REVIEW ARTICLE

T-cell and natural killer cell therapies for hematologic malignancies after hematopoietic stem cell transplantation: enhancing the graft-*versus*-leukemia effect

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ABSTRACT

Hematopoietic stem cell transplantation has revolutionized the treatment of hematologic malignancies, but infection, graft-*versus*-host disease and relapse are still important problems. Calcineurin inhibitors, T-cell depletion strategies, and immunomodulators have helped to prevent graft-*versus*-host disease, but have a negative impact on the graft-*versus*-leukemia effect. T cells and natural killer cells are both thought to be important in the graft-*versus*-leukemia effect, and both cell types are amenable to *ex vivo* manipulation and clinical manufacture, making them versatile immunotherapeutics. We provide an overview of these immunotherapeutic strategies following hematopoietic stem cell transplantation, with discussions centered on natural killer and T-cell biology. We discuss the contributions of each cell type to graft-*versus*-leukemia effects, as well as the current research directions in the field as related to adoptive cell therapy after hematopoietic stem cell transplantation.

Introduction

Hematopoietic stem cell transplantation (HSCT) has revolutionized the treatment of hematologic malignancies, bringing substantial improvements to survival outcomes for many patients.[1] However, infection,[2] graft-*versus*-host disease (GVHD),[3] and relapse[4] are still the most challenging sequelae to address to improve the outcomes of all patients after allogeneic transplantation.[5] Over the last several decades, the introduction of calcineurin inhibitors, T-cell depletion strategies, and immunomodulators has helped to prevent GVHD, but at a cost - with inhibition of the donor-specific immune response including the graft-*versus*-tumor/leukemia (GVL) effect.[3] Efforts have been made alone or in combination to increase GVL without increasing GVHD by: (i) optimizing conditioning regimens; (ii) selecting better matched donors; and (iii) administering GVHD prophylaxis.[6] Perhaps the most direct method to restore the GVL effect would be to administer T cells (manipulated or unmanipulated), which mediate the GVL effect,[7] and donor-derived natural killer (NK) cells.[8] Both T and NK cells are thought to be the principal effector cells mediating the GVL effect (Figure 1):[6] directly killing tumor cells through the Fas and perforin pathways, but also indirectly contributing to tumor lysis through the secretion of cytokines.[9] From a therapeutic perspective, both cell types are amenable to *ex vivo* manipulation and clinical manufacture, thus making them versatile immunotherapeutics. We provide an overview of these two immunotherapeutic strategies following HSCT, with discussions centered on NK and T-cell biology and contributions to the GVL effect as well as the current research directions in the field as related to adoptive cell therapy after HSCT.

T-cell therapy after hematopoietic stem cell transplantation

Biology

T cells, along with B cells, comprise the major cellular components of the adaptive immune system (Figure 1). By rearranging gene segments during T-cell development, a large number of T cells with different T-cell receptors (TCR) are made that can potentially recognize an unlimited number of peptides in the context of MHC molecules. These T cells are primed to recognize foreign proteins expressed on malignant and non-self cells. Following recognition, T cells either directly lyse their targets by secreting powerful perforins and granzymes, or orchestrate a more potent immune response by secreting inflammatory cytokines and chemokines.[10]

Evidence of a graft-versus-leukemia effect

The role of T cells in the GVL effect has long been established. An analysis of 2254 patients receiving bone marrow transplants for acute myeloid leukemia (AML), acute lymphoblastic leukemia, and chronic myeloid leukemia showed lower rates of relapse in patients with non-T-cell-depleted allografts with GVHD, compared to those receiving T-cell-depleted allografts without GVHD.[11] This evidence was further supported by studies using donor lymphocyte infusions (especially in the setting of chronic myeloid leukemia).[12] However, GVHD remains a problem with donor lymphocyte infusions, thereby necessitating the use of more specific populations of T cells to enhance the GVL effect, such as T cells targeting minor histocompatibility antigens, or leukemia-specific antigens.[12]

Exploiting the graft-versus-leukemia effect: manufacturing T cells for immunotherapy

The two general strategies to manufacture T cells to exploit a GVL effect in the setting of HSCT are: (i) *ex vivo* expansion and (ii) genetic modification.

(i) *Ex vivo* expansion (Figure 2). This involves the selective proliferation of T cells expressing endogenous TCR that recognize tumor cells. This approach exploits repeated stimulation with antigens to expand large numbers of T cells.[13] *Ex vivo* expansion of T cells has the advantage of decreasing alloreactivity *in vitro*,[14] as a result of cell death or outgrowth by the antigen-specific T cells.[15] Two important parameters

 doi:10.3324/haematol.2014.113860
Manuscript received on January 7, 2015. Manuscript accepted on May 6, 2015.
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involved in *ex vivo* expansion are the target antigens and the culture conditions. Target antigens include minor histocompatibility antigens and leukemia-specific antigens, which include, in some settings, viral antigens.[16] Minor histocompatibility antigens are proteins that are expressed differently across individuals as a result of genetic polymorphisms.[17] Leukemia-specific antigens, on the other hand, are proteins that are either mutated (e.g. bcr-abl), lineage-restricted (e.g. CD19), or overexpressed in malignancies while concomitantly absent or minimally expressed in healthy tissues.[18] Culture conditions are optimized to present the best priming environment for T cells to encounter antigen, involving different antigen-presenting cells,[19] stimulatory cytokines,[20] and selection of subpopulations.[21]

(ii) Genetic modification (Figure 3). Using various gene therapy vectors (retroviruses,[22] lentiviruses,[23] transposons[24]), investigators have been able to introduce new specificities onto T cells to allow for HLA-independent targeting of hematologic malignancies. Chimeric antigen receptor-modified T cells, in particular, have been used as both a bridge to transplant and as adjuvant therapies after HSCT. These modified cells are discussed in more detail below.

