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Licensed to Kill: Towards Natural Killer Cell Immunotherapy

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1. Introduction

Allogeneic stem cell transplantation (SCT) is often the final treatment modality for patients with leukemia or other hematological malignancies (Schaap et al., 1997; Thomas & Blume, 1999). 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 tumour cells (Barge et al., 2003; Peggs et al., 2004; Schaap et al., 2001). Unfortunately, DLI treatment using donor-derived T cells does not only provoke graft-versus-leukemia (GVL) reactivity, but also increases the risk for the development of graft-versus-host disease (GVHD). Thus, post allogeneic SCT, long-term remission is still greatly dependent on effective 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 (Cook et al., 2004; Giebel et al., 2006; Ruggeri et al., 2002; van der Meer et al., 2008). Ruggeri *et al.* showed that alloreactive donor NK cells were able to lyse recipient tumour cells *in vitro*, implying that these NK cells may be able to provide immune reactivity by targeting residual tumour cells still present in the recipient (Ruggeri et al., 1999). Moreover, fast recovery of NK cells and predicted GVL reactivity towards host tumour cells has been associated with reduced GVHD, decreased relapse rates, and better overall survival of the patient (Kim et al., 2005a; Kim et al., 2006; Ruggeri et al., 2007; Savani et al., 2007). Altogether, this makes NK cells important candidates for immunotherapeutic use in the treatment of leukemia and other malignancies.

2. Natural Killer cells

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 tumour surveillance. Their name was based on their ability to kill target cells without prior sensitization (Kiessling et al., 1975a; Kiessling et al., 1975b). As they are morphologically recognized as relatively large lymphocytes containing azurophilic granules, they have also been known as large granular

lymphocytes (LGL) (Herberman, 1986). 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 (Vivier, 2006). NK cells are believed to originate from the same common lymphoid progenitor lineage as T and B cells in bone marrow (Spits et al., 1995). 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 NKT cells. NK cells can be further characterized based on their expression of CD56 in combination with CD16, a low affinity Fc receptor (Figure 1) (Cooper et al., 2001).

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 behaviour 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 T_H1/T_H2 bias of the adaptive immune response by activating T_H1 cells. Thereby, NK cells form a bridge between innate and adaptive immunity (Seaman, 2000).

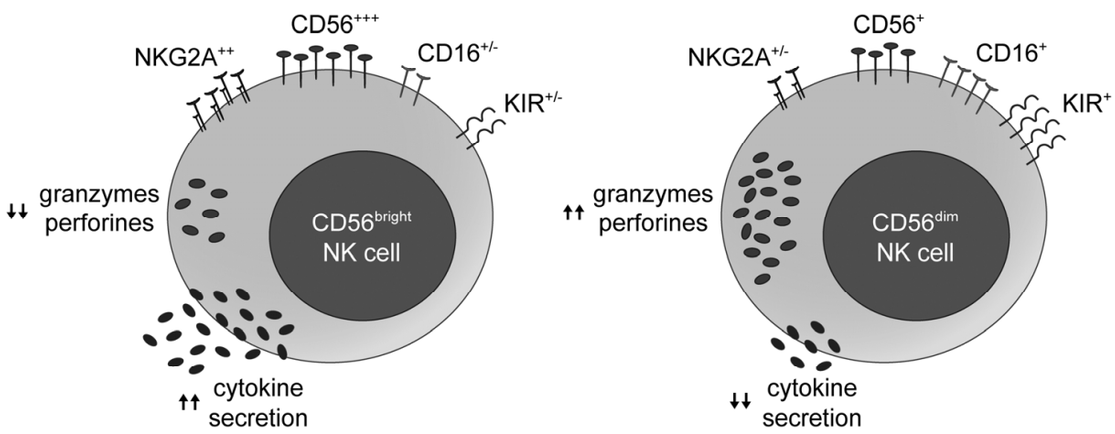

Fig. 1. Natural Killer cell subsets. NK cells can be divided in two major subsets based on their expression of CD56 and CD16. $CD56^{bright}$ NK cells have a high CD56 and low CD16 expression profile, and are specialized in cytokine secretion (e.g. TNF-α, IFN-γ). In addition they highly express inhibitory 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.

