Published in final edited form as:
Curr Opin Oncol. 2010 March ; 22(2): 130–137. doi:10.1097/CCO.0b013e328335a559.

Biology and clinical effects of natural killer cells in allogeneic transplantation

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Abstract

Purpose of review—Following allogeneic hematopoietic cell transplantation, donor-derived natural killer (NK) cells target recipient hematopoietic cells, resulting in an antileukemia effect and a lower incidence of graft rejection. NK cells do not mediate and may diminish graft versus host disease. Here we review the determinants of NK cell alloreactivity and their implications for adoptive NK cell therapy.

Recent findings—NK cell alloreactivity has been defined by the absence of recipient MHC class I ligands for donor inhibitory killer immunoglobulin-like receptor (KIR) receptors, as predicted by a number of algorithms. Recently, the role of activating NK receptors and their cognate ligands has received more attention. The beneficial clinical effect of NK-cell alloreactivity has not been uniformly demonstrated, likely reflecting differences in conditioning regimens, graft components and posttransplant immune suppression. Investigations of NK cell phenotype and function after transplantation have helped demonstrate which NK cell subsets mediate the graft versus leukemia effect. These advances have proceeded in parallel with increasing facility in GMP-grade bulk purification and administration of NK cell preparations.

Summary—NK cells are a heterogeneous population of lymphocytes with diverse patterns of target-cell recognition and effector function. Further clinical and functional correlations will help maximize their potential for clinical benefit.

Keywords

adoptive immunotherapy; hematopoietic transplantation; natural killer cells

Introduction

Natural killer (NK) cells differentiate self from nonself by gauging the expression of MHC class I molecules on potential target cells. In the context of allogeneic hematopoietic cell transplantation (HCT), particularly with HLA mismatched transplants, donor-derived NK cells have been shown in some studies to influence the outcome by a direct antitumor effect as well as by mitigating graft versus host disease (GVHD) and reducing the incidence of graft rejection. The optimal way to harness NK cell alloreactivity remains the subject of vigorous debate. In this article, we review the basic biology of NK cells in the context of recent clinical trials of HCT.

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Human natural killer immunophenotype

NK cells do not express a rearranged germline-antigen receptor and they are identified by expression of CD56 in the absence of CD3. Two subsets of mature NK cells are recognized, defined by the brightness of CD56 expression and the presence or absence of the low affinity IgG receptor CD16. The $CD56^{bright}CD16^{-}$ subset is enriched in lymphoid organs, secretes cytokines to help coordinate adaptive immunity and is the major subtype recruited to sites of inflammation (including malignancy) [1,2]. In contrast, the $CD56^{dim}CD16^{+}$ subset circulates in the peripheral blood and shows potent cytotoxicity [3]. Several lines of evidence suggest that $CD56^{bright}$ NK cells may differentiate into $CD56^{dim}$ NK cells under some conditions [4, 5]. NK cells are reported to comprise 5–25% of peripheral blood lymphocytes, or approximately 100–600 cells/μl [6].

Effector functions: lytic machinery

NK cell cytotoxicity requires target-cell recognition and the receipt of an activating signal in the absence of an inhibitory signal. Killing is mediated by several pathways; whereas immature NK cells rely on tumor necrosis-related apoptosis-inducing ligand (TRAIL)-mediated killing [7], mature NK cells preferentially utilize the granule-exocytosis pathway (requiring perforin and granzymes) and the Fas-Fas ligand pathway [8]. In order to achieve their maximal cytotoxic potential *in vivo*, NK cells must be activated, with evidence in mice that this step requires the transpresentation of IL-15 by dendritic cells [9]. The observed enhanced cytotoxic potential of activated cells is due in part to the translation of pre-existing pools of granzyme B and perforin mRNA [10].

Effector functions: cytokines

Cytokine-producing NK cells link the innate and adaptive immune responses. Interferon-γ has protean effects including Th1 polarization [11,12], dendritic-cell maturation and activation [13], direct antiviral effect [14,15], as well as various antiproliferative effects on transformed cells. TNFα enhances dendritic cell maturation [16] and also leads to increased IFNγ production [17]. GM-CSF can stimulate phagocytosis by monocytes and contributes to dendritic-cell maturation [18].

