMC fractions of the CD34 group (n=6) and the CD3/19 group (n=5) were stimulated overnight with IL-2 (100 U/ml) and IL-15 (10 ng/ml) and subsequently incubated for 2 h at a NK:target cell ratio of 1:2 in a redirection assay using P815 target cells and NKG2A, NKG2C, NKp46 or isotype control mAbs. Specific degranulation was determined within the $CD56^+CD3^-$ NK cell population as the percentage of $CD107^+CD56^+CD3^-$ NK cells with specific mAbs minus the percentage of $CD107^+CD56^+CD3^-$ NK cells in the presence of isotype control mAbs. Negative values are displayed as equal to 0. Specific degranulation was determined for recipients at 1, 3 and 6 months after SCT. Significance of inhibition through NKG2A was analyzed using the Wilcoxon signed rank test; NS = not significant, $*P<.05$

Notably, from one month post-SCT onwards, only in the CD3/19 group the inhibitory effect of NKG2A triggering was diminished, corresponding with an increased frequency of $CD94/NKG2C^+$ NK cells and a decreased percentage of $CD94/NKG2A^+$ NK cells. In the CD34 group the inhibitory effect of NKG2A triggering was maintained. In addition, the percentage of degranulating NK cells as induced by NKG2C and NKp46 triggering tended to be higher in the CD3/19 group as compared to the CD34 group (3 months: median of 16 vs. 3%; at 6 months: median of 9 vs. 3%). The level of degranulation, induced by NKG2C and NKp46 triggering was also reflected in the IFN-γ production (data not shown), as the NK cells within the CD3/19 group produced higher levels of IFN-γ.

In conclusion, we demonstrate that the use of CD3/19 depleted grafts for allogeneic HLA-matched SCT leads to a preferential development of NKG2C expressing NK cells, paralleled by a loss of NKG2A-mediated inhibition of NK cell degranulation at later stages after SCT.

Discussion

Immediately after stem cell transplantation, alloreactive NK cells have been shown to be beneficial not only for boosting the anti-tumor response, but also for the prevention of GVHD as well as infections. In these cases, full functional activity of NK cells already in the early phase after SCT is essential, and therefore the presence of NK cells in the graft appears to be beneficial for transplant outcome.[15;23] In the current study, we show that patients that received a $CD3^+/CD19^+$ cell depleted graft, which contains NK cells in substantial numbers, exhibited a faster recovery of a functional NK cell receptor repertoire as compared to patients that received a conventional $CD34^+$ graft while both groups were treated under the same immunosuppressive regimen as part of their GVHD prophylaxis. Furthermore, transplantation with a $CD3^+/CD19^+$ cell depleted graft resulted in the development of a functionally different NK cell population that was more prone to activation via the NKG2C/CD94 receptor complex and less sensitive to inhibition via the NKG2A/CD94 receptor complex.

Several studies have shown that the beneficial effect of NK cell activity after SCT is dependent on the fast reconstitution of a cytolytic NK cell repertoire that is regulated by KIR[24] and that a slower reconstitution towards a KIR dependent repertoire is associated with poor clinical outcome.[14;20;21;25] In the group receiving a $CD3^+/CD19^+$ cell depleted graft, we observed functional NK cells already at 14 days after SCT, with KIR expressing NK cell numbers similar to those found in the donor before SCT. After 14 days, the cytolytic activity and number of KIR expressing cells increased even further and remained high during follow up. In contrast, in patients that received a $CD34^+$ cell selected graft KIR reconstitution took much longer, whereby during the first six months after SCT the repertoire was skewed towards NKG2A expressing cells rather than KIR expressing cells, a phenomenon also described by others.[21]

So it would appear that the conditions created after SCT using a $CD3^+/CD19^+$ cell depleted graft support the development of a KIR based repertoire. The clinical relevance of such a finding is exemplified by the fact that in the clinical studies that reported a role for NK cells in the anti-tumor response after SCT, the nature of the interaction between KIR and HLA class I appeared decisive for the outcome.[8;9;25-27] The most straightforward example hereof is the haploidentical SCT setting, whereby donor NK cells expressing KIR specific for self HLA class I ligands sense the absence of this ligand in the recipient. This results in cytolytic responses against the recipient tumor cells and reduced risk of relapse ("missing self").[8;28-30] A similar phenomenon can also be observed in HLA-matched SCT for AML, when the donor expresses a KIR for which the ligand is missing in the patient ("missing ligand").[27] Although it is appealing to incorporate the receptor-ligand model into the criteria for donor selection in HLA-matched SCT, recent findings in the haploidentical setting demonstrated that this model is more complex as not only inhibitory KIR, but also certain activating KIR play a crucial role in the process of tumor cell recognition and eradication.[31]

The high NK cell numbers observed in the CD3/19 group may be the result of NK cells present in the graft. Indeed it has been shown that alloreactive NK cells can expand and persist for at

least 28 days.[32] Alternatively and not mutually exclusive, the high numbers may be a result of de novo generation from CD34$^+$ progenitor cells since the dose of CD34$^+$ cells was much higher in the CD3/19 group as compared to the CD34 group.

Caligiuri and Freud[33] have proposed a model for NK cell development based on different maturation stages of NK cells in secondary lymphoid organs. According to this model, NK cells go through four maturation stages before they become mature peripheral NK cells (stage V). In the last stage (IV) before reaching maturity, the NK cells have high expression of CD56 and express NKG2A. In stage V, the NK cells have a lower level of CD56 expression and are KIR and/or NKG2A positive.[34] This fits the observations made after SCT using a CD34$^+$ cell selected graft. We and others[18,21] observed that most of the NK cells found in peripheral blood in the first six months after SCT resemble those in stage IV (CD56^{bright} and NKG2A$^+$), and that gradually over time more NK cells reach stage V. Clinical data suggest that a sustained high number of NK cells resembling stage IV (CD56^{bright} and NKG2A$^+$) is associated with poor clinical outcome.[14;20] Interestingly, the formation of the NK cell receptor repertoire in case of CD3$^+$/CD19$^+$ cell depleted grafts is clearly different. Not only is there a faster shift to a KIR based repertoire, but the reduction in NKG2A expressing cells appears to coincide with the occurrence of a population expressing the activating NKG2C receptor. At one year after SCT as much as 50% of the NK cells express NKG2C. Normally, in peripheral blood only few mature NK cells express NKG2C. Also, only low numbers of stage IV NK cells express NKG2C.[33] The occurrence of the NKG2C expressing NK cell population may have important consequences for the anti-tumor response after SCT. The stimulatory receptor NKG2C, like NKG2A binds to HLA-E, although the affinity of NKG2C for HLA-E appears to be lower.[35] The NK cells in the CD3/19 group were more prone to activation by NKG2C and NKp46 triggering in terms of cytolytic activity and IFN-γ production. Furthermore, the inhibitory effect of NKG2A triggering on the whole population was lost at later stages after SCT, coinciding with the occurrence of NKG2C expressing NK cells. In contrast, NK cells in the CD34 group maintained a high frequency of NKG2A expressing cells and NKG2A triggering completely inhibited cytolytic NK cell activity. A similar observation was made for NK cells after CD34$^+$ haploidentical SCT.[14] Also in this patient group practically all NK cells expressed NKG2A during the first four months after SCT and expression of HLA-E on the tumor cells completely blocked the cytolytic activity of NKG2A$^+$ NK cells. This may suggest that a shift in balance from a CD94/NKG2A$^+$ to a more CD94/NKG2C$^+$ phenotype in the CD3/19 group, and a subsequent overruling of NKG2A-mediated inhibition, may lead to a stronger anti-tumor response and a better clinical outcome.

Although the two patient groups are yet too small to compare clinical outcomes, we did observe that human cytomegalovirus (CMV) reactivation was more common in the CD3/19 group (4 out of 9 patients) than in the CD34 group (1 out of 14 patients). In previous studies it was demonstrated that CMV has an impact on the expression of NK cell receptors on NK cells and CD8$^+$ T cells irreversibly resulting in increased CD94/NKG2C expression levels and subsequently the loss of CD94/NKG2A expression.[36-38] However, when we excluded the CMV positive patients from our analyses, the change from an overall NKG2A$^+$ to a more NKG2C$^+$ phenotype was also seen in

patients that did not suffer from CMV reactivation (data not shown). The alternative reconstitution of the NK cell receptor repertoire, characterized by the change in balance of CD94/NKG2A$^+$ NK cells to more CD94/NKG2C$^+$ NK cells, and its impact on clinical outcomes after SCT is therefore a subject for further study.

In summary, in this study we show that the use of selectively depleted grafts (CD3$^+$/CD19$^+$ cell depletion) as compared to CD34$^+$ cell selected grafts in HLA-matched SCT leads to faster reconstitution of the KIR repertoire resulting in a longer time frame after SCT for NK cell cytolytic activity. Moreover, at a later stage after SCT, there was a large population of NKG2C expressing cells and NK cells appeared to be more prone to activation. Extended studies are underway to reveal whether these findings have an effect on clinical outcome.

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Supplemental material

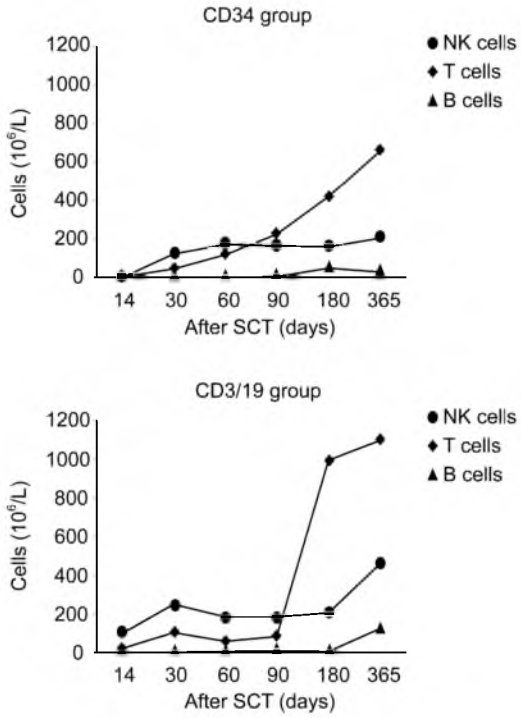

Figure S1. Reconstitution of T, B, and NK cells for the CD34 and CD3/19 group. Shown is the reconstitution for T, B, and NK cells (x10^6/L) within the CD34 (upper panel) and CD3/19 group (lower panel) after SCT. Concentrations of cells are depicted as median concentrations at day 14, 30, 60, 90, 180 and 365 after SCT.

Chapter 3

In vitro skewing of the human KIR repertoire leads to enhanced NK cell alloreactivity

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Abstract

In allogeneic stem cell transplantation (SCT), NK cells are able to perform graft-versus-leukemia (GVL) responses without the induction of graft-versus-host disease (GVHD), a feature that can be exploited for cellular immunotherapy. NK cell alloreactivity is induced when inhibitory killer immunoglobulin-like receptors (KIR) fail to recognize HLA molecules on potential target cells. In this study, we examined the plasticity of the KIR repertoire and the cytolytic response of mature human peripheral blood NK cells in the presence of specific KIR receptor-ligand (i.e. HLA-C) mismatches. We show that the introduction of specific KIR-ligand mismatches (KIR vs. HLA-C) *in vitro* favors the outgrowth of KIR^+ NK cells lacking their cognate ligand leading to an increased frequency of alloreactive KIR^+ NK cells within the whole NK cell population. Furthermore, after culture, these KIR^+ NK cells are more cytolytic on a per cell basis towards KIR-ligand mismatched K562 target cells and primary leukemic target cells. This study demonstrates that HLA class I can skew the mature NK cell phenotype and enrich the alloreactive NK cell population towards leukemic target cells, which may be important for future clinical applications of NK cell-based immunotherapies in transplantation settings.

Introduction

Circumventing graft-versus-host disease (GVHD) while boosting graft-versus-leukemia (GVL) responses after hematopoietic stem cell transplantation (SCT) is one of the major challenges within the field of transplantation today.[1-4] In this respect, natural killer (NK) cells are of interest for use in immunotherapeutic strategies as they form the first line of defense in mediating immunity against microbial pathogens and are efficient effectors in eradicating tumor cells without inducing severe GVHD.[5-8]

NK cells survey potential target cells for the absence or loss of expression of HLA class I molecules or other non-classical HLA class I specific signals through killer cell immunoglobulin-like receptors (KIR) and the inhibitory CD94/NKG2A receptor complex.[9,10] Overall, HLA class I expression is surveyed by the lectin-like receptor CD94/NKG2A through its recognition of the ubiquitously expressed HLA-E molecule.[11] KIR allow for a more subtle surveillance as these receptors recognize specific epitopes present on HLA-A, -B, or -C molecules. HLA-A3 and -A11 are recognized by KIR3DL2, HLA-Bw4 is recognized by KIR3DL1 and receptors KIR2DL1 and KIR2DL2/3 are able to distinguish HLA-C into HLA-C1 and HLA-C2 molecules.[12]

In allogeneic SCT, anti-leukemic NK cell alloreactivity can be facilitated by allowing mismatches for specific KIR-ligands (HLA), i.e. HLA-B and/or HLA-C, between donor and recipient (KIR ligand-ligand model). The introduction of certain HLA mismatches has been shown to induce NK cell-mediated GVL responses, without inducing severe GVHD, and to contribute to decreased relapse, better engraftment and improved overall survival.[13-15] However, others state that the induction of NK cell alloreactivity is not dependent on HLA mismatching, but is rather induced by the presence of an inhibitory KIR in the donor's genotype with the absence of the corresponding KIR-ligand in the recipient's HLA repertoire (receptor-ligand model).[16-18] In SCT, the KIR repertoire of NK cells was shown to be important for the induction of alloreactive NK cell responses.[19-22] However, studies have focused on the KIR repertoire and its reconstitution of NK cells emerging from $CD34^+$ cells after SCT and less is known about the plasticity of the KIR repertoire of mature human NK cells when used for immunotherapeutic strategies.

In this study, we set up an *in vitro* culture system based on the receptor-ligand model and investigated the plasticity of the phenotype and cytolytic response of mature human peripheral NK cells within a KIR receptor-ligand (KIR vs. HLA-C) mismatched environment. Results demonstrate that the presence of a specific KIR-ligand (HLA-C) present on feeder cells skews the response of healthy donor NK cells by favoring the outgrowth of NK cells expressing the KIR that lacks its cognate KIR-ligand, resulting in an oligoclonal increase of specific alloreactive NK cells bearing improved cytolytic ability. These results may be important for future clinical applications of NK cell-based immunotherapy within the field of transplantation.

Materials and methods

Cell isolation and genotyping

Buffy coats from healthy human donors were purchased from Sanquin Blood Bank, Nijmegen, The Netherlands, upon written informed consent with regard to scientific use according to Dutch law. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). NK cells were negatively selected (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in a purity of more than 95%. Parallel to the experiments, HLA-C and KIR genotyping were performed on small samples from all buffy coats (Table 1). Data analysis was focused on NK cell donors having KIR2DL1, 2 and 3 in their KIR repertoire.

Table 1. KIR genotype and HLA-C type of NK cell donors.

	KIR genotype for KIR2DL/S1/2/3 and KIR3DL/S1[a]								HLA-C[a]	
Donor[b]	2DL1	2DL2	2DL3	2DS1	2DS2	2DS3	3DL1	3DS1	C1	C2
1	1	0	1	1	0	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	0	1	1
4	1	1	1	0	1	1	1	0	1	1
5	1	1	1	0	1	1	1	0	1	1
6	1	1	1	1	1	1	1	1	2	0
7	1	1	1	1	1	1	1	1	1	1
8	1	0	1	0	0	1	1	0	1	1
9	1	1	1	1	1	1	0	1	2	0
10	1	1	0	1	1	1	1	1	1	1
11	1	1	1	1	1	0	1	1	2	0
12	1	1	0	1	1	1	1	0	1	1
13	1	1	1	1	1	1	1	1	2	0
14	1	1	1	0	1	*ND*	1	*ND*	1	1

[a]Coding for KIR genotype and HLA-C type; 1 = present, 0 = not present, ND = not able to determine. [b]The following donors were selected for detailed analysis based on the presence of KIR2DL1/2/3; 2, 3, 4, 5, 6, 7, 9, 11, 13, 14.

Cell lines

Single-cell-derived clonal K562 cell lines expressing HLA-Cw*0301 (K562-C1) and HLA-Cw*0403 (K562-C2) were used as feeder cells during culture and as target cells in functional assays. Briefly, HLA class-I deficient K562 cells were transfected with full length HLA-Cw0301 cDNA inserted into the EcoRI and HindIII sites of expression vector pcDNA3.1(-) (Invitrogen, Paisley, UK) and HLA-Cw*0403 cDNA ligated into the pEF6/V5-His expression vector (pEF6/V5-His TOPO TA Expression Kit; Invitrogen, Paisley, UK). The cell lines K562-Cw*0301 and K562-Cw*0403 showed stable and strong HLA class I expression (>95%) analyzed by flow cytometry using the HLA class I antibody W6/32 (Sigma, Steinheim, Germany). The expression

of HLA-Cw*0301 and HLA-Cw*0403 was confirmed by mRNA profiling (sequencing of HLA-Cw cDNA). As a control, HLA class I-negative K562 cells transfected with empty vector pcDNA3.1 were used (K562-Cneg).

In vitro culture system

Freshly isolated NK cells were cultured with irradiated K562-C1, K562-C2, and K562-Cneg feeder cells in a 1:1 ratio in the presence of rhIL-2 (10 U/ml; Chiron, Amsterdam, the Netherlands) and rhIL-15 (1 ng/ml; BioSource International, Camarillo, CA, USA) in culture medium consisting of RPMI 1640 medium supplemented with pyruvate (0.02 mM), glutamax (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% human pooled serum (HPS), in a 37 °C, 95% humidity, 5% CO_2 incubator.

CFSE based division analysis

Cell division was studied by CFSE dilution patterns. Freshly isolated NK cells were labeled with 0.1 µM CFSE (Molecular Probe, Eugene, OR, USA), aliquoted in CFSE labeling buffer (PBS containing 0.02% HPS), for 10 minutes at RT in the dark. The reaction was stopped by addition of equal volumes of cold HPS. Subsequently, cells were washed three times with CFSE labeling buffer and resuspended in culture medium. CFSE-labeled NK cells were cultured as described above and were analyzed using flow cytometry.

Flow cytometry

Non-CFSE labeled NK cells were phenotypically analyzed on the FC500 (Beckman Coulter, Miami, FL, USA) using the following conjugated mAbs: NKAT2-FITC (KIR2DL/S2/3), CD158a-PE (KIR2DL/S1), NKB1-PE (KIR3DL1), CD3-PC5, CD56-PC7 (Beckman Coulter). CFSE-labeled NK cells were analyzed on the Gallios™ using the following conjugated mAbs: CD16-FITC (Dako), CD3-ECD, CD56-APC-A750, CD158b-PC7 (KIR2DL/S2/3), CD158e1-APC (KIR3DL1), CD158a-APC-A700 (KIR2DL/S1), CD45-PO (all provided by Beckman Coulter). For 10-color flow cytometry, fluorochrome combinations were balanced to avoid antibody interactions, sterical hindrance and to detect also dimly expressing populations. Before 10-color analyses were performed, all conjugates were titrated and individually tested for sensitivity, resolution and compensation of spectral overlap. Isotype controls were used to define marker settings.

Functional analysis

The functionality of NK cells was studied by degranulation patterns (CD107a expression) upon target encounter. To this end, NK cells were harvested from culture and viable NK cells were plated in 96-well V-bottom plates in culture medium supplemented with fluorochrome-

conjugated anti-CD107a (1:200; BD Biosciences, Erembodegem, Belgium). Subsequently, non-irradiated K562-C1, K562-C2, K562-Cneg, or Kasumi-1 (HLA-C1 homozygous) target cells were added at an E:T ratio of 1:2 and incubated at 37 °C, 95% humidity, 5% CO_2. After 4 hours, the degranulation of NK cells was phenotypically analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Results of different conditions were compared using paired student t-tests or repeated measures ANOVA analysis with the Tukey multiple comparison test for post-testing. *P*-values <.05 were considered statistically significant.

Results

To study the phenotypical changes and cytolytic response of mature peripheral NK cells within a KIR-ligand mismatched environment, we set up an *in vitro* culture system in which KIR-ligand mismatches were introduced through HLA-C transfected K562 cells. To this end, freshly isolated NK cells were cultured in the presence of IL-2 and IL-15 together with irradiated K562 feeder cells transfected with HLA-C1 (K562-C1), HLA-C2 (K562-C2), or an empty vector (K562-Cneg) in a 1:1 ratio. Subsequently, NK cells were phenotypically and functionally analyzed.

NK cell phenotype is skewed *in vitro* by mismatched KIR-ligands

At day 7 of culture, NK cells were phenotypically analyzed for KIR2DL/S1/2/3 and KIR3DL1. As all donors showed a KIR3DL1 expression between 0 to 2% (data not shown), we omitted this from further analysis and focused on the expression of KIR2DL/S1$^+$KIR2DL/S2/3- (single KIR2DL/S1$^+$) and KIR2DL1-KIR2DL/S2/3$^+$ (single KIR2DL/S2/3$^+$). Skewing of the NK cell phenotype was seen for both KIR subsets in the presence of a specific KIR-ligand mismatched environment (e.g. KIR vs. HLA-C) (Figure 1). Single KIR2DL/S1$^+$ NK cells were significantly more present within cultures containing “mismatched” HLA-C1$^+$ feeder cells compared to cultures containing “matched” HLA-C2$^+$ or HLA-C negative feeder cells (Figure 1A). Visa versa, percentages of single KIR2DL/S2/3$^+$ NK cells showed elevated levels in the presence of “mismatched” HLA-C2$^+$ feeder cells compared to cultures containing “matched” HLA-C1$^+$ or, although not in all cases, HLA-C negative feeder cells (Figure 1B). Thus, *in vitro* data suggest that a specific KIR-ligand mismatched environment leads to a favored NK cell phenotype in which NK cells preferably express the KIR receptor for which the cognate ligand is lacking.

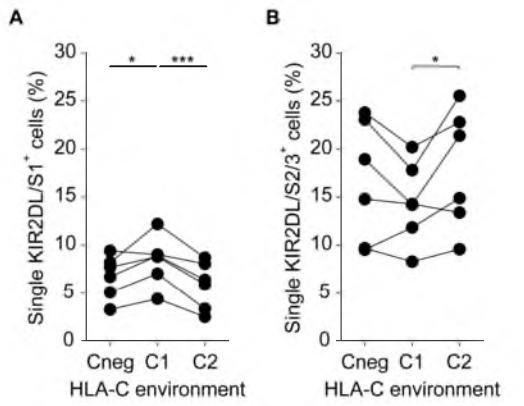

Figure 1. Distribution of KIR2DL/S1 and KIR2DL/S2/3 on KIR3DL1- NK cells at day 7 of culture in a non-specific (HLA-Cneg), HLA-C1 and HLA-C2 environment. Freshly isolated NK cells were cultured in the presence of irradiated HLA-Cneg, HLA-C1$^+$, and HLA-C2$^+$ K562 feeder cells. The phenotype of NK cells was analyzed at day 7 of culture. (A) Shown are the percentages of single KIR2DL/S1$^+$ cells within the total NK cell population after non-specific (HLA-Cneg), HLA-C1 and HLA-C2 stimulation at day 7 of culture. Differences between the different stimulation settings were analyzed using repeated measures ANOVA analysis; *P<.05, ***P<.001. (B) Shown are the percentages of single KIR2DL/S2/3$^+$ cells within the total NK cell population after non-specific (HLA-Cneg), HLA-C1 and HLA-C2 stimulation at day 7 of culture. Differences between the different stimulation settings were analyzed using repeated measures ANOVA analysis; *P<.05.

Mismatched KIR-ligand induces oligoclonal division of specific KIR positive NK cells

To investigate whether the skewed NK cell phenotype through a KIR-ligand mismatched environment was due to clonal expansion of specific KIR$^+$ subsets, we performed CFSE based division analyses. To this end, CFSE-labeled NK cells were cultured within a KIR-ligand mismatched environment. At day 7, the cell division of the cultured CFSE-labeled NK cells was analyzed (Figure 2). In the presence of both HLA-C1$^+$ and HLA-C2$^+$ feeder cells, KIR negative NK cells were able to divide within culture. However, in the presence of HLA-C1$^+$ feeder cells, 2 out of 3 donors showed that single KIR2DL/S1$^+$ NK cells had a higher division percentage than single KIR2DL/S2/3$^+$ NK cells (Figure 2; open bullets). The opposite effect was seen in the presence of HLA-C2$^+$ feeder cells as single KIR2DL/S2/3$^+$ NK cells were triggered to divide more than single KIR2DL/S1$^+$ NK cells (Figure 2; closed bullets). Thus, within a KIR-ligand mismatched environment, KIR$^+$ NK cells that lack their cognate ligand are more prone to expand in culture compared to the KIR$^+$ NK cells that are able to recognize their proper ligand *in vitro*. This suggests that KIR-ligand mismatching guides the proliferation of allospecific KIR$^+$ NK cells. Overall, these results suggest that the introduction of NK cells to a specific KIR-ligand mismatched environment triggers the outgrowth of KIR$^+$ NK cells lacking their cognate KIR-ligand and therefore skews the NK cell phenotype towards a specific KIR repertoire. As these reactive NK cells can be cytolytic towards the same KIR-ligand mismatched cells, we further hypothesized that a specific KIR-ligand mismatched environment may lead to an increase of specific cytolytic KIR$^+$ NK cells within the whole NK cell population.

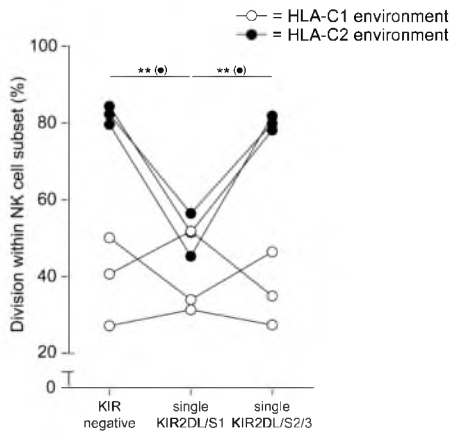

Figure 2. Division of single KIR2DL/S1$^+$, single KIR2DL/S2/3$^+$ and KIRneg NK cell subsets at day 7 of culture in an HLA-C1 and HLA-C2 environment. CFSE-labeled NK cells were cultured in the presence of irradiated HLA-C1$^+$ and HLA-C2$^+$ K562 feeder cells and analyzed at day 7 of culture. Shown are the results for 3 donors of which NK cells were cultured in an HLA-C1 (o) and an HLA-C2 (●) environment. Depicted are the percentages of divided cells within the KIR negative, single KIR2DL/S1$^+$ and single KIR2DL/S2/3$^+$ NK cell subsets at day 7 of culture. Differences in division between the different NK cell subsets were analyzed using repeated measures ANOVA analysis; $^{**}P<.01$.

Skewing of KIR$^+$ NK cells is associated with increased functional alloreactivity

To investigate whether a KIR-ligand mismatched environment leads to an increase of specific cytolytic KIR$^+$ NK cells within the whole alloreactive (CD107$^+$) NK cell population, HLA-C1 and HLA-C2 stimulated NK cells were analyzed for their degranulation potential against K562-C1 and K562-C2 target cells at day 7 of culture (Figure 3). Results showed that stimulation with "mismatched" HLA-C1$^+$ feeder cells led to an increase of cytolytic KIR2DL/S1$^+$ NK cells against K562-C1 target cells as compared to the percentage of KIR2DL/S1$^+$ NK cells stimulated by "matched" HLA-C2$^+$ feeder cells (Figure 3A,B). Vice versa, stimulation of NK cells with "mismatched" HLA-C2$^+$ feeder cells increased the percentage of KIR2DL/S2/3$^+$ NK cells within the alloreactive CD107$^+$ NK cell pool against K562-C2 target cells compared to the percentage of KIR2DL/S2/3$^+$ NK cells stimulated by "matched" HLA-C1$^+$ feeder cells (Figure 3A,C). These results show a clear trend, albeit not significant, that stimulation of NK cells with a specific KIR-ligand mismatch may lead to an increase of specific cytolytic KIR$^+$ NK cells within the whole alloreactive NK cell pool against target cells expressing the same KIR-ligand mismatch. As the percentage of the specific cytolytic KIR$^+$ NK cells increased, we further investigated whether this would lead to an enhanced cytolytic alloresponse on a per cell basis.

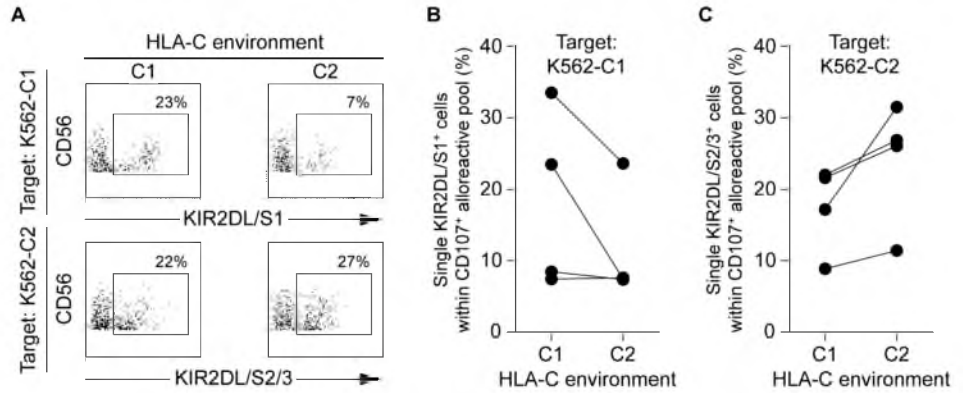

Figure 3. Distribution of single KIR2DL/S1$^+$ and single KIR2DL/S2/3$^+$ NK cells within the alloreactive (CD107$^+$) NK cell population upon target encounter. Freshly isolated NK cells of 4 donors were cultured in an HLA-C1 and HLA-C2 environment and analyzed for degranulation (CD107$^+$) at day 7 of culture against K562-C1 and K562-C2 target cells. (A) Shown are the results for one representative donor. The upper panels depict the percentages of alloreactive (CD107$^+$) single KIR2DL/S1$^+$ NK cells within the whole CD107$^+$ NK cell pool upon K562-C1 target encounter at day 7 of culture in an HLA-C1 and HLA-C2 environment. The lower panels depict the percentages of alloreactive (CD107$^+$) single KIR2DL/S2/3$^+$ NK cells within the whole CD107$^+$ NK cell pool upon K562-C2 target encounter at day 7 of culture in an HLA-C1 and HLA-C2 environment. (B) Shown are the percentages of single KIR2DL/S1$^+$ NK cells within the alloreactive (CD107$^+$) NK cell population upon K562-C1 target encounter at day 7 of culture in an HLA-C1 and HLA-C2 environment. Differences between the different stimulation settings were analyzed using paired t-tests. (C) Shown are the percentages of single KIR2DL/S2/3$^+$ NK cells within the alloreactive (CD107$^+$) NK cell population upon K562-C2 target encounter at day 7 of culture in an HLA-C1 and HLA-C2 environment Differences between the different stimulation settings were analyzed using paired t-tests

Increased specific cytolytic alloresponse upon KIR-ligand mismatching

As we were interested to know whether specific cytolytic KIR$^+$ NK cells show enhanced alloreactivity when pre-stimulated by target cells lacking the inhibitory KIR-ligand, we tested the degranulation potential of KIR2DL/S1$^+$ NK cells against KIR-ligand mismatched (K562-C1) target cells after 7 days of pre-stimulation with “mismatched” HLA-C1$^+$ feeder cells (K562-C1) and compared this with pre-stimulation with HLA-negative feeder cells (K562-Cneg) or unstimulated NK cells (D0) (Figure 4A). Stimulating the cells non-specifically for 7 days (K562-Cneg) led to an enhanced cytolytic alloresponse (% degranulating cells) of KIR2DL/S1$^+$ NK cells as compared to freshly isolated NK cells (D0) against K562-C1 target cells. Thus, stimulation of NK cells by co-culture with K562 cells leads to increased alloreactivity. More importantly, stimulation of NK cells in the presence of the specific KIR-ligand mismatch for KIR2DL/S1$^+$ (C1) showed an even stronger cytolytic response of KIR2DL/S1$^+$ NK cells towards K562-C1 target cells as compared to non-specifically stimulated NK cells (Cneg).