Ex vivo-*expanded T cells*

The first *ex vivo*-expanded T cells for therapy in HSCT began with studies of antiviral T cells and T cells recognizing minor histocompatibility antigens. The use of donor-derived Epstein Barr virus-specific T cells successfully prevented and treated Epstein Barr virus-associated post-transplant lymphoproliferative disease[25-28] Furthermore, gene marking of the infused cells demonstrated that these tumor-specific T cells persist in the long term.[25] Outside of the context of targeting viral tumor-associated antigens, investigators from Leiden University Medical Center and Seattle pioneered the use of T cells targeting minor histocompatibility antigens, demonstrating that T-cell clones recognizing these antigens can kill myeloid leukemic cells and inhibit leukemia growth *in vitro*.[29] Moreover, T cells specific for minor histocompatibility antigens were expanded *in vitro* and infused into a patient with accelerated phase chronic myeloid leukemia after allogeneic transplant. Complete eradication of leukemic cells was achieved after three infusions of leukemia-reactive T cells.[30] A more recent trial used *ex vivo*-expanded donor derived CD8$^+$ T cells against unknown minor histocompatibility antigens to treat leukemia relapse following allogeneic transplant. Donor T cells stimulated with recipient peripheral blood mononuclear cells, depleted of CD4$^+$ T cells, and selected for reactive clones capable of lysing transformed cells but not fibroblasts were administered to seven patients; four of whom had transient complete remissions.[31]

While the approaches described above show promise, very few hematologic malignancies have viral epitopes as targets, and often, identification of minor histocompatibility antigens that will not elicit GVHD is difficult. For these reasons, attempts have shifted towards generating leukemia-associated antigen-specific T cells. Studies have suggested that expansion of leukemia-associated antigen-specific T cells in the peripheral blood of patients after allogeneic HSCT contribute to the GVL effect.[32,33]

Leukemia-associated antigens include an eclectic mix of proteins seen in hematologic malignancies,[18] including cancer/testis antigens such as MAGE A3, developmental proteins such as WT1, and prosurvival/antiapoptotic proteins such as survivin. Several studies have shown that T cells targeting leukemia- and lymphoma-associated antigens can be generated from donors' and patients' periph-

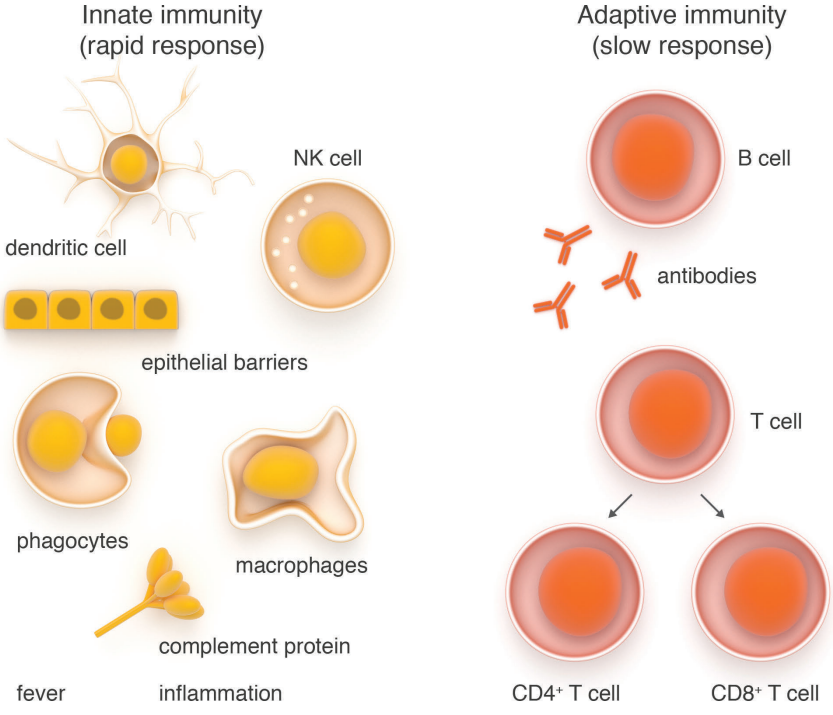

Figure 1. Effector cells of Innate and adaptive Immunity. T cells (right) and NK cells (left) are among the principal cellular effectors of the adaptive and innate immune responses, respectively. Compared to other cells, both T cells and NK cells are amenable to *ex vivo* manipulation, making them excellent sources of biological therapeutics.