Natural killer receptors

MHC class I molecules are critical determinants of NK cell activity; NK cells effectively lyse cells lacking expression of some or all MHC molecules. Karre *et al*. [19] recognized that NK cytotoxicity was abrogated if the effectors and targets shared MHC class I molecules. The ‘missing-self’ hypothesis correctly predicted the existence of receptors for self-MHC that, when engaged, would inhibit cytotoxicity. In humans, inhibitory receptors that recognize HLA-A, B, and C molecules belong to the killer immunoglobulin-like receptor (KIR) family [20, 21]. Another inhibitory receptor, the C-type lectin NKG2A pairs with CD94 and recognizes the nonclassical MHC molecule HLA-E [22–24]. The expression patterns of inhibitory receptors create a repertoire of NK cells with nonoverlapping specificities.

The human KIR gene cluster is located on chromosome 19q13.4 and contains 14 KIR genes and 2 pseudogenes. Inhibitory receptors possess long cytoplasmic tails with immunoreceptor tyrosine-based inhibitory motifs (ITIM) that allow docking of tyrosine phosphatase molecules, whereas activating KIRs have short cytoplasmic tails that enable pairing to adapter molecules with immunoreceptor tyrosine-based activating motifs (ITAM) [25]. The extracellular domains of the activating KIR share sequence similarity with the corresponding inhibitory KIR and may share HLA-binding specificities, though the ligands for most activating KIRs remain unknown

[26,27]. It is thought that inhibitory KIRs bind class I molecules with greater affinity than the corresponding activating KIR.

KIR proteins recognize allotypic motifs in the class I alpha helix, as described in Table 1. Two KIR haplotypes have been defined, A and B [28]. The A haplotype has five inhibitory genes (2DL1, 2DL3, 3DL1, 3DL2, 3DL3) and one activating gene (KIR2DS4). The B haplotypes are less uniform in gene content and have a greater number of activating receptors. HLA genes on chromosome 6 and KIR genes on chromosome 19 assort independently, meaning that KIR genotype, rather than HLA type, is the major determinant of KIR expression pattern. KIR expression is stochastic, generating an NK cell compartment with individual members expressing one or more inhibitory receptors. NK cells that express only inhibitory KIRs for absent class I molecules are detectable but rather than being autoreactive, as would be predicted by the missing-self model, such cells are hyporesponsive. The licensing theory of NK cell maturation suggests that engagement of an inhibitory KIR by the cognate class I molecule is necessary to acquire effector function [29,30]. Another model proposes that the NK cells become functionally anergic without self-inhibitory signals [31].

Licensing notwithstanding, failure to engage an inhibitory KIR is insufficient to elicit an NK attack. Rather, a cell-surface derived activating signal is necessary. In addition to activating KIR molecules, a number of activating NK cell receptors have now been characterized (see Table 2).

Predictors of alloreactivity

According to the missing-self model, mismatch between donor KIR and recipient MHC class I molecule expression predicts the existence of a subset of donor NK cells that are not inhibited and are thought to be alloreactive. The Perugia group brought renewed attention to NK cell alloreactivity in their pioneering studies of haploidentical transplantation. Recipients received myeloablative chemoradiotherapy and ATG. In order to avoid GVHD, donor grafts were T-cell depleted and 'megadose' $CD34^+$ (e.g. 10^7/kg) cells were infused in order to overcome the resistance to engraftment observed with T-cell depleted transplants [32]. No posttransplant immune suppression was used. Under these conditions, KIR ligand mismatching in the GVH direction (observed in 30% of unselected haploidentical related donors) benefited patients with myeloid leukemias [33,34]. Patients receiving KIR ligand mismatched transplants had better overall survival, improved rates of engraftment and a reduced incidence of GVHD. Protection against GVHD has been explored in a mouse model, where it was found that donor NK cell alloreactivity was linked to the depletion of recipient antigen-presenting cells [34].

