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Differences in the Phenotype, Cytokine Gene Expression Profiles, and In Vivo Alloreactivity of T Cells Mobilized with Plerixafor Compared with G-CSF


Plerixafor (Mozobil) is a CXCR4 antagonist that rapidly mobilizes CD34+ cells into circulation. Recently, plerixafor has been used as a single agent to mobilize peripheral blood stem cells for allogeneic hematopoietic cell transplantation. Although G-CSF mobilization is known to alter the phenotype and cytokine polarization of transplanted T cells, the effects of plerixafor mobilization on T cells have not been well characterized. In this study, we show that alterations in the T cell phenotype and cytokine gene expression profiles characteristic of G-CSF mobilization do not occur after mobilization with plerixafor. Compared with non-mobilized T cells, plerixafor-mobilized T cells had similar phenotype, mixed lymphocyte reactivity, and Foxp3 gene expression profiles characteristic of G-CSF mobilization do not occur after mobilization with plerixafor. Compared with non-mobilized T cells, plerixafor-mobilized T cells had similar phenotype, mixed lymphocyte reactivity, and Foxp3 gene expression levels in CD4+ T cells, and did not undergo a change in expression levels of 84 genes associated with Th1/Th2/Th3 pathways. In contrast with plerixafor, G-CSF mobilization decreased CD62L expression on both CD4 and CD8+ T cells and altered expression levels of 16 cytokine-associated genes in CD3+ T cells. To assess the clinical relevance of these findings, we explored a murine model of graft-versus-host disease in which transplant recipients received plerixafor or G-CSF mobilized allograft from MHC-matched, minor histocompatibility–mismatched donors; recipients of plerixafor mobilized peripheral blood stem cells had a significantly higher incidence of skin graft-versus-host disease compared with mice receiving G-CSF mobilized transplants (100 versus 50%, respectively, \( p = 0.02 \)). These preclinical data show plerixafor, in contrast with G-CSF, does not alter the phenotype and cytokine polarization of T cells, which raises the possibility that T cell–mediated immune sequelae of allogeneic transplantation in humans may differ when donor allografts are mobilized with plerixafor compared with G-CSF. The Journal of Immunology, 2013, 191: 000–000.

For adults with malignancies, mobilized peripheral blood progenitor cells (PBPC) are the preferred source of hematopoietic stem cells (HSCs) for autologous transplants and matched sibling allogeneic transplants because PBPCs engraft faster than bone marrow–derived progenitor cells and are technically easier to collect (1). The administration of G-CSF results in successful mobilization of PBPCs in most healthy donors. Although most regimens using G-CSF succeed in collecting adequate numbers of peripheral blood stem cells (PBSCs) from healthy donors, 5–10% of patients will mobilize poorly, necessitating multiple large-volume apheresis or bone marrow harvesting (2). Although G-CSF is generally well tolerated in healthy donors, it frequently causes bone pain, headache, myalgia, and in rare circumstances, life-threatening adverse effects such as stroke, myocardial infarction, and splenic rupture (3). The number of lymphocytes contained in G-CSF mobilized PBPC grafts is ~10–15 times higher than BM grafts (4, 5). Despite containing substantially higher numbers of T cells, several prospective, randomized trials have shown that the incidence of acute graft-versus-host disease (GVHD) is not higher in recipients of G-CSF mobilized PBPC allografts compared with BM allografts (4, 6–10). Although a number of mechanisms have been postulated to account for this phenomenon, alterations in cytokine-related genes in T cells mobilized with G-CSF including a polarization toward a type 2 cytokine (Th2) response is thought to play a pivotal role in reducing the ability of T cells to trigger acute GVHD (11–14). The distinct cytokine production profile of Th2 cells has also been shown in animal models to contribute to the pathogenesis of chronic GVHD, which has been shown in randomized trials to occur more commonly with G-CSF mobilized transplants compared with bone marrow transplants.

Plerixafor (Mozobil) is a bicyclam compound that inhibits the interaction of stromal cell–derived factor-1 (SDF-1) with its cognate receptor CXCR4 (15, 16). CXCR4 is present on CD34+ hematopoietic progenitor cells, and its interaction with SDF-1 plays...
a pivotal role in the homing of CD34+ cells in the bone marrow (17, 18). Inhibition of CXCR4–SDF-1 binding by plerixafor releases CD34+ cells into the circulation, allowing them to be collected easily by apheresis (19). The addition of plerixafor to G-CSF has a potent synergistic effect on the mobilization PBPCs in patients undergoing autologous transplantation. Plerixafor is approved in the United States in combination with G-CSF for hematopoietic stem cell mobilization and collection for subsequent autologous transplantation in non-Hodgkin’s lymphoma and multiple myeloma patients.