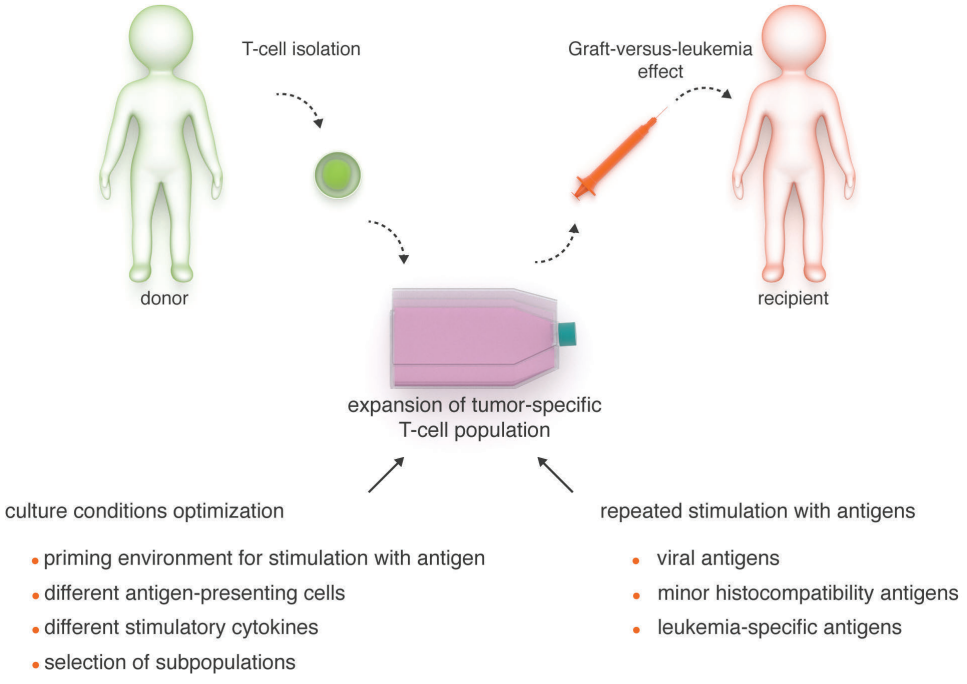

Figure 2. Schematic of *ex vivo* expansion of T cells. T cells are isolated from autologous or allogeneic donor sources, and are kept in culture under different conditions, with the eventual goal of expanding a tumor-specific T-cell population that is then infused back to the patient to elicit a GVL response.

eral blood mononuclear cells and show cytolytic activity against lymphoma cell lines and primary tumor cells *in vitro*.[34-37] For example, preliminary findings of WT1-specific T-cell immunotherapy from a phase I clinical trial show that these T cells are well tolerated and effective. Infusions of WT1-specific cytotoxic T lymphocytes into patients with AML, acute lymphoblastic leukemia, or myelodysplastic syndrome following allogeneic HSCT were able to transiently reduce or eliminate cells expressing WT1, without mediating toxicities or GVHD. These cells were generated from healthy donors of hematopoietic stem cells, using dendritic cells pulsed with a pool of overlapping peptides spanning the WT1 protein as antigen-presenting cells to stimulate and expand WT1-specific T cells.[38] In a more recent study, T cells directed against the leukemia-associated antigens BCR-ABL, PR1, and WT1 infused after transplantation resulted in 7/14 patients with chronic myeloid leukemia remaining in molecular remission a median of 45 months after prophylactic infusion of cells.[39]

Genetically modified T cells

While the use of *ex vivo*-expanded T cells has advantages, especially given the ability of this approach to generate a product which targets multiple tumor antigens, they are still limited by the low affinity of their antigens as well as the HLA dependence of tumors which can downregulate their MHC. To overcome these limitations, T cells can be genetically modified to redirect their specificity. Two promising gene transfer technologies include: (i) optimized high affinity T-cell receptors and (ii) chimeric antigen receptors (CAR).[40]

(i) High affinity T-cell receptors. Certain clones from patients or healthy donors develop high affinity TCR purely by chance, and such TCR can confer high specificity onto their T cells. Cloning this TCR complex onto other cells is the basis of TCR gene transfer, and the technology has shown encouraging results against hematologic malignancies.[41] However, a significant concern with TCR gene transfer is the potential mispairing that may occur between endogenous TCR and the introduced high affinity TCR. At best, the mispairing decreases effectiveness of the therapy,[42] but at worst, the new TCR may recognize self-proteins[43] and potentially cause harmful GVHD. Several methods have been proposed to address this, one approach being the use of short interfering RNA to silence endogenous TCR genes, which has shown promise in the setting of leukemia immunotherapy. A TCR recognizing WT1 was transduced into T cells along with short interfering RNA for endogenous TCR genes. The resultant TCR-transduced T cells derived from leukemia patients were cytolytic against autologous leukemia cells but not normal hematopoietic progenitor cells. Furthermore, these T cells were capable of eliciting anti-leukemia activity in mouse xenograft models without impairing hematopoiesis.[44]

(ii) Chimeric antigen receptors. Another highly promising technology is the CAR approach, which circumvents some of the limitations of both TCR gene transfer T cells and *ex vivo*-expanded T cells since HLA restriction is not required. CAR-modified T cells can theoretically recognize any target (not just proteins) in an HLA-independent manner with significantly enhanced potency.[45] CAR are composed of an extracellular recognition domain (usually derived from the variable regions of an antibody) coupled to intracellular signaling domains that combine both signal 1 (TCR complex) and signal 2 (co-stimulatory molecule signaling) from the T cells.[46] CAR were first designed by Eshhar *et al.* who evaluated whether the antibody complex can confer new specificity onto T cells.[47] The best experience to date with CAR-modified T cells in HSCT involve CAR that recognize CD19, present on B-cell malignancies.[48] Although CD19 is also present in healthy