Receptor-ligand

The Ruggeri model assumes that NK cells expressing inhibitory KIR for ligands absent on both donor and recipient are nonalloreactive. The licensing model predicts that developing NK cells that fail to receive a signal through an inhibitory receptor are hyporesponsive to activating stimuli. However, licensing can be bypassed in a proinflammatory environment [29], as is seen following myeloablative conditioning. Leung *et al*. [35] argues that alloreactivity may be predicted by considering only the expression of donor-inhibitory KIR (as determined by flow cytometry) and recipient ligand, thus eliminating donor ligand from the equation. In an analysis of 36 pediatric patients receiving T-cell depleted haploidentical transplants, the receptor-ligand model was a better predictor of leukemia relapse than the ligand–ligand model.

Missing killer immunoglobulin receptor ligand

The missing ligand model is an extension and simplification of the receptor-ligand model. Regardless of HLA expression, most individuals express the full complement of inhibitory

KIR. An individual who is homozygous for KIR ligand epitopes (e.g. HLA-C1 or HLA-Bw4) would be predicted to have a subset of NK cells expressing an inhibitory KIR for the absent ligand (KIR-L). Hsu *et al*. [36] analyzed the impact of missing ligand in 1770 myeloablative, T-cell replete transplants from unrelated donors. Recipients were grouped according to homozygosity of HLA-C group 1, HLA-C group 2, or HLA-Bw6. Patients homozygous for KIR epitopes who received transplants from HLA mismatched donors had a lower rate of relapse than heterozygotes. This effect was greater among patients with AML than those with CML or ALL. However, among patients receiving HLA-matched unrelated donor transplants, homozygosity of these epitopes was associated with a slightly higher risk of relapse. In a similar analysis Miller *et al*. [37] analyzed 2062 unrelated donor transplants with myeloid malignancies. Of these patients, 70% were missing one or more KIR-L. For patients with early stage myeloid malignancies, that is AML in CR1, early stage MDS, or early chronic phase CML, absence of KIR-L was protective against relapse, but this effect was lost in more advanced stage disease. Unlike the data of Hsu, this effect was seen in HLA matched and mismatched transplants alike. In a further analysis of patients receiving haploidentical transplants for myeloid malignancies, Ruggeri *et al.* [38] compared the predictive value of the missing ligand model with the original mismatched ligand model, finding the latter to be more informative.

Activating killer immunoglobulin receptor

Increased numbers of activating KIR molecules have correlated with increased propensity to autoimmune disorders as well as to resistance to CMV reactivation in kidney-transplant recipients [39]. In the setting of allo-HCT, higher numbers of activating KIR genes has been correlated with increased frequency of acute and chronic GVHD. In a multivariate analysis of 448 unrelated transplants for AML, Cooley *et al.* [40•] found that presence of a donor, KIR-B haplotype, which contains a higher number of activating KIR genes than the A haplotype, predicted improved relapse free and overall survival in which there was no KIR ligand mismatch. This benefit was not observed in patients with KIR ligand mismatched transplants. An interesting recent study showed that in the setting of TCD haploidentical transplantation with KIR-L mismatch, NK cells co-expressing the activating KIR2DS1 with inhibitory KIR2DL2/3 or NKG2A were able to kill recipient leukemia blasts, highlighting that in some settings, recognition by activating KIR is able to overcome inhibitory signals [41].

Clinical outcomes

The potential benefits of NK cell alloreactivity have been explored in cohorts of patients receiving mismatched unrelated donor transplants, often with discordant conclusions. These results have been reviewed previously [42–45].

