Preservation of Immune Repertoire by Selective Depletion of Haploidentical Grafts

Eva Guinan,1 Leo Luzmik,2 Rupert Handgretinger,3 Ann Woolfrey4

An important barrier to the success of transplanting haploidentical hematopoietic stem cells is delayed reconstitution of immune cells that provide protection from opportunistic infections and recurrent malignancy. In recent years a large research effort has been directed toward improving immune reconstitution through methods that potentially spare these cells while simultaneously reducing the alloreactive lymphocytes that cause graft-vs.-host disease. The basic concepts that support three very different approaches to selective depletion of haploidentical grafts are described in this section. Two methods take advantage of the proliferation of donor T cells after encountering alloantigen, and the third method exploits newer technology to engineer a graft that excludes alloreactive T cells while preserving other immunomodulatory cells.

INTRODUCTION

Initial attempts to transplant unmodified (T-replete) haploidentical bone marrow were associated with severe, often lethal, graft rejection or graft-versus-host disease (GVHD) as a consequence of excessive alloreactivity of host and donor T cells [1,2]. Tremendous strides have been made since, such that a haploidentical donor is now a reasonable option for the many patients without a closely matched related or unrelated donor. In the 1970s and 1980s, various methods were perfected to deplete T cells from the donor graft, which markedly reduced the risk for GVHD. However, it soon became apparent that donor T cells play an important role in preventing graft rejection by eliminating or inactivating recipient alloactive cells that remain functionally active after the pretransplant conditioning. Graft rejection, resulting from the paucity of T cells in the graft, became the main obstacle to success; but by the late 1990s this complication largely was overcome in children by administration of very intensive immunosuppressive regimens, and in adults by transplantation of very large numbers of hematopoietic stem cells found in mobilized peripheral blood (PB) [3,4].

Currently the most important barrier to the success of haploidentical hematopoietic cell transplant (HCT) is the lack of expedient immune reconstitution, leading to opportunistic infections and relapse. Severe depletion of T lymphocytes removes virus-specific cytotoxic T lymphocytes (CTLs) and other immunomodulatory cells important in controlling malignancies[5]. This article focuses on the development of selective depletion processes with the aim of enhancing immune reconstitution following haploidentical HCT. Two methods take advantage of the proliferation of donor T cells after encountering alloantigen: 1 of these induces anergy in alloreactive T cells within the graft by blocking costimulatory signals, whereas the other takes advantage of the relative sensitivity of alloreactive T cells, compared to hematopoietic stem cells (HSC), to high-dose cyclophosphamide given in vivo. The third method exploits newer technology to engineer a graft that includes high numbers of natural killer (NK) cells and other immunomodulatory cells, as well as high numbers of HSCs.
Induction of alloantigen-specific tolerance in the reactive cells offers an alternative approach [8]. There are multiple mechanisms for inducing tolerance in human T cells. One well-studied approach derives from recognition that 2 different signals are required to activate T cells, the first resulting from major histocompatibility complex (MHC) restricted antigen binding to a specific T cell receptor and the second resulting from nonspecific but requisite costimulation by the antigen-presenting cell (APC) [9]. In humans, costimulation provided by the ligands B7.1 and B7.2 (CD80 and CD86) on APC with the CD28 receptor (present on ~95% of CD4+ T cells) appears sufficient to produce full activation and function. Blockade of the APC costimulatory signal concurrent with delivery of a first antigen specific signal to T cells results in antigen-specific hyporesponsiveness on rechallenge [10]. This is termed anergy or tolerance. Anergy does not require ongoing costimulatory blockade, and leaves the remaining T cell repertoire (ie, the function of T cells that had not received a first signal during anergy induction) intact. In the transplant setting, donor T cells can be stimulated ex vivo with a source of recipient alloantigens by performing a donor:recipient MLR in the presence of molecules blocking B7 family ligation of CD28. This can be achieved by use of first- or second-generation Ig-fusion proteins of the high affinity ligand for B7.1/2, CTLA4, or by antibodies to B7.1 and B7.2 [10,11].