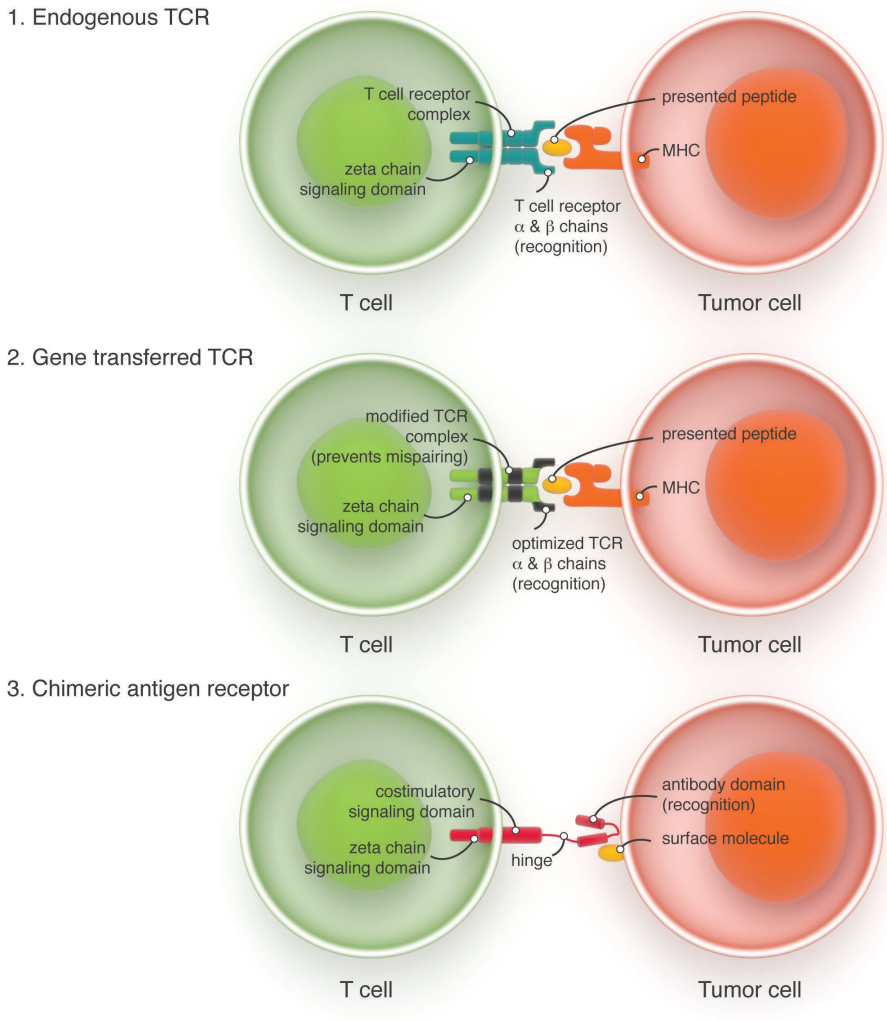

Figure 3. Different T-cell receptors featured in T-cell immunotherapies. T cells utilized for immunotherapies employ one of three receptors depicted from top to bottom: (1) native/endogenous T-cell receptors, which usually have low affinities and recognize tumor peptides in the context of MHC; (2) gene-modified T-cell receptors, which have high affinities using a TCR optimized for enhanced binding and intracellular complexes modified to prevent mispairing; and (3) chimeric antigen receptors, using antibodies as recognition domains capable of recognizing any surface molecule (protein, carbohydrate, lipid) independent of HLA restriction.

B cells, clinical experience with patients with common variable immune deficiency and patients treated with rituximab suggest that B-cell depletion is manageable.[49]

CD19 chimeric antigen receptor T cells

The earliest experiences with CD19 CAR T cells were disappointing due to a general lack of persistence (less than a week) that coincided with poor clinical responses. These cells incorporated so-called "first generation" CAR, which only used the TCR zeta chain as the sole signaling domain.[50] Subsequent studies suggested that the addition of co-stimulatory molecules in the construct (i.e. "second generation" CAR) could provide improved persistence and antitumor activity in murine models and in patients.[51] The group at the Baylor College of Medicine infused two populations of T cells: (i) T cells expressing a first-generation CD19 CAR and (ii) T cells expressing a second-generation CD19 CAR (including the CD28 co-stimulatory domain). Second-generation CAR T cells persisted longer than their first-generation counterparts, demonstrating the ability of co-stimulation to enhance T-cell proliferation and persistence. However, the efficacy of the second-generation CAR T cells in this study was limited.[52] Several key improvements then paved the way for the remarkable antitumor efficacies reported in literature. One improvement involved utilizing a lymphodepletion regimen to enhance persistence of CAR T cells by eliminating competing endogenous cells.[53] Using a chemotherapy regimen comprising cyclophosphamide and fludarabine followed by infusion of CD19 CAR T cells, Rosenberg's group at the National Cancer Institute observed remissions from progressive B-cell malignancies in six of eight patients.[53] Another change introduced at the University of Pennsylvania was a lentivirus vector expressing 41BBL instead of CD28 as the co-stimulatory signaling domain. Infusion of these lentivirus-modified T cells following customized chemotherapy regimens initially produced impressive responses in two of three patients with chronic lymphocytic leukemia.[23,54] Estimates suggested that each infused CAR-expressing T cell eradicated more than a 1,000 chronic lymphocytic leukemia cells.[54] Subsequently, even more dramatic successes have been observed using second-generation CAR-CD19 transduced T cells for patients with acute lymphoblastic leukemia.[55] Several groups have demonstrated response rates ranging from 70-100% in some patients with poor prognosis ALL.[55-57] It is, however, still difficult to determine the optimal CAR approach since each protocol varies in terms of CAR design, T-cell production, prior conditioning chemotherapy, and tumor burden.[58]

Future directions

Several lines of inquiry are being explored in our efforts to improve the use of antitumor T cells after HSCT, including: (i) improving safety; (ii) improving activity and persistence *in vivo*; (iii) decreasing manufacturing time; (iv) increasing the number of antigens being targeted; and (v) conferring protection against immune suppression.

