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Use of natural killer cells as immunotherapy for leukaemia

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Abstract

Natural killer (NK) cells potentially play a significant role in eradicating residual disease following allogeneic haematopoietic cell transplantation, and have been explored as tools for adoptive immunotherapy for chemotherapy-refractory patients. NK cell cytotoxicity is modulated by multiple activating and inhibitory receptors that maintain a balance between self-tolerance and providing surveillance against pathogens and malignant transformation. The functional characteristics of NK cells are dictated by the strength of inhibitory receptor signalling. Major histocompatibility complex (MHC)-specific inhibitory receptor acquisition occurs sequentially during NK cell development, and is determined by the nature of immunological reconstitution after allogeneic haematopoietic cell transplantation. Polymorphisms of inhibitory receptors [killer immunoglobulin-like receptors (KIRs)] and their ligands (MHC) contribute to interindividual variability. As a result, the functional NK cell repertoire of individual donors has variable potential for graft-vs-leukaemia reactions. Models predicting NK cell alloreactivity, including KIR ligand mismatch and missing KIR ligand strategies, are discussed as algorithms for optimal NK cell donor selection. Future modifications to improve NK cell adoptive immunotherapy by means of increasing target recognition and reducing inhibitory signalling are being explored.

Keywords

immunotherapy; allogeneic hematopoietic cell transplant; adoptive therapy; NK cells; graft-vs-leukaemia reaction; killer immunoglobulin-like receptors

Ever since the original observation that freshly isolated lymphocytes could kill malignant cells,¹ the search to understand and harness these so-called natural killer (NK) cells has been ongoing. NK cells have been defined as CD56⁺CD3[−] lymphocytes that often have a granular morphology.² Unlike T cells and B cells, NK cells lack rearranged antigen-specific receptors and are therefore incapable of antigen-specific recall responses. Such properties place NK cells within the innate immune system. NK cells recognize targets by displaying a variety of surface

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receptors that transmit either activating or inhibitory signals. These receptors are not uniformly expressed on NK cells, and this heterogeneous receptor expression creates a diverse repertoire of NK clones with varying ability to recognize targets. Thus, as opposed to T and B cells, NK cell diversity is not a result of a single rearranged antigen receptor, but is due to the collective influence of multiple, germline encoded receptors.³

FUNCTIONAL CHARACTERISTICS OF NK CELLS

NK cell function is partially determined by receptors which transmit inhibitory intracellular signals when ligated by major histocompatibility complex (MHC) expressed on target cells. NK cells express inhibitory receptors such as Ly49 (in mice),⁴ killer immunoglobulin-like receptors (KIRs, in humans)⁵ and CD94/NKG2A (in both mice and humans).⁶ These inhibitory receptors help to explain two seminal characteristics of NK cell function: target cell killing and hybrid resistance. In both circumstances, NK cell cytotoxicity occurs when inhibitory receptors are not engaged, either because of a reduction (or lack) of MHC on malignant or virally infected cells⁷ or due to the absence of 'self-MHC' on transplanted parental bone marrow (i.e. hybrid resistance⁸). In either situation, the lack of inhibitory receptor signalling leaves NK cells unrestrained, resulting in NK cell activation and target elimination. This mode of action has been termed 'missing self-recognition'.⁹

In addition to inhibitory receptors, other receptors that activate NK cells are required for target recognition and killing. A number of activating NK receptors have been identified, including NKG2D, NKp30, NKp44, NKp46, activating KIR, CD94/NKG2C, DNAM-1, CD96, CRTAM, 2B4, NTB-A and CD69.¹⁰ It appears that activating receptors recognize ligands which increase following cellular stress (i.e. malignant transformation, infection, oxidative stress or irradiation). This led to the 'induced self-model' where NK cells use activating receptors to recognize perturbed cells.¹¹ Additionally, several activating receptors directly recognize components of viral infections.^{12,13} The net balance of signals from inhibitory and activating receptors determines whether NK activation will lead to the release of cytotoxic granule contents (granzymes and perforin¹⁴) and the display of surface death receptors (FasL and TRAIL¹⁵). NK cell receptor signalling may also result in cytokine release [interferon gamma (IFN- γ), tumour necrosis factor alpha, interleukin (IL)-5, IL-10, IL-13 and granulocyte-macrophage colony-stimulating factor¹⁶]. The majority of human peripheral blood NK cells express CD16, an immunoglobulin receptor which can trigger cytotoxicity and cytokine release when bound to antibody-coated targets [i.e. antibody-dependent cellular cytotoxicity (ADCC)].¹⁷

