Review

Natural Killer Cells: Biology and Clinical Use in Cancer Therapy

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Natural killer (NK) cells have the ability to mediate both bone marrow rejection and promote engraftment, as well as the ability to elicit potent anti-tumor effects. However the clinical results for these processes are still elusive. Greater understanding of NK cell biology, from activating and inhibitory receptor functions to the role of NK cells in allogeneic transplantation, needs to be appreciated in order to draw out the clinical potential of NK cells. Mechanisms of bone marrow cell (BMC) rejection are known to be dependant on inhibitory receptors specific for major histocompatibility complex (MHC) molecules and on activating receptors that have many potential ligands. The modulation of activating and inhibitory receptors may hold the key to clinical success involving NK cells. Pre-clinical studies in mice have shown that different combinations of activating and inhibitory receptors on NK cells can reduce graft-versus-host disease (GVHD), promote engraftment, and provide superior graft-versus-tumor (GVT) responses. Recent clinical data have shown that the use of KIR-ligand incompatibility produces tremendous graft-versus-leukemia effect in patients with acute myeloid leukemia at high risk of relapse. This review will attempt to be a synthesis of current knowledge concerning NK cells, their involvement in BMT, and their use as an immunotherapy for cancer and other hematologic malignancies. *Cellular & Molecular Immunology*. 2004;1(1):12-21.

Key Words: NK cell, BMT, GVHD, GVT

Introduction

Natural killer (NK) cells were originally described in terms of function in 1971 when Cudkowizc and Bennett observed that mice that had received lethal irradiation were capable of rejecting allogeneic or parental strain bone marrow cell (BMC) allografts (1, 2). Classical transplantation laws state that transplantation antigens are inherited co-dominantly, (i.e. parental antigens are coexpressed in the F1 hybrid) and offspring are obligately tolerant towards parental major histocompatibility complex (MHC) determinants (1). However, Cudkowizc and Bennett observed that irradiated F1 hybrid H-2-heterozygous (A×B) mice were capable of rejecting parental H-2-homozygous BMC (A or B). This phenomenon was termed "hybrid resistance". It was later seen that the cells mediating this phenomenon were radioresistant and identical to lymphoid cells characterized in 1975 by their ability to mediate spontaneous killing of tumors *in vitro* in an MHC-unrestricted manner (3-6). Later, these effector cells became known as "natural killer" or "NK" cells. Graft rejection generally occurs by radioresistant T-cells, though further work in 1987 showed that NK cells alone could mediate the specificity of marrow graft rejection. This NK-mediated rejection of BMC was seen when mice with severe combined immune deficiency (SCID), which cannot develop B or T cells due to a failure in B and T cell receptor rearrangement, displayed normal NK cell function and the ability to reject BM grafts (7). However, the clinical data to support the role of NK cell-mediated BMC rejection after allogeneic BMT are lacking. Years of NK research since then have greatly increased knowledge of these effectors, yet the why and how of NK cell mediated BMC rejection remain unclear.

NK cell biology

NK cells are of lymphoid origin and are found in the peripheral blood, spleen, and BM, as well as other tissues. They represent an important arm of innate immunity and are thought to play a critical role in the immune surveillance against tumors and virally infected cells. Unlike other cells of the immune system, especially T cells, NK cells cannot be sensitized by repeated stimulation nor do they develop long-lasting memory to antigens. Due to the lack of any NK specific and restricted markers, NK cells of mouse and man are characterized by both positive and negative criteria. Many markers that are thought to be NK specific, such as NK1.1, DX5, asialo-GM1 in the mouse, and CD56 and CD16 in humans are also present on certain T-cell subsets or other cell types. Therefore, in order to classify NK cells, one must define them as cells that are positive for these

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Abbreviations: NK, natural killer; BMT, bone marrow transplantation; GVHD, graft versus-host-disease; GVT, graft-versus-tumor; MHC, major histo-compatibility complex; TRAIL, tumor necrosis factor related apoptosis inducing ligand; KIRs, killer cell Ig-like receptors; MCMV, murine cytomegalovirus.