Umbilical cord transplantation

As with haploidentical transplantation, umbilical cord blood (UCB) transplants are frequently characterized by a high degree of HLA mismatching. UCB has as its advantage the relatively low risk of acute GVHD due to a lower number of mature donor T cells and thus an increased ability to use HLA mismatched units. Willemze *et al*. [46•] reviewed the impact of KIR ligand mismatching in UCB transplants for acute leukemia (lymphoid or myeloid) in first complete remission (CR1). Of 218 transplants, 10% were HLA mismatched and 47% had greater than one mismatch. Thirty-two percent of donor recipient pairs were KIR-ligand incompatible in the graft versus host direction. Patients with KIR-ligand incompatible donors had improved overall survival (57 vs. 40%) and decreased relapse (20 vs. 37%) when compared with those without these incompatibilities. As was seen in the Perugia studies, benefits of KIR ligand incompatibility were most striking among patients with AML although UCB recipients with ALL also had a trend toward improved leukemia-free survival. However, Brunstein *et al*.

[47] failed to observe any benefit of KIR-L mismatch in 155 recipients of UCB after myeloablative conditioning. In fact, in 102 patients who had received UCB after nonmyeloablative conditioning, KIR-L mismatch was associated with an increased rate of acute GVHD and higher treatment-related mortality.

Nonmyeloablative transplantation

Nonmyeloablative conditioning is marked by a period of mixed chimerism in which recipient NK cells may exert antidonor effects. KIR-L mismatch in the host versus graft direction in 31 patients conditioned with fludarabine and 2Gy TBI was found to predict for increased risk of graft rejection and lower incidence of complete donor chimerism [48•].

The impact of NK cell alloreactivity in the graft versus host direction was studied in a series of 282 donor–recipient pairs treated with 2Gy of TBI with or without fludarabine, where the majority (88%) received HLA-matched grafts [49•]. High donor NK cell chimerism before day 100 was associated with low relapse rates. The risk of relapse was lower for patients expressing ligands for all donor KIR, though this did not reach statistical significance. Of note, a lower risk of relapse was not significantly associated with high donor T-cell chimerism. In contrast, the risk of acute GVHD (grade II–IV) was associated with high levels of donor T-cell chimerism, whereas NK cell chimerism did not correlate with GVHD.

Natural killer reconstitution posttransplant

During the first few months post HCT, NK cells are the predominant circulating lymphoid cell subset with the potential to control disease relapse. NK cells are the first lymphoid cells to repopulate, reaching normal numbers within 1 month regardless of donor type or patient age in adults [50,51] and children [52]. Similar findings are shown after UCB transplantation in children [53]. Although some studies have highlighted the ability of NK cells to exert a potent antileukemic effect and reduced relapse in high-risk hematologic malignancy, the question remains whether NK cells reconstituting after HCT manifest an actual antileukemic effect. This issue has been investigated by evaluating the phenotype and cytotoxic potential of NK cells re-isolated at early time-points after transplant.

Phenotypic changes

According to the Perugia KIR-mismatch model, potentially alloreactive NK cells post-HCT are cells that express KIR for missing KIR-L molecules in the absence of the inhibitory receptor CD94/NKG2A. The Perugia data, however, show that the majority of NK cells in the first 5 months post-HCT do not belong to this subset [38]. Similarly, other groups have found that early after both HLA-matched and haploidentical HCT (with or without T cell depletion), NK cells are predominantly $CD56^{bright}$, NKG2A+ and KIR−, a finding which has been described as consistent with an ‘immature phenotype’ [54,55•,56,57] and has been related to increased post-HCT levels of the homeostatic cytokine IL-15 [56]. The remaining $CD56^{dim}$ cells, which normally express low levels of NKG2A, upregulate expression of this receptor post-HCT while expression of CD16 as well as of KIR is lower than in healthy controls [55•,57]. A mature donor-type KIR repertoire appears within 3 months–3 years [35,55•,58].

Expression of NKp46 as well as of the activating receptor NKG2D appear to be increased during the first year post HLA-matched non-TCD HCT, whereas NKp44 and NKp30 do not appear to be upregulated [56].

With regard to the expression of effector molecules, production of both perforin and IFNγ is increased among the $CD56^{bright}$ cells in patients post HLA-matched HCT when compared with the same subset in normal donors [56].