LIGAND SPECIFICITY OF KIRS

KIRs transmit either inhibitory or activating signals depending upon the motif contained within the cytoplasmic tail. KIRs with long cytoplasmic tails have ITIM regions that associate with phosphatases (SHP-1, -2 and SHIP-1) and inhibit NK cell activation.¹⁸ In contrast, ITIM regions are absent in short KIRs, and when ligated, these receptors associate with activating adapter proteins (DAP12).¹⁹

Many inhibitory KIRs are specific for polymorphic domains of MHC class I.^{5,20,21} KIR2DL1 (CD158a) recognizes human leukocyte antigen (HLA)-C alleles with lysine in position 80 (C2 specificity, e.g. Cw2, 4, 5, 6), while KIR2DL2 and KIR2DL3 (CD158b1/b2) recognize HLA-C alleles with asparagine in position 80 (C1 specificity, e.g. Cw1, 3, 7, 8). KIR3DL1 (CD158e1) is specific for HLA-B alleles bearing the Bw4 motif (and some HLA-A alleles). Thus, KIR genes recognize polymorphisms of HLA-C and -B in a bi-allelic manner: C1 vs C2 and Bw4 vs Bw6. Another receptor, KIR3DL2 (CD158k), recognizes other HLA-A epitopes (A3, A11);⁵ however, a precise description of its functional specificity is less clear. Consequently,

KIR3DL2 interactions are not included in most studies evaluating the effect of KIR ligands on transplant outcomes (see below).

POLYMORPHISM OF KIR GENES

KIR genes are encoded by a set of 15 loci, including two pseudogenes, in the leukocyte receptor cluster on chromosome 19q13.4.²² Individuals vary with respect to the number of KIR genes contained in their genome (i.e. KIR gene content).²³ In addition to varying gene content, there are marked allelic polymorphisms of these genes and this may affect both expression and function.^{23,24} Adding to the complexity of this system, each KIR gene is only expressed by a fraction of NK cells.²⁵ KIR gene expression is transcriptionally regulated within the leukocyte receptor cluster locus,²⁶ but the repertoire may be further influenced by the presence or absence of the cognate ligand (i.e. MHC I) encountered during development.²⁷

KIR genes are closely linked and inherited as a haplotype. The variability of KIR gene content can be simply organized into two groups of KIR haplotypes, termed A and B.²⁵ The genes present in both haplotypes (3DL3, 2DL4 and 3DL2) constitute the framework loci. In addition to these framework genes, group A haplotypes contain a variable number of inhibitory receptor genes (KIR2DL1, KIR2DL3, KIR3DL1), only one of which activates KIR2DS4, and do not contain KIR2DL5 or other activating KIRs. In addition to the genes found in the group A haplotypes, group B haplotypes are defined by the presence of one or more of the following: KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR3DS1 or KIR2DL5. Thus, the main difference between group A and group B haplotypes is that group B haplotypes contain several activating receptor genes. Additionally, there is a strong linkage disequilibrium between some KIRs, such as KIR2DS2 (on the group B haplotype) and KIR2DL2. Thus, it has been difficult to define clinical significance to individual KIR genes.²³

CD94/NKG2A INHIBITORY RECEPTOR

NKG2A associates covalently with CD94 and together they recognize HLA-E, a non-classical HLA molecule.^{6,28} The presence of HLA-E on the cell surface depends on the availability of peptides derived from the leader sequence of HLA antigens (HLA-A, -B, -C and -G).^{6,28} Thus, HLA-E expression is a broad indication of HLA expression. HLA-E shows little polymorphism which does not influence the interaction with CD94/NKG2.²⁹ Similarly, CD94 and NKG2A genes themselves are present in all individuals and are conserved among species (unlike KIR genes).³⁰