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markers and negative for T-cell specific markers such as CD3 and CD4, and as cells that do not rearrange receptors for antigen recognition like T-cells (through their T-cell receptors [TCRs]) or B-cells (through the immunoglobulin (Ig) complex (4, 5)). As a member of the innate immune system, NK cells cannot rearrange genes in the germ line that encode receptor components, and therefore cannot recognize antigens displayed in the context of classical MHC molecules. NK cells can kill in a rapid manner and this NK cell-mediated cytotoxicity occurs primarily through the perforin/granzyme-dependent pathway, although NK cells can also use Fas ligand (FasL) and tumor necrosis factor related apoptosis inducing ligand (TRAIL) to kill target cells (8). NK cells also have the ability to secrete a wide range of cytokines upon activation (9) and these cytokines can either promote (granulocyte-monocyte colony stimulatory factor, GM-CSF, and granulocyte colony stimulatory factor, G-CSF) or inhibit (interferon gamma, IFN-γ, and transforming growth factor, TGF-β) hemato-poiesis (9-11). These same cytokines also can play a role in controlling the early immune response and contributing to the delayed T cell response to infection. Cytokines also play a key role in the differentiation of NK cells. Interleukin (IL)-2, IL-15, and IL-21, three cytokines that all share the common gamma (γ) chain of the IL-2 receptor (IL-2R), are capable of inducing proliferation and activation of NK cells. However, only IL-15 and fms-like tyrosine kinase 3 (flt3) ligand have been shown to be critical for NK cell development and maintenance (12-17). It was shown that IL-15 knockout mice, unlike IL-2 deficient mice, lack functional, mature NK cells (15, 16). The necessity of IL-15 for NK cell maintenance was seen when mature NK cells were transferred to IL-15 knockout mice, and they did not survive for more than a few days, while mature NK cells transferred into normal mice survived much longer. This experiment demonstrated that IL-15 provides critical survival signals to the NK cells (18). Stem cell factor (SCF) and fetal liver kinase ligand (flk2L) have also been shown to be important in NK cell differentiation (19). Unlike T-cells that require the thymus for development, NK cell development is critically dependent on the bone marrow (BM) (12, 13, 20). This is not to suggest that T-cells and NK cells are completely independent in differentiation, as it has been shown that common NK and T-cell progenitors can be found in the thymus, thus showing the close association between these two cell types (21). Stromal cells are also thought to be important in NK cell differentiation (22). The function of NK cells is also mediated by cytokines. IL-12 and IFN-α/β exert potent stimulatory effects on NK cells, and IL-12 and IL-18 in combination is particularly effective in augmenting NK cell function (4, 23). IL-2 has also been shown to significantly activate NK cells, and adoptive immunotherapy of IL-2 activated NK cells after autologous BMT has been used in patients with cancer with acceptable toxicities (24). The ability to manipulate NK cell development as well as increasing NK activity using other cytokines, such as IL-15 and IL-21, may provide new options in the treatment of malignant cancer.

The seminal finding in 1985 by Ljunggren and Karre (25) of the "missing-self" hypothesis is based on the observation that tumor cells lacking MHC class I molecules are susceptible to killing by NK cells, unlike T-cells that are MHC dependent. The investigators observed that variants of a certain murine leukemia cell line that had lost expression of the murine MHC molecules, H-2, were more susceptible to NK cell mediated lysis than H-2 bearing leukemia cells (26). In concurrence, it was also shown that human NK cells can lyse MHC class I deficient Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (27). Interestingly, the transfection of H-2 deficient leukemia cells or human HLA class I-deficient B-lymphoblastoid cells with H-27 or human leukocyte antigen (HLA) class I genes, respectively, restored resistance to NK cell-mediated lysis (27, 28). However, the presence of MHC class I is not always necessary for protection from NK cell-mediated cytotoxicity, nor is the absence of MHC class I sufficient for activation of cytolysis by NK cells. NK cells, for instance, are unable to reject MHC class I deficient non-hematopoietic tissues, such as skin grafts, and *in vitro*, NK cells fail to lyse cells lacking MHC class I from β_2-microglobulin null mice (29). Conversely, autologous NK cells can kill some MHC-bearing virally-infected cells (30) and upon IL-2 activation, these effectors can kill NK cell resistant targets (31). The murine cytomegalovirus (MCMV) has established a very clever way to avoid attack by NK cells through the ability of MHC to inhibit NK cell killing. Throughout evolution, the MCMV has pirated genes from host cells in order to evade the immune system, and one MCMV gene that accomplishes this is called m144. m144 is a homolog of a MHC class I gene, and this homolog contributes to MCMV evasion of NK cells (32). To avoid T cell-mediated lysis, the MCMV will down-regulate the host's MHC class I molecules, but it will upregulate m144 to present a non-functional MHC class I molecule that will prevent NK cells from recognizing the cell as MHC class I deficient. However, the NK cell has developed a way to counteract MCMV's evasion by expressing activating receptors to MHC as well. Ly49H is an activating receptor found on NK cells that specifically recognizes MCMV infected cells and its function is blocked by anti-Ly49H antibodies (33). Ly49H recognizes m157, a MCMV glycoprotein that is part of the m145 family of glycoproteins, many of which have immune evasion properties (such as m144 and m152) upon binding inhibitory receptors present on NK cells (34).

NK cell activating and inhibitory receptors

The identification of MHC class I-specific inhibitory and activating receptors in addition to non-MHC class I specific activating and inhibitory receptors has grown substantially in the past decade. This has led to a tremendously complex set of receptors responsible for innate recognition of foreign, abnormal, or virally infected cells by NK cells, and these receptors have become relevant with respect to allogeneic BMT and malignant cancer therapies.