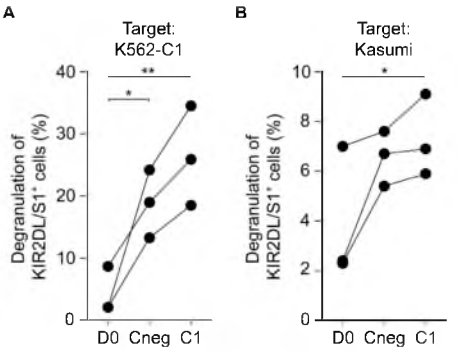

Figure 4. Degranulation of single KIR2DL/S1$^+$ NK cells on a per cell basis upon target encounter. Freshly isolated NK cells of 3 donors were cultured in a non-specific (HLA-Cneg) and HLA-C1 environment. NK cells were analyzed for degranulation before culture (D0) and at day 7 of culture upon the encounter of (A) K562-C1 target cells and (B) the HLA-C1 homozygous primary tumor cell line Kasumi. Differences in degranulation within the single KIR2DL/S1$^+$ NK cell subset before and after culture were analyzed repeated measures ANOVA analysis; **P*<.05, ***P*<.01.

As priming of NK cells towards their target is of interest for future NK cell therapies against hematological malignancies or for treatment of solid tumors, we further investigated if the specific KIR-ligand mismatched stimulation would trigger the cytolytic KIR$^+$ NK cell subset towards increased killing of a primary tumor cell line. To this end, we tested freshly isolated NK cells before culture (D0) and non-specific stimulated (Cneg) and specific KIR-ligand mismatched stimulated NK cells (C1) at day 7 of culture against a primary HLA-C1 homozygous tumor cell line (Kasumi) (Figure 4B). Comparable to the alloreactivity towards K562-C1 target cells, results show that overall stimulation of NK cells within culture (Cneg) leads to enhanced alloreactivity of KIR2DL/S1$^+$ NK cells towards the primary HLA-C1 homozygous Kasumi tumor cell line. In addition, the alloreactivity of the KIR2DL/S1$^+$ NK cells is further improved when NK cells are specifically stimulated with "mismatched" HLA-C1$^+$ feeder cells. Thus, by specifically introducing a KIR-ligand mismatch within the culture system, specific KIR$^+$ NK cells can be primed to give a better cytolytic alloresponse towards non-previously encountered primary tumor cell lines bearing the same KIR-ligand mismatch.

Discussion

Regulation of cytolytic responses of alloreactive NK cells through interactions between inhibitory KIR and HLA class I ligands has well been described.[12] In this study, we show that the phenotype of mature human NK cells from healthy donors can be skewed through KIR-ligand (KIR vs. HLA-C) mismatches resulting in an enrichment of specific alloreactive NK cells bearing higher cytolytic responses towards specified targets. A simplified model summarizing our findings is shown in Figure 5. This is the first study that uses a designed *in vitro* culture system, in which specific KIR-ligand mismatched feeder cell lines skew mature human NK cell phenotype and its cytolytic response.

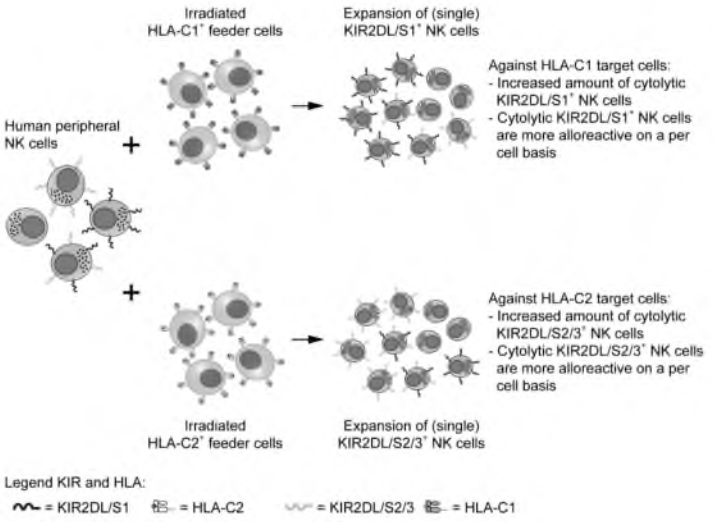

Figure 5. Skewing of the human KIR repertoire of mature peripheral NK cells leads to enrichment of specific cytolytic KIR$^+$ NK cells against HLA-C1$^+$ or HLA-C2$^+$ target cells. Proposed model showing a simplified summary on how the human NK cell KIR repertoire and the NK cell cytolytic response can be skewed *in vitro* in the presence of an HLA-C1 or HLA-C2 environment. Here, a human peripheral NK cell population is shown containing KIR negative, KIR2DL/S1$^+$ (red), KIR2DL/S2/3$^+$ (blue), and KIR2DL/S1$^+$/KIR2DL/S2/3$^+$ (red and blue) NK cells. In the presence of "mismatched" irradiated HLA-C1$^+$ feeder cells (upper scheme), KIR2DL/S1$^+$ NK cells expand in culture leading to an increase in cytolytic KIR2DL/S1$^+$ NK cells within the whole alloreactive NK cell population upon HLA-C1$^+$ target encounter. In addition, these cytolytic KIR2DL/S1$^+$ cells are more alloreactive on a per cell basis as compared to freshly isolated or non-specific stimulated KIR2DL/S1$^+$ NK cells. Vice versa, in the presence of "mismatched" irradiated HLA-C2$^+$ feeder cells (lower scheme), KIR2DL/S2/3$^+$ NK cells expand in culture leading to an increase in cytolytic KIR2DL/S2/3$^+$ NK cells within the whole alloreactive NK cell population upon HLA-C2$^+$ target encounter. In addition, although not analyzed in this study, these cytolytic KIR2DL/S2/3$^+$ cells may be more alloreactive on a per cell basis as compared to freshly isolated or non-specific stimulated KIR2DL/S2/3$^+$ NK cells.

Previous research by Rose *et al.*[23] showed that the KIR repertoire of human NK cells can be skewed according to the KIR ligand-ligand model[13], but not by the receptor-ligand model.[16] They showed that when feeder cells were mismatched with NK cells for a specific KIR-ligand (ligand-ligand mismatch), the frequency of the cognate KIR in the NK cell population would increase compared to autologous or allogeneic HLA-matched cultures. Our results, however, when using HLA-C1 homozygous donor NK cells, support the receptor-ligand model.[16] In this setting, the NK

cell population from these donors showed an increase in the frequency of KIR2DL/S1$^+$ cells when cultured in the presence of HLA-C1 feeder cells and of KIR2DL/S2/3$^+$ cells in the presence of HLA-C2 feeder cells. Thus, we show that as well in an HLA-matched as in an HLA-mismatched situation, KIR positive NK cells lacking their cognate ligand in culture are able to increase in frequency. The difference in these findings is most likely explained by the substantially different culture methods used in these two studies. The former study tested polyclonal NK cells starting from CD3$^+$-depleted PBL which were broadly activated by PHA and cultured in the presence of high amounts of IL-2 together with HLA-mismatched PBMC and later PHA-blasts for a duration twice as long as our culture protocol.[23] Our culture setup may be more subtle as we used highly purified and freshly isolated NK cells and cultured them directly, without pre-activation, in the presence of low amounts of IL-2 and IL-15 with HLA-homozygous or HLA-negative K562 tumor cell lines for only 7 days. Although the initial amount and increase of cell numbers are not that high as in the former study, without severe pre-activation and cytokine stimulation, this setup, that focuses purely on the interaction between KIR and HLA-C, may allow for the detection of a more genuine reaction of mature human NK cells *in vitro*.

Remarkably, in our CFSE based division analyses, the effect through HLA-C2 mismatching tended to be stronger than HLA-C1 mismatching. However, this might be explained by the results of the HLA-C typing of the donors. Of the 3 donors used for these experiments (Table 1; donors 11, 13 and 14), 2 out of 3 were HLA-C1 homozygous, whereas only one was HLA-C1/C2 heterozygous. According to the NK cell "licensing/education" model[24-26], KIR2DL/S1$^+$ NK cells of HLA-C1 homozygous donors may be hyporesponsive as these cells lacked their cognate ligand *in vivo.* This subsequently results in a damped response within a mismatched HLA-C1 environment. From these same donors, the KIR2DL/S2/3$^+$ NK cells are properly armed as their ligand, HLA-C1, has been present *in vivo* and thus these cells are perfectly able to respond within a mismatched HLA-C2 environment. The KIR2DL/S1$^+$ and KIR2DL/S2/3$^+$ NK cell subsets from the HLA-C1/C2 donor are both properly armed and thus able to respond within, respectively, a mismatched HLA-C1 or HLA-C2 environment (Figure 2B). Thus, these results suggest that the self HLA-C background of NK cells is an important determinant for the strength of their response in the presence of a KIR-ligand mismatch *ex vivo*. Concerning adoptive transfer of mature NK cells for immunotherapeutic purposes, the results of this study suggest that the presence of inhibitory KIR on donor NK cells in absence of its cognate ligand in the recipient as well as the HLA-background of the donor NK cells are two key factors that determine the alloreactive NK cell response within the recipient.

During NK cell maturation, the KIR repertoire is pre-dominantly formed by the KIR genotype of the cells and is only mildly influenced by the HLA class I type.[27;28] The minor role of HLA class I in shaping the KIR repertoire is also reflected in the setting of allogeneic SCT, as reconstitution of the KIR repertoire mainly reflects that of the donor and not the recipients'.[19;29] This *in vitro* study, however, shows that the KIR repertoire of human mature NK cells can be reshaped in the presence of a specific KIR-versus-HLA mismatch. Moreover, *in vitro* reshaping of the KIR repertoire of mature NK cells, within a specific KIR-versus-HLA mismatched environment,

increases the cytolytic response of the alloreactive KIR$^+$ NK cells against target cells lacking the cognate ligand. Thus, as the KIR genotype is important in the formation of the basic KIR repertoire during NK cell maturation, the HLA class I environment is pre-dominant in reshaping the KIR repertoire and determining the strength of the cytolytic NK cell response after NK cell maturation. Overall, these findings hold promise for future transplantation strategies using mature NK cells as effectors and future research is warranted to optimize and exploit the skewing of NK cell responses towards specific targets for the immunotherapeutic treatment of hematological malignancies.

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CHAPTER 4

Rapamycin and MPA, but not CsA, impair human NK cell cytotoxicity due to differential effects on NK cell phenotype

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Abstract

Cyclosporin A (CsA), rapamycin (Rapa) and mycophenolic acid (MPA) are frequently used for GVHD prophylaxis and treatment after allogeneic stem cell transplantation (SCT). As NK cells have received great interest for immunotherapeutic applications in SCT, we analyzed the effects of these drugs on human cytokine-stimulated NK cells *in vitro*. Growth-kinetics of CsA-treated cultures were marginally affected, whereas MPA and Rapa severely prevented the outgrowth of $CD56^{bright}$ NK cells. Single-cell analysis of NK cell receptors using 10-color flow cytometry revealed that CsA-treated NK cells gained a similar expression profile as cytokine-stimulated control NK cells, mostly representing $NKG2A^{+}KIR^{-}NCR^{+}$ cells. In contrast, MPA and Rapa inhibited the acquisition of NKG2A and NCR expression and NK cells maintained an overall $NKG2A^{-}KIR^{+}NCR^{+/-}$ phenotype. This was reflected in the cytolytic activity, as MPA- and Rapa-treated NK cells, in contrast to CsA-treated NK cells, lost their cytotoxicity against K562 target cells. Upon target encounter, IFN-γ production was not only impaired by MPA and Rapa, but also by CsA. Overall, these results demonstrate that CsA, MPA and Rapa each have distinct effects on NK cell phenotype and function, which may have important implications for NK cell function *in vivo* after transplantation.

Introduction

Graft-versus-host disease (GVHD) is a major complication after allogeneic stem cell transplantation (SCT) resulting in high mortality rates. Various immunosuppressive drugs (ISD) are applied to efficiently prevent GVHD. These drugs mainly focus on targeting and suppressing unwanted adaptive immune responses. However, the effect on NK cell responses after SCT remains largely unknown.

NK cells have shown to possess immunotherapeutic activity in SCT. NK cells form the first line of defense in mediating immunity against microbial pathogens, directly through cytolysis of virus-infected cells and indirectly by the production of inflammatory cytokines such as IFN-γ.[1,2] Furthermore, NK cells of donor origin are efficient effectors in eradicating tumor cells without inducing severe GVHD.[3-5] In HLA-haploidentical SCT, Ruggeri *et al.*[6] showed that donor alloreactive NK cells isolated from peripheral blood of recipients were able to lyse tumor cells derived from the recipient. This implicates that NK cells may provide immune reactivity after SCT by targeting residual tumor cells. For HLA-matched SCT, we have previously shown that the use of NK cell-enriched stem cell grafts leads to early NK cell repopulation with concomitant high cytolytic capacity within the first months after SCT.[7]

The cytolytic and cytokine-producing activities of NK cells are regulated through a range of inhibitory and stimulatory receptors. Killer immunoglobulin-like receptors (KIR), including inhibitory (KIR-DL) and stimulatory receptors (KIR-DS), specifically recognize HLA-A,-B and -C molecules.[8] The CD94/NKG2 heterodimeric complex, with its inhibitory (NKG2A) and stimulatory (NKG2C) form, is part of the C-type lectin family and recognizes HLA-E class I molecules.[9] Other stimulatory NK cell receptors include the natural cytotoxicity receptors (NCR; NKp30, NKp44 and NKp46), NKG2D (a C-type lectin homodimer) and 2B4 (CD244).[10] The balance between the inhibitory and stimulatory signals triggers and modulates NK cell effector function.[11,12] Although NK cells are exposed to ISD administered to the patient post-SCT, they have to exert optimal anti-tumor reactivity and produce cytokines. Among the various ISD, cyclosporin A (CsA), rapamycin (Rapa) and mycophenolic acid (MPA; the active metabolite of MMF) have been applied successfully for the prevention of GVHD.[13-17] However, these drugs may influence NK cell reactivity, which could have implications for their use in clinical immunotherapeutic settings. Due to distinct experimental designs, previous studies on the effect of CsA on human and murine NK cells have shown contradictory results with respect to the functional activity of NK cells after treatment.[18-23] In contrast to CsA, the effect of Rapa and MPA has been studied less extensively. A previous study on rat NK cells directly comparing CsA with Rapa, showed that Rapa, in contrast to CsA, significantly inhibited the growth and cytotoxicity of rat NK cells.[22] Also, in a mouse SCT model, it has been shown that MMF does not inhibit graft-versus-leukemia responses or the activity of lymphokine-activated killer (LAK) cells, suggesting that MPA may not affect functional NK cell responses after SCT.[24] Here, we tested the effects of CsA, Rapa and MPA on peripheral human NK cells and show that these drugs each have a distinct impact on NK cell phenotype and function, which may have important implications for NK cell function *in vivo* after transplantation.

Materials and methods

Cell isolation

Buffy coats from healthy human donors were purchased from Sanquin Blood Bank, Nijmegen, The Netherlands, upon written informed consent with regard to scientific use. PBMC were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). NK cells were negatively selected (Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in a purity of more than 95%.

Culture conditions and ISD

Freshly isolated NK cells were cultured in the presence of rhIL-2 (100 U/ml; Chiron, Amsterdam, the Netherlands) and rhIL-15 (10 ng/ml; BioSource International, Camarillo, CA, USA) in culture medium (RPMI 1640 medium supplemented with pyruvate (0.02 mM), glutamax (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% human pooled serum (HPS)), in a 37 °C, 95% humidity, 5% CO_2 incubator. CsA (Novartis Pharma B.V., Arnhem, The Netherlands), MPA (Sigma-Aldrich, Zwijndrecht, The Netherlands), or Rapa (LC Laboratories, Woburn, MA, USA) was added at final concentrations ranging from 0.01 to 10 µg/ml (CsA and MPA) or 0.1 to 100 ng/ml (Rapa) based on therapeutic serum levels handled at our medical centre and previously published data.[25-28]

Flow cytometry

Cells were phenotypically analyzed using the FC500 and the Gallios™ Flow Cytometer (Beckman Coulter, Miami, FL, USA). The following conjugated mAbs were used for four-color stainings (analyzed on the FC500): CD16-FITC (Dako, Glostrup, Denmark), CD69-PE, CD3-ECD, CD56-PC7 (Beckman Coulter). To exclude dead cells from analysis, 7-amino-actinomycin-D (7-AAD; Sigma-Aldrich) was added to cells prior to acquisition. For 10-color analyses on the Gallios™, the following conjugated mAbs were combined: CD16-FITC (Dako), CD159c-PE (NKG2C; R&D Systems, Minneapolis, CA, USA), CD3-ECD, CD56-APC-A750, CD158b-PC7 (KIR2DL/S2/3), CD158e1-APC (KIR3DL1), CD158a-APC-A700 (KIR2DL/S1), CD159a-PB (NKG2A), CD45-PO, CD336-PE (NKp44), CD337-PC5.5 (NKp30), CD335-PC7 (NKp46), CD314-APC (NKG2D), CD244-APC-A700 (2B4) (all provided by Beckman Coulter). The combinations were balanced in fluorochrome combinations to avoid antibody interactions, sterical hindrance and to detect also dimly expressing populations. Before 10-color analyses were performed, all conjugates were titrated and individually tested for sensitivity, resolution and compensation of spectral overlap. Isotype controls were used to define marker settings.

Proliferation assays

CFSE based proliferation analysis

Freshly isolated NK cells were labeled with 0.1 µM CFSE (Molecular Probe, Eugene, OR, USA),

aliquoted in CFSE labeling buffer (PBS containing 0.02% HPS), for 10 minutes at RT in the dark. The reaction was stopped by addition of equal volumes of cold HPS. Subsequently, the cells were washed three times with CFSE labeling buffer and resuspended in culture medium. The CFSE-labeled NK cells were cultured as described above and were analyzed using flow cytometry.

Quantitative flow cytometric analysis

Freshly isolated NK cells were incubated with CD16-FITC (Dako) and CD56-PC5 (Beckman Coulter) for 20 minutes at RT in the dark. $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^{+}$ cells were separated using high purity FACS (Altra flow cytometer, Beckman Coulter). After 5 days of culture, cells were stained for CD56 and CD16 and counted by flow cytometry using Flow-Count fluorospheres (Beckman Coulter). 7-AAD (Sigma-Aldrich) was used to exclude dead cells from analyses.

Cytotoxicity assay

HLA class-I deficient K562 target cells were labeled with 3.7 MBq (100 µCi) chromium-51 (^{51}Cr; Perkin Elmer, Boston, MA, USA) for 2 hours in a 37 °C, 95% humidity, 5% CO_2 incubator. Labeled target cells were washed four times with culture medium and plated in triplicate in 96-well V-bottom plates (10^3 cells/well). NK cells were harvested from culture and viable NK cells were added in a 1:1 ratio and incubated for 4 hours at 37 °C, 95% humidity, 5% CO_2. Supernatant was harvested and analyzed using a gamma counter. The percentage specific lysis was calculated as follows: % specific lysis=((experimental release-spontaneous release)/(maximum release-spontaneous release))x100%. To assess the IFN-γ production of NK cells during target encounter, parallel incubations with unlabeled K562 target cells were performed.

ELISA

IFN-γ production of NK cells during incubation with K562 cells was measured by ELISA (Sanquin (CLB), Amsterdam, The Netherlands). Supernatants were collected after 4 hours of incubation and stored at -80 °C until further use. IFN-γ ELISA was performed according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0. For comparisons between cytokine-stimulated control NK cells and NK cells cultured in the presence of ISD, we used ANOVA analysis with Dunnett's multiple comparison test for post-testing. Paired t-tests were used to analyze the statistical difference between freshly isolated NK cells and cytokine-stimulated control NK cells. The Wilcoxon signed rank test was used for non-normally distributed data. *P*-values <.05 were considered statistically significant.

Results

Dose-dependent inhibition of NK cell proliferation

To study the effect of CsA, Rapa, and MPA on the proliferation capacity of isolated NK cells, CFSE-labeled NK cells were cultured in the presence of 100 U/ml IL-2 and 10 ng/ml IL-15 with increasing concentrations of the drugs. After a 5-day culture, CFSE dilution patterns showed a median proliferation rate of 44% (range 22–78%) of cytokine-stimulated control NK cells (Figure 1A), in five different division cycles (Figure 1B). Rapa and MPA significantly inhibited NK cell proliferation in a dose-dependent way. In contrast, CsA only showed a trend towards reduced proliferation.

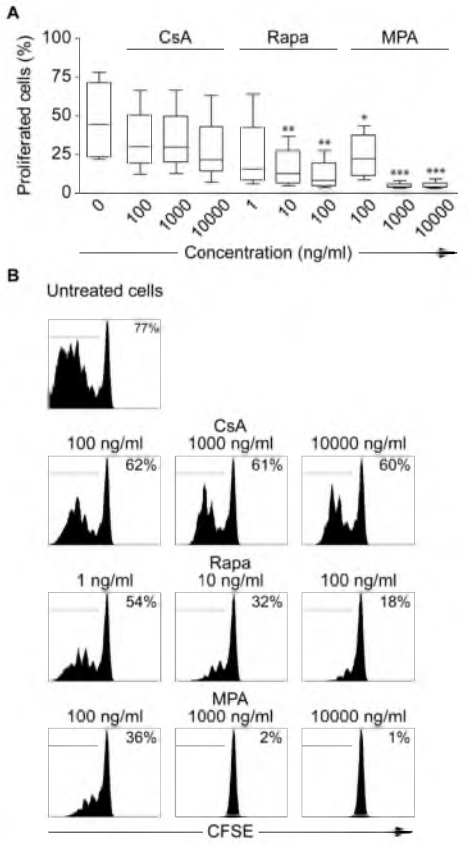

Figure 1. Proliferation of NK cells at day 5 of culture after CsA, Rapa and MPA treatment. (A) The proliferation of NK cells isolated from 4 different donors with increasing amounts of CsA, Rapa and MPA. Results are shown in median with range. Differences between the cytokine-stimulated control and drug-treated NK cells were analyzed using ANOVA; **P*<.05, ***P*<.01, ****P*<.001. (B) CFSE division patterns of cytokine-stimulated control and CsA-, Rapa- and MPA-treated NK cells from one representative donor. Numbers in the upper right are the percentage of divided cells.

Freshly isolated NK cells can generally be separated into two distinguishable subsets. The CD56^{bright}CD16$^{+/-}$ subset comprises 5-10% of all peripheral blood NK cells, while the rest has a CD56^{dim}CD16^{+} phenotype.[29] In steady state, the CD56^{bright}CD16$^{+/-}$ subset is described to be more immunoregulatory through cytokine production (e.g. IFN-γ, TNF-α), whereas the CD56^{dim}CD16^{+} subset is more cytotoxic. After activation, both subsets are able to produce cytokines and possess cytolytic activity. We analyzed the proliferation capacity of both subsets under influence of the different ISD. The enriched CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} subsets, having equal expression patterns of the IL-2 and IL-15 receptor (Figure S1, Table S1), were incubated separately in the presence or absence of CsA, Rapa and MPA (Figure 2).

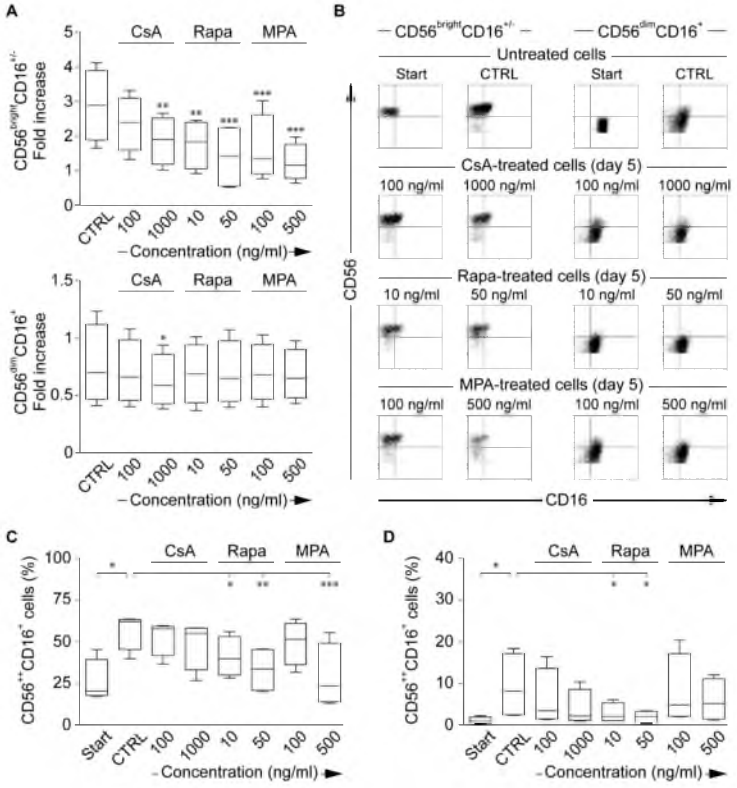

Figure 2. CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cell subsets at day 5 of culture after CsA, Rapa and MPA treatment. (A) Fold expansion of sorted CD56^{bnght}CD16$^{+/-}$ NK cells (upper panel) and sorted CD56^{dim}CD16^{+} NK cells (lower panel) isolated from 4 different donors with increasing amounts of CsA, Rapa and MPA. Results are shown in median with range. (B) CD56/CD16 phenotype of CD56^{bnght}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cells before and after culture of one representative donor. The upper panels show the CD56/CD16 phenotype of both NK cell subsets before and after culture without ISD. The lower panels show the phenotype of the NK cell subsets after CsA, Rapa and MPA treatment. (C) The percentage of CD56^{++}CD16^{+} cells within the sorted CD56^{bnght} subset before (start) and after culture in the absence (CTRL) or presence of CsA, Rapa and MPA. Results are shown in median with range. (D) The percentage of CD56^{++}CD16^{-} cells within the sorted CD56^{dim} subset before (start) and after culture in the absence (CTRL) or presence of CsA, Rapa and MPA. Results are shown in median with range. The difference cytokine-stimulated control NK cells before (Start) and after culture (CTRL) was analyzed using paired t tests; *P<.05, **P<.01, ***P<.001. Differences between the cytokine-stimulated control (CTRL) and drug-treated NK cells were analyzed using ANOVA; *P<.05, **P<.01, ***P<.001.

The cytokine-stimulated control $CD56^{bright}CD16^{+/-}$ cells showed a significant increase in cell number after 5 days of culture as compared to the initial amount of cells (P=.014), whereas the amount of $CD56^{dim}CD16^{+/-}$ cells decreased (P=.065) suggesting that the proliferation seen in the whole NK cell population (Figure 1) is only due to the proliferation of the $CD56^{bright}CD16^{+/-}$ NK cell subset (Figure 2A). Treatment of either NK cell subset with ISD showed that Rapa and MPA impaired the growth of the $CD56^{bright}CD16^{+/-}$ cells, whereas CsA only inhibited the growth of these cells at higher concentrations. In contrast to the $CD56^{bright}CD16^{+/-}$ subset, Rapa and MPA did not affect the amount of $CD56^{dim}CD16^{+}$ cells. However, CsA showed a significant decrease at 1000 ng/ml. Thus, Rapa and MPA only influence the amount of NK cells within the $CD56^{bright}CD16^{+/-}$ subset, whereas CsA is capable of affecting NK cell numbers of both subsets.

Rapa and MPA, but not CsA, affect the $CD56^{bright}/CD56^{dim}$ balance

Interestingly, while maintaining their $CD56^{bright}$ phenotype, the sorted $CD56^{bright}CD16^{+/-}$ subset increased the expression of CD16 during culture (P<.05), whereas the $CD56^{dim}CD16^{+/-}$ cells elevated their CD56 expression (P<.05) and lowered their CD16 expression (Figure 2B (upper panels), C, D). In the $CD56^{bright}CD16^{+/-}$ subset, Rapa (10 ng/ml, P<.05; 50 ng/ml, P<.01) and MPA (500 ng/ml, P<.001) dose-dependently inhibited the upregulation of CD16 expression, whereas CsA-treated $CD56^{bright}CD16^{+/-}$ cells revealed a similar phenotype as compared to cytokine-stimulated control cells at day 5 of culture (Figure 2B (left panels), C, D). As cytokine-stimulated control $CD56^{dim}CD16^{+/-}$ cells elevated their CD56 expression, especially Rapa inhibited the upregulation of CD56 with increasing concentrations (10 ng/ml and 50 ng/ml, P<.01; Figure 2B (right panels), C, D). This inhibition was also seen after treatment with CsA and MPA, although to a lesser extent.

The differential immunosuppressive effect on the proliferation and phenotype of the separately cultured NK cell subsets was also reflected on the phenotype in cultures of the whole NK cell population. During culture, the balance between the $CD56^{bright}$ and $CD56^{dim}$ subsets shifted towards a more $CD56^{bright}$ phenotype during culture (Figure 3A,B). As CsA showed to have a marginal effect on the growth of $CD56^{bright}CD16^{+/-}$ cells and did not strongly affect the changing phenotype of the NK cell subsets (Figure 2), the whole NK cell population showed the same shift towards a $CD56^{bright}$ phenotype as compared to cytokine-stimulated control cells (Figure 3A,B). In contrast, mainly due to their strong inhibitory effect on the proliferation of the $CD56^{bright}$ subset, the majority of the NK cell population after Rapa and MPA treatment maintained a $CD56^{dim}$ phenotype. Thus, under immunosuppressive treatment, Rapa and MPA, but not CsA, inhibit the outgrowth of $CD56^{bright}$ NK cells and thereby affect the $CD56^{bright}/CD56^{dim}$ distribution of the whole NK cell population.

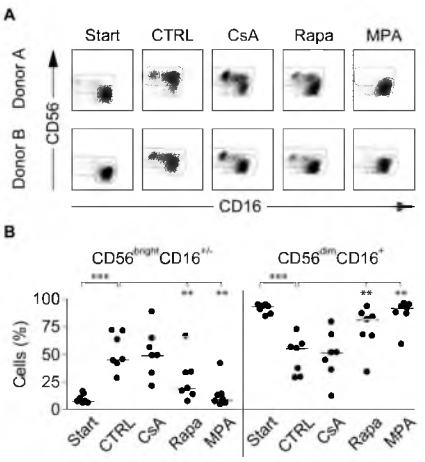

Figure 3. CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} distribution within the NK cell population before and after culture with CsA, Rapa and MPA. (A) Shown are two representative donors before (start) and after culture in the absence (CTRL) or presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). Gated are the CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cells. (B) The percentage of CD56^{bright}CD16$^{+/-}$ (left panel) and CD56^{dim}CD16^{+} (right panel) NK cells within the NK cell population. Shown are the results for 7 different donors before and after culture. The difference between cytokine-stimulated control NK cells before (S) and after culture (CTRL) was analyzed using paired t tests; ***P<.001. Differences between the cytokine-stimulated control (CTRL) and drug-treated NK cells were analyzed using ANOVA; **P<.01.