(i) Improving safety. One concern with T-cell immunotherapies is toxicity. This is especially a concern when gene-modified T-cell approaches are utilized (e.g. using CAR with potent co-stimulatory signaling domains). Severe adverse events including cytokine storms have been observed in patients who have antitumor responses following CAR T-cell therapies.[53,55] In an attempt to address such "predictable" adverse events, investigators have developed management plans aimed at curtailing the effects of cytokines. For example, it was known that interleukin (IL)-6 mediates the cytokine release syndrome and immunosuppression with an IL-6 receptor antibody was shown to be capable of reversing the syndrome, so treatment algorithms have been proposed.[59] Safety switches have also been incorporated into T cells. One recent suicide gene approach is the use of the inducible caspase 9 system, in which the administration of an inert dimerizing agent that brings together two halves of a protease involved in initiating and perpetuating the apoptotic cascade results in rapid elimination of the gene-modified T cells and rapid reversal of clinical symptoms.[60,61]

(ii) Improving activity and persistence. Currently, efforts are underway to determine the efficacy of CD19-CAR T cells in phase II/III studies. Attempts to develop CAR targeting other tumor targets are now a substantial focus of this field.[62] Additionally, to further improve the efficacy of CAR-modified T cells some groups are exploring the incorporation of two or more co-stimulatory domains (so-called third-generation CAR)[62,63] or combining them with other antibody recognition domains (so-called tandem CAR).[64] Addition of cytokine signals such as IL-21[65] and IL-15[66] has also been explored. Other attempts to improve *in vivo* persistence involve utilizing the endogenous signaling provided by latent viruses[67,68] which have been explored clinically in neuroblastoma and B-cell leukemias/lymphomas.[69,70] Most recently, improvements within the construct itself have also allowed improvements in CAR T-cell persistence and efficacy.[71]

(iii) Decreasing production time. One active avenue of research involves improvements in the *ex vivo* expansion of both antigen-specific T cells and CAR T cells. Prolonged culture times cause anergy in cell populations, hence efforts are underway to decrease the amount of time cells remain in culture by using novel bioreactors that allow improved gas exchange and surface area (and consequently more rapid expansion)[72] or by using different cell populations such as central memory-derived,[73] naïve-derived,[74] or stem-cell memory-derived T cells.[74,75]

(iv) Increasing target antigens. One of the advantages of *ex vivo* expansion using peptide mixes is the ability to increase the number of target antigens used to stimulate T cells. T cells targeting multiple proteins have been generated using this method,[20] and further increases in antigen can be accomplished *in vivo* using epigenetic modifying drugs.[76,77]

(v) Engineering resistance to immune suppression. Finally, efforts are also underway to confer greater resistance to cells against the immunosuppressive microenvironment mediated by tumors. To counteract the suppressive cytokine transforming growth factor-beta (TGFβ), for example, T cells can be genetically modified with a mutated dominant negative TGFβ type II receptor (DNR) that prevents the formation of the functional tetrameric TGFβ.[78] Modification of T cells expressing this DNR allows them to negate signals from the inhibitory cytokine. DNR-transduced cytotoxic T lymphocytes were resistant to the anti-proliferative effects of TGFβ and adoptive transfer of TGFβ-DNR transduced T cells resulted in eradication of tumor cells *in vivo*.[79] Other current limitations of T-cell therapies are summarized in Table 1.

Natural killer cell therapy after hematopoietic stem cell transplantation

Biology

NK cells, in contrast to T cells and B cells, are lymphocytes of the innate immune response (Figure 1). These cells have receptors that have been predetermined by the germline (i.e., no further rearrangements/increased diversity occur during development).[10] NK cells have diverse activating and inhibitory receptors (another unique feature, in contrast to T cells) – with the corresponding ligands providing signals for activation or inhibition, respectively, of NK cell activity. A balance of these opposing signals determines whether NK cells exert their powerful activities or remain tolerant (Figure 4).[80] The absence of a corresponding matched inhibitory ligand, for example, is often enough signal for the NK cell to eliminate the defective target (the "missing self" hypothesis of NK cell activation).[81] But in a remarkable evolutionary design, only NK cells with inhibitory receptors that have previously encountered self ligands (from interacting with their own hematopoietic cells) are "licensed" to kill non-self-expressing target cells.[8] As components of the innate immune response, most NK cells are already licensed for activity, and can directly mediate cytotoxicity or cytokine secretion upon recognizing their allogeneic targets.[10]

Evidence of the graft-versus-leukemia effect

It is now understood that killer cell immunoglobulin-like receptor (KIR)-ligand mismatches (with the "missing" ligand present on donor NK cells and absent on recipient cells, including malignant cells) account for the GVL effect exhibited by NK cells.[6] An added benefit to the GVL effect elicited by NK cells is the proposed concomitant protection against GVHD. NK cells also target the recipient's antigen-presenting cells, thereby decreasing T-cell mediated GVHD,[82] while "ignoring" healthy cells which do not express ligands for the NK cells' activating receptors.[83] Evidence for a NK-cell-mediated GVL effect was seen in both murine models of leukemia, in which allogeneic NK cells eradicated disease and caused myeloablation without causing GVHD,[82] as well as in clinical experience with haploidentical donor transplants, where patients with AML achieved higher survival rates in the presence of alloreactivity than in the absence of alloreactive mismatches.[82,84] NK cells are, therefore, believed to be the potent effector cells responsible for the antitumor efficacy of haploidentical stem cell transplants against myeloid leukemias,[84,85] and most "immunotherapies" utilizing NK cells are being developed in the context of a haplotype-mismatched HSCT.[8] Nevertheless, not all patients benefit from an NK-mediated GVL effect following engraftment,

possibly because of delayed reconstitution of functional NK cells.[86] As a result, groups are exploring the direct expansion and subsequent infusion of NK cells after HSCT. This has been made possible by advances in purification and selection methods of these innate effectors.