The first class of MHC class I-specific receptors was identified in mouse and is termed Ly49 (Table 1). These receptors are type II integral membrane homodimers, lectin-related molecules, that recognize MHC class Ia molecules H-2^d and/or H-2^k (35-37). In any given mouse, an NK cell population can express a variable combination of the 20 or more Ly49 receptors characterized. Some of these receptors have different affinities for the different MHC class I molecules; Ly49C/I has strong affinity for H-$2K^b$ but can also bind H-$2K^d$ to a lesser extent (38, 39). Of the Ly49

Table 1. *Effects of in vivo depletion of NK cell subsets in the rejection of BMC.*

Donor→ Host	Effect of Depletion				
	Ly49A	Ly49G2	Ly49C/I	Ly49D	Ly49H
d→b	−	−	+(++)[b]	+(++)[b]	?
b→d	−(++)[a]	+(++)[a]	−	−	?
b→k	+(++)[a]	−(++)[a]	−	−	?
dxb→b	−	−	−	+	?
dxb→d	−	−	−	−	?
b→dxb	−(++)[a]	+(++)[a]	−	−	?
d→dxb	−	−	+	−	?

Ly49 subsets were depleted from bone marrow of donor mice prior to transfer to recipients in either H-2-mismatched or parent-offspring BMT settings.
"+", reflects extent of growth/engraftment with "–", representing rejection
[a] Synergistic effect if Ly49A and Ly49G2 depleted together
[b] Additive effect if Ly49C/I and Ly49D depleted together

molecules characterized so far, of which there are more than 20, 13 have been found to be inhibitory while 8 are activating, a conclusion based on the cytoplasmic domains of these receptors and effects on NK cell function (40).

The next class of receptors are the killer cell Ig-like receptors (KIRs) which are primarily expressed in man. These receptors are type I transmembrane molecules belonging to the Ig superfamily and are structurally characterized by 2 or 3 extracellular Ig-like domains that specifically recognize groups of HLA-C (p58 or KIR2DL1-KIR3DL3) (41-44), HLA-B (p70 or KIR3DL1) (45-48) and HLA-A alleles (p140) (49, 50). Each single KIR recognizes determinants shared between members of a group of HLA alleles (51). For example, the inhibitory receptors KIR2DL2 and KIR2DL3, and the activating receptors KIR2DS2 and KIR2DS3 recognize group 1 HLA-C alleles (Cw1, Cw3, Cw7 and Cw8). The inhibitory receptor KIR2DL1 and activating receptor KIR2DS1 recognize the group 2 HLA-C alleles (Cw2, Cw4, Cw5 and Cw6). It appears that HLA-C is a predominant class I isotype involved in the inhibitory and activating regulation of human NK cells. In contrast to Ly49 molecules, KIRs display a greater degree of complexity and different specialization (52).

Both the Ly49 and KIR multi-receptor families contain members that have either activating or inhibitory action. Inhibitory receptors mediate their action through the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (53, 54) upon binding the appropriate MHC determinant. The tyrosines on the ITIMs are then phosphorylated and attract tyrosine phosphatase-1 (SHP-1), which inhibits activating signals. The activating receptors of the Ly49 and KIR families have truncated cytoplasmic domains lacking ITIMs. These activating receptors associate with the immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor molecule DAP12 through a charged residue in the transmembrane region which results in an activating signal (55, 56).

Recently, the first rodent homologue to the human KIR receptor, KIR-Like 1 (KIRL1), was found (57). KIRL1 shares 40% amino acid homology with primate KIR family members, with the majority of the equivalence contained in the Ig-like ectodomains. KIRL1 was found to be expressed at low levels, including in IL-2 expanded NK cells, but is highly expressed in immature thymocytes. Unlike the diverse KIRs found in humans, however, KIRL1 lacks the ITIM and ITAM motifs. The precise function of this receptor remains unknown.

Another class of MHC class I-specific receptors expressed both in humans and in mice is comprised of the C-type lectin molecule CD94, which is covalently associated with a member of the NKG2 family. Like Ly49 and KIR receptors, these receptors also have been shown to exert inhibitory (NKG2A or NKG2B) or activating (NKG2C) signals upon binding Qa-1b (mouse) or non-classical HLA-E (human) molecules on targets (58-61). There is less understanding of CD94/NKG2 receptor specificity than KIR receptors (62).

Another receptor that has only recently been found is the novel lectin-like receptor KLRE1. KLRE1 is an inhibitory receptor found in both mice and rats. A type II transmembrane receptor, it appears to form a functional heterodimer with an as of yet unidentified ITIM-bearing partner that can recruit SHP-1 to generate an inhibitory receptor complex.

NK cells also have Ig-like transcript (ILT) receptors that interact with HLA-G (63). The non-classical (class Ib) HLA-G molecule, which is primarily expressed on fetal cells on the human placenta, interacts with the ILT receptors on maternal decidual NK cells and appears to protect the fetus and placenta from rejection.