Differential phenotypical changes of cultured NK cells under immunosuppressive treatment

Using 10-color flow cytometry, we analyzed the effect of the different ISD on the phenotype of cultured NK cells. After a 5-day culture, cytokine-stimulated control NK cells showed a significant increase in the percentage of activating receptors (NKG2D, NCR) as compared to freshly isolated NK cells (Table 1). In addition, the percentage of KIR^{+} cells was reduced, paralleled by a significant increase in the percentage of NKG2A^{+} cells. Thus, in a cytokine-driven NK cell culture, the NK cell population appears to reach a more activated state and shifts the inhibitory control by the KIR repertoire towards control by the NKG2A receptor complex.

Cytokine-stimulated NK cells treated with CsA only showed minor changes in the percentages of NK cell receptor-positive cells as compared to cytokine-stimulated control NK cells (Table 1). With respect to KIR expression, the percentage of KIR2DL/S2/3^{+} NK cells was significantly lower (P<.05) and there was significant less increase of the percentage of NKG2A^{+} NK cells (P<.05). With regard to NCR expression, the percentage of NKp30^{+} NK cells was significantly higher as compared to cytokine-stimulated control NK cells (P<.05). In contrast to the minor changes observed for CsA treatment, MPA treatment had a major impact on the NK cell receptor repertoire, i.e. the percentage of KIR^{+} NK cells remained significantly higher (P<.01 for KIR2DL/S1 and KIR2DL/S2/3) and the percentage of NKG2A^{+} NK cells was significantly lower (P<.01) as compared to cytokine-stimulated control NK cells. Furthermore, the KIR/NKG2A distribution

within the NK cells much resembled the situation of freshly isolated NK cells. Nevertheless, MPA allowed for the activation of NK cells since the percentage of NK cells positive for NCR and NKG2D increased during NK cell culture. For all NK cell receptors analyzed, Rapa showed to have intermediate effects on the NK cell receptor repertoire as compared to CsA and MPA. In summary, MPA, and Rapa to a lesser extent, inhibited reformation of the NK cell receptor repertoire during culture, whereas CsA only had mild effects on the NK cell receptor repertoire.

Table 1. NK cell phenotype after culture.

	Isolation	CTRL		CsA		Rapa		MPA	
	%	%	[1]P	%	[2]P	%	[2]P	%	[2]P
KIR2DL/S1	21 (13-28)	17 (8-24)	*ns*	16 (5-21)	*ns*	20 (9-31)	*<.01*	25 (16-36)	*<.01*
KIR2DL/S2/3	40 (16-62)	31 (14-43)	*ns*	24 (7-44)	*<.05*	30 (15-50)	*ns*	48 (19-61)	*<.01*
KIR3DL1	15 (0.0-28)	5 (0.1-16)	*<.01*	3 (0.3-15)	*ns*	3 (0.3-16)	*ns*	4 (0.4-21)	*ns*
NKG2A	44 (16-58)	84 (68-94)	*<.001*	72 (45-89)	*<.05*	69 (44-81)	*<.01*	50 (34-74)	*<.01*
NKG2C	14 (4-50)	13 (7-34)	*ns*	14 (7-31)	*ns*	13 (6-27)	*ns*	20 (6-38)	*ns*
NKG2D	59 (46-81)	95 (44-98)	*<.05*	94 (56-99)	*ns*	84 (19-97)	*<.05*	77 (14-92)	*<.01*
2B4 (CD244)	99 (96-100)	96 (88-98)	*<.05*	97 (86-98)	*ns*	86 (78-95)	*<.01*	88 (78-96)	*<.01*
NKp30	51 (29-57)	83 (28-94)	*ns*	93 (69-99)	*<.05*	74 (17-95)	*ns*	55 (11-88)	*<.05*
NKp44	1.1 (0.3-2)	64 (37-96)	*<.001*	65 (29-95)	*ns*	33 (10-87)	*<.01*	19 (5-59)	*<.01*
NKp46	69 (36-78)	90 (56-94)	*<.05*	81 (50-88)	*ns*	85 (56-93)	*ns*	85 (48-92)	*ns*

NK cells were isolated and cultured for 5 days in the absence (CTRL) or presence of immunosuppressive drugs (CsA; 1000 ng/ml, Rapa; 50 ng/ml, MPA; 500 ng/ml). Isolated NK cells and (un)treated NK cells were analyzed by flow cytometric analysis using anti-KIR, -NKG2A, -NKG2C, -NKG2D, -CD244 and -NCR mAbs. Medians (with ranges) are shown (n=7). [1]The difference between cytokine-stimulated control NK cells before (Isolation) and after culture (CTRL) was analyzed using the Wilcoxon signed rank test. [2]Differences between the cytokine-stimulated control (CTRL) and drug-treated NK cells were analyzed using ANOVA. *ns* indicates no significant difference.

Differential distribution of KIR/NKG2A and NCR upon immunosuppressive treatment

By using KIR and NKG2A/C monoclonal antibodies (mAbs) in one 10-color panel and NKG2D, 2B4, NCR mAbs in a second 10-color panel, we could analyze the expression of multiple combinations of different NK cell receptors on a single-cell level.

As there were no significant changes in NKG2C expression (Table 1), we focused our analysis on NK cell subsets positive for KIR and/or NKG2A. This analysis showed that freshly isolated NK cells largely consist of subsets that either express NKG2A without KIR (median:26%) or KIR without NKG2A (median:42%) (Figure 4Ai). Only a small proportion of cells expressed both receptors (median:10%) and the remaining cells had no expression of NKG2A and KIR. Culturing NK cells with IL-2 and IL-15 significantly changed the distribution of these subsets as almost all NK cells expressed NKG2A either in combination with KIR expression (median:25%)

or without KIR expression (median:58%). The percentages of NKG2A negative NK cell subsets were significantly decreased after culture. As compared to cytokine-stimulated control cells, CsA-treated cells showed a similar distribution pattern of NKG2A$^+$ and/or KIR$^+$ NK cells after culture and most of the cells were NKG2A$^+$KIR$^-$ (median:58%) (Figure 4Aii). However, there were significant less NKG2A$^+$KIR$^+$ NK cells and significant more NKG2A$^-$KIR$^-$ NK cells present within the whole NK cell population. In contrast to CsA treatment, MPA-treated, and to a lesser extent Rapa-treated, NK cells had a similar distribution of NKG2A and KIR as freshly isolated NK cells and in this respect significantly differed from cytokine-stimulated control cells.

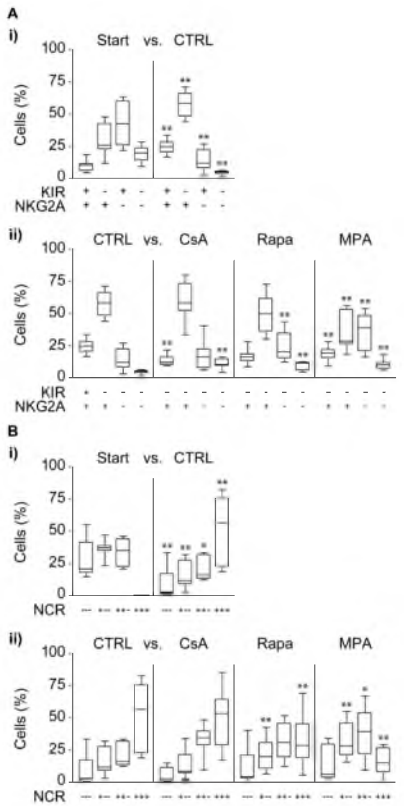

Figure 4. KIR/NKG2A and NCR distribution within the NK cell population. Shown are the results for 7 different donors in median with range. (A) The distribution of KIR and NKG2A expression within the NK cell population from (i) freshly isolated NK cells (start) versus cytokine-stimulated control NK cells (CTRL) cultured for 5 days and (ii) the KIR and NKG2A expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). (B) The expression of the different NCR (NKp30, NKp44, NKp46) within the NK cell population: --- = NCR negative; +-- = single NCR$^+$ (cells expressing only one of the NCR); ++- = double NCR$^+$ (cells expressing two different NCR); +++ = triple NCR$^+$ (cells expressing all three NCR). Shown are the distribution of NCR expression within the NK cell population from (i) freshly isolated NK cells (start) and cytokine-stimulated control NK cells (CTRL) cultured for 5 days and (ii) the NCR expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). The difference between cytokine-stimulated control NK cells before and after culture (CTRL) was analyzed using the Wilcoxon signed rank test; *P<.05, **P<.01. Differences between the cytokine-stimulated control (CTRL) and drug-treated NK cells were analyzed using ANOVA; *P<.05, **P<.01.

Further analysis of the stimulatory NCR receptors (NKp30, NKp44, NKp46) and their distribution within the NK cell population, showed that freshly isolated NK cells either contained single (one NCR; median:37%) or double (combination of two different NCR; median:35%) NCR^+ NK cells or did not express any NCR (median:21%) (Figure 4Bi).

After culture, the NK cell population showed a more activated phenotype as they primarily consisted of triple NCR^+ NK cells (expression of all three NCR; median:56%), only 2% remained NCR-. CsA treatment had no effect on the acquisition of NCR by NK cells and showed the same distribution pattern as cytokine-stimulated control NK cells (Figure 4Bii). As MPA-treated NK cells were hampered in their upregulation of NKp30 and NKp44 (Table 1), most NK cells were single (median:28%) or double (median:39%) NCR^+ and only a median of 27% expressed all three NCR (Figure 4Bii). As compared to MPA-treated NK cells, Rapa inhibited NCR upregulation to a lesser extent and revealed a median expression distribution of 20% single, 31% double, and 28% triple NCR^+ NK cells. Thus, while KIR/NKG2A data suggest that the NK cells maintain their original status, NCR data indicate that the cells are able to upregulate NCR expression, albeit it significantly lower than the cytokine-stimulated control NK cells.

In summary, MPA and Rapa inhibited the NK cell receptor repertoire from shifting towards an overall $NKG2A^+KIR^-NCR^+$ phenotype and maintained an overall $NKG2A^-KIR^+NCR^{+/-}$ phenotype, whereas CsA-treated NK cells were hardly different from cytokine-stimulated control NK cells. Detailed analysis on KIR/NKG2A and NCR modulation within sorted and separately cultured $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^+$ subsets, revealed that both subsets are subjected to change in phenotype after culture (Figure S2, S3). Thus, the net results for the whole NK cell population are based on the KIR/NKG2A and NCR modulation of both NK cell subsets together with the proliferation of the $CD56^{bright}$ subset.

Cytolytic activity and IFN-γ production after immunosuppressive treatment

As the balance between the inhibitory and stimulatory receptors of the NK cell receptor repertoire was differentially affected by CsA, Rapa and MPA, we assessed the overall cytotoxicity against MHC class I-negative K562 target cells in a 1:1 ratio. In parallel, we analyzed the IFN-γ production after the encounter with K562 target cells. To analyze the overall activation of NK cells before target encounter, we analyzed the expression of CD69, an early lymphocyte activation marker that plays a role in various NK cell functions.[30]

Upon NK cell isolation from healthy donor PBMC fractions, only few NK cells express CD69 (data not shown). After culture, Rapa showed a dose-dependent inhibition of CD69 expression with a significant lower percentage of $CD69^+$ cells at 100 ng/ml. Although not significant, CsA- and MPA-treated NK cells showed a trend towards a dose-dependent inhibition in the median percentage of CD69 expression (Figure 5A).

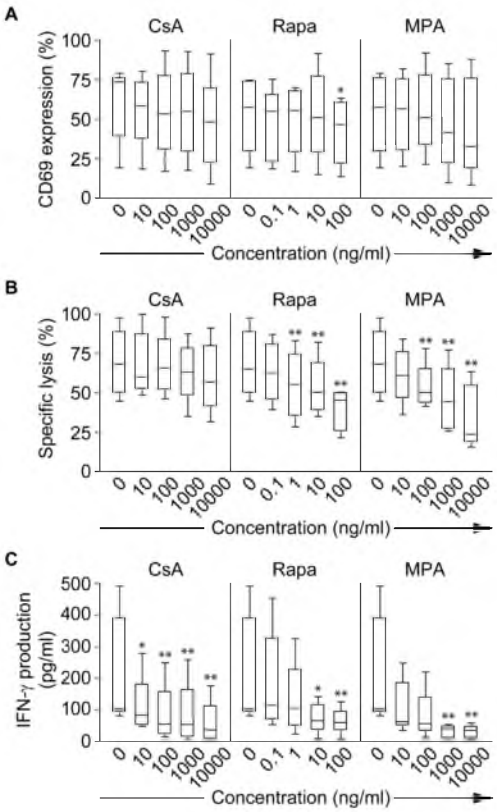

Figure 5. Activation, cytolytic activity and IFN-γ production after culture with CsA, Rapa and MPA. After culture in the absence or presence of CsA, Rapa and MPA, the expression of CD69, the cytolytic NK cell activity and the IFN-γ production was analyzed. (A) Expression of the early activation marker CD69 at day 5 of culture after CsA, Rapa and MPA treatment. Shown are the results for 5 different donors in median with range. (B) The cytolytic activity (specific lysis) was measured in a 4 hr ^{51}Cr-release assay containing NK cells in a 1:1 ratio with K562 target cells. Shown are the results for 6 different donors in median with range. (C) Supernatant of parallel 4 hr co-cultures, containing NK cells from the same donors (n=6) and K562 target cells in a 1:1 ratio, was analyzed in ELISA assays for the production of IFN-γ. Differences between the cytokine-stimulated control (0 ng/ml) and drug-treated NK cells were analyzed using ANOVA; *P<.05, **P<.01.

After a 5-day culture, NK cells showed to be significantly impaired in their cytolytic activity after Rapa and MPA treatment, but not after CsA treatment (Figure 5B). However, as CsA did not affect the cytolytic activity of the NK cells, IFN-γ production was severely impaired (Figure 5C). Thus, upon target encounter, Rapa- and MPA-treated NK cells, but not CsA-treated NK cells, are impaired in their cytolytic activity. IFN-γ production was affected by all three ISD.

Discussion

Within the first months after SCT, NK cells contribute to graft-versus-leukemia (GVL) responses without the induction of GVHD and form a first line of defense against infectious agents. This makes NK cells attractive candidates for immunotherapeutic purposes in SCT. The presence of NK cells in the graft proved to be beneficial for clinical outcome.[31,32] In these cases, NK cells need to be fully functional in the early phase after SCT, despite high level of immunosuppressive treatment. Therefore it is important to study the influence of ISD on NK cell activity in order to select the drug with less influence on NK cell function. In this study, we show that CsA, Rapa and MPA have diverse effects on NK cell phenotype and function, which may have important implications for their use in GVHD prophylaxis.We show that CsA had minor effects on NK cell proliferation, whereas Rapa and MPA severely inhibited the outgrowth of $CD56^{bright}CD16^{+/-}$ NK cells. As we showed that CsA-treated NK cells had a similar $CD56^{bright}/CD56^{dim}$ distribution as cytokine-stimulated control NK cells, Wang *et al.*[21] revealed that CsA could even increase the percentage of $CD56^{bright}$ NK cells as compared to cytokine-stimulated control cells. This difference might be explained by our distinct gating strategy, as we, in contrast to Wang *et al.*[21], also included the $CD56^{bright}CD16^{+}$ cells within our $CD56^{bright}$ subset, in consensus with gating strategies described by Cooper *et al.*[29] When excluding the $CD16^{+}$ cells from the $CD56^{bright}$ subset, we also noticed a tendency towards a higher percentage of $CD56^{bright}CD16^{-}$ NK cells after CsA (Figure 3A). Treatment of NK cells with CsA had a minimal effect on the changes within the NK cell receptor distribution during culture, resembling the NK cell receptor repertoire of cytokine-stimulated control NK cells. However, MPA- and Rapa-treated NK cells were inhibited in their shift towards a $NKG2A^{+}KIR\text{-}NCR^{+}$ phenotype and largely maintained a $NKG2A^{-}KIR^{+}NCR^{+/-}$ phenotype, also observed in freshly isolated NK cells. However, in contrast to freshly isolated or cytokine activated NK cells the MPA or Rapa treated cells were significantly inhibited in their cytolytic capacity. Thus, MPA and Rapa are able to inhibit shifts in the NK cell receptor repertoire and also inhibit cytolytic capacity, whereas CsA does not interfere with changes in the NK cell receptor repertoire within an *in vitro* culture system and leaves the cytolytic capacity intact. Recently, we also demonstrated that CsA does not affect NK cell receptor repertoire shifts and function *in vivo*, as we showed, in a randomized phase III study, that transplantation with $CD3^{+}/CD19^{+}$ cell depleted grafts, in contrast to $CD34^{+}$ enriched grafts, resulted in the development of a different NK cell population which was more prone to activation due to equal distribution of NKG2A and NKG2C.[7] Both patient groups were treated with the same immunosuppressive regimen, using CsA as a first-line agent, as part of their GVHD prophylaxis. Thus, MPA and Rapa, but not CsA, are likely to inhibit the outgrowth of functional NK cells and therefore could have a negative effect on NK cell mediated GVL responses *in vivo* after SCT. Clearly, MPA and Rapa showed a dose-dependent inhibition in NK cell cytotoxicity against K562 target cells, whereas CsA did not affect cytotoxicity. In addition, each of the ISD severely inhibited the IFN-γ production upon target encounter. As NK cells are a major source of IFN-γ, wich plays a major role in facilitating GVL effects and preventing GVHD[33], this could have clinical implications for patients after SCT.

A recent study, however, showed that CsA-treated NK cells sustain their IFN-γ production after IL-12 and IL-18 stimulation.[21] This suggests that CsA selectively inhibits IFN-γ production upon target encounter, but sustains the IFN-γ production capacity after IL-12 and IL-18 stimulation. Fauriat *et al.*[34] recently described that upon target encounter the $CD56^{dim}$ subset, and not the $CD56^{bright}$ subset, is responsible for IFN-γ production, whereas the $CD56^{bright}$ subset is the major source of IFN-γ during IL-12 and IL-18 stimulation. Thus, our data suggest that CsA, Rapa, and MPA treatment clearly inhibit the $CD56^{dim}$ cells from producing IFN-γ, leaving the $CD56^{bright}$ cells as the only remaining NK cells left for IFN-γ production. As MPA, and Rapa to a lesser extent, prevent the outgrowth of $CD56^{bright}$ cells, this could have important implications for the overall IFN-γ production and therefore the IFN-γ-mediated GVL effects after SCT. In contrast, as CsA does not inhibit the outgrowth of $CD56^{bright}$ NK cells, the overall IFN-γ production after SCT might be less affected and IFN-γ-mediated GVL effects driven by cytokines should remain intact. As the immunotherapeutic applications for NK cells have received huge interests in the field of SCT, it is important to gain insight in the effects of the limiting conditions in the different settings of transplantation in which NK cells need to be able to function. Immunosuppression is one of the conditions that may influence NK cell functionality. In this study, we have demonstrated that CsA, in contrast to Rapa and MPA has the least effect on NK cell phenotype and function *in vitro*. Thus, this study clearly suggests that the choice of immunosuppressive treatment might affect the outcome of NK cell therapy *in vivo* after transplantation. Additional studies on NK cell phenotype and function of patients after SCT using different immunosuppressive strategies are warranted to survey the *in vivo* effect of the different immunosuppressive regimens in more detail.

Acknowledgements

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Supplementa material

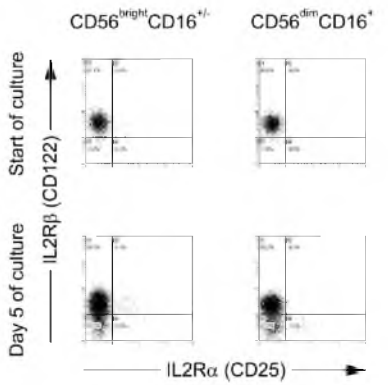

Figure S1. Expression of IL2Rα (CD25) and IL2Rβ (CD122) on sorted CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cell subsets. Sorted CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} subsets were cultured separately in the presence of 100 U/ml IL-2 and 10 ng/ml IL-15. The expression and distribution of IL2Rα (CD25, x-axis) and IL2Rβ (CD122, y-axis) were analyzed at the start of culture and at day 5 of culture. Shown are the results for one representative donor (n=4).

Table S1. Expression of IL2Rα (CD25) and IL2Rβ (CD122) on sorted CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} NK cells.

	IL2Rβ (CD122) (%)		IL2Rα (CD25) (%)	
	CD56^{bright}	CD56^{dim}	CD56^{bright}	CD56^{dim}
Start	98.2 ± 1.2	99.0 ± 0.9	1.4 ± 0.8	0.1 ± 0.09
Day 5	83.1 ± 2.2	79.5 ± 8.3	2.1 ± 1.0	1.3 ± 0.8

CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16^{+} subsets were sorted out of freshly isolated NK-cells and cultured in the presence of 100 U/ml IL-2 and 10 ng/ml IL-15. Shown are the mean percentages +/- error of CD25 and CD122 expression of 4 different donors at the start and at day 5 of culture.

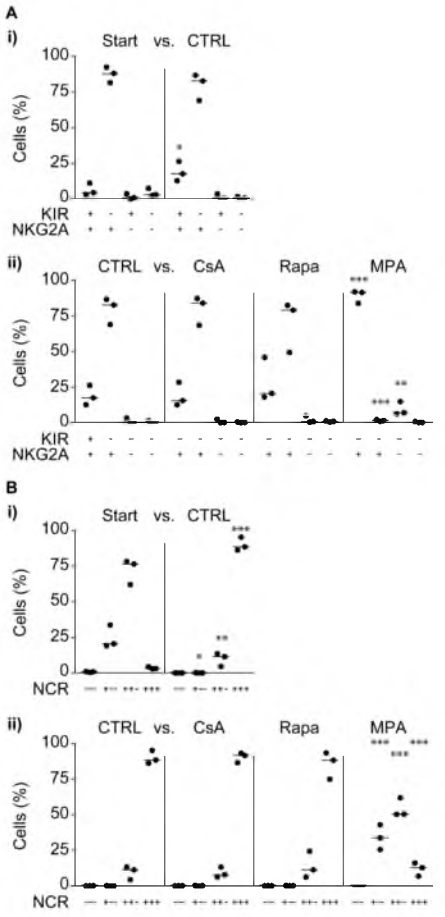

Figure S2. KIR/NKG2A and NCR distribution within the CD56^{bright}CD16$^{+/-}$ NK cell subset. Shown are the results of sorted CD56^{bright}CD16$^{+/-}$ cells of 3 different donors in median with range. (A) The distribution of KIR/NKG2A expression at (i) the start of culture (Start) versus cytokine-stimulated CD56^{bright}CD16$^{+/-}$ cells cultured for 5 days (CTRL) and (ii) the KIR/NKG2A expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). (B) The expression of the different NCR (NKp30, NKp44, NKp46) by CD56^{bright}CD16$^{+/-}$ cells before and after culture: --- = NCR negative; +-- = single NCR^{+} (cells expressing only one of the NCR); ++- = double NCR^{+} (cells expressing two different NCR); +++ = triple NCR^{+} (cells expressing all three NCR). Shown are the distribution of NCR expression at (i) the start of culture (Start) versus cytokine-stimulated CD56^{bright}CD16$^{+/-}$ cells cultured for 5 days (CTRL) and (ii) the NCR expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). The difference between cytokine-stimulated control CD56^{bright}CD16$^{+/-}$ cells before and after culture (CTRL) was analyzed using the Wilcoxon signed rank test; **P*<.05, ***P*<.01, ****P*<.001. Differences between the cytokine-stimulated control (CTRL) and drug-treated CD56^{bright}CD16$^{+/-}$ cells were analyzed using ANOVA; **P*<.05, ***P*<.01, ****P*<.001.

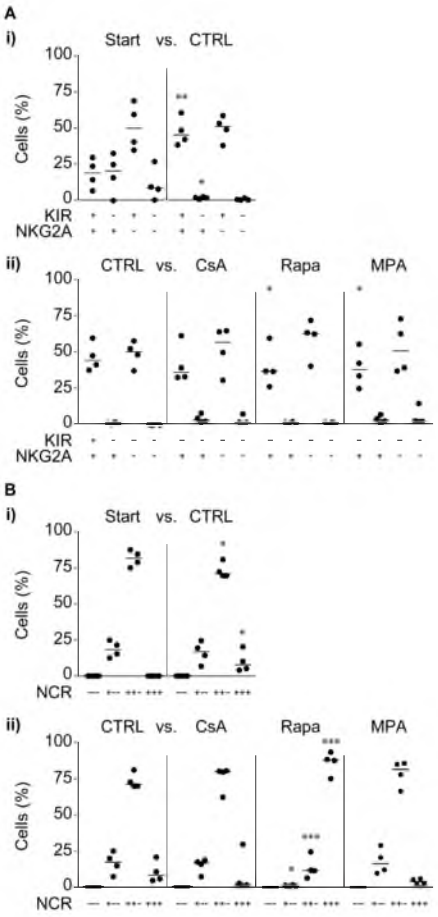

Figure S3. KIR/NKG2A and NCR distribution within the CD56^{dim}CD16^{+} NK cell subset. Shown are the results of sorted CD56^{dim}CD16^{+} cells of 4 different donors in median with range. (A) The distribution of KIR/NKG2A expression at (i) the start of culture (Start) versus cytokine-stimulated CD56^{dim}CD16^{+} cells cultured for 5 days (CTRL) and (ii) the KIR/NKG2A expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). (B) The expression of the different NCR (NKp30, NKp44, NKp46) by CD56^{dim}CD16^{+} cells before and after culture: --- = NCR negative; +-- = single NCR^{+} (cells expressing only one of the NCR); ++- = double NCR^{+} (cells expressing two different NCR); +++ = triple NCR^{+} (cells expressing all three NCR). Shown are the distribution of NCR expression at (i) the start of culture (Start) versus cytokine-stimulated CD56^{dim}CD16^{+} cells cultured for 5 days (CTRL) and (ii) the NCR expression after 5 days of culture in the absence (CTRL) versus presence of CsA (1000 ng/ml), Rapa (50 ng/ml) and MPA (500 ng/ml). The difference between cytokine-stimulated control CD56^{dim}CD16^{+} cells before and after culture (CTRL) was analyzed using the Wilcoxon signed rank test; *P<.05, **P<.01, ***P<.001. Differences between the cytokine-stimulated control (CTRL) and drug-treated CD56^{dim}CD16^{+} cells were analyzed using ANOVA; *P<.05, **P<.01, ***P<.001.

CHAPTER 5

High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy

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Abstract

Immunotherapy based on natural killer (NK) cell infusions is a potential adjuvant treatment for many cancers. Such therapeutic application in humans requires large numbers of functional NK cells that have been selected and expanded using clinical grade protocols. We established an extremely efficient cytokine-based culture system for *ex vivo* expansion of NK cells from hematopoietic stem and progenitor cells from umbilical cord blood (UCB). Systematic refinement of this two step system using a novel clinical grade medium resulted in a therapeutically applicable cell culture protocol. $CD56^{+}CD3^{-}$ NK cell products could be routinely generated from freshly selected $CD34^{+}$ UCB cells with a mean expansion of >15,000 fold and a nearly 100% purity. Moreover, our protocol has the capacity to produce more than 3-log NK cell expansion from frozen $CD34^{+}$ UCB cells. These *ex vivo*-generated cell products contain NK cell subsets differentially expressing NKG2A and killer immunoglobulin-like receptors. Furthermore, UCB-derived $CD56^{+}$ NK cells generated by our protocol uniformly express high levels of activating NKG2D and natural cytotoxicity receptors. Functional analysis showed that these *ex vivo*-generated NK cells efficiently target myeloid leukemia and melanoma tumor cell lines, and mediate cytolysis of primary leukemia cells at low NK:target ratios. Our culture system exemplifies a major breakthrough in producing pure NK cell products from limited numbers of $CD34^{+}$ cells for cancer immunotherapy.

Introduction

Natural killer (NK) cells are $CD56^+CD3^-$ large granular lymphocytes that exert innate immunity against viral infections and cancer.[1] NK cells recognize and subsequently react to virus-infected and transformed cells without prior immunization, basically through the balance of signals from inhibitory and activating receptors.[2] Therefore, NK cells have been previously described as promising effectors for adoptive immunotherapy against cancer.[3] The anti-tumor potential of NK cells has been best demonstrated during therapy of leukemia patients with allogeneic stem cell transplantation (SCT). Ruggeri *et al.*[4] demonstrated that NK cell alloreactivity can control relapse of acute myeloid leukemia (AML) without causing graft-versus-host disease (GVHD) in the setting of HLA-mismatched haploidentical allogeneic SCT. In addition, haploidentical NK cell infusions together with IL-2 in a non-transplantation setting have been associated with complete hematologic remission in poor-prognosis patients with AML.[5] These encouraging results point out that allogeneic NK cell-based immunotherapy may be a promising therapeutic strategy for AML in both the non-transplant and post-transplant setting.[5;6]

To date, most clinical studies exploiting allogeneic NK cells for adoptive immunotherapy have been performed with NK cells selected from leukapheresis products by immunomagnetic beads selection protocols.[7-12] In order to circumvent limitations in cell numbers, purity and state of activation of such blood-derived NK cells, *ex vivo* expansion of NK cells with higher purity will facilitate the infusion of a greater number of activated NK cells in patients with a relatively large tumor burden or permits multiple NK cell infusions.[8;13;14] Therefore, development of innovative strategies enabling the generation of clinically relevant NK cell products with high cell numbers, high purity and functionality promises a major breakthrough in NK cell-based immunotherapy.

In this study, we developed a cytokine-based method for large scale expansion of functional $CD56^+$ NK cells from hematopoietic stem and progenitor cells. Similar studies have been performed previously focusing on either $CD34^+$ hematopoietic progenitor cells (HPC) from bone marrow (BM) or umbilical cord blood (UCB).[15-17] However, most of these culture systems are unsuitable for clinical application because of the use of animal sera, animal-derived proteins and supportive feeder cell lines. Furthermore, most of these methods yielded only limited NK cell numbers for successful adoptive immunotherapy in cancer patients. In order to surmount these shortcomings, we established a two-step culture scheme in which we used a novel clinical grade medium to generate more than 3 to 4-log fold-expanded functional $CD56^+$ NK cells from freshly selected or cryopreserved $CD34^+$ UCB cells, respectively. The $CD56^+$ NK cell products generated by this method have a very high purity, contain various NKG2A and killer immunoglobulin-like receptor (KIR) expressing mature subsets and efficiently lyse AML and solid tumor cells. These findings exemplify that this culture system could hold great promise for the *ex vivo* generation of clinical grade NK cell products for cellular immunotherapy against cancer.