*Exploiting the graft-*versus*-leukemia effect: manufacturing natural killer cells for immunotherapy*

The most common method of purifying NK cells from donor sources involves the selective depletion of T-cell populations (e.g. using magnetic depletion of $CD3^+$ populations), followed by a further purification step for $CD56^+$ populations[87,88] and/or subsequent enrichment with cytokines (Figure 5). Mononuclear cells are subjected to immunomagnetic selection to remove unwanted $CD3^+$ T-cell populations. Although subsequent purification steps yielded higher purity, a substantially increased processing time (and consequent lower recovery) was needed.[87] Enrichment steps involve the cytokines IL-2, IL-15, or various feeder cells (K562 cells of myeloid lineage modified to express IL-15,[89] irradiated autologous peripheral blood mononuclear cells in the presence of IL-2,[90] or autologous mesenchymal stromal cells[91]). Both IL-2 and IL-15 appear equally efficacious cytokines for expanding NK cells *ex vivo* and no added benefit was seen when both were combined or combined with other cytokines (e.g. IL-7).[92] The use of mitogen-activated feeder cells has previously been shown to increase proliferation, lytic activity, and purity of expanded NK cells.[93] Some groups have also investigated the *ex vivo* differentiation of NK cells from stem cell progenitors by first expanding $CD34^+$ cells using static culture bags, and subsequently differentiating the stem cells using a cytokine cocktail comprising stem cell factor, IL-7, IL-15, and IL-2.[94]

Ex vivo*-expanded natural killer cells*

Numerous groups have evaluated the safety and feasibility of infusing allogeneic NK cells in the HSCT setting, particularly against AML. A group in Korea infused *ex vivo-*

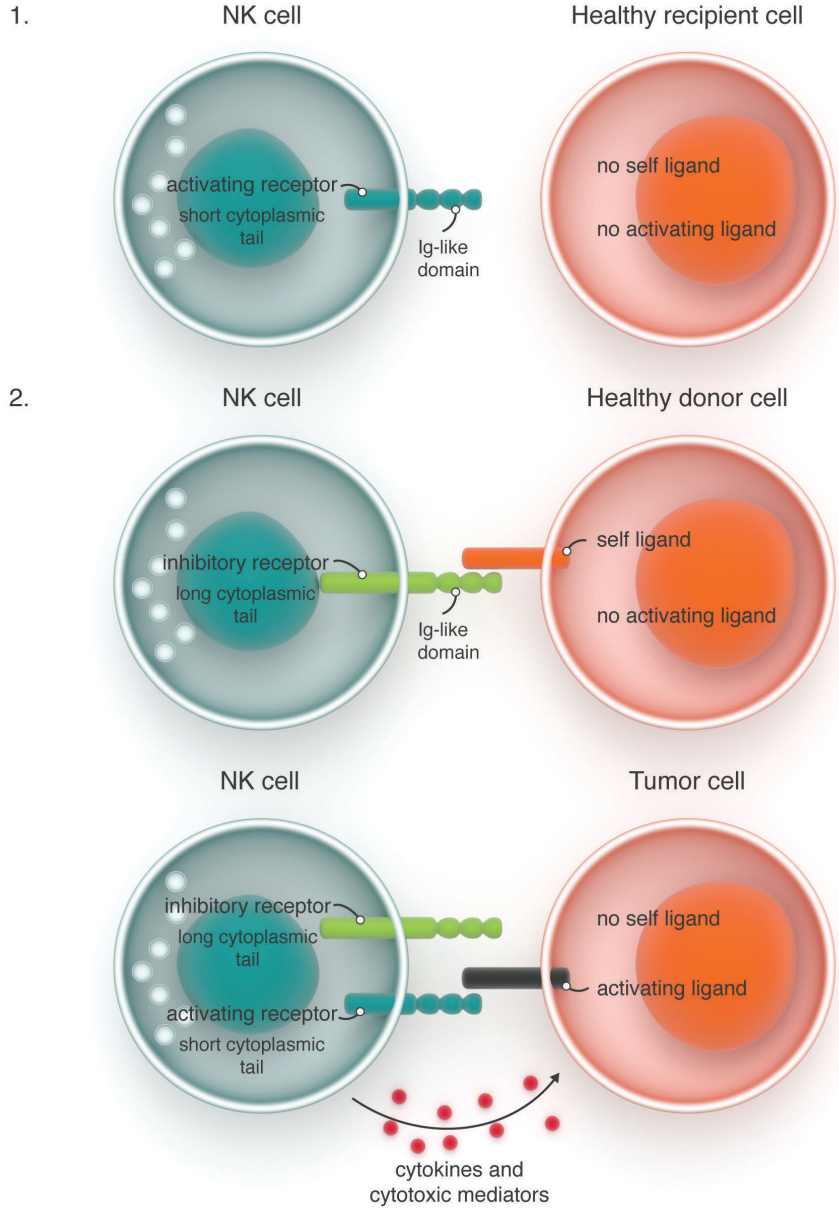

Figure 4. Different NK cell receptors used in NK cell immunotherapies. Two general receptor groups are used by NK cells to discriminate their targets: (1) activating receptors and (2) inhibitory receptors. NK cells recognize tumors from the recipient because of the absence of inhibitory receptor ligands (missing self) and the presence of danger signals that are ligands to activating receptors. In addition, they remain tolerant to both healthy cells from the donor (which possess inhibitory ligands/self-ligands) and healthy cells from the recipient, which do not have activating receptor ligands.