Another group of NK cell receptors comes from a more diverse family of receptors of NK-cell-specific Ig-like molecules that are known as natural cytotoxicity receptors, or NCRs. NCRs include NKp30, NKp46, and NKp44 as well as NKG2D (64, 65). NKG2D is a member of the NKG2 family expressed by NK cells and cytotoxic lymphocytes (CTLs) (66, 67). Unlike activating KIRs, Ly49s, and CD94/NKG2C, NCRs and NKG2D mediate cytotoxicity against MHC class I-deficient or negative targets. The targets for NCRs remain undefined, but the targets for NKG2D include the MHC class I polypeptide-related sequence A/B (MICA and MICB), and the UL16-binding proteins (ULBPs) in man (66, 67), and retinoic acid inducible genes 1 (RAE-1) and minor histocompatibility antigen H-60 in mice (68). MIC genes in humans are under the expression of a heat-shock promoter and may be upregulated in response to cellular stress (69). MIC genes have been found on *ex vivo* tumors and on gut epithelia, though the biological significance of expressing a ligand for the NK cell activating receptor NKG2D by tumors is poorly defined (68). Unlike NCRs, NKG2D is expressed as a homodimer along with its adaptor molecule DAP10, forming a receptor that is similar to the activating Ly49 receptors (58). The function of NKG2D appears to be to mediate the killing of "stressed" or transformed cells that have altered MHC class I expression. All peripheral blood NK cells constitutively express NKp46 and NKp30 (70, 71), while IL-2 activated NK cells express NKp44 (72). The NCRs signal through coupling DAP12 or ITAM-containing CD3ζ and/or FcεRIγ adaptor proteins may be involved in the recognition and killing of various tumor cells.

Finally, most NK cells can express the FcγRIII (CD16) molecule, which recognizes the Fc component of bound Ig molecules and initiates cytolysis by the antibody dependent

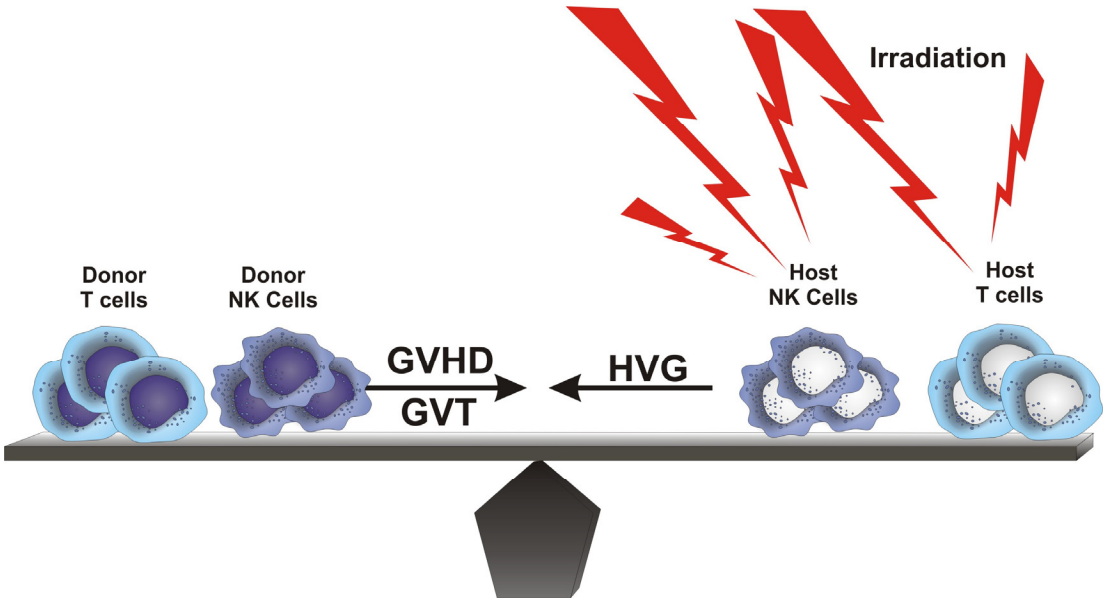

Figure 1. Roles of donor and host effector cells during allogeniec BMT. Host effector cells can resist engraftment by producing a host-versus-graft (HVG) response. Donor T cells can initiate graft-versus-host-disease (GVHD) and produce a graft-versus-tumor/leukemia (GVT/GVL) response. Donor NK cells appear to only be capable of initiating a GVT response but can contribute to GVHD pathology if GVHD has already begun. Recipient conditioning in the form of irradiation or chemotherapy contributes to host immunosuppression (i.e. elimination of host T and NK cells which mediate resistance to the donor marrow graft), tipping the balance towards the donor.

cellular cytotoxicity (ADCC) pathway (73), thus giving the NK cell another method of target recognition.