NK CELL TOLERANCE AND REPERTOIRE FORMATION

Critical to exploiting NK cells therapeutically is an understanding of how the NK cell repertoire is formed and how tolerance is maintained. The finding that every NK cell will express at least one inhibitory receptor able to recognize self-MHC has been termed the 'at least one rule'.^{31,32} This has been established by demonstrating that all NK clones were tolerant of 'self' by means of MHC-specific receptors.^{31,32} Even though the 'at least one rule' clearly emerged from these studies, both murine and human NK cells lacking self-MHC-specific inhibitory receptors have been identified. Importantly, they are functionally impaired and do not result in autoreactivity.^{33–35} Conversely, NK cells expressing inhibitory receptors (KIRs in humans or Ly49 in mice) for which there is an appropriate MHC ligand have more robust function.³⁶ Therefore, the strength of inhibition by self-MHC may ultimately dictate the functional capacity of NK cells. Theories explaining such findings include descriptions of: licensing,³⁶ disarming³⁷ and the continuum of rheostat adjustment.³⁸ The licensing theory implies that inhibitory signalling through MHC-specific receptors endows an NK cell with functional capacity, and that lack thereof renders them unresponsive. While the concept of arming is principally similar, disarming would be an active process, leading to unresponsiveness of NK

cells if they lack functional inhibitory receptors. Finally, the rheostat concept posits that the functional state of NK cells is not binary (licensed or not), but rather is a continuum. Under this model, the NK cell activation threshold is set at different levels that balance the potency of activation with the strength of inhibition.

The term ‘licensed’ has been used to describe NK cells responding in a particular manner (IFN- γ production, cytotoxic granule release) to a particular stimulus, such as co-incubation with target cells or triggering of a single activating receptor.³⁶ Not surprisingly, the activation requirements for freshly isolated, resting NK cells show that a combination of activating receptors is more effective than a single activating receptor.³⁹ Moreover, the requirement for NK cell triggering is lower when NK cells are pre-incubated with cytokines such as IL-2. Therefore, whether a given NK cell is considered licensed or not may depend on the circumstances such as the mode of triggering and/or cytokine prestimulation. Perhaps the rheostat model, whereby NK cells have differing set points of activation, may best explain these findings. In this scenario, cytokines, such as IL-2 and IL-15, lower the NK cell activation threshold.

ACQUISITION OF INHIBITORY RECEPTORS DURING NK CELL DEVELOPMENT

In-vitro studies on the development of NK cells from haematopoietic precursors show that inhibitory receptor acquisition occurs sequentially, with CD94/NKG2A acquisition prior to KIR.⁴⁰ Similar stages of NK cell development have been described in vivo.⁴¹ Furthermore, cytotoxic function and cytokine production are acquired concomitantly with the expression of CD94/NKG2A.⁴⁰ Such a mode of inhibitory receptor expression emerges as a possible strategy, ensuring that every NK cell is self-tolerant. As described above, CD94/NKG2A and its ligand, HLA-E are present in every individual and form a functional receptor–ligand pair. In contrast, subsequent KIR expression is seemingly a more random event. As stated above, KIR expression is regulated by multiple promoters with an on and off function,⁴² as well as by epigenetic factors. Studies on reconstitution of the NK repertoire after haematopoietic cell transplantation recapitulate in-vitro studies by showing that the NK cells express CD94/NKG2A early and acquire KIRs later.⁴³

NON-MHC INHIBITORY RECEPTOR SYSTEMS

The necessity of MHC-specific receptors for NK cell self-tolerance is challenged by observations in MHC-class-I-deficient individuals. NK cells from such patients do not kill autologous T cells and do not cause overt autoimmunity.⁴⁴ This suggests a role for other non-MHC inhibitory receptors. It has been shown that CEACAM-1 serves as a non-MHC-restricted inhibitory receptor in class-I-deficient patients.⁴⁵ In addition, CD161 binds to homologous ligands, providing self-tolerance in mice.⁴⁶ 2B4 (CD244) may also transmit inhibitory signals in NK cells, assuring tolerance when binding its ligand, CD48.⁴⁷ Thus, the process of NK cell education can result in tolerance to self-tissues, even in the absence of MHC. However, such NK cells are unable to recognize allogeneic cells through ‘missing self-MHC’.⁴⁸