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 (Bossi & Griffiths, 1999).

2.1 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.

2.1.1 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 cell immunoglobulin-like receptors (KIRs) and lectin-like receptors (Farag & Caligiuri, 2006; Papamichail et al., 2004). The cytoplasmic domains of all inhibitory NK cell receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) (Burshtyn et al., 1996; Fry et al., 1996; Vivier & Daeron, 1997). These domains recruit intracellular tyrosine phosphatases SHP-1 or SHP-2 that mediate the inhibition of cytotoxicity and cytokine release (Burshtyn et al., 1996; Fry et al., 1996; Le et al., 1998; Olcese et al., 1996).

The lectin-like receptor complex CD94:NKG2A forms an inhibitory receptor that recognizes non-classical HLA-E molecules (Braud et al., 1998). 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 (Braud et al., 1997). 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 (Parham, 2005). 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 L (long) or S (short) (Vilches & Parham, 2002). 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 (Vilches & Parham, 2002).

2.1.2 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 (Braud et al., 1998; Houchins et al., 1997). KIR2DS and KIR3DS are stimulatory members of the KIR family (Biassoni et al., 1996; Bottino et al., 1996; Moretta et al., 1995). 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) (Lanier et al., 1998). There is evidence that the stimulatory KIRs bind self-HLA class I molecules with lower affinity as compared with the inhibitory receptors (Vales-Gomez et al., 1998a; Vales-Gomez et al., 1998b). 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 (Raulet et al., 2001).

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 (Arnon et al., 2001; Bauer et al., 1999; Mandelboim et al., 2001; Sivori et al., 1997). 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 (Bottino et al., 2000; Pende et al., 1999). 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 (Arnon et al., 2001; Arnon et al., 2005; Mandelboim et al., 2001). NCR have also been shown to mediate lysis of tumour cells and that NK cells with a NCR^{dull} phenotype are unable to kill tumour cells, suggesting that their ligands may be upregulated or induced upon malignant transformation of cells (Bottino et al., 2005; Fauriat et al., 2005; Fauriat et al., 2007). NKp30 and NKp46 are both uniquely expressed on resting and activated NK cells, whereas NKp44 is only present on IL-2 activated NK cells (Cantoni et al., 1999).

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 (Wu et al., 1999). NKG2D recognizes HLA class I-like molecules, such as MIC A and MIC B. It has been shown that the expression of NKG2D ligands, i.e. MIC A/B, ULPB1, ULPB2, and ULPB3, are upregulated by cells in times of stress, virus infection, and malignant transformation (Bauer et al., 1999; Cosman et al., 2001; Farrell et al., 2000). NKG2D is constitutively expressed on all human NK cells and can be upregulated through stimulation by IL-15, IL-12 and IFN-α (Diefenbach et al., 2000; Sutherland et al., 2006). Stimulation of NKG2D complements NCR activation in mediating NK cell lysis of tumour cells (Pende et al., 2001). Similarly, cooperation between NKG2D and stimulatory KIRs has been shown for both cytolytic activity and IFN-γ secretion (Wu et al., 2000). 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 (Das et al., 2001; Groh et al., 2001; Ugolini & Vivier, 2001).

Other stimulatory receptors that are involved in NK cell activation are co-stimulatory receptors NKp80 and 2B4 (CD244) (Biassoni et al., 2001). NKp80 and 2B4 both function synergistically with NCR (Sivori et al., 2000; Vitale et al., 2001). In addition, CD16, CD69 and DNAM-1 have been shown to trigger NK cell-mediated lysis in redirected cytotoxicity assays (Lanier et al., 1988; Moretta et al., 1991; Shibuya et al., 1996).

2.2 NK cell allorecognition

2.2.1 The "missing self" hypothesis

In 1976, Snell *et al.* observed a correlation between the susceptibility of target cells to NK cell lysis and the absence or loss of expression of HLA class I molecules on the target cells (Snell, 1976). 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 Kärre and Ljunggren demonstrated the regulation of NK cell activity (Kärre et al., 1986a). 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 (Kärre et al., 1986b). Resistance to NK cell-mediated lysis of tumours with low MHC class I expression could be restored by reintroduction of MHC class I molecules (Franksson et al., 1993; Ljunggren et al., 1990). Based on these data, they proposed the "missing self" hypothesis, which is nowadays still appreciated as the basic model for NK cell activation (Figure 2) (Ljunggren & Kärre, 1990).