Costimulatory blockade during MLR results in activation of an alternative intracellular signaling cascade than that activated when both signal 1 and 2 are intact [12]. One result of this process is a dramatic decrease in IL-2 and IL-4 transcription and secreted cytokine, manifest as reduced proliferation of donor T cells during this primary culture. Moreover, donor T cells exhibit profound hyporesponsiveness (anergy) when reexposed to APC from the same stimulator, with 85% to 98% inhibition of proliferation. This approach reduces stimulator specific proliferation by both CD4+ and CD8+ responder T cells [13]. Ex vivo anergization of haploidentical family member donor bone marrow, complete with $10^7$-$10^8$ contaminating donor T cells/kg, has been undertaken in clinical trials in which either CTLA4-Ig (n = 19) or anti-B7.1 and anti-B7.2 humanized antibodies (n = 5) were used to provide blockade of CD28-mediated costimulation [13,14]. Irradiated recipient PBMC were used as allostimulators. Patients receiving these alloanergized haploidentical grafts were observed to have less than the expected rate of GVHD despite the large number of mature donor T cells infused. Perhaps more striking, however, in these admittedly small cohorts, was the paucity of opportunistic infection, relapse, and chronic GVHD (cGVHD) observed. The former may be attributable in part to several related factors, such as relative freedom from GVHD and immunosuppressive medication. However, anergization supports persistence of mature donor T cells with repertoire relevant to the immediate port transplant course, such as response to cytomegalovirus (CMV) and other herpes family viruses as well as response to the tumor antigen WT1.6 Functional pathogen and tumor specific responses are retained by both CD4+ and CD8+ cells.

The recipients of anergized bone marrow (BM) transplants also had striking expansion of T regulatory (Treg) cells in the early posttransplant period, and levels of Treg above baseline were retained for an extended period. Treg levels were greater than those shown in human observational studies to be associated with decreased acute GVHD (aGVHD) and cGVHD. Whereas Treg isolated from the PB of conventional allogeneic BMT recipients have demonstrated global suppressive activity, the Treg present in the patients given anergized T cells demonstrated suppression specific to recipient alloantigens. Published in vitro models have strongly suggested a role for Treg in suppression of alloresponses after alloanergization [15]. Exploring this question in samples from our patients and from normal donors disclosed that alloanergization by means of costimulatory blockade indeed generated Treg and that these were recipient allospecific. Reexposure to alloantigen (ie, conduct of a secondary MLR to mimic the reexposure of ex vivo anergized donor T cells to recipient alloantigens upon infusion into the transplant recipient) resulted in significant increases in both the number and the potency of this allospecific Treg population. Thus, there appear to be at least 2 potent and potentially synergistic mechanisms by which blockade of CD28 mediated costimulation controls alloreactivity transplantation of alloanergized donor T cells.

Thus, costimulatory blockade offers a multifaceted approach to control of alloreactive cells with sparing of pathogen and tumor specific repertoire. Both laboratory and clinical exploration will be needed to fully understand the implications of these dual mechanisms in relation to optimal cell-based strategies for prophylaxis and treatment of aGVHD and cGVHD and improving the tempo and breadth of immune reconstitution after HSCT. Additional roles in solid organ
transplantation and treatment of autoimmunity are of further interest.

**POSTTRANSPLANT CYCLOPHOSPHAMIDE: TOLERANCE INDUCTION OF ALLOREACTIVE T CELLS (LEO LUZNIK)**

Studies in the mouse model suggest that allo HSCT induces bidirectional activation and proliferation of alloreactive host-versus-graft (HVG) and graft-versus-host (GVH) T cells, and that these recently activated T cells are more susceptible to cyclophosphamide than resting T cells (Figure 1) [16]. Tolerance to histocompatibility antigens can be induced if cyclophosphamide is administered after allografting; however, it must be given in high doses 2 to 3 days after alloantigen exposure [16,17]. Tolerance is not induced if the same high dose of cyclophosphamide is given 4 or more days after transplantation. This ability of high-dose cyclophosphamide to induce tolerance to histocompatibility antigens provides an example of the phenomenon of drug-induced immunologic tolerance. Strauss et al. [18] have shown that cyclophosphamide and methotrexate are agents used in clinical practice, which can induce apoptosis of alloreactive T cells, a mechanistic prerequisite for the induction of tolerance.