PATHWAY(S) OF NK CELL DEVELOPMENT

Within the human NK cell population, two phenotypically and functionally distinct subsets can be distinguished: CD56^{bright}CD16[−] and CD56^{dim}CD16⁺ NK cells.⁴⁹ CD56^{bright} NK cells constitute ~10% of peripheral blood NK cells. This subpopulation uniformly expresses high-density CD94/NKG2A⁺ and most lack KIRs. CD56^{bright} NK cells have been proposed to be freshly differentiated NK cells, which then give rise to the CD56^{dim} NK cells.⁴¹ In support of this, interaction of CD56^{bright} NK cells with tissue-resident fibroblasts promotes their

maturation, leading to a CD56^{dim}CD16⁺KIR⁺CD94⁻ phenotype.⁵⁰ However, CD94/NKG2A-KIR⁻ NK cells can also be identified in blood. These cells are hyporesponsive, leading to the conclusion that they are developmentally immature.³⁵ Interestingly, they are CD56^{dim} NK cells, thus it is difficult to rectify these findings with the notion that CD56^{dim} NK cells are exclusively derived from CD56^{bright} cells through a process of maturation. Whether CD94/NKG2A-KIR⁻ NK cells are derived from a CD94⁺NKG2A⁺ NK cell or through a separate pathway of NK cell development (where KIR expression is not secondary to CD94/NKG2A acquisition) remains an open possibility. Importantly, CD94/NKG2A-KIR⁺ NK cells are present in the circulation of individuals. As discussed below, cells with this combination of receptors (KIR⁺CD94/NKG2A⁻) are endowed with maximal alloreactive potential and are likely to be involved in the eradication of leukaemia.⁵¹

REPertoire OF NK CELLS AND ALLOREACTIVITY

The main determinants of NK cell alloreactivity are inhibitory receptor interactions (KIRs and CD94/NKG2A) with their HLA ligands.^{26,31} The fraction of NK cells expressing KIRs for which there is an HLA ligand in the host is variable, depending on polymorphisms of both the KIR and HLA genes. Individuals differ in the number of KIR–HLA receptor–ligand pairs they possess. For example, a person homozygous for HLA-Bw6 and -C2 will only have one functional inhibitory KIR–HLA ligand pair (2DL1–HLA-C2). In contrast, other individuals may have up to five functional KIR–HLA pairs: 2DL1–C2, 2DL2–C1, 2DL3–C1, 3DL1–Bw4 and 3DL2–A3,A11. In individuals with only one functional KIR–HLA pair, a larger proportion of NK cells express CD94/NKG2A as their self-specific inhibitory receptor.²⁶ As a result, NK cells expressing CD94/NKG2A would be inhibited by allogeneic HLA-E to the same extent as by autologous HLA-E. Thus NK cells with the highest potential for alloreactivity reside in the fraction relying on KIRs and not CD94/NKG2A. These KIR⁺CD94/NKG2A⁻ NK cells can kill allogeneic cells that lack the MHC ligand for their KIR.³¹ A recent study indicated another factor important for NK cell alloreactivity. The presence of activating KIRs (i.e. haplotype B) was critical for NK cell cytotoxicity against allogeneic Epstein Barr virus (EBV)-transformed B cells.⁵² NK cells from individuals that are homozygous for haplotype A showed minimal cytotoxicity against allogeneic targets. Collectively, the above findings suggest that donors may vary in their ability to eradicate residual leukaemia. Identifying factors that control NK cell alloreactivity may aid in better donor selection.

ROLE OF NK CELLS IN SURVEILLANCE AGAINST MALIGNANT TRANSFORMATION

The potential for NK cells to kill cancer cells has resulted in studies on their role in tumour surveillance. Murine studies show that NK cells can purge leukaemia from bone marrow⁵³ and control lymphoma development.⁵⁴ It is believed that the main target of NK cell activity is within the haematopoietic system, as manifested by a hybrid resistance model in which NK cells reject allogeneic bone marrow but not skin or solid organs.^{8,55} Accordingly, NK cells may play a role in the prevention of haematogenous (metastatic) spread of certain solid tumours. For instance, in ocular melanoma, there is a high predilection for liver metastases which decreases in cases with MHC loss, suggesting NK-cell-mediated elimination of MHC-deficient melanoma from blood.⁵⁶ However, the lack of well-described human immunodeficiencies specifically affecting NK cells limits our knowledge of their role in physiology.⁵⁷ Perforin mutations predispose to EBV-induced lymphoma, but this does not directly implicate NK cells since cytotoxic T cells are also affected.⁵⁸