expanded NK cells derived from CD34+ progenitor cells into patients with acute leukemia and myelodysplastic syndrome after HLA-mismatched HSCT. Although no acute toxicity was observed, six patients developed acute or chronic GVHD after NK cell infusion.[95] A group in Switzerland administered purified allogeneic NK cells on days 3, 40, and 100 after haploidentical T-cell-depleted HSCT. The NK infusions did not, however, seem to have an effect on relapse rates (compared to those in historical controls).[96] A group in France reported *in vivo* expansion of infused alloreactive NK cells in a patient who received NK cells and IL-2 for relapsed AML following haploidentical HSCT and salvage chemotherapy.[97] While the NK cells detected *in vivo* retained their activity as measured in functional *in vitro* assays, the patient still relapsed after NK cell infusion and subsequently died of his disease.[97] A group from the University of Minnesota used a regulatory T-cell-depleting protein, the IL-2 diphtheria fusion protein, to suppress regulatory T-cell activity that limits NK cell function. Patients treated with the toxin and NK infusion had higher IL-15 levels and improved efficacy of haploidentical NK cell therapy for AML.[98] Finally, a group from the University of Arkansas reported the use of KIR mismatched haploidentical NK cells in the setting of an autologous HSCT for multiple myeloma. The donors' NK cells persisted transiently after infusion, but five patients still relapsed early, two patients had progressive disease, one patient had stable disease, and two patients subsequently relapsed.[99] As evidenced from these studies, more research is still needed to identify the optimal NK cell product and the optimal setting for the successful use of these cells after HSCT.

Genetically modified natural killer cells

Efforts to further enhance NK cells have led investigators to explore genetically modified NK cells to both redefine their specificity and/or enhance their potency. To extend NK cell activity against lymphocytic leukemias, for example, one group genetically modified the NK cell line NK-92 with CAR recognizing CD19 or CD20. This redi-

Table 1. Current limitations of T cell and NK cell immunotherapy.

Limitations	Crucial Issues	Potential Solutions
T-CELL IMMUNOTHERAPY		
Toxicity	On-target toxicity such as the cytokine release syndrome results from excessive activation following target recognition; off-target toxicity results from recognition of similar proteins in non-tumor/healthy tissue[111]	Introduce suicide gene to eliminate cells following occurrence of cytosine release syndrome/GVHD[61,112] Administer specific treatments for cytokines responsible[59]
In vivo persistence	Following infusion, clinical effects seen while T cells detectable; however, limited T-cell persistence leads to disease recurrence[113]	Use of lymphodepleting conditioning regimens[53]
Complexity of manufacturing individualized products	Protocols for generating T cells are individualized, complicated, and time-consuming, requiring special facilities and trained personnell[72]	Designate core production facilities[114] Develop simplified bioreactors that require minimal manipulation of cells and decreased production time[72] Maintain banks of third party T cells[115]
Tumor immune escape	Tumors are often heterogenous and display multiple antigens; targeting a single antigen can lead to tumor immune escape (e.g. post CD19 CAR-T cell therapy for ALL, approximately 50% of relapses are CD19 negative)[116]	Develop T cells recognizing multiple antigens[20] Administer two products targeting two separate antigens (e.g. CAR-T-cell products targeting CD19 and CD22)[111]
Tumor immune suppression	Tumors mediate a variety of mechanisms to hinder T-cell function at the disease site[117]	Genetically modify T cells to resist immune suppression[78]
NK CELL IMMUNOTHERAPY		
Large numbers required	Limited size of the alloreactive subset within infused NK cell populations require administration of large numbers of NK cells to achieve clinical efficacy[99]	Increase NK:tumor target ratio by administering NK cells during remission or minimal residual disease states rather than relapse[118] Evaluate improved methods of selection and expansion *ex vivo*[89]
Identification of appropriate donors	Current methods identifying KIR ligand mismatches by genotyping may not be sufficient to select for the best possible donor (i.e. actual phenotype may not reflect genotype through different gene regulation mechanisms)[119,120]	Use of KIR phenotyping and *in vitro* testing to better characterize donor-derived NK cells[85]
In vivo persistence	NK cells do not persist; recipient T cells are believed to reject NK cells or, in the case of regulatory T cells, affect proliferation and function[121]	Eliminate regulatory T cells– e.g. with IL2 immunotoxin[98] Provide cytokine support following infusion[121] Administer lymphodepleting conditioning regimens[122]
Negative effects of cryopreservation	Recent report demonstrated decreased NK cell activity following cryopreservation[89]	Shipment of NK cells "fresh" in four degree media[89]
Tumor immune suppression	Tumors mediate a variety of mechanisms to limit NK cell function at disease sites (e.g. soluble ligands, methylation)[123,124]	Administer immunomodulatory chemotherapies[108] or block soluble ligands[99] Rendering NK cells resistant to tumor microenvironment using gene modification[103]

rection of specificity enabled these cells to eliminate previously NK cell-resistant tumors in murine models.[100] Similarly, another group targeted multiple myeloma cells by transfecting NK cells with an anti-CD138 CAR.[101] Investigators have also modified NK cells to express granzyme B following activation by their ligands to augment NK-mediated killing of tumor cells.[102] Finally, to better protect NK cells from the NK cell-inhibitory cytokine TGFβ, NK cells (NK-92) were genetically modified to express a dominant negative TGFβ receptor designed to neutralize TGFβ signaling. These cells were rendered resistant to the suppressive effects of the cytokine and were able to mediate antitumor activity.[103]

Future directions

Several lines of inquiry are being explored in efforts to expand use of NK cells clinically, including: (i) optimizing the choice of mismatch when selecting donor NK cells; (ii) inclusion of conditioning regimens before NK cell infusion; (iii) exploring co-administration with T cells; and (iv) exploring the use of NK cells outside the HSCT setting.