Materials and Methods

Cell lines

Cell lines (K562, KG1a, Lama, Kasumi, BLM and FM3) were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad CA, USA) containing 50 U/ml penicillin, 50 µg/ml streptomycin and 10-20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands) Characteristics of the cell lines used in functional assays are shown in Table 1. Clonal K562 cell lines expressing HLA-Cw*0301 and/or HLA-Cw*0403 were used as target cells to analyze specificity of the UCB-derived NK cells. Briefly, K562 cells were transfected with full length HLA-Cw0301 cDNA inserted into the EcoRI and HindIII sites of expression vector pcDNA3.1(−) (Invitrogen, Paisley, UK) and HLA-Cw*0403 cDNA ligated into the pEF6/V5-His expression vector using the TOPO TA Expression kit (Invitrogen). The cell lines K562-Cw*0301, K562-Cw*0403 and K562-Cw*0301/0403 showed stable and strong HLA class I expression (>95%) analyzed by flow cytometry (FCM) using the HLA class I antibody W6/32 (Sigma, Steinheim, Germany). The (co-)expression of HLA-Cw*0301 and HLA-Cw*0403 was confirmed by sequencing of HLA-Cw cDNA.

Selection of CD34-positive stem and progenitor cells

UCB units have been obtained at birth after normal full-term delivery with written informed consent with regard of scientific use from the cord blood bank of the Radboud University Nijmegen Medical Center (RUNMC). UCB samples were stored at room temperature and processed within 24 h after collection. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque (1.077 g/ml; GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Alternatively, UCB samples were thawed at 37°C and resuspended in CliniMACS buffer (Miltenyi Biotech, Bergisch Gladbach, Germany) containing 5% HSA, 3.5 mM MgCl2 and 100 U/ml Pulmozyme (Genentech). After 30 min of incubation, thawed UCB cells were washed and used for $CD34^+$ cell selection. $CD34^+$ cells were selected from UCB cells using anti-CD34 immunomagnetic bead separation (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. The cell number and purity of the enriched $CD34^+$ fraction was analyzed by FCM. Purity of the obtained cell populations was 85±13%.

Ex vivo expansion of CD34-positive progenitor cells

$CD34^+$ UCB cells (between 1×10^4 and 3×10^5 per ml) were plated into 24-well tissue culture plates (Corning Incorporated, Corning, NY) using Methods I, II or III (Figure 1) in the following basal media: StemSpan® H3000 (Stemcell Technologies, Grenoble, France), Stemline I™ and Stemline II™ Hematopoietic Stem Cell Expansion Medium (Sigma-Aldrich, Zwijndrecht, The Netherlands) and Glycostem Basal Growth Medium (GBGM®) (Clear Cell Technologies,

Beernem, Belgium). H3000 and Stemline media were supplemented with 20 mg/ml ascorbic acid, 50 µmol/l ethanolamine, 50 µmol/l sodium selenite, 25 µmol β-mercaptoethanol (all Sigma Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/L L-glutamine (all Invitrogen). All media used in Method I and II were supplemented with 10% human serum (HS; Sanquin Blood Bank, Nijmegen, The Netherlands) and a low-dose cytokine cocktail consisting of 10 pg/ml GM-CSF, 250 pg/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-1α (all Stemcell Technologies) and 50 pg/ml IL-6 (CellGenix, Freiburg, Germany), which was based on studies using the fetal liver-derived stromal cell line AFT024.[32] In Method III, LIF and MIP-1α were left out of the low-dose cytokine mixture. In addition, a high-dose cytokine cocktail was added consisting of 27 ng/ml SCF (CellGenix), 25 ng/ml Flt3L (CellGenix), 25 ng/ml TPO (Stemcell Technologies) and 25 ng/ml IL-7 (Stemcell Technologies). During the first 14 days of culture, low molecular weight heparin (LMWH) (Clivarin®; Abbott, Wiesbaden, Germany) was added to the expansion medium in a final concentration of 25 µg/ml. In Method II and III, TPO was replaced by 20 ng/ml IL-15 at day 9-14. Cell cultures were refreshed with new medium every 2-3 days. Cultures were maintained in a 37 °C, 95% humidity, 5% CO_2 incubator.

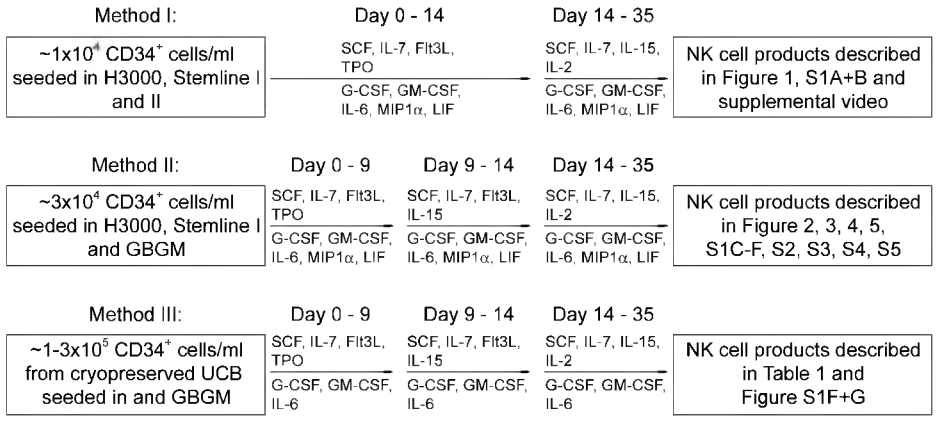


Figure 1. Schematic diagram of the different culture methods used for the *ex vivo* generation of CD56⁺ NK cells from cytokine-expanded CD34⁺ UCB cells. In Method I-III different combinations of cytokines were tested as described in detail in Materials and Methods starting with different numbers of initially seeded CD34⁺ cells. In Method I and II, NK cells were generated from freshly selected UCB donors and the culture duration was 5 weeks. In Method III, CD34⁺ cells were used from cryopreserved UCB donors and the culture period was 6 weeks. Finally, the diagram depicts which NK products were used and displayed as results in figures and supplemental material.

Differentiation of *ex vivo*-expanded CD34-positive cells into NK cells

Expanded CD34⁺ UCB cells were differentiated and further expanded using NK cell differentiation medium. This medium consisted of the same basal medium as used for the CD34 expansion step supplemented with 10% HS, the low-dose cytokine cocktail (as previously mentioned) and a new high-dose cytokine cocktail consisting of 20 ng/ml IL-7, 22 ng/ml SCF, 1000 U/ml IL-2 (Proleukin®; Chiron, München, Germany) and 20 ng/ml IL-15 (CellGenix). Flt3L (20 ng/ml) was

only added to the differentiation medium in Method I (Figure 1). Medium was refreshed twice a week from day 14 onwards. Total and $CD56^+$ cell expansion at each week of culture was calculated by the number of cultured cells divided by the number of seeded cells. The number of seeded cells was reduced by dilution with fresh differentiation medium. The theoretical total cell numbers were calculated by multiplying the expansion rate per week with the number cultured cells.

Flow cytometry

Cell numbers and expression of cell-surface markers were determined by FCM. Briefly, cells were incubated with the appropriate concentration antibodies for 30 min at 4 °C. After washing, cells were resuspended in Coulter® Isoton® II Diluent (Beckman Coulter) and analyzed using the Coulter FC500 flow cytometer (Beckman Coulter). Cell numbers and NK cell purity have been determined by gating on $CD45^+$ cells in combination with forward scatter (FS) and side scatter (SS). For phenotypic analysis of the NK cell products, living cells were gated only on FS/SS and further analyzed with the specific antigen of interest. The following conjugated antibodies were used: anti-CD16-FITC (NKP15), anti-CD94-FITC (HP-3D9), anti-NKG2D-PE (1D11) (BD Biosciences Pharmingen, Breda, The Netherlands), anti-CD161-PE (191B8), anti-NKG2A-PE (Z199), anti-CD122-PE (CF1) (Immunotech, Marseille, France), anti-NKG2C-PE (134591), anti-NKp80-PE (239127) (R&D System, Abingdon, UK), anti-CD45-ECD (J33), anti-CD117-PE-Cy5 (104D2D1), anti-CD34-PE-Cy7 (581), anti-CD56-PE-Cy7 (N901), anti-NKp30-PE (Z25), anti-NKp44-PE (Z231), anti-NKp46-PE (BAB281), anti-CD158a,h-PE (EB6.B), anti-$CD158b_1/b_2$,j-PE (GL183), anti-$CD158e_1/e_2$-PE (Z27), anti-CD158i-PE (FES172), anti-CD3-ECD (UCHT1), anti-CD244-PE (C1.7), anti-CD25-PE-Cy5 (B1.49.9), anti-CD7-PECy5 (8H8.1), anti-CD2-FITC (39.C1.5) (all Beckman Coulter, Woerden, The Netherlands) and anti-HLA-ABC-PE (W6/32) (Dako, Enschede, The Netherlands).

Flow cytometry-based cytotoxicity and degranulation studies

FCM-based cytotoxicity assays were performed as described previously[33] with minor adaptations. Target cells were labeled with 0.5 µM carboxyfluorescein diacetate succimidyl ester (CFSE; Molecular Probes Europe, Leiden, The Netherlands) in a concentration of 1×10^7 cells per ml for 10 min at 37 °C. The reaction was terminated by adding an equal volume of FCS, followed by incubation at room temperature for 2 min and stained cells were washed twice with 5 ml IMDM/10% FCS. After washing, cells were resuspended in IMDM/10% FCS to a final concentration of 2×10^5/ml. $CD56^+$ NK cells were washed with PBS and resuspended in IMDM/10% FCS to a final concentration of 2×10^5/ml. Target cells (2×10^4) were co-cultured with effector cells at different E:T ratio's in a total volume of 200 µl IMDM/10% FCS in 96-wells flat-bottom plates. NK cells and target cells alone were plated out in triplicate as controls. NK cell co-cultures with primary AML cells were supplemented with IL-3 (50 ng/ml), SCF (25 ng/ml),

Flt3L (20 ng/ml), GM-CSF (100 ng/ml) and G-CSF (100 ng/ml). To measure degranulation by NK cells, anti-CD107a (H4A3; BD Biosciences) was added in a 1:200 dilution to the co-cultures. After incubation for 4 or 24 h at 37 °C, 50 µl supernatant was collected and stored at -20 °C for later use to measure cytokine production. Cells in the remaining volume were harvested and the number of viable target cells was quantified by FCM. Target cell survival was calculated as follows: % survival = ((absolute no. viable $CFSE^+$ target cells co-cultured with NK cells)/(absolute no. viable $CFSE^+$ target cells cultured in medium))x100%. The percentage specific lysis was calculated as follows: % lysis = (100-(% survival)). Degranulation of NK cells during co-culture was measured by cell surface expression of CD107a.[34] After 2, 4 or 24 hrs of incubation at 37 °C, the percentage of $CD107a^+$ cells was determined by FCM.

IFN-γ production assay

Production of IFN-γ by target cell-stimulated NK cells was measured in the supernatant of the co-cultures by ELISA (Pierce Endogen, Rockford, IL, USA). Absorbance was measured at 450 nm with a Multiscan MCC/340 ELISA reader (Titertek, Huntsville, Alabama, USA).

Life imaging NK cell-mediated killing

NK cell-mediated killing of solid tumor cell lines was visualized by life imaging on a Zeiss Axiovert 35 M inverted contrast microscope (Zeiss, Sliedrecht, The Netherlands) which was placed in a 37 °C incubator. Images were digitized in 768x512 pixels with a camera (VarioCam, PCO computer Optics GmbH, Kelheim, Germany), coupled with the Pixel Pipeline in a Macintosh-G4. Video recordings of microscopic fields were made continuously (1 image/min) using an in house developed software program version of the IPlab 3.5.5. image program (Scananlytics Inc. USA All). Images were further processed using the NIH Image 1.61 program, resulting in a Quick-Time movie.

Statistics

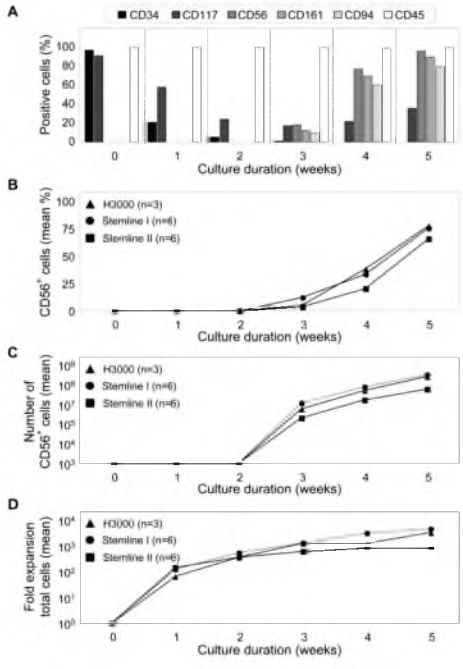
Results from different experiments are described as mean ± standard deviation of the mean (SD). Statistical analysis was performed using ANOVA and Duncan Post-hoc comparison. *P*-values <.05 were considered statistically significant.

Results

Ex vivo progenitor cell expansion and NK cell differentiation

The aim of this study was to develop an efficient cytokine-based *ex vivo* culture system for the expansion of $CD34^+$ cells followed by the subsequent log-scale generation of $CD56^+CD3^-$ NK cells. To identify a suitable medium for clinical applicable expansion and differentiation of NK cells, we tested different basal media using a two-step *in vitro* differentiation scheme (Figure 1). Initially, we compared the media H3000, Stemline I and Stemline II seeding 1×10^4 $CD34^+$ cells using Method I. We detected a strong increase in $CD34^+$ cell numbers in all three media, resulting in a mean expansion rate for all experiments (n=15) of 48±7 fold and 78±27 fold after 1 and 2 weeks of culture, respectively. The total cell expansion was associated with a gradual decline of the frequency of $CD34^+$ cells from 84±16% at day 0 till 47±14% at week 1 and 17±9% at week 2 (Figure S1A and S1B).

Next, we investigated whether the expanded UCB-derived $CD34^+$ cells were able to differentiate into $CD56^+$ NK cells. The differentiation step was monitored by the analysis of the cell surface molecules CD34, CD117, CD56, CD94 and CD161, which have been described to be expressed at different human NK cell developmental stages *in vivo*.[18] We observed after 3 weeks total culture duration that the percentage of $CD34^+$ cells further declined, while the $CD56^+CD161^+CD94^+$ NK cell population increased to 10-18% (Figure 2A). Thereafter, the population of $CD56^+CD161^+CD94^+$ cells rapidly increased to 60-77% after 4 weeks and 80-96% after 5 weeks of culture (Figure 2A).



Expansion UCB-derived NK cells

← **Figure 2. *Ex vivo* generation of CD56$^+$ NK cells from cytokine-expanded CD34$^+$ UCB cells.** CD34-enriched UCB cells were expanded for two weeks using three different media (H3000, Stemline I and Stemline II) and subsequently differentiated into NK cells for three additional weeks in the same basal medium using Method I (Figure 1). Cell cultures were weekly analyzed for cell numbers and phenotype using FCM. (A) Representative example of antigen expression during the two-step culture period using H3000 medium. One week after the onset of the NK cell differentiation step 2 (i.e. after 3 weeks total culture duration) the CD56$^+$CD161$^+$CD94$^+$ NK cell population increases and reaches high purity after 3 weeks of differentiation (i.e. week 5). (B) Mean CD56$^+$ cell frequency during the 5 week culture period for three different media, which have been tested in parallel experiments using 3-6 UCB donors. (C) Mean total CD56$^+$ NK cell numbers after initial seeding of 1x10^4 CD34$^+$ UCB cells during 5 weeks of culture using Method I. Data represent a theoretical calculation based on the actual expansion rates of CD56$^+$ cells. Total yield of CD56$^+$ cells at each week was calculated by multiplying the expansion rate per week with the number of cultured cells. (D) Mean fold expansion of total cells after initial seeding of 1x10^4 CD34$^+$ UCB cells during 5 weeks of culture using Method I. Data represent a theoretical calculation based on the actual expansion rates of total cells.

Although we could not detect significant differences between the different basal media, the purity of the CD56$^+$ cell product appeared slightly higher with H3000 medium (77±24%; n=3) and Stemline I medium (75±21%; n=6) as compared to Stemline II medium (66±17%; n=6) (Figure 2B). Furthermore, a trend was observed towards higher CD56$^+$ cell numbers with Stemline I medium (range 4x10^6-1.1x10^8 CD56$^+$ cells; n=6) followed by H3000 (5.2x10^6-5.4x10^7 CD56$^+$ cells; n=3) and Stemline II medium (range 1x10^6-2x10^7 CD56$^+$ cells; n=6) (Figure 2C). The mean total cell expansion after 5 weeks of culture with Stemline I medium was ~4,400-fold and with H3000 medium ~3,200-fold, but only ~850-fold expansion with Stemline II (Figure 2D). These results indicate that the culture conditions support an effective outgrowth of CD56$^+$ NK cells with a high purity of the final NK cell product after a culture period of 5 weeks using various basal media.

Superior expansion of extremely pure NK cell products was achieved using a novel clinical grade medium

Although high expansion rates and purities could be obtained with current commercially available basal media, we were not satisfied with the purity of the CD56$^+$ NK cell product following 5 weeks of culture using Method I (Figure 1). To further increase the efficiency of our culture method, we tested a newly formulated serum-free medium, designated Glycostem Basal Growth Medium (GBGM®), which is produced under GMP conditions and suitable for clinical applications. In addition, we slightly adjusted the cytokine conditions during the expansion step, since we observed that expansion of CD34$^+$ cells as well as total cells was most prominent during the first week of culture (Figure S1A and S1C). Furthermore, to increase the balance towards expansion of NK cell progenitors we added IL-15 instead of TPO to the expansion medium from day 9 of culture, and left out Flt3L in the differentiation medium (Figure 1). Using this modified Method II, culture in GBGM® resulted in the highest total cell expansion during the first week and subsequent differentiation into CD56$^+$ NK cells (Figure 3 and Figure S1E). The total cell expansion rate in GBGM® increased to >15,000 fold (range 16,991-73,666; n=3) (Figure 3A). More importantly, *ex vivo* generation of CD56$^+$ NK cells in GBGM® yielded a significant higher purity of 99%±1% (n=3) compared to H3000 with 88±3% (n=3; P<.05) and Stemline I with 63±24% (n=3; P<.05), respectively (Figure 3B). These data demonstrate that the inventive

modified culture system using the newly formulated GBGM® medium results in more than 4-log expansion of pure human NK cells from freshly selected CD34$^+$ UCB cells.

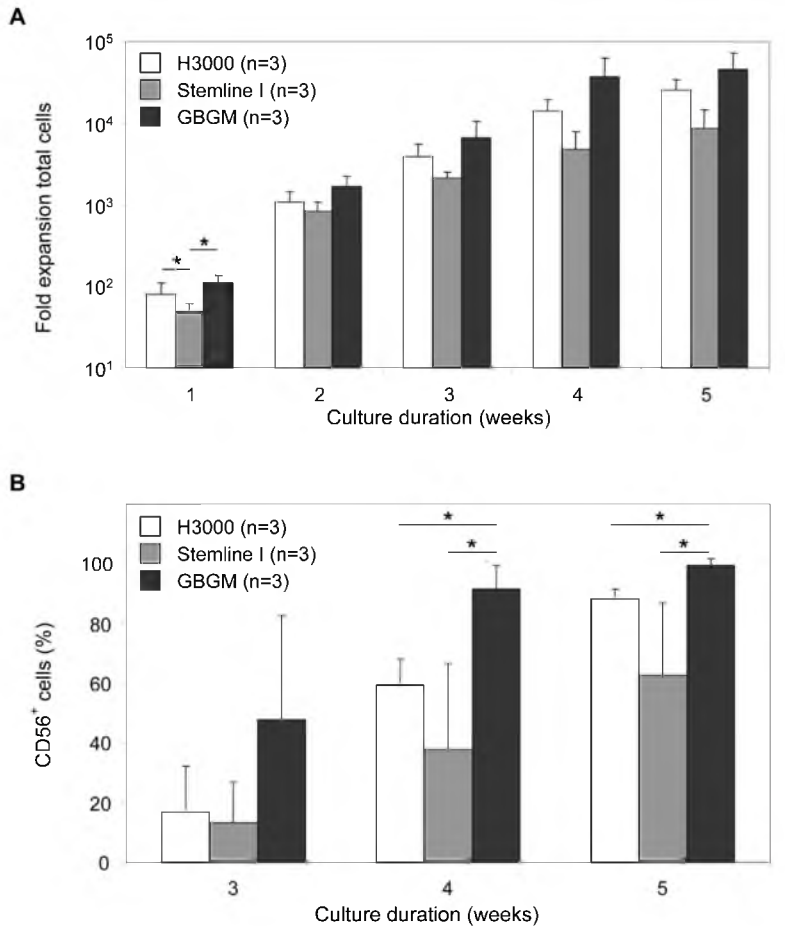


Figure 3. Superior expansion of UCB-derived CD56$^+$ NK cells using a novel clinical grade medium. The new GBGM® medium was compared with two previously tested media in the NK cell generation system according to Method II (Figure 1). Cell cultures were weekly analyzed for cell numbers and phenotype using FCM. (A) Fold expansion of total cells after initial seeding of 1x10^4 CD34$^+$ UCB cells was determined during 5 weeks of culture. Data represent the calculation based on the actual expansion rates of total cells and are displayed as mean±SD of three different experiments. (B) CD56$^+$ cell frequency during the differentiation stage for three different media, which have been tested in parallel experiments using three UCB donors. Data are depicted as mean±SD; *P <.05.

Phenotypic profile of CD56$^+$ NK cells derived from expanded CD34$^+$ cells

Flow cytometric analysis revealed that the *ex vivo*-generated NK cells after 5 weeks of culture contained a homogeneous cell population displaying high expression of CD56 in the absence of CD3 (Figure 4A). A high frequency of this CD56$^+$CD3$^-$ NK cell population displayed expression of CD94, while only a limited subset was positive for CD16. Furthermore, NK cell products displayed homogeneous and relatively high expression of NKG2D and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, whereas NKG2A was more differentially expressed (Figure 4B and Figure S2A). Additionally to these findings we detected high expression of 2B4 (CD244) and NKR-P1 (CD161) as typical NK cell receptors, while NKG2C and NKp80 were absent or expressed at relatively low levels. In addition, we observed high expression of MHC class I (HLA-ABC) and cytokine receptor chains for IL-2 and IL-15 (CD122; IL-2/IL-15Rβ) and SCF (CD117; c-kit-R), which are important for NK cell differentiation, expansion and activation.
KIR repertoire analysis showed significant individual differences in the frequency of KIR-expressing NK cell subsets between various UCB donors. KIR$^+$ NK cell subsets could already be detected at week 4 of culture and remained relatively stable until the end of the culture period at week 5 (Figure 4C and Figure S2B). While some CD56$^+$ NK cell products contained positive cells for all four KIR phenotypes analyzed (Figure 4C), others specifically lacked the KIR2DL1/DS1$^+$ subset (Figure S2B). Generally, the KIR2DL2/DL3/DS2$^+$ subset was present in a higher proportion in all NK cell products analyzed so far, which is in agreement with earlier observations that recovery of KIR2DL2/DL3/DS2$^+$ NK cells is faster compared to the KIR2DL1/DS1$^+$ subset following HSCT.[19]
Taken together, these results illustrate that UCB-derived NK cells generated with our optimized culture system contain developmentally mature NK cell populations expressing NKG2A, KIR and various activating receptors.

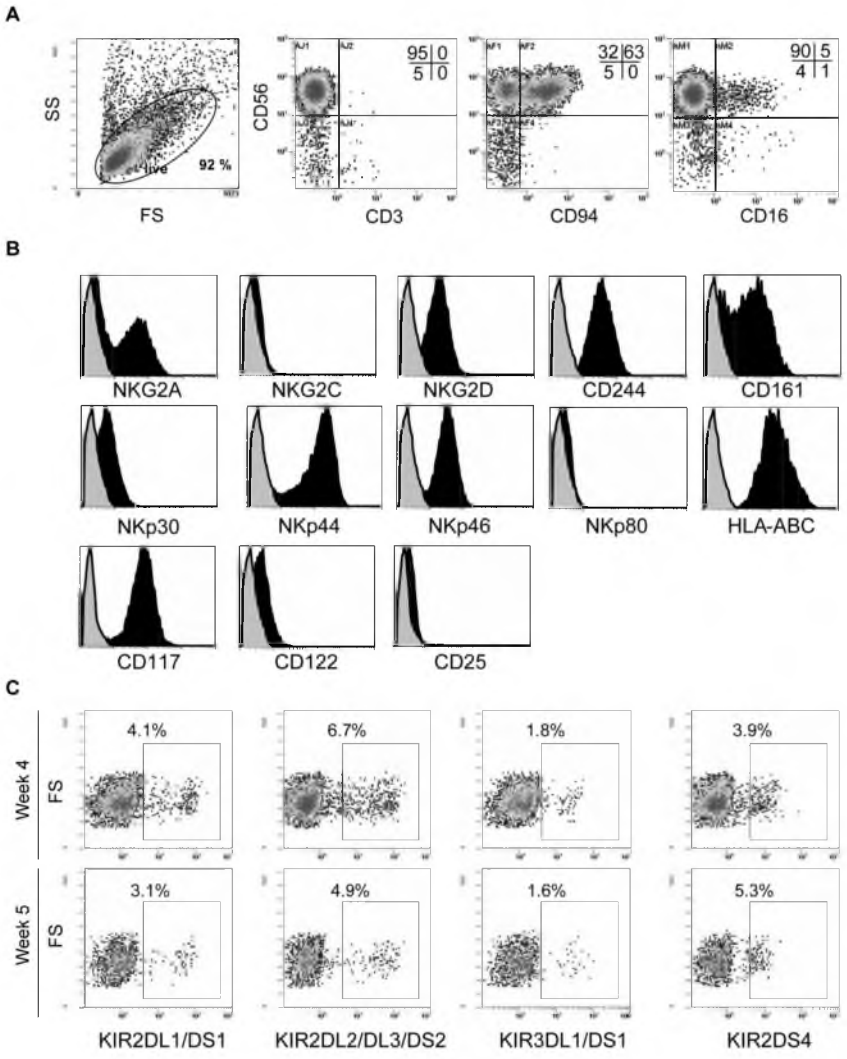


Figure 4. Phenotypical profile of *ex vivo*-generated NK cells using Method II with GBGM. (A) Flow cytometric analysis of a representative NK cell product generated from CD34$^+$ UCB progenitor cells. Cells at 5 weeks of culture were analyzed for expression of CD56, CD3, CD94 and CD16. (B) Expression of a repertoire of receptors important for regulating NK cell activity, including C-type lectin receptors, natural cytotoxicity receptors and cytokine receptors. Histograms show expression of the antigen of interest (black histogram) compared to the specific isotype control (grey histogram). (C) Acquisition of KIR$^+$ NK cell subsets during *ex vivo* NK cell generation from expanded CD34$^+$ UCB cells. KIR expression was determined at week 4 and 5 during the differentiation step by FCM.

Ex vivo-generated CD56$^+$KIR$^+$ and CD56$^+$NKG2A$^+$ NK cells are functionally regulated by MHC class I expression

Phenotypic analysis showed that our *ex vivo*-generated NK cell products contained up to 10% CD56$^+$KIR$^+$ cells and a high proportion (40-60%) CD56$^+$NKG2A$^+$ cells. To determine cytolytic activity of these subsets and investigate if this activity is regulated by the expressed inhibitory receptors, we performed CD107a-based degranulation assays using HLA-negative K562 cells and KG1a cells expressing relatively high levels of HLA-A,-B,-C and -E (Table 1).

Table 1. HLA-C subtype, KIR group and cell surface expression of HLA molecules by the different target cell lines.

Cell line	HLA-Cw typing[1]	KIR group	HLA-A,-B,-C[2]	HLA-E[2]
K562	Cw*03, Cw*05	C1 & C2	<1	2
KG1a	Cw*04, Cw*16	C1 & C2	101	5
Lama	Cw*05, Cw*12	C1 & C2	73	5
Kasumi	Cw*03	C1	9	<1
BLM	Cw*07	C1	121	<1
FM3	Cw*05	C2	132	6

[1]HLA-Cw typing was performed by SSO-PCR using sequence specific primers according to ASHI guidelines. [2]Expression of HLA molecules was measured by FCM and expressed as delta MFI (= MFI specific antibody - MFI isotype control). HLA class A and B subtypes of the cell lines were: K562 (A*11, A*31, B*18, B*40), Lama (A*02, A*25, B*18, B*44), Kasumi (A*26, B*40, B*48), KG1a (A*30, B*53, B*78), BLM (A*02, B*07) and FM3 (A*02, B*44).

For these experiments, we used two *ex vivo*-generated NK cell products that contained approximately 15-30% CD56$^+$CD107a$^+$ cells upon co-culture with K562 (Figure 5 and Figure S3). In contrast, KG1a cells hardly stimulated degranulation of these NK cell products, indicating that cytolytic activity is inhibited by HLA expression.

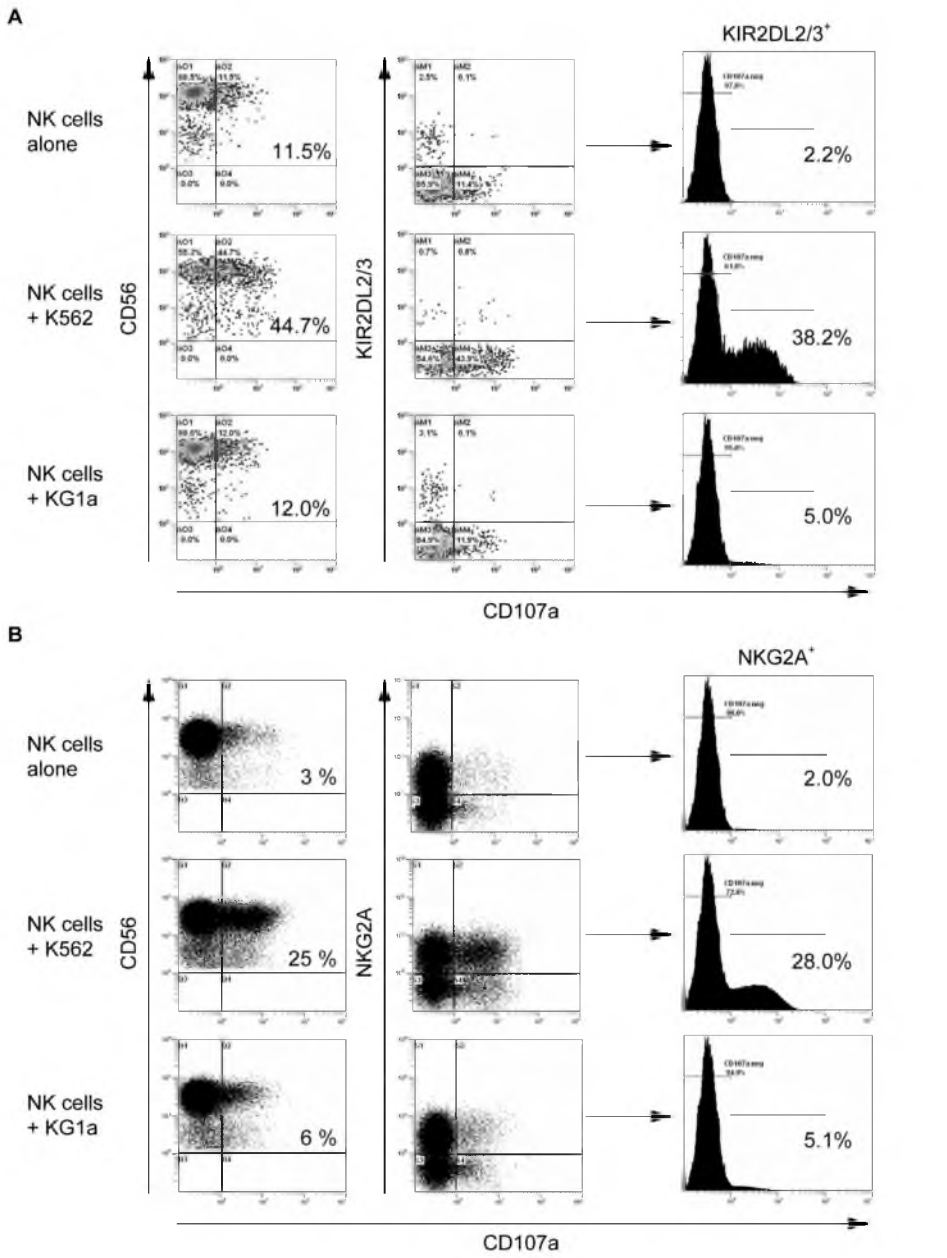


Figure 5. Responsiveness of *ex vivo*-generated KIR$^+$ and NKG2A$^+$ NK cells to MHC class I-deficient target cells. *Ex vivo*-generated NK cells using Method II with GBGM were incubated alone, or 18 hours with MHC class I-negative K562 or MHC class I-expressing KG1a cells at an E:T ratio of 1:1. Cells were then stained for CD56, CD3, KIR or NKG2A, and the degranulation antigen CD107a. (A) Degranulation of total CD56$^+$CD3$^-$ NK cells and KIR2DL2/DL3$^+$ NK cell subset expanded for 5 weeks from CD34$^+$ UCB cells. Density plots are gated on CD56$^+$CD3$^-$ NK cells and the histogram plots show the CD107a degranulation of the KIR2DL2/DL3$^+$ NK cells. (B) Degranulation of total CD56$^+$CD3$^-$ NK cells and NKG2A$^+$ NK cell subset expanded for 6 weeks from CD34$^+$ UCB cells. Density plots are gated on CD56$^+$CD3$^-$ NK cells and the histogram plots show the CD107a degranulation of the NKG2A$^+$ NK cells.