While the exact role of NK cells in the surveillance of malignant transformation is debated, NK cells are functionally impaired in leukaemia patients. Whether this precedes the onset of malignancy or is secondary to cancer development is not known. Considering that at least the

early stages of NK development take place in the bone marrow (which is also the site of leukaemic burden), the milieu required for NK cell development could be perturbed. Likewise, malignant cells can shed MICA, a ligand for activating receptor NKG2D which can negatively affect the cytotoxic capacity of mature NK cells.⁵⁹ Functional NK cell impairment has prognostic significance. Cytotoxicity against autologous leukaemic blasts tested either in vitro^{60–62} or using a xenogeneic in-vivo model⁶³ can correlate with the duration of remission. A reduction in activating receptors (NCR^{dull} phenotype), as well as reduced cytotoxicity against autologous blasts, is also associated with inferior outcomes due to disease recurrence.⁶⁴ Interestingly, impaired NK cell activity⁶⁵ and the NCR^{dull} phenotype⁶⁴ can reverse following chemotherapy, implying a causative role of the leukaemia in NCR downregulation.

SHORTCOMINGS OF AUTOLOGOUS NK CELLS

Based on laboratory observations that IL-2 enhances NK cell activity, numerous studies have tested the efficacy of IL-2 administration to cancer patients.⁶⁶ The limitations of this approach include the side-effects of systemic IL-2, perhaps resulting in incomplete in-vivo NK cell activation. To overcome this, subsequent trials employed ex-vivo activated NK cells infused to patients, followed by IL-2 administration. Although this led to significant in-vivo NK expansion and improved in-vitro function, the clinical results were disappointing. Only modest, non-significant improvements in the time to disease progression were reported.⁶⁷ This could be explained by the finding that low-dose IL-2 may not only activate NK cells but also regulatory T cells, which may promote tolerance and hamper NK cell activity.⁶⁸

Another consideration is that autologous NK cells are self-tolerant by means of inhibitory receptors. Individuals will have a NK cell repertoire that is formed by both KIRs and MHC. Understanding how KIR and HLA genotypes influence the ability of autologous NK cells to prevent relapse would open up the possibility of selecting patients that may potentially benefit from autologous NK cell therapy. In one small study, KIR and HLA types correlated with outcomes after high-dose chemotherapy and autologous haematopoietic cell transplantation. Patients with more inhibitory receptors (KIRs) for which there was no HLA ligand were protected from relapse.⁶⁹

BREAKING NK CELL SELF-TOLERANCE

To improve autologous NK cell immunotherapy, strategies are being developed to break self-tolerance and/or increase malignant cell recognition. Attempts at reducing inhibitory signalling include KIR blocking antibody⁷⁰ and agents, such as proteasome inhibitors (Bortezomib), that reduce MHC expression on target cells. Bortezomib also sensitizes malignant cells to NK cell killing via upregulation of the TRAIL receptor.⁷¹ Antibodies recognizing receptors present on malignant cells (e.g. CD20, Her2/neu) can lead to NK lysis through ADCC.⁷² In support of the role of ADCC in antibody therapy, polymorphisms of the low-affinity Fc receptor (CD16) influence clinical outcomes.⁷³ A further step forward may be antibody–cytokine conjugates that could activate NK cells upon recognition of antibody-coated targets,⁷⁴ or the combined use of systemic cytokines with antibody therapy. Other cytokines are now being tested to overcome the lack of efficacy seen with IL-2. IL-21 deserves particular attention,⁷⁵ as it supports the development of NK cells.⁷⁶ Activation of toll-like receptors using CpG is also being tested therapeutically in cancer patients. CpG stimulates NK cells directly⁷⁷ or via IFN- α release from plasmacytoid dendritic cells (DCs), which in turn triggers NK cell activity.⁷⁸ Interestingly, a recent report indicates that DCs may acquire lytic activity typical for NK cells under the influence of CpG.⁷⁹ In summary, attempts to either increase target cell recognition or decrease inhibitory signalling may interfere with NK cell tolerance to autologous malignant cells.