Based on these studies, it is reasonable to hypothesize that administration of high-dose cyclophosphamide after transplant can induce tolerance simultaneously in both recipient and donor T cells, thus facilitate engraftment and reduce the incidence of GVHD. In mice conditioned with a reduced-intensity regimen of fludarabine and 200 cGy total-body irradiation (TBI), a single dose of posttransplantation cyclophosphamide (200 mg/kg) augmented the engraftment of major histocompatibility complex (MHC)-mismatched bone marrow [19]. In addition, posttransplantation cyclophosphamide reduced the incidence and severity of GVHD across MHC barriers after myeloablative conditioning. This latter observation was not entirely new, because cyclophosphamide administration was one of the first strategies shown to be effective in controlling aGVHD in rodent models. However, in an early clinical trial, cyclophosphamide given in multiple small doses to patients allografted with HLA-matched sibling bone marrow was found to be inferior to cyclosporine in preventing GVHD [20]. In retrospect, cyclophosphamide may have been given at the wrong time or at a dose that was too low to be maximally effective at suppressing GVHD. Results of the more recent preclinical work, however, indicates that the primitive HSC is fairly resistant to high-dose cyclophosphamide, and provides the rationale to test this approach in the clinical setting [21].

Two independent clinical trials evaluated the safety and efficacy of high-dose, posttransplantation cyclophosphamide, given in conjunction with mycophenolate mofetil (MMF) and tacrolimus after nonmyeloablative conditioning [22,23]. All patients were treated in outpatient setting and received conditioning modified from the regimen developed by Storb and colleagues, followed by T cell-replete donor bone marrow on day 0, and 50 mg/kg of cyclophosphamide.
on day 3 (28 patients) or on days 3 and 4 (40 patients). Administration of tacrolimus and MMF was not initiated until the day following to avoid blocking cyclophosphamide-induced tolerance. The median times to neutrophil and platelet recovery were 15 and 24 days, respectively. Graft failure occurred in 9 of 66 (13%) evaluable patients, and was fatal in 1. The cumulative incidences of acute grade II-IV and grade III-IV GVHD were 34% and 6%, respectively. The cumulative incidence of nonrelapse mortality (NRM) at 1 year was 15%. There was a trend toward a lower incidence of extensive cGVHD among recipients of 2 versus 1 dose of posttransplant cyclophosphamide (5% versus 25%, P = .05), the only difference in outcomes between these 2 groups of patients. Serious infections were relatively infrequent; there were no cases of CMV disease and only 5 cases of invasive fungal infection, 2 of which were fatal. These encouraging outcomes in patients with advanced hematologic malignancies served as a rationale to examine the applicability of this strategy in patients with life-threatening nonmalignant hematologic diseases [24]. Engraftment was achieved in 2 patients with thrombotic paroxysmal nocturnal hemoglobinuria (PNH), 1 of whom also had sickle cell disease, demonstrating proof of principle. Currently, a clinical trial is testing the efficacy of this approach in adult patients with sickle cell disease (Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins) and in children with primary immunodeficiency states (Fred Hutchinson Cancer Center).

Several conclusions can be made from these early studies. First, posttransplantation immunosuppression with high-dose cyclophosphamide, tacrolimus, and thrice daily MMF is associated with an acceptably low incidence of graft rejection after nonmyeloablative conditioning. Of equal importance, nearly all patients with graft rejection eventually recovered autologous hematopoiesis. Second, this approach is associated with a low incidence of aGVHD and cGVHD. The low incidence of cGVHD is especially appealing to patients with nonmalignant disorders. Third, it also appears that this strategy may allow the preservation of fertility in young females. Finally, this approach may improve immune reconstitution, which may reduce the incidence of life-threatening opportunistic infections.