Similarly, around 35% of the CD56$^+$KIR2DL2/DL3$^+$ subset degranulated upon triggering by K562, while only a small percentage (<5%) expressed CD107a following co-culture with KG1a (Figure 5A and Figure S3). In agreement with these results, degranulation by the CD56$^+$KIR2DL2/DL3$^+$ subset could be specifically inhibited by K562 cells transfected with the HLA-C group 1 allele HLA-Cw3, while transfection of the HLA-C group 2 allele HLA-Cw4 allele had no effect (Figure S4). Finally, we observed that also the dominant CD56$^+$NKG2A$^+$ subset was able to efficiently degranulate in response to K562 (28% CD107a$^+$ cells), while degranulation towards KG1a was low probably due to relative high expression of HLA-E molecules (Figure 5B and Table 1). These data demonstrate that the KIR$^+$ and NKG2A$^+$ subsets within the UCB-derived NK cell products are fully responsive mediating strong degranulating activity, which is regulated by the expression of MHC class I molecules on the engaged target cells.

Ex vivo-generated NK cells efficiently target leukemia and solid tumor cells

To determine the cytotoxic potential of the *ex vivo*-generated NK cell products, we performed flow cytometry-based cytotoxicity assays using various myeloid leukemia cell lines (K562, Lama, Kasumi and KG1a), primary AML cells and two melanoma cell lines (BLM and FM3). *Ex vivo*-generated NK cells in GBGM® mediated efficient lysis of K562 cells (~40% and ~90% after 4 and 24 hrs, respectively) at a very low E:T ratio of 2:1 (Figure 6A). Furthermore, profound lysis could be observed against MHC class I-expressing KG1a cells (~20% and ~40% after 4 and 24 hrs, respectively). Interestingly, NK cells cultured in GBGM® showed higher cytotoxic and degranulating activity as compared to NK cells cultured in H3000 medium from the same UCB donor (Figure S5A-C). Furthermore, we found that GBGM-derived NK cells showed higher expression of the activating receptors NKG2D, NKp30, NKp44 and NKp46 (Figure S5D).
In addition to effective lysis of K562, also the leukemia cell lines Lama and Kasumi as well as the BLM and FM3 melanoma cells were efficiently lysed by the NK cells (Figure 6D). Interestingly, a monolayer of FM3 melanoma cells was completely destroyed within one hour of co-culture with NK cells (Movie S1). High NK cell-mediated cytolytic activity against leukemia and melanoma cell lines was associated with a high percentage of CD107a$^+$ NK cells (Figure 6B and 6E) and significant production of IFN-γ (Figure 6C and 6F).

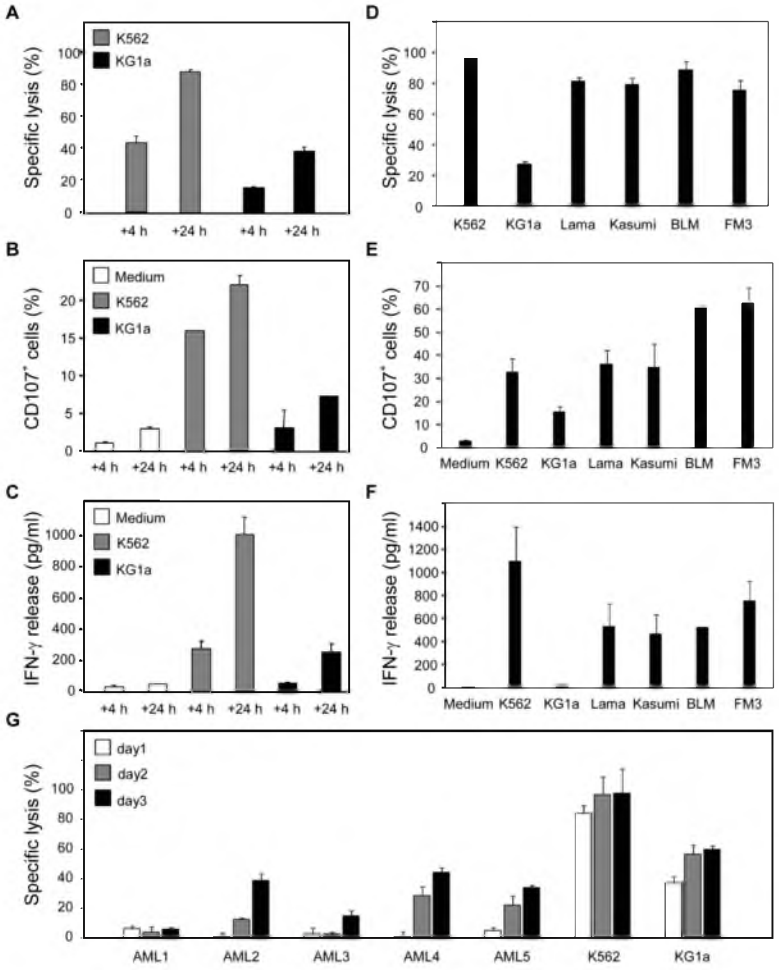


Figure 6. Functional activity of *ex vivo*-generated CD56⁺ NK cells using Method II with GBGM. (A) Specific cytotoxicity of a CD56⁺ NK cell product (98% purity) against the myeloid leukemia cell lines K562 and KG1a. Specific lysis was determined after 4 and 24 hours of co-culture in a FCM-based cytotoxicity assay at an E:T ratio of 2:1. Data are displayed as mean±SD of triplicate wells. (B) Degranulation of CD56⁺ NK cells was determined by FCM as the percentage of CD107a⁺ cells. Results are depicted as mean±SD of triplicate wells. (C) IFN-γ production was determined by ELISA and depicted as mean±SD of triplicate measurements. (D) Specific cytotoxicity of another CD56⁺ NK cell product (95% purity) against the myeloid leukemia cell lines K562, Lama, Kasumi and KG1a, and the melanoma cell lines BLM and FM3. Specific lysis was determined after 18 hours of co-culture in a FCM-based cytotoxicity assay at an E:T ratio of 1:1. Data are displayed as mean±SD of triplicate samples. (E) Degranulation of CD56⁺ NK cells was determined by FCM as the percentage of CD107a⁺ cells after overnight stimulation with different targets. Data are depicted as mean±SD of triplicate samples. (F) IFN-γ production was determined by ELISA and depicted as mean±SD of triplicate measurements. (G) Specific cytotoxicity of a third CD56⁺ NK cell product (97% purity) against primary AML cells from 5 different patients. Specific lysis was determined after 24, 48 and 72 hours of co-culture in a FCM-based cytotoxicity assay at an E:T ratio of 3:1. Data are displayed as mean±SD of triplicate samples

Finally, we investigated whether primary leukemia cells are susceptible to killing by the *ex vivo*-generated NK cells. Therefore, we performed cytotoxicity assays with AML cells from five different patients. Primary AML cells from three patients (Pt 2, 4 and 5) were substantially killed within 3 days of co-culture at a low E:T ratio of 3:1 albeit less potent than K562 cells (Figure 6G). These results indicate that UCB-derived NK cells generated by our efficient culture method have the ability to kill myeloid leukemia and solid tumor cells.

Ex vivo generation of NK cell products from frozen $CD34^+$ UCB cells

To investigate whether our NK cell expansion protocol could be adapted to a clinically applicable procedure, we have performed experiments using GBGM® medium in which we left out LIF and MIP-1α in the low-dose cytokine mixture because these cytokines are not available clinical grade. Furthermore, we performed these experiments using $CD34^+$ cells selected from thawed UCB according to Method III depicted in Figure 1. Thawing and $CD34^+$ cell selection resulted in obtaining $1.30\pm0.61x10^6$ (range $0.84\text{-}2.50x10^6$) $CD34^+$ UCB cells from six donors (Table 2). *Ex vivo* generation using Method III resulted in a calculated NK cell yield of $4.6\pm2.4x10^9$ (range $1.9\text{-}7.8x10^9$) NK cells with a purity of >95% after 6 weeks of culture (Table 2). The expansion rate ranged between 1,500 and 6,500 fold starting with $CD34^+$ cells from cryopreserved UCB. These data demonstrate that the transfer of our described procedure to clinical applicable conditions is feasible, and generates pure NK cell products with a more than 3-log expansion potential.

Table 2. Overview of CD34⁺ cell selections and NK cell culturing procedures.

			Method I - H3000		Method I - Stemline I		Method I - Stemline II	
	CD34 (%)	CD34 cells ($x10^6$)	CD56 (%)	CD56 cells ($x10^9$)	CD56 (%)	CD56 cells ($x10^9$)	CD56 (%)	CD56 cells ($x10^9$)
UCB1	78	1.10	50	0.57	74	1.20	87	2.20
UCB2	55	0.32	88	0.68	65	0.15	82	0.15
UCB3	93	0.52	94	3.10	43	0.55	71	0.50
UCB4	69	0.45	*ND*	*ND*	99	3.50	57	0.12
UCB5	87	0.20	*ND*	*ND*	75	0.40	48	0.02
UCB6	83	0.94	*ND*	*ND*	97	10.0	49	0.12
Mean	78	0.59	77	1.50	75	2.70	66	0.51
SD	14	0.36	24	0.14	21	4.00	17	0.82
Range	55-93	0.20-1.10	50-94	0.57-3.10	43-99	0.15-10.0	48-87	0.02-2.20
Median	81	0.49	88	0.68	75	0.90	64	0.14
			Method II - H3000		Method II - Stemline I		Method II - GBGM	
	CD34 (%)	CD34 cells ($x10^6$)	CD56 (%)	CD56 cells ($x10^9$)	CD56 (%)	CD56 cells ($x10^9$)	CD56 (%)	CD56 cells ($x10^9$)
UCB7	68	0.50	91	11.0	40	0.69	98	7.70
UCB8	79	0.20	89	6.1	60	0.66	99	8.50
UCB9	96	0.24	85	3.4	88	3.20	100	18.0
Mean	81	0.31	88	6.9	63	1.53	99	11.0
SD	14	0.16	3	4.0	24	1.50	1	5.50
Range	68-96	0.20-0.50	85-91	3.4-11.0	40-88	0.69-3.20	98-100	7.70-18.0
Median	79	0.24	89	6.1	60	0.69	99	8.50
			Method III - GBGM					
	CD34 (%)	CD34 cells ($x10^6$)	CD56 (%)	CD56 cells ($x10^9$)				
UCB10	98	0.84	99	5.50				
UCB11	96	1.20	96	1.90				
UCB12	95	1.00	97	2.00				
UCB13	88	0.92	95	4.00				
UCB14	98	1.30	97	6.60				
UCB15	98	2.50	97	7.80				
Mean	96	1.30	97	4.60				
SD	4	0.61	1	2.40				
Range	88-98	0.84-2.50	95-99	1.90-7.80				
Median	97	1.10	97	4.70				

A summary of all different experiments and the corresponding UCB donors using the different culture methods I-III and media (Figure 1). The purity and the total number of CD34⁺ cells after immunomagnetic selection determined by FCM are depicted for each UCB donor. The CD56 content after 5 weeks (Method I and II) or 6 weeks (Method III) of culture was analyzed by FCM as described in the Materials and Methods. Numbers of NK cells represent a theoretical calculation based on the actual expansion rates of CD56⁺ NK cells and were calculated using the initial numbers of CD34⁺ cells from each UCB multiplied by the NK cell expansion rate from each culture procedure.

Discussion

Here, we report a novel and highly potent two-step culture method for the *ex vivo* generation of functional NK cells for clinical application in the treatment of patients with AML and other malignancies. We implemented a new clinical grade basal medium, designated GBGM®, which facilitates efficient *ex vivo* HPC and NK cell expansions. Our method enables the generation of functional human NK cells more than 4-logs from $CD34^+$ cells enriched from freshly collected UCB units and more than 3-log from frozen UCB. To the best of our knowledge, this is the first demonstration that human $CD56^+$ NK cells can be efficiently generated *ex vivo* at such high log-scale magnitude.

Similar studies have been reported previously using different combinations of cytokines with or without feeder cell lines and use of animal and human sera.[15-17;20-24] For instance, a recent study by Kao *et al.*[16] showed that serum-free expanded $CD34^+$ cells could be differentiated into a NK cell product with an average purity of 40%-60% after 5-7 weeks of culture. They reached a calculated mean expansion rate of 300-fold, but used fetal bovine serum during the NK cell generation phase.Compared to these recently published data, our novel culture system holds great promise, resulting in more than 3-log-scale *ex vivo* generation of NK cell products with nearly 100% purity using clinical grade medium, human serum, and cytokines. The only cytokines that are currently or in the near future not available as clinical grade reagents are LIF and MIP-1α, which are part of the low-dose supporting cytokine cocktail. However, experiments using $CD34^+$ selected cells from cryopreserved UCB revealed that these two factors can be discarded without the loss of the NK cell differentiation potential of our refined protocol (Table 2). Taking the high expansion potential, our system allows the generation of an average number of $4.6x10^9$ highly pure NK cells from frozen $CD34^+$ UCB cells (Table 2). Moreover, preliminary upscaling experiments in cell culture bags revealed that the established culture procedure generates up to $2x10^9$ NK cells with the same phenotype and function as shown for the NK cell populations generated in tissue culture plates (data not shown). These findings indicate that our refined *ex vivo* expansion protocol has the potential to produce more NK cells (i.e. $>2x10^9$ with a purity between 95-99%) as compared to purification of mature NK cells from a 15 liter lymphapheresis procedure, which yields $0.25x10^9 \pm 0.14x10^9$ (range $0.01\text{-}0.47x10^9$) NK cells according to the published data of McKenna *et al.* in 2007.[7-12]

The NK cell product generated by our method displayed reproducibly an activated phenotype with a high expression of CD56 and various activating receptors, such as NKG2D, NKp30, NKp44 and NKp46. In line with earlier observations, our *ex vivo*-generated NK cells cultured in the presence of IL-2 and IL-15 showed a $CD56^{bright}$ phenotype of which only a subset expressed CD16.[20;21;25;26] The resulting NK cell products contained 40-60% $CD56^+NKG2A^+$ NK cells and up to 10% of the $CD56^+$ population expressed various KIRs, though there was considerable variation in the frequency of KIR^+ NK cell subsets between the different UCB donors. According to the NK cell differentiation model of Freud and Caligiuri[27], our *ex vivo*-generated NK cell products predominantly contain developmentally mature NK cells expressing high CD94/NKG2A, CD117

and/or KIR mediating potent cytolytic activity against K562 cells. A minority of the $CD56^+$ NK cells in our products are phenotypically immature lacking NKG2A and KIR. However, these immature NK cells may have the potential to maturate *in vivo* following adoptive transfer. Interestingly, Cooley *et al.*[28] have shown that $CD56^+NKG2A^-KIR^-$ NK cells are able to mature *in vitro* upon culture with IL-15 and a stromal feeder cell line. Phenotypic analysis have revealed that UCB-derived NK cells generated with our protocol display expression of cytokine receptors including IL-2/IL-15Rβ (CD122), which may promote *in vivo* survival, expansion and maturation upon transfer following immunosuppressive therapy.

The strong cytotoxic activity of our UCB-derived NK cells against various tumor cell lines as well as the cytolysis of primary AML cells were displayed by specific lysis, CD107a-mediated degranulation and the production of IFN-γ. Most experiments were done with AML cell lines and primary AML cells, which has been shown to be an attractive target for NK cell-mediated immunotherapy.[5;6] Interestingly, we also observed efficient lysis of melanoma cell lines, which strengthens the therapeutic potential of our NK cells product also towards non-hematological cancers. Most recently, several studies demonstrated, that NK cell-mediated cytotoxicity and the use of NK cell-based immunotherapy could be an efficient approach for the treatment of melanoma, which was also demonstrated in a phase I trial using the NK-92 cell line.[29-31]

In conclusion, the method presented here provides an important advance for generating clinically relevant NK cell products from hematopoietic stem and progenitor cells with high cell numbers, high purity and functionality for use in NK cell-based immunotherapy. These *ex vivo*-generated NK cell products can be exploited for adoptive immunotherapy either following haploidentical SCT for boosting NK cell-mediated graft-versus-leukemia reactivity or in the non-transplant setting following lymphodepleting immunosuppressive regimens. Our current NK cell generation protocol has been optimized for the use of $CD34^+$ cells from UCB, which can be readily obtained from cord blood banks. Furthermore, the described modifications of our protocol and use of the GBGM® medium have also enabled the generation of NK cells from bone marrow or mobilized peripheral blood $CD34^+$ cells (preliminary data not shown). Currently, our first aim is to explore the feasibility of adoptive transfer of *ex vivo*-generated NK cell products from KIR-ligand mismatched UCB donors in elderly patients with AML following an intensive immunosuppressive regimen. Important aspects to study in this trial are whether *ex vivo*-generated NK cell products are able to survive, migrate and expand *in vivo* following infusion into preconditioned patients.

Acknowledgements

The authors thank Lucy Sonnenberg of the Sanquin Blood Bank (Nijmegen, The Netherlands) for collecting UCB samples after normal full-term baby delivery. We thank Rob Woestenenk and Jeroen van Velzen for assistance in FCM, Peter Linssen for live imaging video experiments and Jan Boezeman for statistical analysis.

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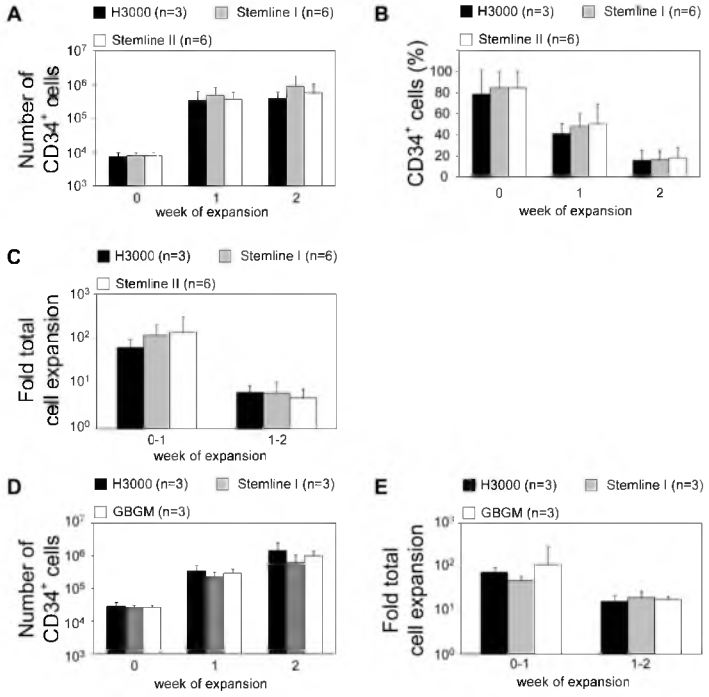

Figure S1. Expansion of CD34-enriched UCB cells using a cytokine-based culture method. $CD34^+$ UCB cells were selected by immunomagnetic beads and cultured for 2 weeks in three different basal media supplemented with 10% HS, a low-dose cell supporting cytokine cocktail, a high-dose cell expansion cocktail and clinical grade low molecular weight heparin (see details in Materials and Methods and Figure 1). Absolute $CD34^+$ cell numbers (A), fold expansion of total cells (B) and CD34 content (C) were determined by FCM after one and two weeks of culture using Method I. $CD34^+$ cell numbers and fold expansion of total cells using Method II (D,E). Data are depicted as mean±SD for the different media, which have been tested in parallel experiments with $CD34^+$ cells from 3-6 UCB donors.

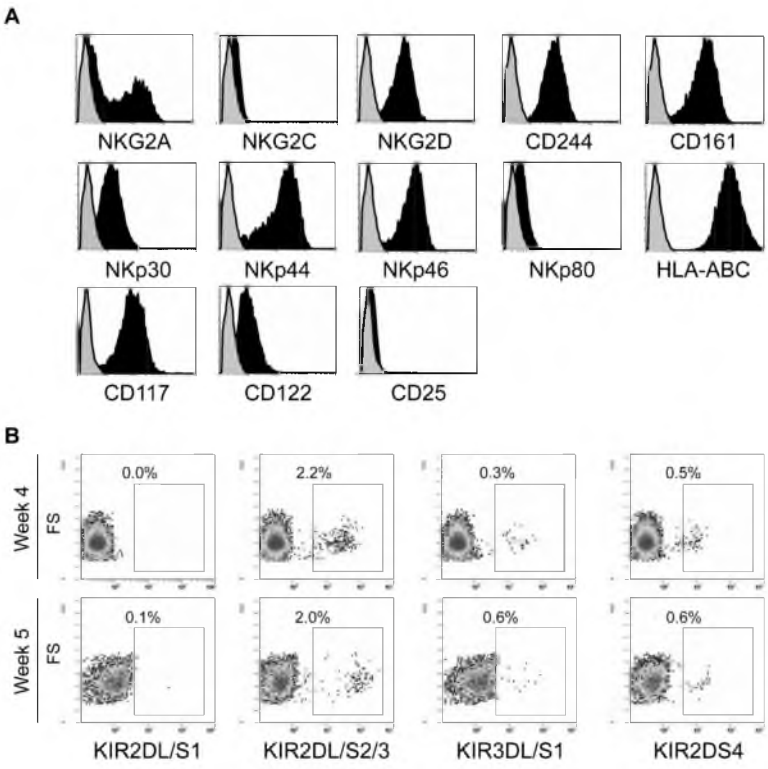

Figure S2. Phenotypical profile of *ex vivo*-generated NK cells using Method II with GBGM. Flow cytometric analysis of a second NK cell product generated from CD34$^+$ UCB progenitor cells. Cells at 5 weeks of culture were analyzed for expression of CD56, CD3, CD94 and CD16. (A) Expression of a repertoire of receptors important for regulating NK cell activity, including C-type lectin receptors, natural cytotoxicity receptors and cytokine receptors. Histograms show expression of the antigen of interest (black histogram) compared to the specific isotype control (grey histogram). (B) Acquisition of KIR$^+$ NK cell subsets during *ex vivo* NK cell generation from expanded CD34$^+$ UCB cells. KIR expression was determined at week 4 and 5 during the differentiation step by FCM.

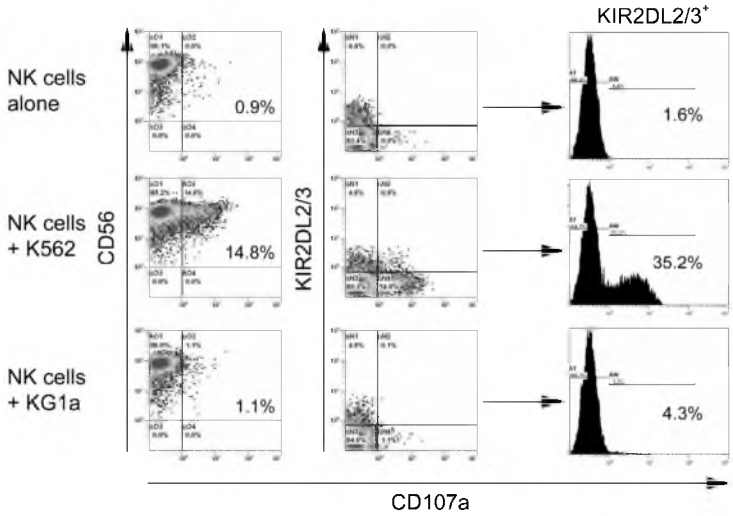

Figure S3. Responsiveness of *ex vivo*-generated KIR$^+$ NK cells generated using Method II with GBGM to MHC class I-deficient target cells. *Ex vivo*-generated NK cells were incubated alone, or 18 hours with MHC class I-negative K562 or MHC class I-expressing KG1a cells at an E:T ratio of 1:1. Cells were then stained for CD56, CD3, KIR, and the degranulation antigen CD107a. Shown are the degranulation of total CD56$^+$CD3$^-$ NK cells and KIR2DL2/DL3$^+$ NK cell subset expanded for 5 weeks from CD34$^+$ UCB cells. Density plots are gated on CD56$^+$CD3$^-$ NK cells and the histogram plots show the CD107a degranulation of the KIR2DL2/DL3$^+$ NK cells.

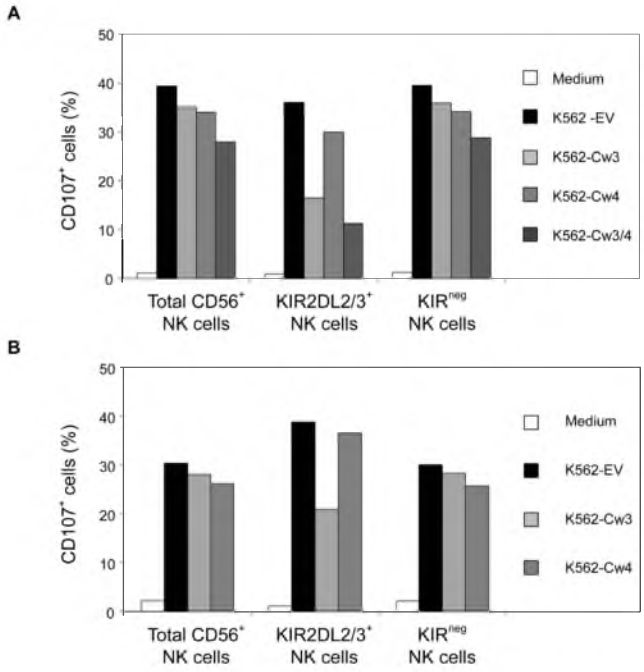

Figure S4. Responsiveness of *ex vivo*-generated KIR$^+$ NK cells generated by Method II to target cells expressing different KIR ligands. Activity of UCB-derived NK cells derived from two different donors (A and B) were tested in a 2 hour CD107a degranulation assay against K562 cells transfected with empty vector (EV), HLA-Cw3 cDNA, HLA-Cw4 cDNA or both.

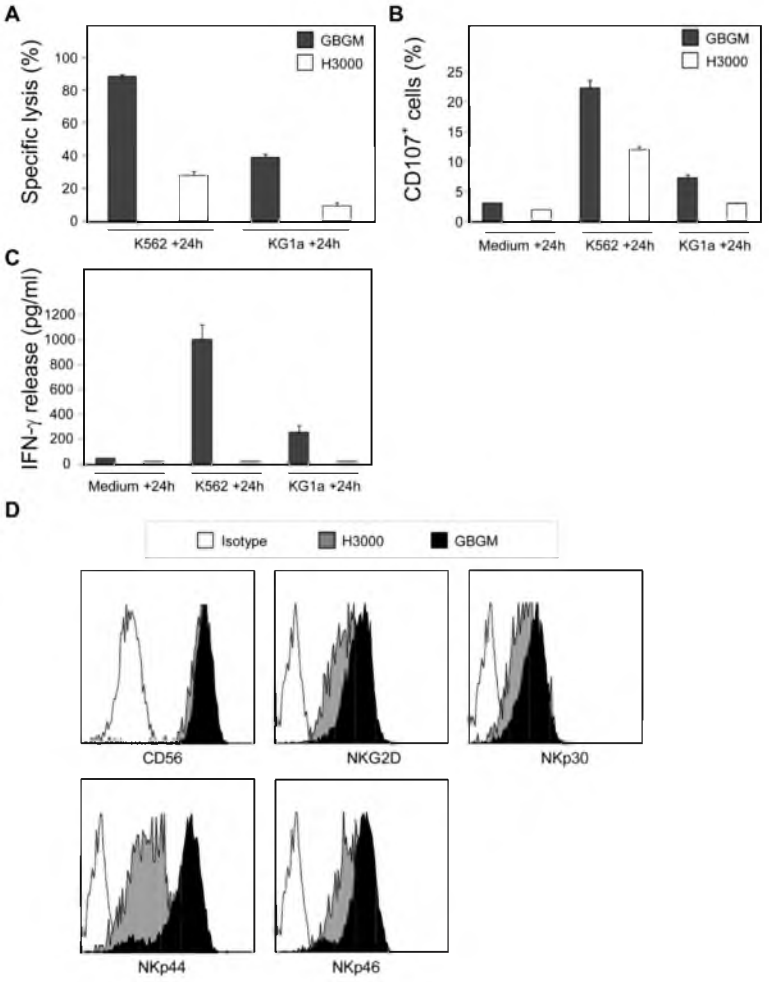

Figure S5. Functional activity of *ex vivo*-generated CD56$^+$ NK cells using Method II. (A) Specific cytotoxicity of two CD56$^+$ NK cell products from the same UCB donor but cultured either in H3000 or GBGM against the myeloid leukemia cell lines K562 and KG1a. Specific lysis was determined after 24 hours of co-culture in a FCM-based cytotoxicity assay at an E:T ratio of 2:1. Data are displayed as mean±SD of triplicate wells. (B) Degranulation of CD56$^+$ NK cells was determined by FCM as the percentage of CD107a$^+$ cells. Results are depicted as mean±SD of triplicate wells. (C) IFN-γ production was determined by ELISA and depicted as mean±SD of triplicate measurements. (D) Expression of activating receptors important for NK cell activity. Histograms show antigen expression of GBGM-derived NK cells (black histogram) compared to H3000-derived NK cells (grey histogram) and the specific isotype control (white histogram).

Movie S1. Movie showing the strong killing potential of *ex vivo*-generated NK cells (bright spots) towards the adherent growing melanoma cell line FM3 within one hour of co-culture. NK cells were added to the melanoma tumor cell layer at an E:T ratio of 1:1. The movie can be found at: doi:10.1371/journal.pone.0009221.s006 (1.43 MB AVI).