ALLOGENEIC TRANSPLANTATION

The potential benefit of NK cells has been realized in the setting of allogeneic haematopoietic cell transplantation. Allogeneic NK cells are advantageous to autologous NK cells in that: (1) they may not be tolerant to patient MHC, and (2) they are not functionally impaired (as is in the case of NCR^{dull} cells). In the setting of CD34⁺-selected, MHC-haplo-identical transplants, the MHC specificities as recognized by KIRs can differ between donor and recipient. Following transplant from a haplo-identical donor, NK cells could be identified that expressed KIRs for which the ligand was not present (or 'missing') in the recipient.⁸⁰ Recipients that were 'missing KIR ligands' had a lower relapse rate and better survival.⁵¹ This analysis was based on the MHC of the donor and recipient, without investigating the donor KIR genotype or phenotype. Such analysis is referred to as the 'KIR ligand mismatch model'. The superior control over acute myeloid leukaemia (AML), but not lymphoid leukaemia in these 'KIR ligand mismatched transplants' is most likely to be due to NK cell activity.

These findings led to retrospective studies testing whether they could be extended to unrelated donor transplants. Such transplants do not typically involve CD34⁺ selection or T-cell depletion. Most large registry studies failed to show a survival benefit following unrelated transplantation from KIR ligand mismatched donors,^{81–83} although some did.⁸⁴ These contradicting results highlight several variables between transplant platforms. Among them was the variable use of anti-thymocyte globulin (ATG) in the conditioning regimen. Additionally, when used, ATG formulations differed (horse vs rabbit). Interestingly, in-vitro studies show that each ATG preparation has differential effects on NK cells.⁸⁵ Moreover, while pretransplant administration of ATG results in recipient T-cell elimination, the antibodies can persist in the circulation for over 2 weeks, and are likely to deplete donor-derived cells (referred to as 'in-vivo T-cell depletion'). This may allow for enhanced NK cell reconstitution and proliferation due to less lymphocyte competition for cytokines, such as IL-15 and IL-7.^{86,87} In-vivo T-cell depletion may also result in less graft-vs-host disease (GvHD) and hence better NK cell reconstitution. Furthermore, the immunosuppressive prophylaxis and treatment of acute GvHD is different for patients who receive T-cell depletion and this may also account for the differences between the above studies. This refers to both pre-emptive immunosuppression [with cyclosporine A (CSA)] and acute GvHD treatment with corticosteroids. CSA has a detrimental effect on NK cell proliferation in vitro; however, the KIR[−] CD56^{bright} NK cells are relatively resistant to CSA compared with the KIR⁺ CD56^{dim} population.⁸⁸ In contrast, recent studies have shown that high-dose steroids have a negative effect on both NK cell proliferation and function.⁸⁹ Finally, MHC mismatches, including those beneficial for NK activity, may also be recognized by T cells and they could lead to acute GvHD. Thus, in the T-cell-replete transplant setting, the negative effect of acute GvHD on patient survival may offset any benefit of NK cell alloreactivity.⁹⁰ A study addressed the hypothesis that T cells present in the graft influence NK cell recovery. When T cells were not depleted, recovering NK cells expressed less KIRs and produced more IFN- γ . Interestingly, the fraction of NK cells expressing KIRs was an independent predictor of survival in this patient group.⁹¹ Thus, there are multiple mechanisms by which T cells, ATG and immunosuppression may affect NK cell recovery and function after transplantation.

MISSING KIR LIGAND MODEL

As mentioned above, the KIR ligand mismatch model compares the HLA typing of recipient and donor (ligand–ligand comparison). An alternative view is the missing KIR ligand model (receptor–ligand comparison).⁹² In this analysis, the HLA typing of the donor is not taken into account. Instead, the repertoire of donor KIRs and patient HLA typing are considered. KIR expression by donor NK cells is tested on the protein level (by flow cytometry), and the presence of KIR ligands is ascertained by patient HLA typing. This model may better predict

relapse compared with the KIR ligand mismatch model, and may extend to lymphoid as well as myeloid leukaemia.⁹² As mentioned above, this analysis does not take into account the presence of KIR ligands in the donor; thus, it considers NK cells expressing KIRs which are both 'licensed' and 'not licensed' (Figure 1). One drawback of this model is that retrospective analysis of the KIR repertoire in donors is sometimes not possible at a protein level. A more feasible approach is to test for the presence of KIR genes, assuming that the protein will be expressed. Patients with AML or myelodysplastic syndrome had significantly less relapse if they lacked an HLA ligand for the KIR present in the donor.⁹³ Since the donor–recipient pairs in this study were HLA-identical siblings, the KIR ligand mismatch model would predict no NK alloreactivity. Thus, the potential for leukaemia surveillance is not restricted to 'licensed', alloreactive NK cells as analysed by the KIR ligand mismatch model, and may be extended to other NK cell subsets (Figure 1).