2.2.2 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.* proposed that an individual NK cell can simultaneously express multiple inhibitory KIRs in a stochastic fashion (Raulet et al., 2001). 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 (Raulet et al., 2001). 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 (Salcedo et al., 1997; Sentman et al., 1995; Valiante et al., 1997). 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.* 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 (Kim et al., 2005b). 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 (Cooley et al., 2007; Fernandez et al., 2005). Thus, in order for NK cells to get their "license to kill", they need to fulfil the requirement of HLA class I-

specific receptor engagement by self-HLA. There appears to be one exception to this rule: Orr *et al*. showed in a mouse model that, contrary to the licensing hypothesis, unlicensed NK cells were the main mediators of NK cell-mediated control of mouse cytomegalovirus infection (MCMV) *in vivo* (Orr et al., 2010). It would be highly relevant to check whether such a cell population harbours increased allo- or tumour reactivity.

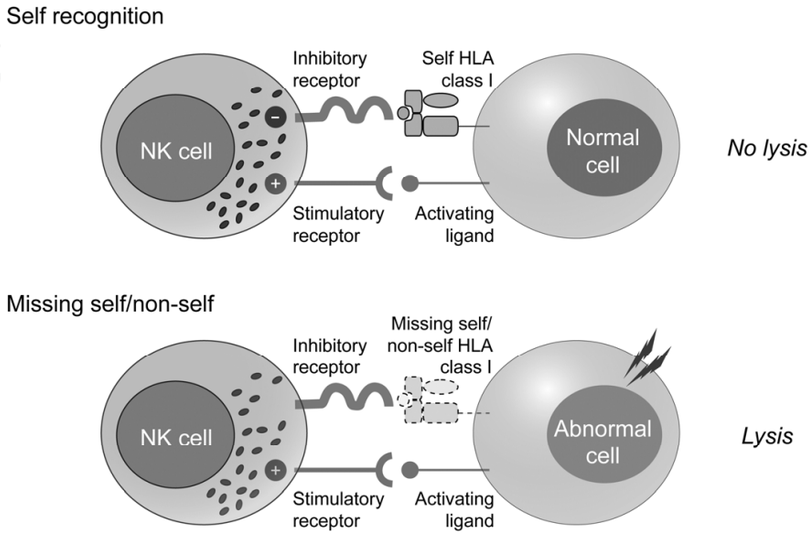

Fig. 2. "Missing self" hypothesis. NK cell activity depends on a balance between inhibitory (i.e. KIR, CD94/NKG2A) and stimulatory (e.g. NCR, NKG2D) signals. In steady state, NK cells are inhibited from activation by the recognition of self-HLA class I molecules, which overrules potential stimulatory 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.

3. NK cells and their therapeutic role in SCT

3.1 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 (Pawelec et al., 1995; Pierson & Miller, 1996). Moreover, activated autologous NK cells were shown to suppress the growth of primitive CML progenitors in long-term *in vitro* cultures (Cervantes et al., 1996). 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}) (Fauriat et al., 2007). 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 (Fauriat et al., 2005). Allogeneic SCT may overcome the impairment of NK cell-mediated lysis.

In different allogeneic SCT settings, NK cells have been shown to play an important role in the anti-tumour response within the first months after transplantation (Cook et al., 2004; Giebel et al., 2006; Ruggeri et al., 2002; van der Meer et al., 2008). In haploidentical SCT, Ruggeri *et al.* showed that donor alloreactive NK cells isolated from peripheral blood of the recipient were able to lyse tumour 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 tumour cells still present in the recipient (Ruggeri et al., 1999). Moreover, fast recovery of NK cells and predicted GVL reactivity towards host tumour cells has been associated with decreased relapse rates and better overall survival of the patient (Kim et al., 2006; Ruggeri et al., 2007; Savani et al., 2007). Altogether these data suggest that NK cells play an important role in the control and clearance of leukemic cells after allogeneic SCT.