CHAPTER 6

CD244 and CD33 expression refine description of early human NK cell developmental stages in vivo and reveal distinctive maturation patterns in situ

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Abstract

A better understanding of human NK cell development *in vivo* is crucial to exploit NK cells for immunotherapy. Here, we identified seven distinctive NK cell developmental stages in bone marrow of single donors using 10-color flow cytometry and found that NK cell development is accompanied by early expression of stimulatory co-receptor CD244 (2B4) *in vivo*. Further analysis of cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) samples showed diverse distributions of the NK cell developmental stages. In addition, distinctive expression profiles of early development marker CD33 and C-type lectin receptor NKG2A between the tissues, suggest that differential NK cell differentiation may take place at different anatomical locations. Differential expression of NKG2A and stimulatory receptors (e.g. NCR, NKG2D) within the different subsets of committed NK cells demonstrated the heterogeneity of the $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^{+}$ subsets within the different compartments and suggests that microenvironment may play a role in differential in situ development of the NK cell receptor repertoire of committed NK cells. Overall, differential in situ NK cell development and trafficking towards multiple tissues may as a whole give rise to a broad spectrum of mature NK cell subsets found within the human body.

Introduction

Natural killer (NK) cells are large $CD56^{+}CD3^{-}$ granular lymphocytes and are considered part of the innate immune system. NK cells can kill infected or malignant-transformed cells without prior sensitization, and through the production of cytokines, such as IFN-γ, they form a bridge between innate and adaptive immune responses.[1,2] NK cell reactivity is tightly regulated through a balance of signals between stimulatory and inhibitory receptors, a feature that is being exploited today for NK cell-based immunotherapy against cancer.[3] For this, a thorough understanding of human NK cell development *in vivo* is crucial.

Bone marrow (BM) is generally considered as the primary site for human NK cell development.[4-7] However, a complete pathway for NK cell development and maturation in BM has not been described and it may be possible that precursor NK cells traffic from BM to other tissues for terminal differentiation *in situ*.[8] In 2005, Freud *et al*.[9] identified a BM-derived $CD34^{+}$ hematopoietic precursor cell (HPC) residing in lymph nodes (LN) where further differentiation into $CD56^{bright}$ could take place. Subsequently, they identified four discrete stages for human NK cell development within secondary lymphoid tissues (SLT) based on cell surface expression of CD34, CD117 and CD94: stage 1; $CD34^{+}CD117^{-}CD94^{-}$, stage 2; $CD34^{+}CD117^{+}CD94^{-}$, stage 3; $CD34^{-}CD117^{+}CD94^{-}$, and stage 4; $CD34^{-}CD117^{+/-}CD94^{+}$.[10] Following NK cell development, commitment to the NK cell lineage takes place at stage 3, in which CD56 appears on the cell surface and gives rise to $CD56^{bright}$ NK cells in stage 4. These data confirmed previous research describing the abundant presence of $CD56^{bright}$ NK cells in SLT.[11;12] In addition, we and others have shown that $CD56^{bright}$ cells are the first mature NK cells to arise after hematopoietic stem cell transplantation (SCT).[13;14] Overall, these data support a model of *in vivo* human NK cell development in which $CD34^{+}$ NK cell precursors traffic from BM to SLT where further differentiation into $CD56^{bright}$ NK cells can take place. However, how these NK cell developmental stages correlate with NK cell subsets in other compartments of the human body (e.g. peripheral blood (PB), spleen (SPL)) remains unclear.

In this study, we identified seven distinctive NK cell developmental stages in bone marrow using 10-color flow cytometry and found that NK cell development is accompanied by early expression of stimulatory co-receptor CD244 (2B4) *in vivo*. Furthermore, distinctive expression profiles of early development marker CD33 and the C-type lectin receptor NKG2A between the different tissues, suggest that differential *in situ* NK cell differentiation may take place at different anatomical locations. Thus, differential *in situ* NK cell development and trafficking towards multiple tissues may as a whole give rise to a broad spectrum of mature NK cell subsets found within the human body.

Materials and methods

Tissue collection and mononuclear cell isolation

Bone marrow (BM), peripheral blood (PB), spleen (SPL) and cord blood (CB) samples were obtained at the Radboud University Nijmegen Medical Centre (RUNMC; Nijmegen, The Netherlands). BM and PB samples were obtained from healthy stem cell transplantation donors before mobilization treatment with G-CSF. SPL samples were obtained from deceased liver or kidney transplantation donors. CB samples, obtained at birth after normal full-term delivery, were provided by the cord blood bank of RUNMC. At the Erasmus Medical Centre (Rotterdam, The Netherlands), liver draining lymph node (LiLN) samples were obtained from deceased liver transplantation donors and inguinal lymph node (inLN) samples from kidney transplant recipients (not treated with immunosuppressive drugs prior to lymph node excision). After collection, each tissue sample was stored at room temperature and processed within 24h. Lymph node and spleen samples were first forced through 74 µm netwell filters (Costar, Corning International, NY, and USA) to obtain single cell suspensions. Mononuclear cells (MNC) were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark) and cryopreserved in liquid nitrogen until further use. At least 5 independent samples of each tissue were collected. This study was performed in accordance with the regulations as set by the Medical Ethical Committees for human research of the RUNMC and the Erasmus MC, and written informed consent with regard of scientific use was obtained from all study participants or their representatives.

Multi-color flow cytometry

For detailed flow cytometric (FCM) analysis of the different developmental stages and phenotype of NK cells, we designed three different 10-color FCM panels using conjugated mAbs kindly provided by Beckman Coulter (Marseille, France) with the exception of CD16-FITC (Dako, Glostrup, Denmark) and CD159c-PE (R&D Systems, Minneapolis, CA, USA). Detailed description of the panels is shown in Table 1.

Table 1. Panels used for flow cytometry.

	FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PB	PO
1	CD34	CD133	CD3	CD159a	CD117	CD33	CD244	CD56	CD94	CD45
	581	*AC133*	*UCHT1*	*Z199.1.10*	*104D2D1*	*D3HL60.251*	*C1.7.1*	*N901*	*HP-3B1*	*J33*
2	CD16	CD159c	CD3	-	CD158b	CD158e1	CD158a	CD56	CD159a	CD45
	DJ130c	*134522*	*UCHT1*	-	*GL183*	*Z27.3.7*	*EB6.B.3.1.1*	*N901*	*Z199.1.10*	*J33*
3	CD16	CD336	CD3	CD337	CD335	CD314	CD244	CD56	-	CD45
	DJ130c	*Z231*	*UCHT1*	*Z25*	*BAB281*	*ON72*	*C1.7.1*	*N901*	-	*J33*

← Displayed are the combinations of conjugated monoclonal antibodies (mAb) against specific antigens within each panel. In addition, the clone for each specific mAb is shown. Each panel was used for flow cytometric (FCM) analysis of bone marrow, cord blood, peripheral blood, inguinal LN, liver LN, and spleen samples of human donors (all n=5). Thawed MNC fractions of the human tissue samples were assessed on a Navios™ 10-color flow cytometer and analyzed using Kaluza Software® 1.0 (Beckman coulter). Panel 1 was used to identify different NK cell developmental stages based on CD34, CD117, CD94 and CD56 expression profiles.[10] Additionally, expression of early development markers CD133 and CD33, stimulatory co-receptor 2B4 (CD244), and C-type lectin NKG2A were analyzed to refine the definition of the different NK cell developmental stages. Panel 2 and 3 were used to analyze the NK cell receptor repertoire of $CD45^+CD56^{bright}CD16^{+/-}CD3^-$ and $CD45^+CD56^{dim}CD16^+CD3^-$ NK cells consisting of inhibitory and stimulatory receptors. Inhibitory receptors contain KIR (CD158a, CD158b, $CD158e_{1}$) and NKG2A (CD159a). Stimulatory receptors contain NCR (CD335/336/337), NKG2C (CD159c), NKG2D (CD314), and CD244 (2B4).

For 10-color FCM, combinations of mAb-fluorochrome were balanced to avoid antibody interactions, sterical hindrance and to detect also dimly expressing populations. Before multi-color analyses, all conjugates were titrated and individually tested for sensitivity, resolution and compensation of spectral overlap. Isotype controls were used to define marker settings. Thawed MNC fractions of collected human tissues were assessed on a Navios™ 10-color flow cytometer and analyzed using Kaluza Software® 1.0 (Beckman coulter). To define NK cell developmental stages, samples were gated on the $CD45^+CD3^-$ population within CD45+/SS gated cells to exclude T cells and endothelial cells (which may express CD34 but are CD45 negative[15]) from analysis. To analyze the NK cell receptor repertoire (Table 1) of committed NK cells expressing CD56, cells were further gated on $CD56^+$ cells within the $CD45^+CD3^-$ population. Cell populations > 0.1% of the $CD45^+CD3^-$ population with a threshold of more than 50 cells were considered reliable. Cell populations were considered to be present in a specific tissue when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. An overview of analyzed sample sizes is shown in Table S1.

Statistical analysis

To compare percentages of cells positive for single markers between the different tissues, a random effect logistic regression model was used that accounted for the biological diversity between samples of each tissue and for the fact that several samples of each tissue type were taken. Mean fluorescence (MFI) of specific markers between the different tissues were analyzed using ANOVA analysis with Tukey post testing. *P*-values <.05 were considered significant.

Results

To identify human NK cell developmental stages within the different tissues and to analyze the distribution of different NK cell subsets and their NK cell receptor repertoire, we designed three 10-color flow cytometry (FCM) panels (Table 1). As BM is considered the origin of NK cell development[4-7], we first analyzed BM for the presence of NK cell developmental stages.

Identification of seven NK cell developmental stages in BM

Distinct NK cell developmental stages can be characterized through expression analysis of CD34, CD117, CD94 and CD56 antigens.[10] Based on that, we gated our samples on the $CD45^+CD3^-$ population within $CD45^+$/SS gated cells to exclude T cells and endothelial cells from analysis. Subsequently, cell subsets were first divided based on the expression of CD34 and CD117. From there, in a second step, each subset was analyzed for CD56 and CD94 expression. Using this gating strategy, we were able to identify seven distinctive developmental stages in BM (Figure 1).

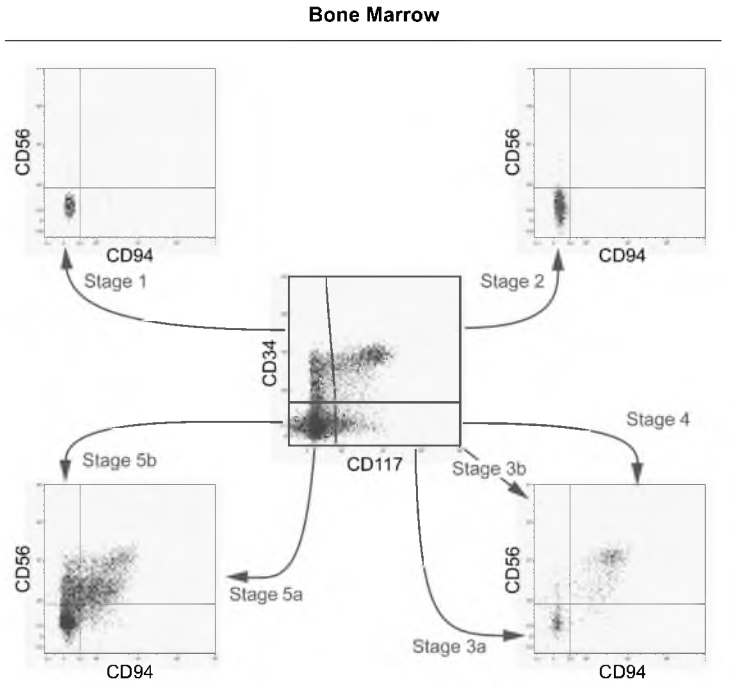

Figure 1. Identification of seven NK cell developmental stages in bone marrow (BM). Based on the stages defined in Table 1, we analyzed the presence of the different NK cell developmental stages in BM. Shown is one representative example (n=5). Cells were gated on the $CD45^+CD3^-$ population within $CD45^+$/SS gated cells to exclude T cells and endothelial cells from analysis. Subsequently, cell subsets were divided based on the expression of CD34 and CD117. From there, each subset was analyzed for CD56 and CD94 expression, leading to the identification of seven NK cell developmental stages: 1, 2, 3a, 3b, 4, 5a, 5b.

On this basis and in concert with NK cell developmental stages as identified in secondary lymphoid tissues (SLT)[10], we now propose the following model of NK development, starting from $CD34^+CD117^-CD56^-CD94^-$ cells (stage 1), followed by the gain of CD117 (stage 2; $CD34^+CD117^+CD56^-CD94^-$). Subsequently, CD34 expression is lost in stage 3a ($CD34^-CD117^+CD56^-CD94^-$) followed by NK cell lineage commitment through CD56 acquisition in stage 3b ($CD34^-CD117^+CD56^+CD94^-$). After NK cell lineage commitment, cells gain CD94 expression

and develop into immature $CD56^{bright}$ NK cells (stage 4; $CD34^{-}CD117^{+}CD56^{+}CD94^{+}$). Through loss of CD117 expression, $CD56^{dim}$ cells start to develop (stage 5a; $CD34^{-}CD117^{-}CD56^{+}CD94^{+}$), followed by loss of CD94 expression in stage 5b ($CD34^{-}CD117^{-}CD56^{+}CD94^{-}$). The acquisition/ loss of the different antigens and the presence of $CD56^{bright/dim}$ cells within each stage in BM are summarized in Table 2.

Table 2. Developmental stages of NK cells in BM.

	CD34	CD117	CD56	CD94	CD56: bright or dim
Stage 1	+	-	-	-	-
Stage 2	+	+	-	-	-
Stage 3a	-	+	-	-	-
Stage 3b	-	+	+	-	$CD56^{dim}$
Stage 4	-	+	+	+	$CD56^{bright}$
Stage 5a	-	-	+	+	$CD56^{bright < dim}$
Stage 5b	-	-	+	-	$CD56^{bright << dim}$

Main stages of NK cell development in BM based on expression profiles of CD34, CD117, CD56 and CD94.

Early and sustained CD244 expression during *in vivo* NK cell development

By using 10-color FCM, we were able to further specify the identified NK cell developmental stages in BM by analyzing additional antigen expression. For this purpose, we analyzed the cell surface expression of CD133, CD33, CD244 and NKG2A within each defined stage (Figure 2). CD133 is known as a stem cell antigen that may provide an alternative to CD34 for the selection and expansion of hematopoietic cells for transplantation.[16] Together with CD34, this antigen was only expressed within stages 1 and 2. CD33 has been described as an antigen for early NK cell development[17] and was expressed in stages 2 and 3a. The CD244 receptor is suggested to be a co-receptor in activation of mature NK cells.[18] Interestingly, we found that CD244 was already expressed on $CD34^{+}CD117^{+}$ stage 2 cells in BM. During stages 3a and 3b, CD244 expression remained present and the amount of $CD244^{+}$ cells was increased to more than 98% in stages 4 to 5b. Until now, CD244 expression was only shown to be present at early stages of NK cell differentiation during *in vitro*-induced human NK cell maturation.[19] The inhibitory NKG2A receptor, shown to be expressed early during NK cell maturation[13], was detected starting from stage 4 just after NK cell commitment (stage 3b) till stage 5b. In summary, as the different assessed antigens showed different expression profiles during NK cell development, we were able to further define the NK cell developmental stages (Table 3), in which CD133 expression is specific for stages 1 and 2, followed by CD33 expression in stages 2 and 3. From stage 2, CD244 is continuously expressed and NKG2A is found in stages 4 to 5b on part of the cells.

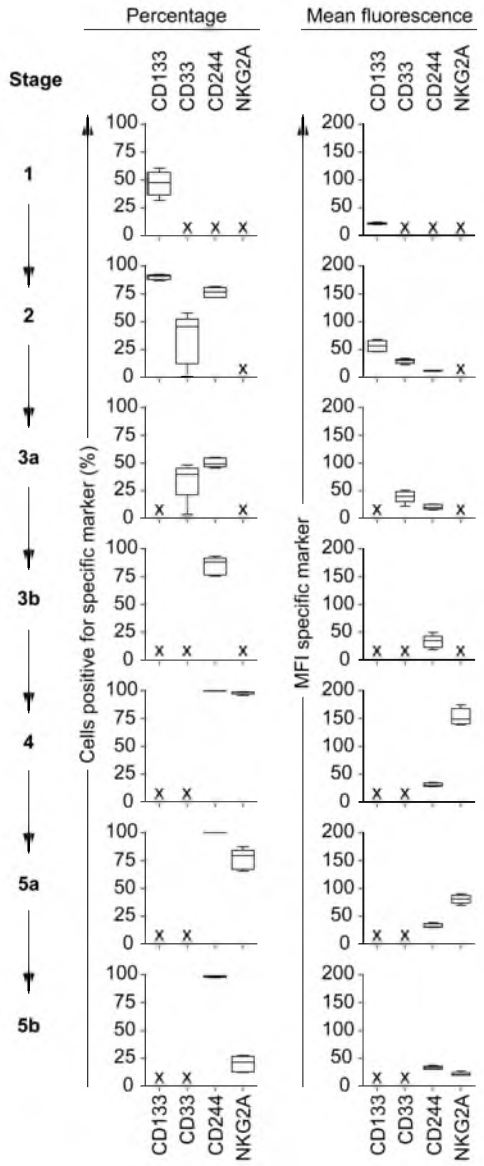

Figure 2. Expression of CD133, CD33, CD244 and NKG2A within the NK cell developmental stages in bone marrow (BM). Cells were gated on the $CD45^{+}CD3^{-}$ population within $CD45^{+}$/SS gated cells to exclude T cells and endothelial cells from analysis. Next, cell subsets were divided based on the expression of CD34 and CD117. From there, each subset was analyzed for CD56 and CD94 expression. Subsequently, the expression of CD133, CD33, CD244 and NKG2A was analyzed within the different NK cell developmental stages in BM (n=5). Left panels show the percentages of cells positive for the specific markers. Right panels show the mean fluorescence (MFI) of each specific marker. Cell populations > 0.1% of the $CD45^{+}CD3^{-}$ population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. Shown in this figure are all NK cell developmental stages within each tissue.

Table 3. Developmental stages of NK cells in BM (continued).

	CD133	**CD34**	CD33	**CD117**	CD244	**CD56**	**CD94**	NKG2A	CD56: bright or dim
Stage 1	+/-	+	-	-	-	-	-	-	-
Stage 2	+	+	+/-	+	+/-	-	-	-	-
Stage 3a	-	-	+/-	+	+/-	-	-	-	-
Stage 3b	-	-	-	+	+	+	-	-	$CD56^{dim}$
Stage 4	-	-	-	+	+	+	+	+	$CD56^{bright}$
Stage 5a	-	-	-	-	+	+	+	+/-	$CD56^{bright < dim}$
Stage 5b	-	-	-	-	+	+	-	+/-	$CD56^{bright << dim}$

Further identification of developmental NK cell stages in BM based on expression of CD133, CD34, CD33, CD177, CD244, NKG2A, CD56 and CD94. Indicated is the presence of each specified marker within each stage (based on the percentage of positive cells present): + = 100-80%; +/- < 80%; - = below reliable detection limits.

NK cell development starts in BM, followed by further maturation in LN, SPL and PB

To assess whether the NK developmental stages can be found in other human tissues besides BM, we further analyzed samples of cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (Figure 3).

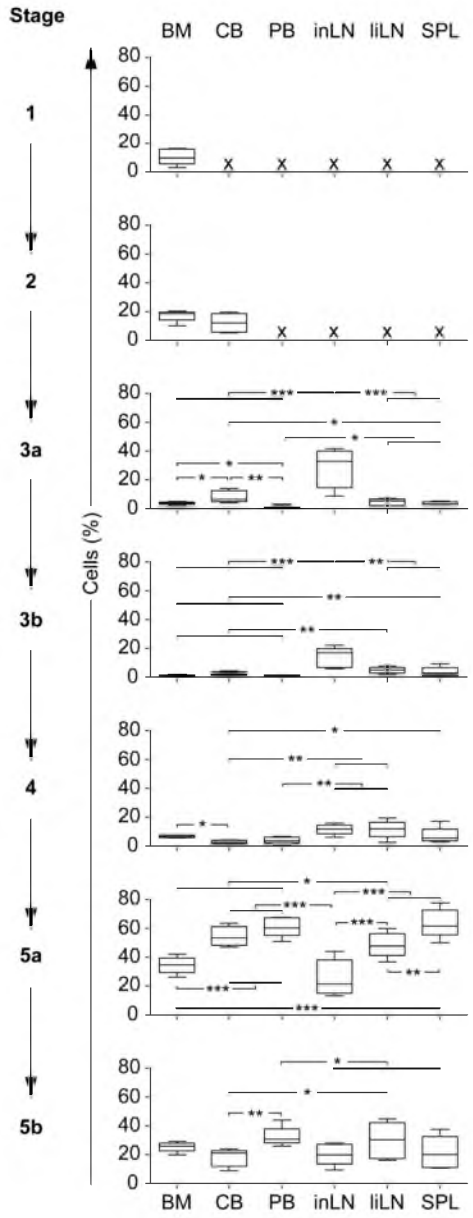

Figure 3. Distribution of the NK cell developmental stages within different human tissues. The distribution of the seven NK cell developmental stages was analyzed within samples of bone marrow (BM), cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (all n=5). For identification of the NK cell developmental stages, cells were gated on the $CD45^+CD3^-$ population within $CD45^+$/SS gated cells to exclude T cells and endothelial cells from analysis. Subsequently, cell subsets were divided based on the expression of CD34 and CD117. From there, each subset was analyzed for CD56 and CD94 expression. Cell populations > 0.1% of the $CD45^+CD3^-$ population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. Shown in this figure are all NK cell developmental stages within each tissue. Comparison between the different tissues was analyzed using a random effect logistic regression model; **P*<.05, ***P*<.01, ****P*<.0001.

Results showed a differential distribution of the NK cell developmental stages within the different tissues. The NK cell developmental stages in BM mainly consisted of stage 5a and 5b cells. In addition, stages 1 and 2 were only detected in BM, confirming BM as the origin of NK cell development. In CB, stage 2 cells were found, but not in PB, showing that blood of fetal origin contains more early NK progenitor cells as compared with adult blood. However, the main NK cell developmental stages in CB were stage 5a followed by stage 5b. In PB, the NK cell developmental stages mainly consisted of stage 5a and 5b cells. In contrast to other tissues, the distribution of NK cell developmental stages in inLN primarily contained stage 3a and stage 3b cells, and showed lower, but similar, frequencies of stages 4 to 5b. In contrast, NK cell developmental stages in liLN and SPL consisted primarily of stages 4, 5a and 5b cells. Following the presence of the different NK developmental stages within the different tissues analyzed, these results suggest that early NK progenitor cells migrate from BM to SLT, after which pre-NK cells (stage 3a) may further develop in LN leadings to NK cell commitment (stage 3b), followed by further maturation in splenic tissue and the release of mature NK cells into the blood stream. The presence of different stages within one tissue, for instance stages other than stage 1 and 2 in BM or stage 3 in LN, indicates that *in situ* differentiation of remaining cells also occurs besides trafficking of developmental stages towards other tissues.

Sustained CD33 expression in liLN during *in vivo* NK cell development

To asses potential differences of the NK cell developmental stages within the human tissues, we further analyzed the expression of CD133, CD33, CD244 and NKG2A within the stages present in the human tissues (Figure 4).

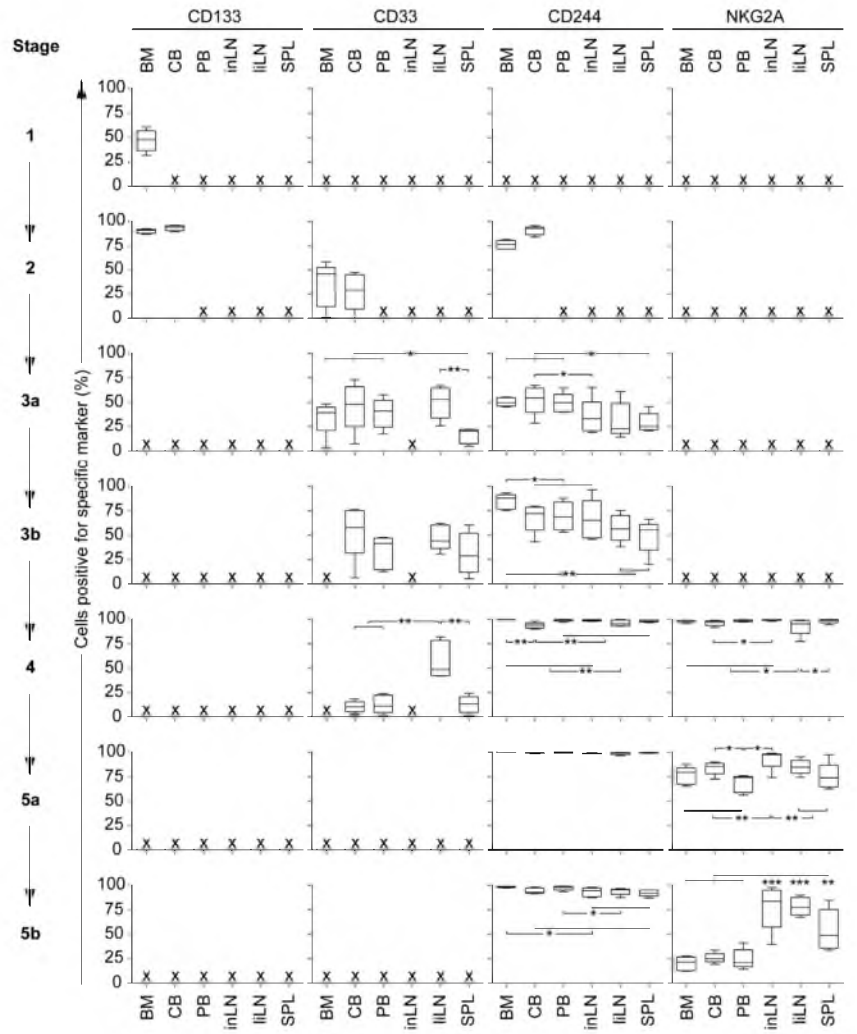

Figure 4. Expression of CD133, CD33, CD244 and NKG2A within the NK cell developmental stages present in different human tissues. Cells were gated on the $CD45^{+}CD3^{-}$ population within $CD45^{+}$/SS gated cells to exclude T cells and endothelial cells from analysis. Next, cell subsets were divided based on the expression of CD34 and CD117. From there, each subset was analyzed for CD56 and CD94 expression. Subsequently, the expression (%) of CD133, CD33, CD244 and NKG2A was analyzed within the different NK cell developmental stages in bone marrow (BM), cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (all n=5). Cell populations > 0.1% of the $CD45^{+}CD3^{-}$ population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. Comparison between the different tissues was analyzed using a random effect logistic regression model; **P*<.05, ***P*<.01, ****P*<.0001.

Between BM and CB, there were no significant differences in expression of CD133, CD33 and CD244 within stage 2. The subsequent trend of CD244 acquisition was the same for each tissue and all tissues showed more than 98% CD244$^+$ cells in stages 4 and 5a/b. Significant differences were seen in the expression profile of the early CD33 antigen within the different human tissues. As we previously characterized CD33 expression to be specific for stage 2 and 3a cells in BM (Table 3), CD33 expression was prolonged in CB, PB, and SPL until stage 3b. Furthermore, in liLN, CD33 expression was even sustained after NK cell commitment until stage 4. The prolonged expression of CD33 in some distinct stages and tissues suggests tissue specific NK cell developmental subsets *in situ*.

NKG2A expression revealed an impaired NK cell maturation profile in lymphoid tissues

Having described tissue specific NK cell subsets, by the expression profile of CD33, we further analyzed if there are also tissue specific differences in the NK cell maturation pattern. As the level of NKG2A expression may be representative for the level of NK cell maturation[20,21], we analyzed the NKG2A expression profile on "committed" NK cells. Besides the significant differences in the CD33 expression profile, the expression profile of NKG2A also showed a distinction between the different human tissues (Figure 4). In stage 4, all tissues contained more than 95% NKG2A$^+$ cells. Following NK cell developmental stages, BM, CB and PB showed a decrease in the percentage of NKG2A$^+$ cells up to approximately 25% NKG2A$^+$ cells in stage 5b, whereas in inLN and liLN a median of 75-80% remained NKG2A$^+$ and SPL kept a median of 50% NKG2A$^+$ cells. The stronger decrease of cells expressing NKG2A in BM, CB and PB as compared with other tissues was also reflected in the mean fluorescence intensity of NKG2A expression following stage 4 to 5b (Figure S1). Overall, these data suggest that the committed NK cells in LN and SPL have a more immature phenotype as compared with cells present in BM, PB and CB.

In order to better define NK cell maturation, we extended our analyses with regard to "committed" NK cells. Therefore, we subsequently analyzed the expression of additional NK cell receptors to further asses the maturity status of the committed NK cells within the different human tissues.

Differences in the NK cell receptor repertoire suggests distinct in situ NK cell development within LN and CB

Phenotypically committed NK cells (CD45$^+$CD3$^-$CD56$^+$) can generally be divided into two distinguishable subsets: the CD56^{bright}CD16$^{+/-}$ and the CD56^{dim}CD16$^+$ subset.[22] Our data, confirmed the heterogeneity of the CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16$^+$ subsets within BM, CB, PB, and LN, showing balances of CD56^{bright}>>CD56^{dim} in LN, and CD56^{bright}<<CD56^{dim} in BM, CB and PB (Figure S2). Additionally, we identified a CD56^{bright}≈CD56^{dim} balance in SPL.

To further assess the maturity of the committed NK cell subsets, we analyzed the expression of various inhibitory and stimulatory NK cell receptors by using FCM panels 2 and 3 (Table 1). For

analysis of the committed NK cell population, we gated on $CD56^+$ cells within the $CD45^+CD3^-$ population and subsequently analyzed the expression of killer immunoglobulin-like receptors (KIR), NKG2A/C, NKG2D, CD244 and natural cytotoxicity receptors (NCR; NKp30, NKp44, NKp46). These receptors trigger and modulate mature NK cell effector function through a balance between inhibitory (KIR, NKG2A) and stimulatory signals (NKG2C, NKG2D, CD244, NCR).[2;23]

We first analyzed the NK cell receptor repertoire of the $CD56^{bright}CD16^{+/-}$ subset within the committed NK cell population of each tissue (Figure 5). Results showed that there was no difference in the amount of KIR^+ cells between the tissues. Nevertheless, the mean fluorescence of KIR2DL/S2/3 and KIR3DL1 was lower in both LN and SPL, suggesting a more immature phenotype of $CD56^{bright}$ cells as compared with BM, CB and PB. Surprisingly, the proportion of $NKG2A^+$ cells was significantly lower in liLN as compared to other tissues. This may be explained by a different NK cell development *in situ*, as suggested by the prolonged expression of CD33 (Figure 4). Furthermore, the amount of activating receptor positive cells, with the exception of NKp44, was also lower in liLN as compared with other tissues (Figure 6). This was also reflected within the $CD56^{dim}CD16^+$ subset of liLN, showing lower amounts of $NKG2D^+$, $CD244^+$ and $NKp30^+$ cells as compared with other tissues. Thus, these results show that NK cell development in lymphnodes may differ *in situ* between LN at different anatomical locations and also other tissues.