It can be seen that one of the main targets of the inhibitory and activating receptors are the MHC molecules; however, the biological significance of having paired inhibitory and activating receptors for MHC class I molecules remains unclear. These receptors may play a pivotal role in the regulation of NK cell function. In the case of both the KIRs and the CD94/NKG2 receptors, the inhibitory forms of the receptors have a higher affinity for the corresponding HLA alleles than do the activating forms (74, 75), which causes NK cells expressing both forms to be preferentially inhibited from killing. With this in mind, the activating receptors specific for MHC class I molecules may only signal when the target cell has lost the expression of the HLA molecule that is recognized by the inhibitory receptor, thereby allowing the activating receptor to engage. This would allow the preferential killing only of target cells that have down-regulated or lost a certain MHC class I molecule while normal cells would be left unaffected (76). Even though the NK cells do not have the diversification power of gene recombination to enhance their receptor/ligand potential, the fact that both Ly49 and KIR receptors on NK cells and MHC class I molecules on target cells can be modulated (giving rise to many receptor-ligand combinations) grants NK cell the ability to recognize and react to numerous potential target cells.

NK cells in BMT

For various hematologic malignancies, BMT has become a useful therapeutic approach. During BMT, a patient will receive a conditioning regimen either through radiation or chemotherapy. This conditioning regimen can either be myeloablative or of reduced intensity, which consists of less cytoreductive conditioning but involves more extensive immunosuppression treatments involving steroids or cyclosporin A. Following the conditioning regimen, hematopoietic stem cells (HSCs) are then administered to repopulate the individual. These can either be isolated from the recipient prior to conditioning (autologous) or from related or unrelated HLA-matched or mismatched donors (allogeneic). There are many potential problems that can influence the outcome of allogeneic BMT including, but not limited to: rejection of the marrow graft, the occurrence of graft-versus-host-disease (GVHD), relapse from the original tumor, and the susceptibility of patients to opportunistic infections due to the immunosuppressive effects of the conditioning regimen.

NK cells have shown to be capable of rejecting allografts and that they alone can mediate the specificity of BMC rejection in mice (7, 77-80). Experiments performed in SCID mice that lack T and B cells but have normal NK-cell function showed that these mice were capable of rejecting BMC allografts, but not solid tissue allografts, following lethal irradiation (7). Such conclusive results of NK cells and BMT have not been reported in man. Clinically, allograft rejection was seen in SCID patients who received HLA-mismatched, T cell-depleted BM transplants (81). There is new evidence from clinical studies showing that the presence of certain HLA-C alleles can correlate with an increased risk for BM allograft rejection (82).

There are many variables that need to be taken into consideration during the process of BM allograft rejection. Complicating the issue is the fact that since T cells and NK cells share many cell surface markers, it can be difficult to

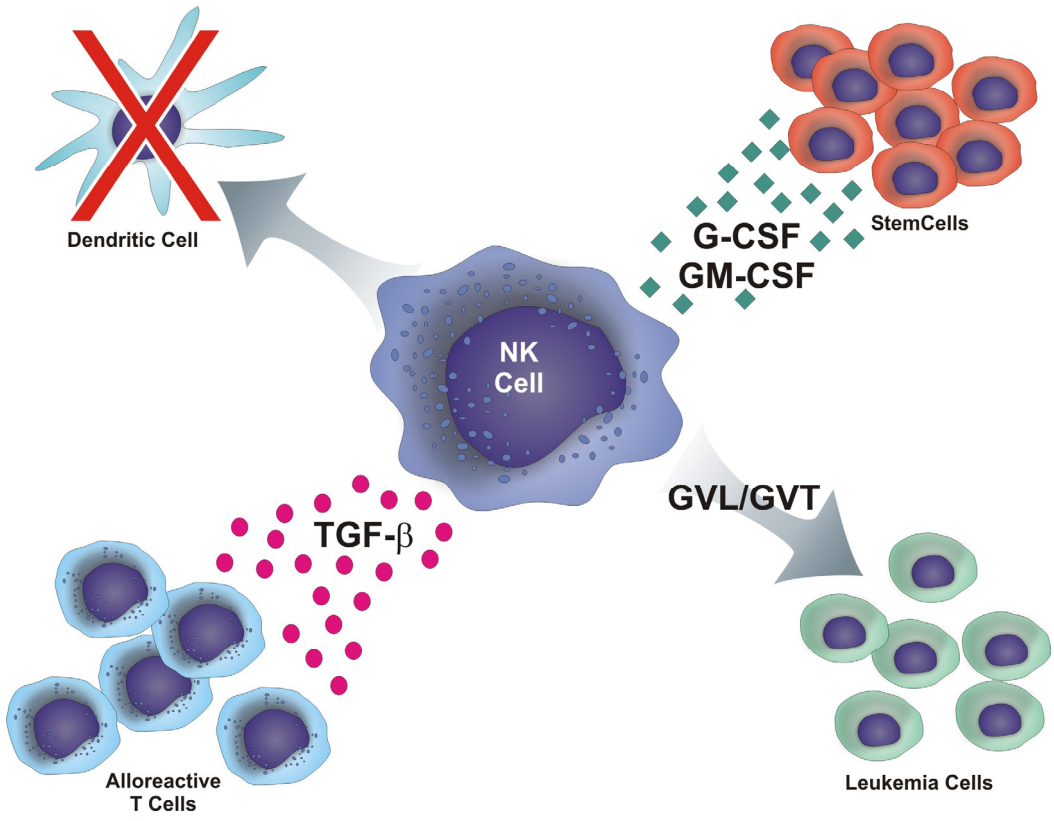

Figure 2. Proposed model of natural killer cells in allogeneic BMT. NK cells can inhibit alloreactive T cells by the production of TGF-β, as well as potentially eliminate host antigen presenting cells (i.e. dendritic cells) that may initiate GVHD. Furthermore, NK cells can promote the growth of donor stem cells through G-CSF and GM-CSF and provide anti-tumor effects by killing residual cancer cells in the host.