3.2 Evidence for the prevention of GVHD

The haploidentical transplantations performed by Ruggeri *et al.* additionally suggested that NK cells may prevent the development of GVHD (Ruggeri et al., 2002). 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 antigen presenting cells (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 (Olson et al., 2010). Overall, these studies demonstrate that alloreactive NK cells may, directly or indirectly, reduce or prevent the occurrence of GVHD while retaining GVL reactivity.

4. Exploitation of NK cell alloreactivity

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

Immediately after allogeneic SCT, alloreactive NK cells have been shown to be beneficial not only for boosting the anti-tumour 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 (Bethge et al., 2006; Kim et al., 2005a).

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 HLA-matched SCT (Eissens et al., 2010a). 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:NKG2A 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 (Guma et al., 2004; Guma et al., 2006; van Stijn et al., 2008), 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 (Federmann et al., 2011). Data showed similar results as compared with our study, including fast recovery of NK cells and fast 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.* 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 (Gentilini et al., 2007).

Overall, these studies suggest that the use of NK cell rich grafts is favourable 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 (Eissens et al., 2010a). 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.

4.2 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 (Ruggeri et al., 1999; Ruggeri et al., 2004; Ruggeri et al., 2007). 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) (Hsu et al., 2005; Leung et al., 2004; Leung et al., 2005). This makes the exploitation of NK cell alloreactivity not only feasible for HLA-mismatched settings, but may also be promising for HLA-matched settings.

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 (Parham, 2006; Raulet & Vance, 2006; Vivier et al., 2008). 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 (Brodin et al., 2009). 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.

4.3 Interference by immunosuppressive drugs

For optimal NK cell-mediated GVL reactivity, NK cells need to be fully functional in the early phase after allogeneic SCT, despite that at this stage a high level of immunosuppressive treatment is given. Among the various immunosuppressive drugs (ISDs), cyclosporin A (CsA), rapamycin (Rapa) and mycophenolate mofetil (MMF) have successfully been applied for the prevention of GVHD (Cutler et al., 2007; Haentzschel et al., 2008; Neumann et al., 2005; Schleuning et al., 2008; Vogelsang & Arai, 2001). Therefore, 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 (Eissens et al., 2010b). 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 (Fauriat et al., 2010). 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 (Wang et al., 2007), 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 (Ohata et al., 2011). 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 (Alyea et al., 2008; Cutler et al., 2007; Ho et al., 2009). 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 immunotherapy *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.

4.4 Clinical grade NK cell products for adoptive cancer immunotherapy

4.4.1 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 (Iyengar et al., 2003; Klingemann & Martinson, 2004; Koehl et al., 2005; McKenna, Jr. et al., 2007; Meyer-Monard et al., 2009; Passweg et al., 2004). 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 (Carayol et al., 1998; Kao et al., 2007; Miller et al., 1992). 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.

Previously, in our centre 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 was developed (Spanholtz et al., 2010). 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 tumour 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 setting of the "receptor-ligand" model (Burke et al., 2010; Igarashi et al., 2004; Lakshmikanth et al., 2009). Overall, further exploitation of this culture system may provide a broad clinical application for NK cell-based immunotherapy against hematological and non-hematological malignancies.

4.4.2 Clinical feasibility of *ex vivo* generated NK cells: a phase I trial

Recently, the NK cell generation protocol described by Spanholtz et al. was transferred to clinical applicable conditions (Spanholtz et al., 2011) 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 (Spanholtz et al., 2010). In the second quartile

of 2011, a phase I dose-escalating study has started 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.

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 3).