Functional changes

Several groups, with somewhat inconsistent results, have investigated the functional consequences of the phenotypic changes described earlier. The Perugia group found that NK alloreactive cells (defined as NK cells of donor origin capable of killing cryopreserved host PHA blasts) are detectable in about half of patients 1 month after haploidentical HCT and are no longer detectable after 12 months [38]. After haploidentical HCT NK cells have poor effector function against primary leukemia cells, a finding which has been related to the increased frequency of NKG2A-bearing NK cells, although results of blocking studies of this inhibitory receptor have yielded conflicting results [54,55•]. Evaluation of cells that are NKG2A− and single-KIR+ (thus predicted to have alloreactive potential under the models described earlier) after haploidentical HCT has demonstrated that these cells are first detectable approximately 75 days after HCT, their frequency is highly variable and shows no correlation with predicted donor NK cell alloreactivity and that their functional capacity was reduced [55•]. This finding is somewhat out of keeping with the conclusions from the Perugia group and may be related to the lack of licensing of these cells, as they are repopulating in the absence of an HLA ligand for their single KIR.

Furthermore, a small study in TCD HLA-matched HCT has shown that NK cells exhibiting KIR for nonself class I ligands (predicted to be alloreactive according to the 'missing ligand' model but nonlicensed according to the licensing hypothesis) are capable of mounting a robust cytotoxic and cytokine response in the first months posttransplant when incubated with MHC class I deficient cell lines [59•]. The authors explain this finding by postulating that NK cell tolerance to host cells takes some time to develop, during which time patients may benefit from NK cell alloreactivity, although it may be that licensing requirements are bypassed in the inflammatory posttransplant environment.

The presence of T cells in the graft has been found to impair NK-cell reconstitution and function posttransplant [43,60]. This finding may in part explain the importance of T-cell depletion for ensuring an optimal NK-cell effect. In addition, peritransplant immunosuppression could impact NK cell function, as exposure of in-vitro differentiated NK cells to cyclosporine A has been shown to lead to preferential expansion of CD56+KIR− NK cells [61].

Conclusions drawn from these studies must be tempered by the recognition that different NK cell functional assays may yield discordant results. Most studies evaluate NK cells by their capacity to degranulate (using the CD107a mobilization assay) or produce IFNγ after exposure to tumor cell lines known to be susceptible to NK cell killing, a somewhat artificial experimental. In addition, studies using as targets patient PHA blasts rather than cryopreserved primary patient leukemia cells likewise may not be representative of the actual antitumor potential.

Natural killer adoptive therapy

As follows from the earlier discussion, NK cells repopulating the host after transplantation may be functionally defective as a result of immaturity, immunosuppression, or other unidentified factors. Thus, it has been proposed that the adoptive transfer of mature NK cells may be beneficial by providing both a cytolytic effect and an immunomodulatory effect, the latter by utilizing NK-dendritic cell cross talk to promote NK-cell activation and subsequent Th1 polarization [14].

GMP grade production of NK cells from apheresis products for adoptive therapy is feasible, with reported yields of up to 10×10^8/kg with recovery of approximately 40–50% [62–65].

In the most important study of adoptive NK-cell therapy to date, haploidentical NK cells were given to 43 patients with advanced malignancies (AML, metastatic melanoma and metastatic renal carcinoma) together with IL-2. There was minimal hematologic and nonhematologic toxicity, the latter mostly attributable to IL-2. Results from this study revealed the following conditions for persistence in the host of donor NK cells: a high-dose conditioning regimen is given to achieve significant lymphodepletion; high levels of IL-15 levels are present; and the addition of nonmyeloablative doses of TBI, cyclophosphamide and fludarabine and infusion of CD34+ progenitor cells leads to better NK cell expansion and higher AML remission rates [66,67]. There was no GVHD, but the protocol was complicated by fatal infections and EBV-PTLD; interestingly, no correlation was found between efficacy and predicted NK alloreactivity according to KIR ligand mismatch.

Questions remain regarding the optimal purity of the product, as there is evidence from early studies that monocytes may be required for optimal NK-cell proliferation [68], and more recent studies show that monocyte-derived dendritic cells promote NK cell effector function [69]. Similarly, whether the optimal source of NK cells should be from nonmobilized or G-CSF mobilized peripheral blood collections needs to be addressed, due to data suggesting that NK cells expanded from G-CSF mobilized PBPC collections are functionally abnormal and possess reduced numbers of NK progenitors [70–72].