discern T cell from NK cell mediated resistance. In order to reduce the severe toxicities associated with conventional BMT procedures, many BMT centers are using more non-myeloablative or reduced intensity conditioning transplant regimens (83). These non-myeloablative regimens involve less conditioning and thus less toxicities, but can result in a greater survival of host NK cells, leading to BM allograft rejection (84). However, it is possible to override NK cell-mediated rejection by increasing BMC number following total body irradiation (TBI) (20). Interestingly, the mechanism of BMC rejection in mice has been shown to depend on the condition of the mouse colony housing the animals. In one study, perforin-deficient mice and FasL mutant mice were found to be incapable of rejecting BMC allografts at one institution yet were capable of rejecting the exact same BMC allograft at another institution (85). Therefore, other factors, such as subclinical infections, need to be taken into consideration when examining NK cell function and dependence on certain cytotoxic pathways. Further complicating the issue, the precise target in the BMC that the NK cells attack, whether it is the stem cells, committed progenitors, or stromal elements, is not known. Taken together, one can see that all these variables make it challenging not only to determine the mechanism of NK cell-mediated BMC rejection but also to extrapolate these data to the clinical perspective.

The earliest *in vivo* activity associated with NK subsets was the demonstration that removal of Ly49C/I abrogated the ability of lethally irradiated H-2^b and other non-H-2^d mice to reject H-2^d BMC allograft (86). Studies using antibodies to deplete particular Ly49 subsets have resulted in a clear picture of a subset's role in BMC rejection, and the patterns that were seen *in vivo* correlated with the *in vitro* specificity seen in cytotoxicity assays using tumors and mitogen-stimulated lymphoblast targets (87-90). NK cells expressing both inhibitory and activating receptors appear to interact with BMC during rejection depending on the MHC expression of the BMC. It is still unclear whether activating receptors are required for BMC rejection or whether the absence of inhibitory receptors would suffice. However, in the case of the ability of a H-2^b mice to reject H-2^d BMC, there remains a significant amount of overlap between activating and inhibitory receptors, in that 98% of the NK cells that express Ly49C/I, (which receive inhibitory signals by H-$2D^b$) also express Ly49D (which receive an activating signal from H-$2D^d$) (88). Surprisingly, the depletion of a single subset has often resulted in negative data, and multiple subsets (i.e. Ly49A and Ly49G2 for H-2^b BMC rejection) need to be depleted in order to observe a biological effect (which is often synergistic (90)) (Table 1). It is not yet known if this result is due to different NK cell subsets having the ability to work in concert or if simply a more efficient depletion is occurring. Another surprising finding was that the removal of a particular NK cell subset responsible for rejection results in a greater engraftment compared to removing all of the NK cells with a pan-NK-specific antibody, such as NK1.1 (90). This suggests that other NK subsets may be beneficial to donor

engraftment such that their removal resulted in less myeloid repopulation. This is in agreement with current data suggesting that NK cells have a dual role in hematopoiesis and are capable of both inhibiting and stimulating myeloid growth, depending on the conditions of the assay and the activation state of the NK cells (11). This can be in part due to the numerous cytokines that are secreted by NK cells. BMC growing under optimal conditions are inhibited by activated synergistic NK cells as determined by CFU-C growth, in part through the production of IFN-γ (11, 88, 91). Conversely, when BMC are placed under limiting conditions, the addition of NK cells can promote myeloid proliferation, in part through the production of GM-CSF (92). It has also been shown that the transfer of activated NK cells can promote hematopoietic reconstitution after syngeneic BMT in mice (93). Some evidence shows that NK cells have differential effects on resting hematopoiesis *in vivo* (93, 94), and that this may be one of the NK cell's normal physiological functions. These data suggest that NK cells can regulate myeloid growth in both syngeneic and allogeneic situations through the production of inhibitory (including lytic) or stimulatory cytokines. The NK cells present in the host have the ability to be both deleterious and beneficial after allogeneic BMT, depending on their inhibitory/activating receptors expression and activation status.