Analysis of CB showed that both the $CD56^{bright}CD16^{+/-}$ (Figure 5) and the $CD56^{dim}CD16^+$ (Figure 6) subset contained significantly more $NKG2A^+$ cells as compared with other tissues. In addition, the level of NKG2A expression (MFI) in the $CD56^{bright}CD16^{+/-}$ subset was also significantly higher, which confirmed previous results.[24] NKG2C, which is the stimulatory lectin-like counterpart of NKG2A, also showed elevated expression within the $CD56^{bright}CD16^{+/-}$ subset of committed NK cells in CB (Figure 5). Together, these data suggest that the fetal micro-environment of CB may provide prevalence for the expression of lectin-like antigens as compared with other human tissues.

Overall, the data on the NK cell receptor repertoire within the different subsets of the committed NK cells demonstrates the heterogeneity of the $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^+$ within the different compartments and suggests that microenvironment may play a role in differential in situ development of the NK cell receptor repertoire of committed NK cells.

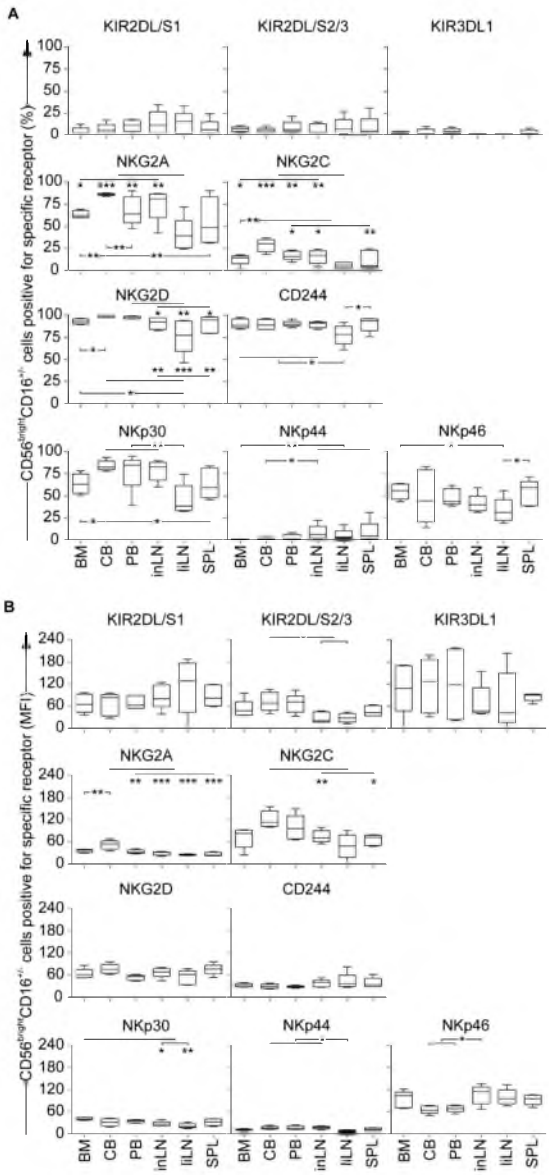

Figure 5. Expression of KIR, NKG2A/C, NCR, NKG2D and CD244 within the CD56^{bright}CD16$^{+/-}$ NK cell subset of different human tissues. Cells were gated on the CD45^{+}CD56^{+}CD3^{-} population within CD45^{+}/SS gated cells to exclude T cells and endothelial cells from analysis. Subsequently, the expression of KIR, NKG2A/C, NCR (NKp30, 44, 46), NKG2D and CD244 was analyzed within the CD56^{bright}CD16$^{+/-}$ NK cell subset present in the committed NK cell population of bone marrow (BM), cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (all n=5). (A) Shown are the percentages of CD56^{bright}CD16$^{+/-}$ cells positive for each specific receptor within each tissue. (B) Shown is the mean fluorescence intensity (MFI) for each specific receptor expressed by CD56^{bright}CD16$^{+/-}$ cells. Cell populations > 0.1% of the CD45^{+}CD3^{-} population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Comparison of percentages of positive cells between the different tissues was analyzed using a random effect logistic regression model. Comparison of MFI of positive cells between the different tissues was analyzed using ANOVA; *P<.05, **P<.01, ***P<.0001.

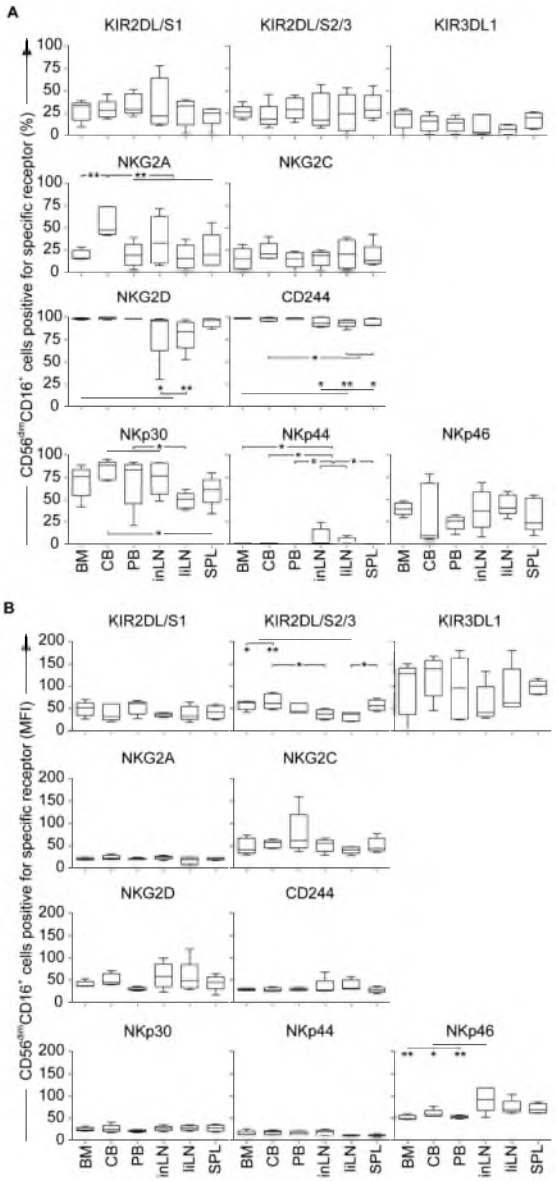

Figure 6. Expression of KIR, NKG2A/C, NCR, NKG2D and CD244 within the CD56^{dim}CD16^{+} NK cell subset of different human tissues. Cells were gated on the CD45^{+}CD56^{-}CD3^{-} population within CD45^{+}/SS gated cells to exclude T cells and endothelial cells from analysis. Subsequently, the expression of KIR, NKG2A/C, NCR (NKp30, 44, 46), NKG2D and CD244 was analyzed within the CD56^{dim}CD16^{+} NK cell subset present in the committed NK cell population of bone marrow (BM), cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (all n=5). (A) Shown are the percentages of CD56^{dim}CD16^{+} cells positive for each specific receptor within each tissue. (B) Shown is the mean fluorescence intensity (MFI) for each specific receptor expressed by CD56^{dim}CD16^{+} cells. Cell populations > 0.1% of the CD45^{+}CD3^{-} population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Comparison of percentages of positive cells between the different tissues was analyzed using a random effect logistic regression model. Comparison of MFI of positive cells between the different tissues was analyzed using ANOVA; *P<.05, **P<.01.

Discussion

In contrast to T and B cells, the developmental pathways and locations for human NK cell development are currently less well defined. Results of this study suggest that this is partly due to the heterogeneity of NK cell subsets and their diverse anatomical distribution over the different compartments within the human body. Using 10-color FCM, we were now able to establish seven NK cell developmental stages, which are numbered in line with the initial 4 stages as described by Freud *et al.*[10], based on expression profiles of CD34, CD117, CD56 and CD94, which were further specified by the expression profiles of CD133, CD33, CD244 and NKG2A. Our results showed that NK cell development is accompanied by early expression of CD244 and that there is a diverse distribution of the NK cell developmental stages among different human tissues. In addition, we provided a detailed phenotypical characterization of the committed NK cell population revealing different NK cell maturation stages (i.e. $CD56^{bright}$:$CD56^{dim}$ ratio and heterogeneity in the NK cell receptor repertoire) within the tissues.

Our data pointed BM to be the origin for NK cell development as stage 1 and 2 cells ($CD34^+CD117^-CD56^-CD94^-$ and $CD34^+CD117^+CD56^-CD94^-$, respectively) were only found in BM and not in LN, SPL or PB. This is in contrast to research done by Freud *et al.*, who identified stage 1 and 2 cells in secondary lymphoid tissues (SLT).[10] This may be due to differences in sample preparations before analysis. In the previous study, for each donor's tissue, cells from multiple tonsil pieces or multiple LN were pooled in order to obtain sufficient numbers within each NK cell developmental stage. In contrast, with the use of 10-color flow cytometry, we were able to study small sample sizes of tissues obtained from single donors. Due to biological variation between donors, pooled samples of each tissue may cause a non-representative view of NK cell subset numbers present within the different tissues analyzed. However, due to the detection limits that we set for our analysis, we cannot rule out that very small populations remained undetected within our samples. Nevertheless, without pooling samples, we believe that this unique collection of tissue samples provides a genuine view on the distribution of the different NK cell developmental stages within the different anatomical locations as they are *in vivo*.

Upon acquiring CD56 expression (NK cell commitment), the cells acquired CD94 expression. Simultaneously, NKG2A positive cells appeared. Later, following NK cell development, CD94 expression decreased during the final maturation steps towards a $CD56^{dim}$ phenotype together with a decrease in NKG2A expression.[17;25] The concomitant downregulation of NKG2A with CD94 may be explained by heterodimer formation that gives rise to a functional inhibitory CD94/NKG2A complex.[26;27] This complex is used for broad immune surveillance as it recognizes the ubiquitously expressed HLA-E molecule. Notably, we also observed a mature *in vivo* NK cell developmental stage, that primarily consisted of $CD56^{dim}$ cells, expressing low levels of NKG2A without CD94 (stage 5b: $CD133^-CD34^-CD117^-CD244^+CD56^+CD94^-NKG2A^+$). The low expression level of NKG2A in combination with the fact that NKG2A only contributes to the binding affinity of the heterodimeric complex and HLA-E solely interacts with CD94[27], suggests that in this case NKG2A is non-functional. Recently, the functional CD94/NKG2A receptor complex was

described to be endocytosed by a macropinocytic-like process, which may be related to the maintenance of its surface expression.[28] However, as it is not clear whether the CD94/NKG2A complex is internalized as a whole or may be first uncoupled before internalization, it may be possible that for a brief period NKG2A may exist on the cell surface without CD94. Together, this suggests that inhibition of cells in stage 5b largely depends on KIR signaling, and that stage 5b is one of the final stages in NK cell development.

Remarkably, the phenotype of committed NK cells (stage 3b-5b) differed between both LN (liver and inguinal sites). The NK cell receptor repertoire in liLN showed significantly less cells expressing NKG2A/C, NKp30, CD244 and NKG2D. All together, these results suggest that committed NK cells in liLN reside in an even more immature state as compared with committed NK cells in inLN. This was confirmed by the expression of CD33, a marker for early NK cell development, which is expressed significantly longer by committed NK progenitor cells in liLN as compared with committed NK progenitors in inLN and other tissues following NK cell development. Similarly, $CD56^{bright}$ NK cells in the liver itself show a more immature phenotype as compared to their counterpart in PB.[29] The differences in NK cell differentiation in situ after NK cell commitment may be due to the regional immune system of the liver, which is characterized by relatively weak cellular immune responses and hyporesponsiveness.[30;31] As NK cells form a bridge between innate and adaptive immune responses, the immature state of committed NK cells in liver and its draining LN may play a role in this. However, as we were not able to perform functional analysis due to small sample sizes, this remains subjected to further study.

Our findings confirm results from previous studies showing that the $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^{+}$ subsets are present in the same proportions in both CB and PB.[32;33] Nevertheless, these studies also reported that CB NK cells have a natural reduced killing ability as compared with PB NK cells. We postulate that this may be due to differences in NKG2A expression as our results showed that NKG2A is significantly more expressed in CB as compared with committed NK cells in PB and also other tissues. Nevertheless, the reduced killing ability of CB NK cells can be reversed after cytokine stimulation.[32;33] Thus, as CB is a source for *ex vivo* generation of NK cell-based immunotherapeutics[34], the high expression of NKG2A and its inhibition on NK cell activity of CB NK cells may not necessarily form a problem for NK cell-based immunotherapeutic strategies.

Although our analysis of the committed NK cells could not distinguish NK cell subsets between stages 3b to 5b, the ratio between the $CD56^{bright}CD16^{+/-}$ and $CD56^{dim}CD16^{+}$ subsets and the overall expression of KIR and NKG2A provided a clear overview on the maturation status of committed NK cells within the different tissues. Committed NK cells in LN represented the most immature status as the NK cells profoundly consisted of $CD56^{bright}CD16^{+/-}$ cells, followed by SPL showing an equal balance of both subsets. BM, CB and PB held the most mature status of NK cells as they abundantly contained the $CD56^{dim}CD16^{+}$ subset. After allogeneic stem cell transplantation (SCT), *in vivo* maturation of the NK cell receptor repertoire is characterized by fast upregulation of NKG2A followed by the acquisition of KIR together with a slow decrease in NKG2A expression.[13;14;20;35] The maturation status of the committed NK cells within the different

tissues analyzed confirm these findings as the overall KIR expression was highest in BM, CB and PB, followed by SPL and was lowest in LN. With the exception of CB, ranges of NKG2A expression were lowest in BM and PB, followed by a higher range of NKG2A expression in LN and SPL.

In summary, these data support a model for *in vivo* NK cell development indicating BM as the origin of NK cell development (Figure 7). Through trafficking of precursor NK cells from BM to LN, commitment to the NK cell lineage takes place in LN followed by in situ differentiation and maturation of NK cells with restricted maturation of the NK cell receptor repertoire. For further differentiation of committed NK cells, stage 3b or stage 4 $CD56^{low/bright}$ cells may traffic towards splenic tissue in which $CD56^{dim}$ cells may develop and further maturation of the NK cell receptor repertoire takes place. Final maturation of NK cells occurs through trafficking of cells (stage 4 to 5) towards the periphery from which NK cells may be further distributed to different compartments in the human body. Further functional analyses of committed NK cells within the different NK cell developmental stages are warranted to obtain a complete view on the NK cell developmental pathway that includes the acquisition of the cytolytic and cytokine producing functions during NK cell development.

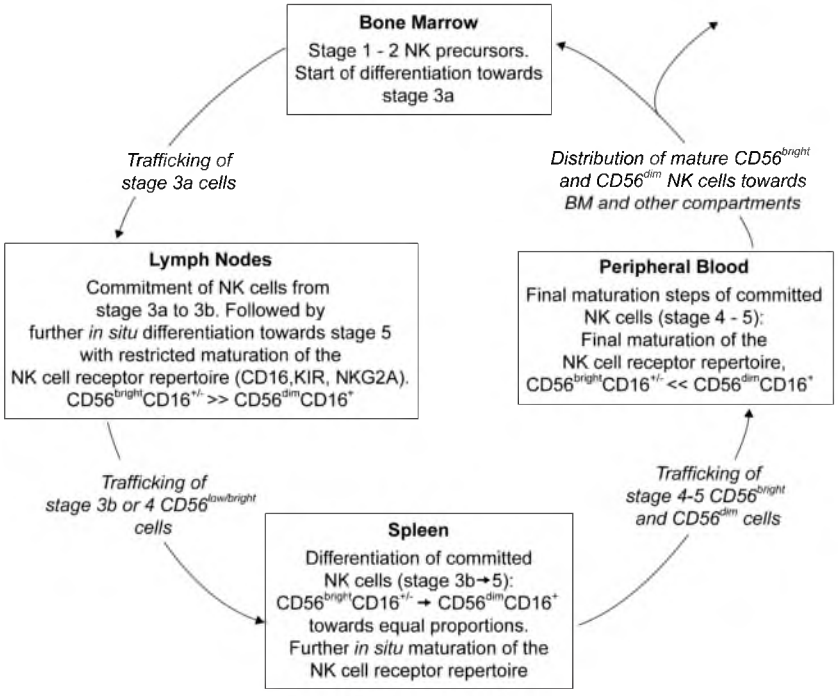

Figure 7. Proposed model for human NK cell development *in vivo*. Based on our data, we propose that precursor NK cells (stage 2) traffic from BM to LN, where commitment to the NK cell lineage takes place (stage 3a → 3b) followed by in situ differentiation of NK cells with restricted maturation of the NK cell receptor repertoire. For further differentiation of committed NK cells, $CD56^{bright}$ cells (stage 4) may traffic towards splenic tissue in which $CD56^{dim}$ cells may develop and further maturation of the NK cell receptor repertoire takes place. Final maturation of NK cells occurs through trafficking of cells towards the periphery from which NK cells may be further distributed to different compartments in the human body.

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Supplemental material

Table S1. Cell numbers in analyzed samples.

	Total cell number[1] (x10^4)	Gated CD45$^+$CD3$^-$ cells[2] (x10^4)	Progenitor cells[3] (x10^4)
BM	16.9 (8.9-34.9)	2.8 (1.9-3.3)	0.98 (0.7-1.2)
CB	39.9 (27.5-99.9)	12.2 (7.1-31.4)	1.88 (0.6-5.0)
PB	37.8 (35.7-38.9)	8.8 (5.4-11.0)	4.15 (1.1-7.7)
inLN	10.0 (21.3-61.9)	6.3 (1.2- 34.4)	2.2 (0.4-5.4)
liLN	4.0 (2.5-39-6)	7.8 (1.5-29.7)	1.1 (0.1-3.0)
SPL	40.8 (27.4-45.8)	25.2 (13-29.8)	3.03 (2.1-4.1)

To define NK cell developmental stages, samples were gated on the CD45$^+$CD3$^-$ population within CD45$^+$/SS gated cells to exclude T cells and endothelial cells from analysis. For each tissue, the following items are indicated: [1]Total cell number within the CD45$^+$/SS gate; [2]the amount of cells within the CD45$^+$CD3$^-$ gate and; [3]the total amount of cells covering all NK cell developmental stages. All cell numbers are shown in median (range).

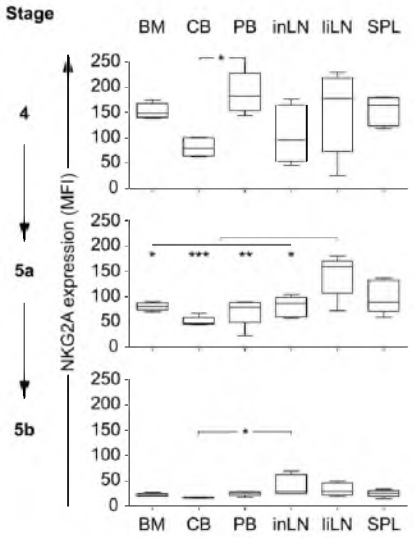

Figure S1. NKG2A expression levels in stages 4, 5a, and 5b in different human tissues. Cells were gated on the CD45$^+$CD3$^-$ population within CD45$^+$/SS gated cells to exclude T cells and endothelial cells from analysis. Next, cell subsets were divided based on the expression of CD34 and CD117. From there, each subset was analyzed for CD56 and CD94 expression. Subsequently, the NKG2A expression level was analyzed within the different NK cell developmental stages in bone marrow (BM), cord blood (CB), peripheral blood (PB), inguinal LN (inLN), liver LN (liLN) and spleen (SPL) (all n=5). Reliable NKG2A expression was seen in stages 4, 5a, and 5b. Cell populations > 0.1% of the CD45$^+$CD3$^-$ population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. Comparison of MFI of positive cells between the different tissues was analyzed using ANOVA; *P<.05, **P<.01, ***P<.0001.

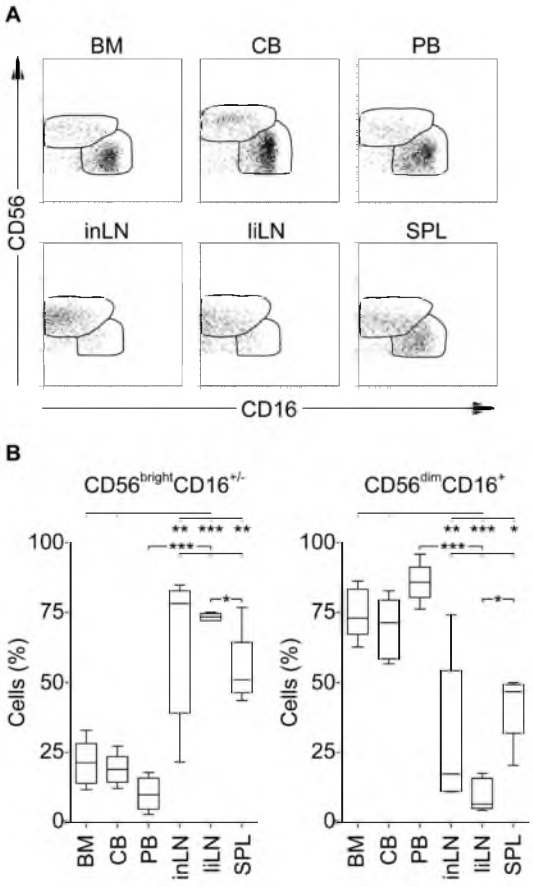

Figure S2. CD56 and CD16 expression patterns of committed NK cells (stage 3b-5b) within different human tissues. Cells were gated on the CD45$^+$CD56$^+$CD3$^-$ population within CD45$^+$/SS gated cells to exclude T cells and endothelial cells from analysis. Based on the CD56 and CD16 expression within the committed NK cell population (stage 3b-5b), NK cells subsets were divided into the major CD56^{bright}CD16$^{+/-}$ and CD56^{dim}CD16$^+$ subset. BM=bone marrow, CB=cord blood, PB=peripheral blood, inLN=inguinal LN, liLN=liver LN, SPL=spleen (all n=5). (A) Shown are representative examples (one of each tissue) for CD56 and CD16 expression patterns within the committed NK cell population. (B) Shown are the distribution of the CD56^{bright}CD16$^{+/-}$ subset (left panel) and the CD56^{dim}CD16$^+$ subset within the different human tissues. Cell populations > 0.1% of the CD45$^+$CD3$^-$ lymphocyte population with a threshold of more than 50 cells were considered reliable. Cell populations were considered tissue specific when at least 3 out of 5 samples showed reliable results. Cell populations that did not suffice to these criteria were excluded from further (statistical) analysis. Comparison between the different tissues was analyzed using a random effect logistic regression model; *P<.05, **P<.01, ***P<.0001.

CHAPTER 7

Summary and Discussion

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7.1 General

Allogeneic stem cell transplantation (SCT) is often the final treatment modality for patients with leukemia or other hematological malignancies.[1,2] However, relapse of the underlying malignancy is still a major complication post SCT. To prevent the occurrence of relapse post SCT, patients are treated with pre-emptive donor lymphocyte infusions (DLI) consisting of donor-derived T cells from the same donor used for allogeneic SCT in order to boost the donor-derived immune system to terminally eradicate residual tumor cells.[3-5] Unfortunately, DLI treatment using donor-derived T cells does not only provoke graft-versus-leukemia (GVL) reactivity, but also increases the risk for GVHD development. Thus, post allogeneic SCT, long-term remission is still greatly dependent on effective graft-versus-leukemia (GVL) reactivity, while strictly controlling GVHD. Therefore, the development of treatment strategies augmenting GVL reactivity while reducing GVHD is of clinical importance.

In allogeneic SCT, natural killer (NK) cells have shown to play an important role in GVL reactivity within the first months after transplantation.[6-9] Ruggeri *et al.*[10] showed that alloreactive donor NK cells were able to lyse recipient tumor cells *in vitro*, implying that these NK cells may be able to provide immune reactivity by targeting residual tumor cells still present in the recipient. Moreover, fast recovery of NK cells and predicted GVL reactivity towards host tumor cells has been associated with reduced GVHD, decreased relapse rates, and better overall survival of the patient.[11-13] Altogether, this makes NK cells important candidates for immunotherapeutic use in the treatment of leukemia and other malignancies.

In this thesis, we studied the alloreactive potential of mature donor-derived NK cells when given as part of the graft in HLA-matched allogeneic SCT (**chapter 2**) and studied the feasibility of skewing NK cell alloreactivity of mature donor NK cells towards leukemic target cells prior to transplantation (**chapter 3**). Bearing in mind that donor NK cells need to exert optimal GVL reactivity after allogeneic SCT, we additionally studied the effects of commonly used immunosuppressive drugs (ISDs), such as CsA, MPA, and rapamycin (**chapter 4**). For exploitation of NK cells for future adoptive cancer immunotherapy, we further developed a cytokine-based culture system for *ex vivo* expansion of NK cells from hematopoietic stem and progenitor cells from umbilical cord blood (**chapter 5**). Additionally, as thorough knowledge of human NK cell development *in vivo* is essential for the development of NK cell-based immunotherapies, we finally studied NK cell development in various human tissues using 10-color flow cytometry (**chapter 6**).

7.2 Facilitation of mature alloreactive donor NK cells in allogeneic SCT

7.2.1 Infusion of mature donor NK cells as part of the graft

Immediately after stem cell transplantation, alloreactive NK cells have been shown to be beneficial not only for boosting the anti-tumor response, but also for the prevention of GVHD as well as infections. In these cases, optimal functional activity of NK cells already in the early phase after SCT is essential, and therefore the presence of NK cells in the graft appears to be beneficial for transplant outcome.[14;15]

In **chapter 2**, as part of a prospective randomized phase III study, we directly compared the alloreactive potential of allogeneic donor NK cells between patients having either received a $CD3^+/CD19^+$ cell depleted graft (containing substantial NK cell numbers) or a conventional $CD34^+$ selected graft (devoid of NK cells) in the setting of in HLA-matched SCT. Results demonstrate that patients having received a $CD3^+/CD19^+$ cell depleted graft, exhibited a faster recovery of NK cells and a functional NK cell receptor repertoire of inhibitory and stimulatory receptors as compared with patients having received a conventional $CD34^+$ graft. Furthermore, transplantation with a $CD3^+/CD19^+$ cell depleted graft resulted in the development of a functionally different NK cell population that was more prone to activation via the CD94/NKG2C receptor complex and less sensitive to inhibition via the CD94/NKG2C receptor complex. Although it was demonstrated that human cytomegalovirus (CMV) infection may result in increased CD94/NKG2C expression levels and subsequent loss of CD94/NKG2A expression[16-18], this phenomenon remained present in the CD3/19 depletion group after exclusion of CMV positive patients from analysis. Unfortunately, later interim analysis on 25 patients per group showed that the primary objectives of this clinical study could not be reached resulting in early termination of the study. Thus, the alternative reconstitution of the NK cell receptor repertoire using $CD3^+/19^+$ depleted grafts, characterized by the change in balance of $CD94/NKG2A^+$ NK cells to more $CD94/NKG2C^+$ NK cells, and its impact on clinical outcomes after HLA-matched SCT remains a subject for further study.

Recently, the reconstitution of allogeneic donor NK cells was evaluated in haploidentical SCT after reduced intensity conditioning (RIC) using $CD3^+/19^+$ depleted grafts.[19] Data showed similar results as compared with our study, including fast recovery of NK cells and immune reconstitution of the NK cell receptor repertoire. In addition, a similar decrease of $NKG2A^+$ NK cells was seen post SCT. However, the expression of NKG2C was not evaluated. Nevertheless, this study confirms our findings that different graft manipulation methods may trigger differential NK cell reconstitution, which may be beneficial for transplant outcome. Previously, Gentilini *et al.*[20] even showed a significant faster and sustained recovery of NK cells in a group of patients after RIC allogeneic SCT with $CD3^+/CD19^+$ depleted grafts in comparison with patients with myeloablative allogeneic SCT with $CD34^+$ selected grafts combined with adoptive NK cell infusion two days post SCT.

Overall, these studies suggest that the use of NK cell rich grafts is favorable for the facilitation of fast and sustained NK cell recovery and differential reconstitution of the NK cell receptor repertoire, which may lead to improved donor NK cell alloreactivity in the GVL direction (suggested by results in **chapter 2**). Further prospective comparisons of the different graft manipulation

methods for allogeneic SCT in the HLA-matched or haploidentical setting are warranted for more detailed analysis of the impact of graft composition on immune reconstitution. Subsequently, the impact of adoptive NK cell infusions after allogeneic SCT for boosting GVL reactivity needs to be studied in further detail.

7.2.2 Skewing donor NK cell alloreactivity before SCT

In allogeneic SCT, donor NK cell alloreactivity can be facilitated by allowing mismatches for specific HLA molecules (e.g. HLA-C) between donor and recipient. This is referred to as the "ligand-ligand" model. The introduction of certain HLA mismatches has been shown to induce NK cell-mediated GVL reactivity, without inducing severe GVHD, and to contribute to decreased relapse, better engraftment and improved overall survival.[10;13;21] However, others state that the induction of NK cell alloreactivity is not dependent on HLA mismatching, but is rather induced by the presence of an inhibitory KIR in the donor's genotype with the absence of the corresponding KIR-ligand in the recipient's HLA repertoire ("receptor-ligand" model).[22-24] This makes the exploitation of NK cell alloreactivity not only feasible for HLA-mismatched settings, but may also be promising for HLA-matched settings.

In **chapter 3**, we examined the plasticity of the KIR repertoire and the cytolytic response of mature human peripheral blood NK cells in an *in vitro* culture system based on the "receptor-ligand" model. Results demonstrate that the presence of a specific KIR-ligand (HLA-C) present on feeder cells skews the response of healthy donor NK cells by favoring the outgrowth of NK cells expressing the KIR that lacks its cognate ligand. Subsequently, this resulted in an oligoclonal increase of specific alloreactive NK cells bearing improved cytolytic ability. The results of this study fully support the "receptor-ligand" model, as skewing of the KIR repertoire was seen in both the HLA-C mismatched setting as in the HLA-C matched setting; the NK cell population from HLA-C1 donors showed an increase in the frequency of KIR2DL/S2/3^{+} NK cells in the presence of HLA-C2 feeder cells and of KIR2DL/S1^{+} NK cells when cultured in the presence of HLA-C1 feeder cells.

Overall, the results of this study hold promise for the exploitation of target-specific alloreactive donor NK cells in both HLA-matched and -mismatched allogeneic SCT settings or adoptive NK cell immunotherapy based on the "receptor-ligand" model. For further exploitation, however, the "licensing/education" model needs to be considered as well. Upon maturation, NK cells obtain their "license to kill" through interactions of inhibitory KIRs with self-HLA class I molecules.[25-27] NK cells that fail to interact with self-HLA class I molecules remain functionally immature and will reside in a hyporesponsive state. Recently, it was shown that the strength of response by an individual NK cell is even quantitatively controlled by the extent of inhibitory signals that are received from HLA class I molecules during NK cell education.[28] Concerning adoptive transfer of mature NK cells for immunotherapeutic purposes, this suggests that the presence of inhibitory KIR on donor NK cells in absence of its cognate ligand in the recipient ("receptor-ligand" model) as well as the HLA-background of the donor NK cells ("licensing/education" model) are two key factors that need to be taken into account for the successful exploitation of alloreactive donor NK cell responses.