Facilitation of NK cell engraftment will likely depend on suppression of the host immune system, eradication of Tregs (which may inhibit transferred NK cells) [73] presence of ‘space’ for homeostatic proliferation following lymphodepletion, and the presence of high levels of cytokines especially IL-15 [74,75]. The assertion that IL-15 is critically required for in-vivo NK cell expansion has been recently challenged in murine models of infection [76].

Receptor and ligand modulation

Modulation of NK cell recognition of tumors may be achieved by blockade of inhibitory KIR as was recently demonstrated using a humanized antibody to KIR2DL-1, KIR2DL-2, and KIR2DL-3 as well as the activating receptors KIR2DS-1 and KIR2DS-2 [77]. A phase I trial of this agent is ongoing.

Additional approaches to augment NK cell activity include the use of bispecific antibodies (such as for HER2/neu and CD16) [78], genetic modification of NK cells to express receptors for tumor-associated antigens not normally part of the NK-receptor ligand repertoire or enforced expression of signaling receptors [79,80]. Other approaches include the indirect pharmacologic modulation of NK receptor ligands on tumor cells by compounds such as bortezomib [81] or histone deacetylase inhibitors [82••]. It may be that several of these techniques may be required in order to realize the full potential of NK cell adoptive therapy.

Conclusion

Successful utilization of adoptive NK therapy may require an available niche as defined either by lymphopenia, selective host NK cell lymphopenia, high IL-15 or other homeostatic cytokine levels, or a combination of these. Other open questions include the appropriate method for collection and activation, optimal cytokine type and schedule, and in-vivo or ex-vivo stimulation, and it will be important to ensure that cells which have been activated for several days *in vitro* do not lose their proliferative potential *in vivo*, as has been found for adoptively transferred T cells [83]. Although it is likely that apheresis products will represent the most easily accessible source of mature NK cells, it is important to keep in mind that NK cells enriched from G-CSF mobilized blood exhibit reduced functional capacity compared with those from unstimulated peripheral blood [70]. It may be that a particular subset of NK cells should be targeted for expansion for adoptive infusion, although studies addressing this

particular point are presently lacking. Finally, the optimal methods to ensure persistent expansion and recognition of target cells still await development, and perhaps will require a combination of cytokines and NK receptor ligand modulating agents.

Acknowledgments

This work was supported by the National Institutes of Health (R01-CA-125276) and the Hematology Society of Australia and New Zealand. We apologize to those whose studies were not cited due to space considerations.

References and recommended reading

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Table 1

Inhibitory and activating receptors for HLA class I molecules

Receptor	Ligand
Inhibitory	
KIR2DL2/3	HLA-C group1 (Cw1, Cw3)
KIR2DL1	HLA-C group 2 (Cw2, Cw4)
KIR3DL1	HLA-A and B with Bw4 motifs at position 77–83
CD94/NKG2A/E	HLA-E
KIR3DL2	HLA-A3/A11
KIR2DL4	HLA-G
Activating	
KIR2DS4	
KIR2DS1	HLA-C2
KIR2DS2	
KIR2DS3	
KIR2DS5	
KIR3DS1	
KIR Haplotype	
A	Inhibitory KIR and KIR2DS4
B	Inhibitory KIR and combinations of activating KIR

KIR genes are organized into diverse haplotypes, which have simplified into Groups A and B.

Table 2

Some activating NK cell receptors

Receptor	Receptor expression	Ligands	Ligand expression
NKG2D	All NK cells, some T cells	MICA, MICB, ULBP1-5	Leukemia, carcinoma cells; induced by viral infection and ionizing radiation (DNA damage response)
NKp30	All NK cells	B7-H6, BAT3	
NKp44	Activated NK cells only	?	
NKp46	All NK cells	Viral hemagglutinin	
DNAM1	All NK cells	CD112, CD155	Melanoma, carcinoma, neuroblastoma

NK, natural killer.