GVT effects of NK cells

NK cells have been shown to have potent anti-tumor effects *in vivo*, particularly with leukemias and metastatic tumors (4, 5), and therefore could be of potential use as an adoptive immunotherapy following BMT for the treatment of various cancers. This may due to the fact that NK cells are the first lymphoid cells to repopulate an individual following BMT. Thus far, the majority of clinical trials using NK cells have been restricted to autologous transplants (24), though current studies are beginning to use NK cells to mediate transplants across haplotype barriers (95). The majority of studies examining the role of NK cells in the allogeneic setting have been in mice. As NK cells have been demonstrated to produce numerous cytokines and to affect immune responses (96, 97), it is attractive to speculate that promotion of immune reconstitution after BMT may also result from the transfer of NK cells. In addition to helping restore the immune system, NK cells can help eliminate any cancer cells remaining after chemotherapy or irradiation. The mechanisms that the NK cells can eliminate the tumor could be by direct cytotoxicity (perforin/granzymes, FasL, or TRAIL) (8) or by the production of inflammatory and suppressive cytokines (TNF-α and/or IFN-γ) (9). As many tumors down-regulate their MHC class I receptors in order to escape T-cell mediated killing, the NK cell is responsible for elimination of these MHC class I-deficient targets. The NK cells ability to kill in an MHC-unrestricted manner has been documented since 1975 (3-5) and was verified in *beige* mice, which lack normal NK cell function due to a deficient in granule-mediated killing pathways in these mice (98). In these experiments, a modified tumor line that had increased NK cell-sensitivity demonstrated an increased growth rate, a faster induction time, and an increased metastatic capability in *beige* mice compared to control mice. When the researchers used an NK-insensitive tumor line, similar tumor growth characteristics of the tumor were seen in *beige* and control mice, suggesting that NK cells play a role in tumor defense by both inhibiting metastases and primary tumor growth. As NK cells have been demonstrated to eliminate hematopoietic cells but not solid tissue allografts, it seems possible that NK cells could be specifically targeted for anti-tumor responses without doing undue collateral damage to the patient after BMT. Optimizing anti-tumor effects by blockade of inhibitory receptor interactions may also provide another means to use NK cells in therapy. As about 50% of murine NK cells were found to have Ly49 and CD94/NKG2A inhibitory receptors, the blockade of these receptors by monoclonal antibodies may augment anti-tumor activity of the NK cells (61, 88). For example, the murine $Ly49C^+/I^+$ NK cell subset has been shown to be inactivated by cells expressing $H\text{-}2D^b$ (35), and these same NK cells have also been shown to reject $H\text{-}2^d$ BMC (99). It has been shown that the blockade of the $Ly49C^+/I^+$ inhibitory receptors with specific $F(ab')_2$ fragments increases anti-tumor activity of NK cells both *in vitro* and *in vivo* (100). NK cells can also be used to eliminate contaminating leukemia cells from bone marrow. Studies by Koh et al. have shown that the use of $F(ab')_2$ fragments can aid in the purging of C1498 leukemia cells from BM in an autologous transplant model (101). These studies also demonstrated that allogeneic NK cells had superior anti-tumor activity compared to syngeneic NK cells. Importantly, the blocking of inhibitory receptors on NK cells resulted in no adverse effects in mice receiving NK cell infusions (100). In humans, it has been shown that KIR-ligand incompatibility resulted in drastically lower relapse rates and graft rejection in patients with acute myeloid leukemia who received mismatched allogeneic BMT (102). Unfortunately, these data only applied to patients who had acute myeloid leukemia, as patients who had acute lymphoid leukemia did not show any significant improvement in the trial, suggesting that KIR-ligand incompatibility may only work for certain kinds of cancers. In this study, patients were defined as having a KIR-ligand incompatibility if they lacked HLA class I alleles that were recognized by KIRs in the BMT. It is important to note that the transplants were not sorted on the basis of KIR receptor expression, but rather based on the absence of the KIR receptors' ligands (i.e. HLA). The data from this trial suggests that the use of inhibitory receptor blockade, or the use of sorted NK cell subsets that lack specific inhibitory receptors, may be a potential immunotherapy for the treatment of cancer.

NK cells and GVHD

The difficulty in separating NK cells from T cells, however, has delayed the clinical use of NK cells in allogeneic BMT for fear of exacerbating GVHD. During GVHD, donor T-cells are able to recognize the host as foreign by both major and minor histocompatibility differences and then attack the host tissues. This leads to the expansion of donor T cells, the release of inflammatory cytokines, and the recruitment of other cell types (i.e. NK cells, macrophages, monocytes) that may mediate tissue injury. For these reasons, T cells have been depleted prior to allogeneic BMT to reduce the incidence and severity of GVHD, but this also results in a greater chance of graft rejection, delayed

immune reconstitution, higher relapse rates, and an increased susceptibility to opportunistic infections. For these reasons, allogeneic BMT is analogized to a balance between the immunoincompetent recipient (due to prior conditioning or immunosuppression but whose T and NK cells can still mediate resistance) and attack from immunocompetent donor T cells, leading to GVHD (Figure 1). This results in a very delicate equilibrium between engraftment and potential GVHD/graft-versus-tumor (GVT) responses or marrow graft failure due to rejection by host effector cells.