Plerixafor given as a single agent can also mobilize substantial numbers of hematopoietic progenitor cells into the circulation. A phase one, dose-escalating study in healthy donors demonstrated that large numbers of CD34+ cells were rapidly mobilized in healthy donors after a single s.c. injection of plerixafor with the peak CD34+ mobilizing effects occurring 6–9 h after 240 µg/kg plerixafor in the absence of any dose-limiting toxicities (20). Importantly, side effects were mild and transient, and did not include bone pain, fever, and myalgias commonly observed after G-CSF administration. Based on these data, investigators have recently begun to explore the use of single-agent plerixafor to mobilize PBPCs for allogeneic transplantation, although its use in this setting remains investigational. The first published study to use plerixafor alone to mobilize PBPCs for allogeneic transplantation showed successful mobilization of CD34+ cells in healthy stem cell donors and sustained trilineage hematopoietic reconstitution in 14 transplant recipients of plerixafor mobilized allografts (21). Leukapheresis products mobilized with plerixafor contained a higher number of CD3+ T cells compared with G-CSF, and in a limited analysis performed on four donors did not appear to alter the phenotype of mobilized T cells. Because of its potential use as a single mobilization agent in this setting, we performed a detailed analysis of the phenotype, cytokine gene expression profiles, and immunological properties of T cells mobilized with plerixafor in healthy donors. Furthermore, we used an MHC-matched model of allogeneic PBSC transplantation to assess whether the phenotypic and genotypic differences observed in T cells mobilized with plerixafor compared with G-CSF would impact transplant outcome.

Materials and Methods

Study design

Eleven healthy subjects had PBMCs collected at baseline and then were mobilized with 5 daily doses of s.c. G-CSF (10 µg/kg daily; National Heart, Lung and Blood Institute [NHLBI] protocols 04-H-0078, 99-H-0050, and 99-H-0064) to analyze G-CSF mobilized cells. Two hours after the fifth dose of G-CSF, subjects had PBMCs collected from the blood followed by a 15- to 25-l apheresis procedure to collect mobilized cells. Twenty healthy subjects underwent plerixafor mobilization on NHLBI protocols 04-H-0078 and 06-H-0179 to analyze plerixafor-mobilized cells. PBMCs were collected at baseline and then 6 h after a single s.c. injection of plerixafor at a dose of 240 µg/kg (n = 12). Eight of these subjects also underwent a 15- to 25-l apheresis beginning 6 h after plerixafor administration. Freshly collected apheresis products were analyzed by flow cytometry for cellular content. Cells were frozen in 10% DMSO for subsequent phenotypic, genotypic, and in vitro and in vivo functional studies. Leukapheresis procedures were performed using the model CS-3000 Plus (Baxter Healthcare, Deerfield, IL) continuous-flow cell separator. Complete blood counts were assayed using an electronic counter, and CD34, CD3, and other cell counts were enumerated by flow cytometry, immediately before and after apheresis, as previously described (23).

Phenotypic analysis of cells

Six-color flow cytometry was used to analyze mononuclear cells obtained from apheresis products or from peripheral blood after mobilization, as well as from premobilization blood and blood obtained immediately before initiation apheresis. Peripheral blood was drawn by venipuncture into tubes containing heparin and stained within 8 h of collection. A 100-µl aliquot of whole blood was incubated for 30 min at 4°C with Abs. Each experiment was performed using the appropriate isotype controls. After incubation, erythrocytes in these samples were lysed (Whole Blood Lysing Reagent; Coulter, Fullerton, CA), washed twice, and then fixed with paraformaldehyde. All samples were analyzed by using a CYAN MLE cytometer (DakoCytomation, Ft. Collins, CO). Summit software (Dako-Cytomation) was used for data acquisition and analysis. Mixtures of six fluorochrome-labeled Abs were used in the same tube to assess the phenotype in different cell populations. CD3-PE-Cy7, CD4-allophycocyanin-Cy7, CD3-PerCP, CD16- allophycocyanin-Cy7, CD56-PE-Cy5, CD19-allophycocyanin, and CD34-FTTC were used to gate T cell, B cell, NK cell, and hematopoietic progenitor cell populations. CD57-FTTC, CD27-FTTC, CD62L-FTTC, HLA-DR–FTTC, CD45RA-FTTC, pan-TCRg-FTTC, CD38-FTTC, CD69-FTTC, CXCR4-PE, CD25-PE, CCR7-PE, CD71-PE, CD45RO-PE, and pan-TCRab-PE were used to assess the phenotype of CD4+ T cells and CD8+ T cells, respectively. PBMCs were purchased from BD Biosciences (San Diego, CA) except for pan-TCRg-FTTC and pan-TCRab-PE (Coulter, Fullerton, CA).