REDUCED-INTENSITY CONDITIONING (RIC) AND CD3/19 DEPLETION: AUGMENTATION OF NK FUNCTION (RUPERT HANDGRETINGER)

An interesting development in the field was the introduction of the megadose concept to overcome the HLA barrier and the use of mobilized peripheral stem cells (PBSCs) instead of bone marrow as the stem cell source [4]. The positive selection of CD34+ stem cells was introduced as an efficient large-scale clinical indirect method for T cell depletion and a number of clinical studies using peripheral CD34+ stem cells were performed in adults and children [4,25]. Although CD34+ positive selection methods have widely been used to indirectly deplete T and B cells from mobilized peripheral stem cell grafts, negative T cell-depletion strategies might offer some advantages in overcoming the HLA barrier with RIC while maintaining an effective graft-versus-malignancy effect. Results of clinical trials using megadose CD34+ selected haploidentical peripheral blood stem cells (PBSC) have pointed to the importance of alloreactive natural killer (NK) cells in the context of HLA-mismatched haploidentical grafts [26]. Alloreactive NK cells not only have significant antileukemic activity in patients but are also able to enhance engraftment in a murine model in the context of RIC. Therefore, it seems reasonable to hypothesize that cotransplantation of large numbers of NK cells together with mobilized PBSCs would support engraftment and exert antileukemic effects, and might facilitate the use of RIC regimens.

The positive selection of CD34+ stem cells results in efficient depletion of all CD34− negative cells, including T and B cells. In contrast, the removal of T and B cells from the PBSCs via CD3/19− negative depletion also leads to an efficient T/B-depletion, but retains all other cells not expressing the CD3 and CD19 antigen. In Figure 2, the fundamental difference between positive selection and negative T and B cell depletion is shown. Although CD34+ positively selected cells are a homogenous population (Figure 2, left panel), the CD3/19-depleted cells are a heterogeneous population consisting of lymphoid and myeloid cells (Figure 2, right panel). Such CD3/19-depleted grafts contain large numbers of CD56+/CD3− NK cells and other monocytic/myeloid cells. Analysis of CD3/19-depleted PBSCs obtained from 27 haploidentical donors for 27 pediatric patients found the average number of contaminating T and B cells to be low, with a median 49 × 10³ CD3+/CD2−/kg recipient body weight (range 7-200 × 10³/kg) and median 20 × 10³ CD19+/CD2−/kg (range: 2-47 × 10³/kg). In contrast, large numbers of CD34+ cells median 16 × 10³/kg, range: 7-41 × 10³/kg), CD56+/CD3− NK cells (median 137 × 10³/kg, range: 9-550 × 10³/kg), and myeloid cells (median 620 × 10³/kg, range: 51-1345 × 10³/kg) were infused in the graft. The myeloid cell compartment consists of large numbers of monocytes, various numbers of dendritic cells, including myeloid and plasmacytoid dendritic cells, and other not yet clearly defined committed precursor cells.

CD3/19-depleted haploidentical PBSCs were transplanted after RIC in 27 patients with various
malignant and nonmalignant diseases treated at the Children's University Hospital Tübingen. The conditioning regimen consisted of Mephalan (140 mg/m²), Thiotepa (10 mg/kg), Fludarabine (160 mg/m²), and the murine anti-CD3 antibody OKT-3 and MMF. Primary engraftment was seen in 23 patients (85%), whereas 4 patients rejected the graft (15%). However, all 4 patients could be rescued by a second transplant using the other haploidentical donor and another RIC regimen including total nodal irradiation, and the final engraftment rate was 100%. A rapid engraftment was seen with a median time to reach $>0.5 \times 10^9$/L neutrophils of 10 days (range: 9-12 days) and a median time to reach independency from platelet transfusion of 8 days (range: 6-188 days). Only 1 patient out of the 27 (4%) died from transplant-related toxicity.

The HLA disparity of donor and recipients allows a simple and detailed analysis of the engraftment status of various lymphocyte and myeloid cell populations [27]. Donor-derived cells can be easily identified using antibodies specific to the disparate HLA. The flow cytometric chimerism analysis is rapid and the results are obtained the same day. Especially in situations with increasing recipient-derived T cell populations, rapid action, such as decrease or stop of immunusuppression or additional donor lymphocyte infusion can be taken.