7.2.3 Interference by immunosuppressive drugs

For optimal NK cell-mediated GVL reactivity, NK cells need to be fully functional in the early phase after SCT, despite that at this stage a high level of immunosuppressive treatment is given. Among the various ISDs, cyclosporin A (CsA), rapamycin (Rapa) and mycophenolate mofetil (MMF) have successfully been applied for the prevention of GVHD.[29-33] In **chapter 2**, patients received CsA as GVHD prophylaxis after transplantation. In **chapter 4**, we studied the influence of CsA, Rapa and mycophenolic acid (MPA; the active metabolite of MMF) on NK cell phenotype and function in an *in vitro* cytokine-based culture system. Results showed that the modulation of the NK cell receptor repertoire during culture was arrested by Rapa and MPA treatment. This was reflected in the cytolytic activity, as MPA- and Rapa-treated NK cells, in contrast to CsA-treated NK cells, lost their cytotoxicity against leukemic target cells. In contrast, IFN-γ production was not only impaired by MPA and Rapa, but also by CsA upon target encounter. A recent study, however, suggested that IFN-γ production upon target encounter may be limited to the $CD56^{dim}$ NK cell subset, whereas the $CD56^{bright}$ NK cell subset produces IFN-γ upon cytokine-stimulation.[34] Thus, as $CD56^{bright}$ NK cells were still abundantly present in the CsA-treated cultures, in contrast to MPA- and Rapa-treated cultures, the IFN-γ production upon cytokine-stimulation may largely be preserved after CsA treatment. This was confirmed in a study showing sustained IFN-γ production by CsA-treated NK cell cultures upon IL-12 and IL-18 stimulation[35], suggesting that IFN-γ-mediated GVL reactivity after allogeneic SCT should remain intact when using CsA as GVHD prophylaxis.

Our findings on the effect of CsA and MPA on the cytolytic response by in vitro cytokine-stimulated NK cells are in concert with previous findings on this subject.[36] Besides CsA and MPA, they also evaluated the effect of tacrolimus (TAC) and methotrexate (MTX), which are also successfully used as GVHD prophylaxis after allogeneic SCT.[31;37;38] Both ISDs did not interfere with NK cell-mediated cytolytic activity against different leukemic cell lines. However, a dose range of each ISD is lacking in this study, which would be more appropriate when studying the effect of ISDs on NK cell functionality.

Overall, these *in vitro* studies clearly suggest that the choice of immunosuppressive treatment might affect the outcome of NK cell therapy *in vivo* after transplantation. Additional studies on NK cell phenotype and function of patients after allogeneic SCT using different immunosuppressive strategies are warranted to survey the *in vivo* effect of the different immunosuppressive regimens in more detail.

7.3 Clinical grade NK cell products for adoptive cancer immunotherapy

7.3.1 Development of clinical grade NK cell products

The facilitation of donor NK cell alloreactivity is not restricted to HLA-matched/mismatched allogeneic SCT, but may also be exploited for adoptive immunotherapy in non-transplantation settings. Previously, several clinical studies have examined the feasibility of allogeneic NK cells for adoptive immunotherapy using allogeneic NK cells selected from leukapheresis products by immunomagnetic beads selection protocols.[39-44] In all these studies, the adoptive transfer of allogeneic NK cells proved to be safe and well tolerated by patients. Nevertheless, for optimal exploitation of NK cell adoptive immunotherapy, the development of innovative strategies producing allogeneic NK cell products with high cell numbers, high purity and functionality are needed. In this respect, recent studies have developed culture systems for large scale *ex vivo* expansion of allogeneic NK cells using either hematopoietic progenitor cells from bone marrow or UCB.[45-47] However, most of these culture systems are unsuitable for clinical application due to the use of animal sera, animal-derived proteins, and/or supportive feeder cells.

In **chapter 5**, we developed a cytokine-based method for high log-scale *ex vivo* expansion of functional allogeneic NK cells from hematopoietic stem and progenitor cells from umbilical cord blood (UCB) using a novel clinical grade medium. The *ex vivo*-generated NK cell products are of high purity and contain developmentally mature NK cell populations expressing inhibitory NKG2A and KIRs, and a variety of stimulatory receptors. Furthermore, these NK cell products show the ability to efficiently kill myeloid leukemia and melanoma tumor cell lines. The findings in this study provide an important advance for the clinical application of *ex vivo*-generated NK cell products to be exploited for adoptive immunotherapy either following allogeneic SCT for boosting NK cell-mediated GVL reactivity or in the non-transplant setting following lymphodepleting immunosuppressive regimens. In addition, human *in vitro* studies and *in vivo* evidence in mice suggest that NK cell-based immunotherapy may also be beneficial for patients with melanoma or renal cell carcinoma when applied in the in the setting of the “receptor-ligand” model.[48-50] Overall, further exploitation of this culture system may provide a broad clinical application for NK cell-based immunotherapy against hematological and non-hematological malignancies.

7.3.2 Clinical feasibility of ex vivo-generated NK cells: a phase I trial

Recently, the NK cell generation protocol, described in **chapter 5**, was transferred to clinical applicable conditions and medical ethical approval was given to study the feasibility of adoptive transfer of *ex vivo*-generated NK cell products in elderly patients diagnosed with poor prognosis AML. In the second quartile of 2011, a phase I dose-escalating study will start for a group of 12 AML patients (age >65 years), not eligible for allogeneic SCT, who have achieved clinical remission after standard remission-induction chemotherapy and who have completed consolidation chemotherapy. Patients will receive allogeneic NK cells generated *ex vivo* from $CD34^+$ UCB cells in a single escalating dose up to $10x10^7$ donor NK cells/kg body weight after completing standard chemotherapy and preparative immunosuppressive conditioning consisting of fludarabine and cyclophosphamide in order to prevent rejection. The primary goal is to evaluate the safety and

dose-limiting toxicity of adoptive transfer of the allogeneic *ex vivo*-generated NK cells. Secondly, the *in vivo* lifespan of the adoptively transferred allogeneic NK cells will be evaluated together with an assessment of NK cell-mediated GVL reactivity in study participants.

7.4 Monitoring NK cell development

To exploit NK cells for immunotherapy, a better understanding of human NK cell development *in vivo* is essential. Freud *et al.*[51;52] were the first to define distinct NK cell developmental stages in secondary lymphoid tissues (SLT) based on expression profiles of CD34, CD117, CD94 and CD5, and provided evidence that supported a model of *in vivo* human NK cell development in which $CD34^+$ NK cell precursors traffic from BM to SLT where further differentiation into mature $CD56^{bright}$ NK cells can take place. We (**chapter 2**) and others have shown that $CD56^{bright}$ cells are also the first mature NK cells to arise after allogeneic SCT followed by further differentiation towards $CD56^{dim}$ NK cells.[53] However, the specific route of NK cell development and how the distinct NK cell developmental stages in SLT correlate with the different NK cell subsets found in other compartments of the human body (e.g. peripheral blood (PB), spleen (SPL)) remains unclear.

In **chapter 6**, we identified seven distinctive NK cell developmental stages in bone marrow using newly developed 10-color flow cytometry panels. We are the first to find that NK cell development is accompanied by early expression of CD244 *in vivo*, which is an important stimulatory co-receptor. Furthermore, distinctive expression profiles of early development antigen CD33 and NKG2A between liver lymph nodes (LN) and inguinal LN, suggest that differential in situ NK cell differentiation may take place in common tissues. Overall, the results of this study support a model of *in vivo* human NK cell development indicating BM as the origin of NK cell development. Commitment to the NK cell lineage takes place in LN followed by *in situ* differentiation and maturation of NK cells ($CD56^{bright}$) with restricted maturation of the NK cell receptor repertoire. Further differentiation of committed NK cells may take place in SPL ($CD56^{bright}$ towards $CD56^{dim}$) followed by trafficking towards the periphery and other anatomical locations of the human body. In contrast to previous NK cell development studies[51;52], we were able to study small sample sizes of tissues obtained from single donors with the use of 10-color flow cytometry. Given the biological variation within human tissues, this creates an improved and more reliable view on human NK cell development within the different locations of the human body. However, further functional analyses of committed NK cells within the different NK cell developmental stages and trafficking studies exploring chemokine and cytokine profiles are warranted to obtain a complete view on the human NK cell developmental pathway. In addition, the use of 10-color flow cytometry should be further exploited for these purposes and may also serve as an important monitoring tool of human NK cell development and functionality *in vivo* after allogeneic SCT or after adoptive NK cell-based immunotherapy.

7.5 Towards NK cell immunotherapy

Within the last decade, different NK cell-based immunotherapy strategies have successfully been developed. They have either already been proved safe in clinical phase I/II trials or are currently under clinical evaluation. Today, applications for NK cell-based immunotherapy can generally be divided into three main categories: (1) the use of enriched NK cell grafts for allogeneic SCT; (2) administering NK-DLI after allogeneic SCT and; (3) adoptive NK cell transfer in non-transplantation settings (Figure 1).

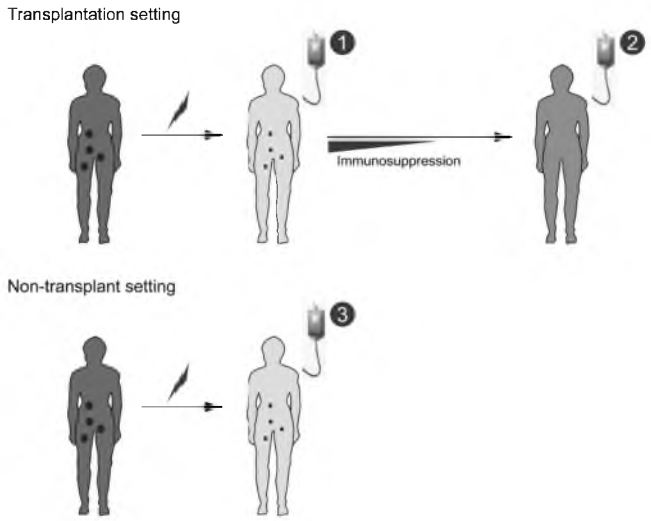

Figure 1. Applications for NK cell-based immunotherapy. In the allogeneic SCT setting, patients are first treated with a conditioning regimen to eradicate healthy hematopoietic recipient cells and to minimize the tumor burden within the recipient. Subsequently, enriched NK cell grafts (1) and/or NK-DLI (2) may be applied in the further course of allogeneic SCT in order to facilitate alloreactive donor NK cell-mediated GVL reactivity. In the non-transplant setting (e.g. patients no longer eligible for allogeneic SCT or with solid tumors), adoptive NK cell transfer can be used as a NK cell-based immunotherapeutic strategy in patients after completing standard chemotherapy and preparative immunosuppressive conditioning in order to prevent rejection.

7.5.1 Allogeneic SCT setting

The use of enriched NK cell grafts for allogeneic SCT has shown to be beneficial for transplant outcome and provides enhanced GVL reactivity directly after transplantation as compared with conventional grafts that lack NK cells.[11;15;chapter 2] In addition, the head start in GVL reactivity may lead to better outcomes in terms of infectious events and overall survival after allogeneic SCT.[14] Altogether, this indicates the immunotherapeutic value of the exploitation of donor NK cells in the allogeneic SCT setting. In order to further increase the beneficial effects of such NK cell-based immunotherapy strategies in the setting of allogeneic SCT, donor NK cells can also be administered as part of the conditioning regimen prior to transplantation instead of, or in combination with, the use of enriched NK cell grafts. This can have three potential beneficial effects. First, NK cell-mediated GVL reactivity could provide anti-tumor activity prior to allogeneic

SCT.[6;54] Second, NK cell-mediated depletion of host dendritic cells before transplantation could prevent the development of acute GVHD allowing for a less stringent depletion of T cells in the graft.[6;54] Third, NK cells may facilitate better engraftment through eradication of host T cells, thereby reducing the need for toxic myeloablative regimens and shortening the neutropenic period.[6] However, to be able to implement the combinatorial strategy of NK cell infusions as part of the conditioning regimen together with the use of enriched NK cell grafts in the clinic, the necessary amount of NK cells used for infusion prior to allogeneic SCT to provoke beneficial immunotherapeutic effects still needs to be established. Insufficient numbers may cause the necessity to choose between the use of NK cell infusions as part of the conditioning regimen and the use of enriched NK cell grafts. In this respect, also in case of the combinatorial strategy, it is important that more research is performed on the effects of conditioning and immunosuppressive regimens on NK cells in exploiting NK cell-mediated (GVL) reactivity in the allogeneic SCT setting. The use donor T cells for DLI after allogeneic SCT has developed into an effective treatment of recurrent hematological malignancy as well as prophylactic treatment in high-risk leukemia and lymphoma.[55] Still, the main risk of T-DLI is the induction of life-threatening GVHD. To minimize the risk of GVHD, studies have been initiated to modify conventional DLI by using donor NK cells instead of donor T cells.[41;56] Additionally, the administration of NK-DLI may facilitate engraftment and induce NK cell-mediated GVL reactivity.[41;56] Although no firm conclusions can be drawn on the clinical efficacy of NK-DLI after allogeneic SCT at this point, data indicate that NK-DLI is safe and well tolerated, and can generate GVL reactivity and long-term remission in some patients after leukemia relapse. As non-malignant tissues generally do not overexpress ligands for activating NK cell receptors, NK-DLI should not cause GVHD.[57;58] Until now, NK-DLI has been tested in the haploidentical SCT setting. Thus, further research on the efficacy of NK-DLI in the HLA-matched SCT setting is warranted.

7.5.2 Non-transplant settings

In non-transplant settings, donor NK cells can be exploited for immunotherapeutic strategies for the treatment of hematological and non-hematological malignancies. Previously, Miller *et al.*[59] showed that haploidentical donor NK cell infusions after high-dose cyclophosphamide and fludarabine treatment resulted in long-term survival and *in vivo* expansion of donor NK cells in patients with metastatic melanoma (n=10), metastatic renal cell carcinoma (n=13), refractory non-Hodgkin's disease (n=1), and poor-prognosis AML (n=19). The *in vivo* NK cell expansion was associated with an increased levels of endogenous IL-15, which were possibly responsible for driving the survival and proliferation of donor NK cells. In general, the donor NK cell infusions were well tolerated without evidence for the induction of GVHD. Furthermore, 5 out of 19 patients with poor-prognosis AML achieved complete remission. Only 4 of the 19 AML patients were KIR-ligand (HLA) mismatched in the graft-versus-host direction. Interestingly, out of these 4 patients, 3 achieved complete remission. These findings indicate that haploidentical donor NK cells can persist and expand *in vivo* and may have a role in the treatment of (non-)hematological malignancies in non-transplant settings or in combination with allogeneic SCT. In addition, when

using haploidentical donors the choice of a KIR-ligand mismatched donor, based on the "ligand-ligand" model, may be needed to obtain successful results in future clinical trials.[59] In case of HLA-matched donors, the choice of a "receptor-ligand" mismatched donor is preferred.

In parallel, adoptive transfers are currently also being performed with the NK cell line NK-92. This cell line can be cultured under good manufacturing practice (GMP) conditions and shows significant cytotoxicity against several tumor cell lines.[60] Infusions of NK-92 cells have been administered to more than 20 patients with advanced renal-cell carcinoma and malignant melanoma. This proved to be safe and generated antitumor effects in some cases.[61] Furthermore, NK-92 cells can easily be obtained in high numbers during GMP culture providing sufficient amounts of cells for adoptive immunotherapeutic strategies. An obvious drawback of relying on a cell line for adoptive immunotherapy is that it may only be successful for certain malignancies or patient groups. Thus, recent studies have developed culture systems for large scale *ex vivo* expansion of allogeneic donor NK cells using either hematopoietic progenitor cells from bone marrow or UCB.[45-47;chapter 5] As mentioned above, our culture system is now being transferred to GMP conditions and will be tested in a clinical phase I trial in the near future.

7.5.3 Crucial issues for NK cell-based immunotherapy

Several issues remain crucial for the development and implementation of successful NK cell-based immunotherapy in the future. In the non-transplant setting , these include issues relating to the type of NK cell preparation to be used (activation, degree of enrichment and possible selection or skewing of specific subpopulations), criteria for donor selection ("ligand-ligand" versus "receptor-ligand" model, KIR genotyping and phenotyping, and size of the alloreactive subset), conditioning of patients prior to immunotherapy, clinical context of therapy, and criteria for patient selection and strategies for the identification of susceptible tumors within patient groups. Besides these issues, the effects of immunosuppressive regimens given after allogeneic SCT are also important when implementing NK cell-based immunotherapy in the setting of allogeneic SCT.

The research described in this thesis focused on the facilitation of alloreactive donor NK cells in HLA-matched allogeneic SCT and elaborated on potential skewing of NK cell alloreactivity towards specific malignant cells. Furthermore, we gave more insight on potential side-effects of immunosuppressive regimens and contributed to the development of a clinical grade NK cell product, which is to be evaluated within the clinic in the near future. The 10-color flow cytometry panels that we developed for our research provided better insight in human NK cell development *in vivo* and may serve as an important and valuable NK cell analysis/monitoring tool for future studies. Altogether, the research described in this thesis contributes to the exploitation of allogeneic NK cells towards NK cell immunotherapy for the treatment hematological and non-hematological malignancies.

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CHAPTER 8

Nederlandse samenvatting
List of publications
Curriculum vitae
Dankwoord

Nederlandse samenvatting

Allogene stamceltransplantatie

Allogene (niet lichaamseigen) stamceltransplantatie (SCT) is vaak de laatste stap in het behandeltraject voor patiënten met hematologische aandoeningen zoals leukemie. Het is een intensieve en zware behandelmethode met als doel het ontspoorde hematopoïetische systeem van de patiënt te vervangen met hematopoïetische stamcellen van een donor. Na transplantatie nestelen donor stamcellen zich in het beenmerg van de patiënt ('engraftment') en vormen ze de basis voor de ontwikkeling van een nieuw en gezond hematopoïetisch systeem. In het transplantaat bevinden zich naast donor stamcellen ook een kleine hoeveelheid volwassen witte bloedcellen van de donor, namelijk T-cellen. Deze cellen voorkomen afstoting van het transplantaat en bestrijden daarnaast de resterende tumorcellen van de patiënt na allogene SCT. Dit laatste wordt ook wel het 'graft-versus-tumor' (GVT) of het 'graft-versus-leukemia' (GVL) effect genoemd. Na allogene SCT kunnen deze alloreactieve T-cellen van de donor echter ook gezond weefsel van de patiënt aantasten, wat resulteert in acute 'graft-versus-host disease' (GVHD). Ter voorkoming dat acute GVHD de overhand neemt en tot ernstige complicaties leidt, wordt de balans tussen GVHD en GVL-reactiviteit direct na transplantatie nauwkeurig onder controle gehouden door toediening van immunosuppressieve medicijnen. Wanneer 'engraftment' van de donor stamcellen in de patiënt heeft plaatsgevonden en zich geen ernstige GVHD heeft voorgedaan, worden de hoeveelheden immunosuppressiva langzaam afgebouwd. Om de kans op terugkeer van de ziekte ('relapse') te verkleinen kan de patiënt vervolgens behandeld worden met een donor lymfocyten infuus (DLI). Dit infuus bevat T-cellen van dezelfde donor en dient als een extra 'boost' aan GVL-reactiviteit. Deze additionele behandeling brengt echter een verhoogd risico op de ontwikkeling van GVHD met zich mee.

GVHD versus GVL

Om de kans op GVHD zo gering mogelijk te houden is het van essentieel belang dat het weefseltype (HLA klasse I en klasse II: 'humane leukocyten antigenen type I en type II') van de donor niet teveel afwijkt van die van de patiënt. Wanneer in de familie geen passende donor gevonden is, kan eventueel een niet-verwante donor met de juiste HLA-typering gevonden worden. Wereldwijd zijn databases van mogelijke stamceldonoren met hun HLA-typering beschikbaar, waarin gezocht kan worden naar een potentieel geschikte donor.
Hoewel het zoveel mogelijk 'matchen' van HLA het risico op GVHD behoorlijk reduceert, is de ontwikkeling van GVHD na allogene SCT niet uitgesloten. Medisch onderzoek richt zich dan ook op de ontwikkeling van nieuwe 'immunotherapeutische' behandelmethoden die vanuit zichzelf voldoende GVL-reactiviteit teweeg brengen zonder overmatige inductie (of zelfs reductie) van GVHD. In dit kader heeft het gebruik van natural killer (NK) cellen grote interesse gekregen.

Natural Killer cellen

NK-cellen zijn witte bloedcellen die tumorcellen en virusgeïnfecteerde cellen bestrijden. Direct na allogene SCT maken NK-cellen deel uit van de eerste defensielinie tegen opportunistische infecties en zijn ze, in tegenstelling tot T-cellen, middels een 'license to kill' in staat om tumorcellen te bestrijden zonder voorafgaande sensitisatie. NK-cellen scannen andere cellen op de aanwezigheid van HLA klasse I met behulp van 'killer immunoglobulin-like' receptoren (KIR), die zich op het oppervlak van hun celmembraan bevinden. Indien deze herkenning tussen NK-cel en potentiële 'targetcel' niet of niet voldoende plaatsvindt, doordat de NK-cel het HLA klasse I als lichaamsvreemd herkent of door verminderde aanwezigheid van het HLA klasse I, kunnen activerende eiwitten (aanwezig op het celmembraan van de targetcel) de NK-cel stimuleren tot het lyseren van de targetcel. In vergelijking met gezonde lichaamscellen hebben tumorcellen en virusgeïnfecteerde cellen vaak een verminderde expressie van HLA klasse I en een hogere expressie van activerende eiwitten. Op deze manier kunnen donor NK-cellen na allogene SCT onderscheid maken tussen de tumorcellen en gezonde lichaamscellen van de patiënt en dus GVL-reactiviteit teweeg brengen zonder inductie van GVHD.
Het uitbuiten van NK-cel gemedieërde GVL-reactiviteit binnen allogene SCT is de focus van dit proefschrift. In **hoofdstuk 1** wordt een algemene inleiding gegeven over allogene SCT en de mogelijke toepassing van donor NK-cellen in het bewerkstelligen van GVL-reactiviteit in afwezigheid van GVHD.

Alloreactieve donor NK-cellen in allogene SCT

In **hoofdstuk 2** wordt het effect van 'NK-cel rijke' stamceltransplantaten vergeleken met het effect van conventionele stamceltransplantaten, die geen NK-cellen bevatten, op de repopulatie en de GVL-reactiviteit van donor NK-cellen na allogene SCT. Wanneer donor NK-cellen worden meegegeven met het stamceltransplantaat vindt direct na allogene SCT een snelle repopulatie van deze NK-cellen plaats met vroege GVL-reactiviteit in vergelijking met conventionele stamceltransplantaten. Tevens lijkt het gebruik van 'NK-cel rijke' stamceltransplantaten tot een meer geactiveerde NK-cel populatie te leiden, wat een gunstig effect kan hebben op de gehele GVL-reactiviteit na allogene SCT.
Vervolgens wordt in **hoofdstuk 3** beschreven dat NK-cel gemedieërde GVL-reactiviteit specifiek 'gestuurd' kan worden in de richting van tumorcellen. De totale NK-cel populatie kan op basis van expressie van verschillende KIR receptoren onderverdeeld worden in verschillende subpopulaties. De NK-cel subpopulaties zullen afhankelijk van de herkenning tussen de specifieke KIR receptoren op NK-cellen en specifieke HLA klasse I eiwitten op de tumorcellen wel of geen GVL-reactiviteit vertonen. Door de gehele donor NK-cel populatie gedurende korte tijd te kweken in aanwezigheid van specifieke HLA klasse I$^+$ tumorcellen vindt er binnen de totale NK-cel populatie een toename plaats van specifieke KIR$^+$ NK-cellen die het HLA klasse I op deze tumorcellen niet herkennen. Naast de toename van deze specifieke KIR$^+$ NK-cellen

zijn deze NK-cellen ook reactiever tegen tumorcellen met dezelfde specifieke HLA klasse I^{+} expressie waarmee ze gekweekt zijn in vergelijking met 'controle' NK-cellen (specifieke KIR^{+} NK-cellen die niet gekweekt zijn of die gekweekt zijn in afwezigheid van de specifieke HLA klasse I^{+} tumorcellen). De resultaten van dit onderzoek geven aan dat de gehele donor NK-cel populatie specifieker gericht kan worden tegen tumorcellen van de patiënt. Dit kan worden gebruikt in de verdere ontwikkeling van specifieke NK-cel immunotherapie.
Voor de toepassing van NK-cel immunotherapie in de setting van allogene SCT is het belangrijk dat NK-cellen hun GVL-reactiviteit behouden onder druk van de immunosuppressieve medicijnen die na allogene SCT worden toegediend aan de patiënt. **Hoofdstuk 4** beschrijft de invloed van verschillende immunosuppressiva, die veelal gebruikt worden na allogene SCT, op het functioneren van donor NK-cellen. Uit de resultaten van deze studie blijkt dat het gebruik van mycofenolaat (MPA; het actieve derivaat van mycofenolaat mofetil (MMF)) en rapamycine (Rapa) de activatie van donor NK-cellen remt, wat resulteert in een verminderde GVL-reactiviteit. Dit in tegenstelling tot het gebruik van cyclosporine A (CsA), dat geen effect lijkt te hebben op de activatie en GVL-reactiviteit van donor NK-cellen. Voor het succes van NK-cel immunotherapie in de setting van allogene SCT is een zorgvuldige keuze uit de beschikbare immunosuppressiva dan ook van essentieel belang voor zover de situatie binnen de kliniek dit toestaat.

Ontwikkeling van klinische NK-cel producten

In **hoofdstuk 5** wordt de ontwikkeling van een immunotherapeutisch NK-cel product beschreven. In deze studie wordt aangetoond dat ruime hoeveelheden donor NK-cellen met GVL-reactiviteit ontwikkeld kunnen worden vanuit hematopoïetische stamcellen uit navelstrengbloed. In een eerste kweekfase zorgt een cocktail van signaalstoffen voor de toename ('expansie') van het beperkte aantal donor stamcellen dat in het navelstrengbloed aanwezig is. Vervolgens worden de geëxpandeerde stamcellen door een andere cocktail van signaalstoffen in een tweede kweekfase 'gedwongen' om zich verder te ontwikkelen tot NK-cellen, welke een sterke GVL-reactiviteit vertonen richting diverse tumorcellijnen en primaire tumoren. Dit proces van het vermeerderen en uitrijpen van hematopoïetische stamcellen tot NK-cellen met voldoende GVL-reactiviteit is een belangrijke stap in het toegankelijk maken van NK-cel immunotherapie. De op deze wijze gegenereerde NK-cel producten zijn niet alleen veelbelovend voor klinische toepassingen binnen de setting van allogene SCT, maar zouden ook toegepast kunnen worden als 'stand alone' immunotherapie voor patiënten die niet (meer) in aanmerking komen voor allogene SCT.

Monitoren van NK-cel ontwikkeling

Voor de ontwikkeling en toepassing van NK-cel immunotherapie is voldoende kennis van de normale NK-cel ontwikkeling in het menselijk lichaam noodzakelijk. Eerder onderzoek heeft laten zien dat NK-cel ontwikkeling onderverdeeld kan worden in vijf stadia. Tot nu toe zijn deze stadia echter alleen in humane secundaire lymfeknopen aangetoond op basis van de detectie van 4 verschillende antigenen (4-kleuren fluorescentieanalyse). In **hoofdstuk 6** zijn de NK-cel ontwikkelingsstadia met behulp van 10-kleuren fluorescentieanalyse in meerdere humane (lymfoïde) weefsels geanalyseerd. Resultaten van deze studie tonen aan dat de NK-cel ontwikkeling onderverdeeld kan worden in acht verschillende stadia. De verspreiding van deze NK-cel ontwikkelingsstadia ondersteunen een NK-cel ontwikkelingsmodel dat start in het beenmerg, waarna voorlopercellen zich tot onvolwassen NK-cellen ontwikkelen in de lymfeknopen. Na deze gedeeltelijke NK-cel rijping differentiëren de onvolwassen NK-cellen zich verder in de milt en vindt via het perifere bloed verspreiding plaats van de verschillende typen volwassen NK-cellen. In de afzonderlijke weefsels zijn tevens weefselspecifieke NK-cel typen te zien. Dit toont aan dat humane weefsels niet alleen ruimte bieden aan NK-cellen in verschillende ontwikkelingsstadia die op doorreis zijn naar het volgende weefsel, maar ook dat NK-cellen weefselspecifieke differentiatie kunnen ondergaan zonder verdere verspreiding naar andere weefsels. Deze combinatie van factoren verklaart de grote hoeveelheid aan verschillende typen NK-cellen, die aanwezig zijn in het menselijke lichaam.

De hierboven beschreven resultaten leveren een belangrijke bijdrage aan het NK-cel ontwikkelingsvraagstuk. Tevens vormt de binnen deze studie ontwikkelde 10-kleuren fluorescentieanalyse een waardevol analyse-instrument voor het monitoren van alloreactieve donor NK-cellen en de verdere ontwikkeling van NK-cel immunotherapie.

NK-cel immunotherapie

In **hoofdstuk 7** wordt tot slot een algemene beschouwing gegeven over de verschillende onderwerpen die in dit proefschrift beschreven staan. In de laatste vijf jaren zijn internationaal verschillende strategieën voor de toepassing van alloreactive donor NK-cellen succesvol ontwikkeld. Deze strategieën zijn inmiddels succesvol toegepast in klinische trials of staan op het punt om klinisch geëvalueerd te worden. Momenteel wordt NK-cel immunotherapie toegepast in drie verschillende en onderling te combineren strategieën: 1) als onderdeel van het stamceltransplantaat; 2) als donor NK-DLI in plaats van donor T-DLI na allogene SCT; en 3) als 'stand alone' NK-cel immunotherapie, los van allogene SCT. Het in dit proefschrift beschreven onderzoek draagt bij aan de ontwikkeling van deze strategieën en aan de succesvolle toepassing van NK-cel immunotherapie voor de behandeling van kanker.

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Curriculum vitae Diana N. Eissens

Diana Noëlle Eissens, daughter of Maureen Patricia van Erven and Jacobus Bernardus Maria Franciscus (Koos) Eissens, was born on September 22 in 1980 (Rotterdam, The Netherlands).

In 1992, she started secondary education at Gymnasium Beekvliet in Sint-Michielsgestel, where she graduated in 1998. Subsequently, she studied Bioprocess Engineering (Biotechnology) at Wageningen University (formerly known as Wageningen Agricultural University, The Netherlands). As a student, she performed research on vaccine development against the White Spot Syndrome virus at the Laboratory of Virology of Wageningen University under the supervision of dr. M.C.W. van Hulten and dr. J. Witteveldt. For her second internship, she joined the group of dr. P.J.M. Leenen at the Department of Immunology of the Erasmus Medical Centre (Rotterdam, the Netherlands). There, she studied the role of β-catenin on the maturation of dendritic cells. Next, she moved to Oxford (United Kingdom) to perform research on the development of HIV-I vaccines within the group of dr. T. Hanke at the MRC Human Immunology Unit of the Weatherall Institute of Molecular Medicine.

After obtaining her MSc. (ir.) degree, Diana pursued her scientific interest in Immunology and Translational Medicine, and started her PhD project in October 2005 as a Junior Researcher at the Laboratory for Medical Immunology (section of the Department of Laboratory Medicine) of the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). Under the supervision of prof. dr. I. Joosten and dr. A. van der Meer, she performed research on the facilitation of alloreactive donor NK cells for NK cell-based immunotherapies within the setting of allogeneic stem cell transplantation. The results of this study are presented in this thesis.

Currently, Diana works as a Postdoctoral Researcher in the laboratory of director dr. M. Taniguchi (Laboratory for Immune Regulation) at the RIKEN Research Centre for Allergy and Immunology (RIKEN RCAI, Yokohama, Japan), where she is involved in research on iNKT cell development and its implication for the development of new strategies for human cancer immunotherapy.

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