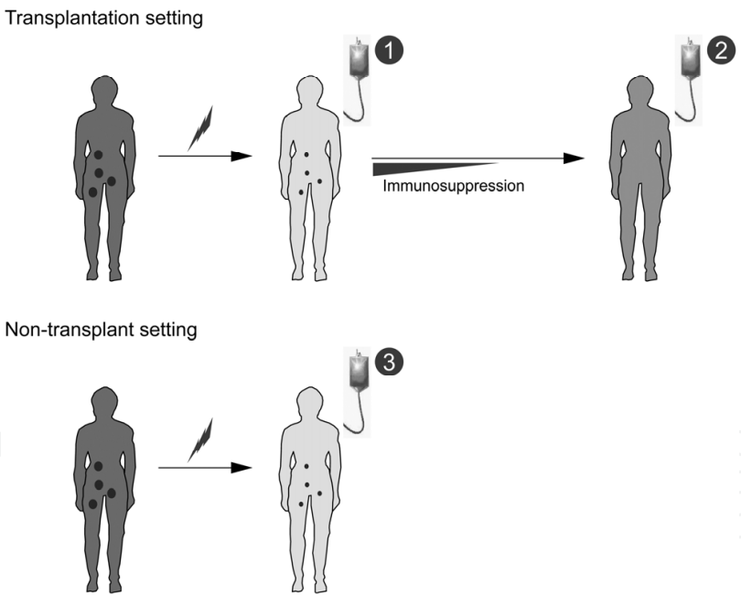

Fig. 3. Applications for NK cell-based immunotherapy. In the allogeneic SCT setting, patients are first treated with a conditioning regimen to eradicate healthy hematopoietic recipient cells and to minimize the tumour burden within the recipient. Subsequently, enriched NK cell grafts (1) and/or NK-DLI (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 tumours), 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.

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 (Bethge et al., 2006; Eissens et al., 2010a; Kim et al., 2006). In addition, the head start in GVL reactivity may lead to better outcomes in terms of infectious events and overall survival after allogeneic SCT (Kim et al., 2005a). 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-tumour activity prior to allogeneic SCT (Lundqvist et al., 2007; Ruggeri et al., 2002). 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 (Lundqvist et al., 2007; Ruggeri et al., 2002). 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 (Ruggeri et al., 2002). 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 (Kolb et al., 2004). 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 (Passweg et al., 2004; Passweg et al., 2005). Additionally, the administration of NK-DLI may facilitate engraftment and induce NK cell-mediated GVL reactivity (Passweg et al., 2004; Passweg et al., 2005). 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 (Bottino et al., 2005; Ruggeri et al., 2004; Ruggeri et al., 2006). 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.

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. 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) (Miller et al., 2005). The *in vivo* NK cell expansion was associated with 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 (Miller et al., 2005). In case of HLA-matched donors, the choice of a "receptor-ligand" mismatched donor is preferred.

In parallel, adoptive transfers are currently 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 tumour cell lines (Tam et al., 2003). 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 anti-tumor effects in some cases (Klingemann, 2005). Furthermore, NK-92 cells can easily be obtained in high numbers during GMP culture providing sufficient amounts of cells for adoptive immunotherapeutic strategies. However, to prevent that the success of NK cell adoptive immunotherapy is solely based on the use of one NK cell line and the use of donor NK cells are still preferred, 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 (Carayol et al., 1998; Kao et al., 2007; Miller et al., 1992; Spanholtz et al., 2010). After transferring these culture systems to GMP conditions, these allogeneic donor NK cells may also be proven safe for use in NK cell adoptive immunotherapy.

6. Conclusion

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, criteria for patient selection, and strategies for the identification of susceptible tumours 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.

7. References

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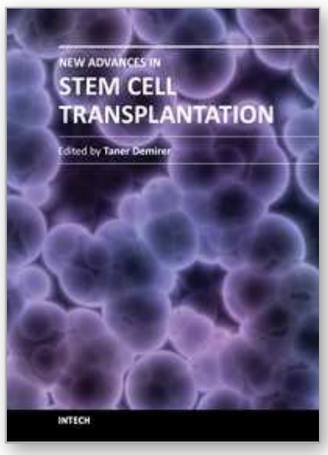

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This book documents the increased number of stem cell-related research, clinical applications, and views for the future. The book covers a wide range of issues in cell-based therapy and regenerative medicine, and includes clinical and preclinical chapters from the respected authors involved with stem cell studies and research from around the world. It complements and extends the basics of stem cell physiology, hematopoietic stem cells, issues related to clinical problems, tissue typing, cryopreservation, dendritic cells, mesenchymal cells, neuroscience, endovascular cells and other tissues. In addition, tissue engineering that employs novel methods with stem cells is explored. Clearly, the continued use of biomedical engineering will depend heavily on stem cells, and this book is well positioned to provide comprehensive coverage of these developments.

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