To determine the functional status of the NK cells in the grafts, the NK activity was measured in 11 donors after mobilisation with granulocyte-colony stimulating factor (G-CSF). As shown in Figure 3, the NK activity of the CD3/19 depleted PBSCs against the K562 standard target cell line is rather moderate, but can be induced by an overnight incubation with IL-2 and most effectively by IL-15. Because IL-15 consistently was most effective stimulating the NK cells in CD3/19-depleted grafts, this approach was used for

**Figure 2.** Flow cytometric analysis of CD34$^+$ positively selected stem cells and CD3/19 negatively depleted PBSCs. Although the CD34$^+$ population is homogenous without any contaminating non-T/B-cells, the CD3/19 depleted PBSC’s contain large numbers of non-T/B-cells, including CD34$^+$ stem cells, NK cells, and myeloid cells.
the large-scale generation and in vivo application of large numbers of haploidentical NK cells using mobilized PBSCs. An aliquot from the CD3/19-depleted PBSCs was incubated overnight with 10 ng/mL IL-15. NK activity of mobilized CD3/19 depleted grafts of 6 donors before and after stimulation with IL-15 increased on average 2- to 3-fold over baseline. Such IL-15-activated NK cells were infused in 6 patients after extensive washing steps to remove IL-15. The median number of activated and infused CD56$^+$ NK cells was $35 \times 10^6$/kg recipient body weight. The infusions were well tolerated and no side effects were observed. To test whether incubation of the CD3/19-depleted PBSCs would influence the proliferative response of the NK cells, the cells were extensively washed and incubated with various stimuli. IL-15 preactivated cells showed a much higher proliferative response to IL-2 compared to nonactivated cells, whereas the proliferative responses to other stimuli (PHA, PWM, Prot-A, OKT-3, ConA) were less impressive.

These studies suggest that the early posttransplant treatment with low dose IL-2 should induce an extensive in vivo proliferation of IL-15-activated NK cells coinfused together with the stem cells at day 0. Previous studies demonstrated that low-dose subcutaneous IL-2 (1-2 $\times 10^6$ IU/m$^2$ 3 times a week) post-T cell-depleted allogeneic transplantation was well tolerated without major side effects and without increase of the risk of GVHD. In addition, there was a significant increase of the NK activity a few weeks after start of the IL-2 treatment. Therefore, clinical studies using ex vivo activation of CD3/19-depleted PBSC-containing NK cells with IL-15 and early in vivo posttransplant treatment with IL-2 are under preparation.

In the future, haploidentical transplantation using RIC and CD3/19-depleted mobilized PBSCs may be viewed as a platform for further immunotherapeutic strategies to enhance engraftment and to increase the antimalignancy effect of the graft exerted by NK cells. The mobilization regimen might have an influence on the NK activity and other mobilization strategies, such as the combination of G-CSF/granulocyte macrophage-colony stimulating factor (GM-CSF) or the combination of growth factors with CXCR4-inhibitors can be envisioned. The T cell depletion methods can be improved, and we have already demonstrated that the depletion of $\alpha/\beta$-positive T-lymphocytes leads to a more efficient T cell depletion compared to CD3-depletion [28]. Such grafts would contain large numbers of NK cells and $\gamma/\delta$-T cells, which do not cause GVHD and might exert significant antileukemic effects[29,30]. These methods could be combined with posttransplant therapeutic strategies to further augment the cytotoxicity of the effector cells, which are cotransfused with T cell-depleted PBSCs. Such an approach could be the early posttransplant treatment with low dose IL-2 or other immune-augmenting substances. Further studies are necessary to determine the clinical importance of other cell populations in such T cell-depleted PBSCs, such as dendritic or committed progenitor cells. Thus, the T cell-depletion approach offers more graft-manipulating and immune-augmenting opportunities compared to the CD34$^+$ positive selection approach. Future

Figure 3. The NK activity of PBSCs without and with overnight stimulation with IL-2 and IL-15 against the standard target cell line K562 is shown.

Biol Blood Marrow Transplant 16:S68-S74, 2010

Selective Depletion of Haploidentical Grafts

S73
clinical trials employing such strategies are warranted to further improve the outcome of haploidentical transplantation.

ACKNOWLEDGMENTS

Financial disclosure: The authors have nothing to disclose.

REFERENCES