The receptor–ligand model can be further simplified by the assumption that all donors express inhibitory genes without testing them. Such an assumption would carry a ~15% probability of error with regards to the presence of KIR genes and an even higher rate of errors when considering cell surface expression.⁹³ This approach only takes recipient HLA into account. However, when applied to a retrospective cohort of >2000 unrelated donor transplants, the lack of KIR ligands reduced relapse significantly in patients with early-stage myeloid malignancies.⁹⁴

RECOVERY OF NK CELLS AND KIRS AFTER TRANSPLANTATION

NK cells form the first wave of immunological recovery after high-dose chemotherapy/radiotherapy. As early as 3–4 weeks after transplantation, the numbers of NK cells in blood reach or exceed those seen in healthy subjects. Patients with higher numbers of NK cells at 30 days after transplantation had a reduced risk of relapse and improved survival.⁹⁵ Additionally, inhibitory receptor expression on recovering NK cells yields several interesting observations. The first NK cells present after transplantation are predominantly CD94/NKG2A⁺ and KIR[−]. In most patients, the pattern of KIR expression approaches the donors >100 days after transplant.⁴³ However, some patients who suffered severe post-transplant complications never recovered a phenotype similar to the donor. As mentioned above, KIR reconstitution is faster in recipients of T-cell-depleted transplants. Not all KIRs are equally abundant on NK cells and thus the dynamics of KIR acquisition differ.⁴³ It has been observed that KIR2DL2 and 2DL3 (CD158b) are expressed earlier and by a higher proportion of NK cells after transplantation compared with KIR2DL1 (CD158a).⁴³ Similar observations have been made in NK cells developing in vitro.⁹⁶ Since KIR2DL2/3⁺ NK cells recognize HLA-C1 alleles, patients with this ligand have a higher proportion of NK cells with competent KIRs in the early post-transplant period compared with HLA-C2 homozygous patients. Superior survival was found in HLA-C1⁺ patients compared with HLA-C2⁺ patients after unrelated donor transplantation.⁹⁷

EFFECT OF KIR GENE CONTENT

Several studies have investigated the role of KIR gene content in allogeneic transplantation. Some studies have shown a benefit to donors expressing activating KIRs and/or B haplotypes, 98–100 while others have not.^{101,102} Importantly, the methodologies of analyses in these studies differ, comparing the KIR repertoire in the donor and recipient,¹⁰³ analysing the effects of the presence vs absence of particular KIR gene(s) in the donor^{100,101} with or without taking into account the recipient's MHC. Perhaps most appropriate from a biological point of view is the analysis of KIR haplotypes (A vs B) in both donors and recipients.¹⁰² The differential effects of KIR gene content on patient survival in these studies were due to treatment-related mortality,⁹⁸ relapse¹⁰⁰ and/or acute GvHD.⁹⁹ It is conceivable that activating KIRs may

differentially affect risk of infection, relapse or GvHD, and thus provide some benefit in certain situations but not others. Large studies with sufficient power to consider multiple factors and modes of analysis are required to resolve this issue.

ADOPTIVE IMMUNOTHERAPY WITH NK CELLS

The ability of NK cells to control leukaemia after allogeneic haematopoietic cell transplantation has prompted clinical studies of NK cell immunotherapy. Adoptive transfer of allogeneic NK cells as a salvage therapy for AML patients can induce remission.⁸⁷ Current studies indicate that chemotherapy prior to NK cell infusion is necessary and allows for transient NK engraftment and in-vivo expansion. The pancytopenia associated with high-dose chemotherapy also increases systemic levels of IL-15, which is critical for NK cell development.⁸⁷ The strategy to isolate sufficient numbers of allogeneic NK cells for adoptive immunotherapy involves leukapheresis followed by immunomagnetic selection.¹⁰⁴ Depletion of CD3⁺ cells was achieved successfully; however, the possibility of EBV-induced B-cell lymphoproliferative disorder also warrants B-cell depletion. Alternatively, positive isolation of CD56⁺ cells is another option; however, CD3⁺CD56⁺ T cells would also be included in such preparation unless it is preceded by T-cell depletion.¹⁰⁵ This two-step approach resulted in significant cell loss (JSM, unpublished).