It is known that host NK cells can resist the donor BMC graft, but the question remains whether donor NK cells attack the recipient during engraftment or during GVHD. NK cells are known not to reject solid tissue allografts, suggesting that donor NK cells should not initiate GVHD. However, NK cells can also contribute to the pathology of GVHD through the release of many inflammatory cytokines, contributing to the so called "cytokine storm". The use of delayed lymphocyte infusions after allogeneic BMT resulting in less GVHD with significant GVT responses suggests that activated NK cells could be safely administered in this situation. In murine studies, it has been shown that the presence of NK cells may be beneficial if contaminating T cells are in the graft, as *in vitro* studies have shown that NK cells can inhibit T cell responses in part through the production of TGF-β (103) (Figure 2). Indeed, it has been shown that donor type activated NK cells can promote marrow engraftment and B cell development during allogeneic BMT (104). This was corroborated in other studies that assessed the effects of activated NK cells after allogeneic BMT in tumor-bearing mice, in which the donor-type NK cells were capable of inhibiting the occurrence of GVHD, in part through the production of TGF-β (105). In this study the donor NK cells also contributed to the anti-tumor effect. Interestingly, if administration of NK cells was delayed until after the initiation of GVHD, the incidence and severity of GVHD was increased, suggesting that only a narrow window of GVHD protection by NK cells exists. It was observed in this study that the administration of NK cells, if timed properly, could inhibit GVHD and preserve GVT in advanced tumor-bearing mice (105). Importantly, the tumor model used in this study was a colonic adenocarcinoma, suggesting that activated NK cells may be of use in BMT for treatment of metastatic solid tumors as well as hematologic malignancies.

A recent study from Ruggeri et al. has shown that the use of NK cell subsets bearing certain inhibitory receptors did not induce GVHD, but they did promote engraftment, prevent T cell-mediated GVHD, and eliminate tumors in a BMT setting. In the study, $Ly49A^{+}/G2^{+}/Ly49C^{-}/I^{-}$ and $Ly49A^{-}/G2^{-}/Ly49C^{+}/I^{+}$ NK cell subsets obtained from mouse $H\text{-}2^{bxd}$ splenocytes were purified and assayed for alloreactivity by using $H\text{-}2^{b}$ or $H\text{-}2^{d}$ recipients as targets (102). They found that the $Ly49A^{+}/G2^{+}/Ly49C^{-}/I^{-}$ alloreactive NK cell subset ($Ly49G2^{+}$ is an inhibitory receptor specific for $H\text{-}2^{d}$, Table 1) was activated to lyse the recipient's $H\text{-}2^{b}$ targets without causing GVHD. The subsequent administration of large numbers of alloreactive NK cells could not initiate GVHD. Similar results were also seen following the administration of $Ly49A^{-}/G2^{-}/Ly49C^{+}/I^{+}$ NK cells, specific for $H\text{-}2^{b}$, to $H\text{-}2^{d}$ mice. It was found that the NK cells were eliminating T cells and granulocytes, and that this elimination of recipient T cells and granulocytes also promoted engraftment of BM. It was then shown that application of non-lethal conditioning with transfer of additional NK cells could promote engraftment to levels similar to those seen when lethal conditioning was used (102). This finding may then allow patients, who normally could not handle the extreme toxicities of myeloablation, to receive one by use of less toxic non-myeloablation and the infusion of donor alloreactive NK cells. The data by Ruggeri et al. also showed that the addition of alloreactive NK cells eliminated the need for T cell depletion of BM. They showed in mice that BM containing T cells plus additional non-alloreactive NK cells caused the mice to die from GVHD, while mice receiving the same treatment plus additional alloreactive NK cells had complete survival. The researchers correlated this protection with the elimination of host dendritic cells (DCs) in mice that received the alloreactive NK cells, suggesting that the NK cells eliminated the host DCs and thus prevented DCs from initiating donor T cell expansion leading to GVHD (Figure 2). Finally, they also showed that a single infusion of human alloreactive NK cells could eliminate bone marrow infiltration by chronic myeloid leukemia myeloid blastic crisis after one week in non-obese diabetic (NOD)/SCID mice. These findings of alloreactive NK cells not causing GVHD, yet promoting engraftment, preventing T cell-mediated GVHD, and eliminating tumors has caused much interest about further uses of NK cells in a clinical BMT setting.

Future implications

As knowledge of NK cell function continues to expand exponentially (as it has the past decade) there will continue to be further understanding of the use of NK cells as an immunotherapy of hematologic malignancies and solid tumor metastases. Greater appreciation of the balance between the activating and inhibitory receptors has led to the successful use of the anti-leukemic potential of NK cells through KIR-ligand mismatches in a haplotype-mismatched stem cell transplantation setting. NK cell alloreactivity can also be exploited in the setting of allogeneic BMT. By selectively depleting host NK cells capable of rejecting a graft prior to pre-transplant conditioning, clinicians could make reduced intensity conditioning regimens available to patients who normally could not tolerate the toxicities associated with current regimens, and this would allow for greater applicability of BMT in cancer.

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