(i) Choice of mismatch. Current mismatch algorithms for NK cell infusions involve three KIR ligands: HLAC1 alleles, HLAC2 alleles, and the Bw4 epitope found in HLA-B alleles. Removal of inhibitory ligands appears to be the primary method of activating NK cells. While mismatches involving inhibitory receptors have normally been used to guide selection of donor NK cells some settings appear to be dominated by activating KIR. Furthermore, some alloreactive clones become functional upon engagement via activating KIR. Consequently, transplants utilizing KIR-B haplotype NK cells have been observed to mediate lower relapse rates and improved survival because they contain more activating than inhibitory receptors.[104]

(ii) Conditioning regimens. Similar to the experience with T cells, lymphodepleting regimens seem to provide a better hematopoietic niche for NK cell expansion and persistence *in vivo*, and several investigators have incorporated such conditioning regimens into NK infusion protocols. The importance of a lymphodepleted state was demonstrated in a study comparing a low intensity regimen and a high intensity regimen. Patients given the low intensity regimen followed by NK cell administration only showed transient NK cell persistence with poor expansion *in vivo*. In contrast, marked *in vivo* expansion of infused allogeneic NK cells with a concomitant induction of hematologic remission was observed in patients with poor prognosis disease who received the high intensity regimen.[105] Other studies, however, suggest that lower intensity regimens may be sufficient, with results depending on the population of patients being treated.[106] Combination with immunomodulatory drugs may also be an option. For example, lenalidomide has been used in patients after HSCT both to activate and increase NK cells,[107] and co-administration with NK cell infusion may lead to better outcomes as the immunomodulation increases CD107 expression, interferon production and degranulation of NK cells.[108]

(iii) Co-administration with T cells. The combination of NK cells and T cells as a single immunotherapeutic strategy is appealing. While NK cells provide rapid, innate activity against tumors, T cells will provide long-lasting adaptive immune activity against the disease. Because of the ability of NK cells to target recipient antigen-presenting cells, it has been suggested that T-cell infusions can be better tolerated with NK infusions with less probability of causing GVHD.[82] However, some studies suggest that memory T cells impair the development and activity of NK cells *in vivo*,[109] and more naïve T-cell populations may be better suited as "partners" for NK cells.[8]

(iv) Use outside the hematopoietic stem cell transplant setting. Finally, several researchers have attempted to extend the benefits of haploidentical transplants in situations in which the procedure is too toxic for potential recipients – by directly substituting allogeneic NK cells in lieu of HSCT. A group from the University of Minnesota induced remissions in patients with poor prognosis AML treated with high-dose cyclophosphamide and fludarabine followed by NK cell infusions and subcutaneous IL-2.[105] A group from Bologna, Italy, infused purified $CD56^{+}CD3^{-}$ NK cells derived from KIR-ligand mismatched donors following fludarabine/cyclophosphamide lymphodepletion into elderly patients with AML not otherwise eligible for allogeneic transplants. Infused cells demonstrated alloreactivity against leukemia blasts, in poor prognosis populations.[110] Finally, the group from St. Jude Children's Research Hospital infused KIR-mis-

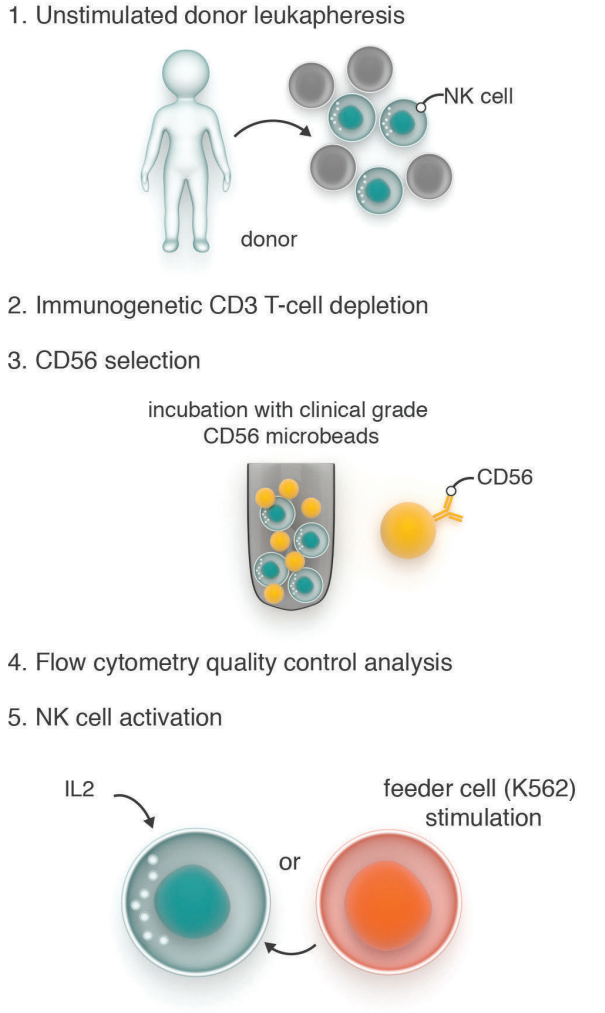

Figure 5. Schematic of *ex vivo* expansion of NK cells. NK cells are enriched from autologous or allogeneic donor sources either following different methods of T-cell depletion or $CD56^{+}$ selection or both, and are then expanded/activated using different combinations of cytokines such as IL-2 and/or artificial antigen-presenting cells (e.g. K562 cells).

matched NK cells into pediatric patients with AML as prophylaxis following chemotherapy. These cell infusions were well tolerated and the NK cells successfully engrafted and patients remained in remission for a median of >2.5 years.[106] Other limitations of NK cell therapies are summarized in Table 1.

Overall summary

Improved methods of generating T cells and NK cells have facilitated the development of novel immunotherapeutic approaches that can augment and potentially even supplant allogeneic HSCT for hematologic malignancies. Advances in genetic modification technologies will only serve to improve the anti-tumor properties of these cells *in vivo*. As indications are broadened, manufacturing protocols optimized, and safety issues addressed, cellular therapies may yet become the standard of care for the treatment of hematologic malignancies as adjunct to, bridge before, or replacement for allogeneic HSCT.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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