Following allogeneic transplantation, donor NK cell infusion could also be performed. NK cells do not pose an increased risk of acute GvHD in animal models;^{51,55,106} however, they can potentially exacerbate ongoing acute GvHD by IFN- γ elaboration. Another point of consideration is the possibility of expanding mature peripheral blood NK cells in vitro prior to infusion.¹⁰⁵ Regardless of the method of expansion, it is not known whether cytokine-stimulated NK cells would function and/or survive following adoptive transfer. Systemic administration of cytokines along with NK cell transfusion (following allogeneic haematopoietic cell transplantation) would be a step further, but requires careful examination of the risk of eliciting GvHD. The selection of the optimal NK cell donor should take into account the current knowledge of NK cell tolerance and alloreactivity briefly summarized above. When planning adoptive NK cell transfer studies, it is critical to consider the possibility that the beneficial effect of NK cell adoptive therapy may be short-lived. Thus, such therapies may be best incorporated into a long-term treatment plan that induces and maintains leukaemia remission.

SUMMARY

The anti-leukaemic activity of NK cells has long been observed in laboratory and animal studies. The beneficial effect of NK cells, first demonstrated in haplo-identical allogeneic stem cell transplantation, is now being tested in other transplantation settings. Adoptively transferred allogeneic NK cells have been used for leukaemia eradication in chemotherapy-refractory myeloid leukaemia patients with encouraging results. Current efforts are focused on optimizing the efficacy of adoptively transferred NK cells by improving their in-vivo engraftment and survival, and by increasing their recognition of malignancy. A more thorough understanding of the basic mechanisms of NK cell development, response to cytokines, homing/migration, and target recognition and survival will aid in these efforts.

Practice points

- at the time of diagnosis, patients with leukaemia show impaired NK cell activity
- allogeneic NK cells can induce remission in a fraction of acute myeloid leukaemia patients who failed other therapies

- chemotherapy and immunodepletion prior to NK cell infusion and cytokine administration are likely to be necessary for optimal survival, expansion and activity of NK cells in the recipient
- donors that express KIRs for HLA ligands that are missing in the recipient (i.e. missing KIR ligand model) offer the greatest chance of NK-cell-mediated anti-leukemia activity, especially if these HLA ligands are present in the donor
- combinations of NK cell therapy and agents that increase target cell recognition (e.g. monoclonal antibody, proteasome inhibitors and others) offer potential for improved efficacy

Research agenda

- studies on the mechanism of leukaemia-induced NK cell impairment
- further studies to determine the importance of KIR genotype, in particular activating KIR genes in the anti-leukemic activity of allogeneic NK cells
- optimization for protocols detailing NK cell preparation (possibly including in-vitro expansion and activation), and post-transfusion administration of cytokines or other agents that increase anti-leukaemic activity of NK cells
- incorporation of NK cell infusion into treatment schemes aimed at long-term cure of leukaemia

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Figure 1. Human leukocyte antigen (HLA) type of the donor affects natural killer (NK) cell repertoire and thereby alloreactive potential of NK cells. Exemplified by two donors, one with a single functional killer immunoglobulin-type receptor (KIR)–HLA pair (2DL1– HLA C2), the other with five KIR–HLA pairs (3DL– HLA Bw4, 2DL1–HLA C2, 2DL2–HLA C1, 2DL3–HLA C1 and 3DL2–HLA A3/A11). In the NK repertoire of such donors, the relative contribution of NK cells expressing KIRs for which there is an HLA ligand (filled black) or expressing KIRs for which there are no ligands (empty) is different (depicted by arrows as relative increase or decrease). The majority of NK cells expressing no functional KIRs (or expressing KIRs for which there are no ligands) rely on CD94/ NKG2A as their major-histocompatibility-complex-

specific inhibitory receptor. The KIR ligand mismatch model predicts NK cell alloreactivity when NK cells express KIRs with their ligands in the donor but missing in the patient. In contrast, the missing KIR ligand model predicts NK cell alloreactivity when NK cells express KIRs for which there are no ligands in the patient, irrespective of the presence of these ligand in the donor.