Assessment of Th1, Th2, and Th3 cytokine gene expression levels in donors mobilized with plerixafor versus G-CSF

Gene expression analysis was performed on CD3+ and CD4+ T cells obtained from the blood or apheresis collections of healthy subjects at baseline and after mobilization with 240 µg/kg plerixafor or G-CSF alone (10 µg/kg/day × 5 d). Fresh samples were Ficolled (LSM; MP Biomedicals), and CD3+ cells were isolated on fresh and thawed samples following manufacturers’ instructions using Human Pan T Cell Isolation Kit II (Miltenyi). CD4+ T cell fractions were positively selected following manufacturers’ instructions using CD4 Microbeads (Miltenyi). Cryopreserved specimens were thawed in a 37°C water bath, washed with 1× RPMI 1640 and 10% heat inactivated FBS, and then were treated with DNsase (Roche) for 30 min at 37°C with the CD3+ and CD4+ T cell fractions being isolated using the same procedure outlined earlier.

RNA was isolated from CD3+ and CD4+ T cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Isolated CD3+ RNA was sent to SuperArray Bioscience Corporation where it was reverse transcribed and analyzed in quantitative real-time PCRs using the Syber Green RT® Profiler PCR Array Human Th1-Th2-Th3 plates. Baseline and postmobilization samples were analyzed by using the appropriate isotype controls. After incubation, RNA (1 µg) was obtained from purified CD4+ T cells. RNA was DNase-treated before reverse transcription using SuperScriptIII Reverse Transcriptase (Invitrogen). Five microliters of cDNA was added to a final volume of 25 µl containing 1× Quantitech Multiplex PCR Kit (Qiagen), 0.4 µM of each forward and reverse primers for actin and Foxp3, and 0.2 µM of each probe. Actin and Foxp3 primer and probe sequences were previously published (24, 25). All samples were run in triplicates on a Peltier Thermal Cycler PTC-200 (MJ Research) with a Chromo4 Continuous Fluorescence Detector 4. Thermal cycling conditions consisted of 95°C for 10 min followed by 94°C for 1 min and 60°C for 1 min (45 cycles). The expression of Foxp3 was normalized to the actin levels within each individual sample, and the fold change from baseline was calculated for each healthy subject.

Mixed lymphocyte reaction

The alloreactivity of mobilized cells collected at baseline and after plerixafor mobilization were tested in an MLR by [3H]thymidine uptake and CFSE dilution assays. Pooled and irradiated PBMCs from three healthy volunteers were used as stimulators. For the thymidine uptake MLR, 5 × 104 responder mononuclear cells in 100 µl culture media were coincubated with 5 × 104 numbers of irradiated, non-stimulated responder cells and were pulsed with [3H]thymidine (Amersham Pharmacia Biotech) at 1 µCi/well. After an additional 18-h incubation, the cells were harvested and thymidine incorporation into DNA was quantified using a beta counter (Beckman Coulter). The results are reported as cpm. For the CFSE dilution assay MLR, responder cells were incubated in prewarmed working concentrations of CFSE (Molecular Probes, Eugene, OR) in Dulbecco’s PBS (0.6 µM) for 15 min at 37°C. The cells were then washed and resuspended in fresh, serum-free RPMI 1640, then were incubated with equal numbers of irradiated (50 Gy) stimulator cells for 5 d, and thereafter harvested and stained with propidium iodide, CD3-PerCP, and CD25-allophycocyanin (Molecular Probes). Four-color flow cytometry was performed on a FACSCalibur dual-laser cytometer (Becton Dickinson, Mountain View, CA). The precursor frequency of alloreactive T cells was estimated using FlowJo analysis software (Tree Star, Ashland, OR).

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MHC-matched murine transplantation model

Animals. Eight- to 12-wk-old BALB/c (H-2\(^b\), Ly9.1\(^-\), 7/4\(^-\)), B10.d2 (H-2\(^d\), Ly9.1\(^-\), 7/4\(^-\)), and C57BL/6 (H-2\(^d\)) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in microisolator cages at NHLBI pathogen-free facilities. Transplanted animals were provided acidified water containing neomycin. All experiments were approved by the NHLBI animal care and use committee (protocol no. NB-0112).

Mobilization. B10.d2 donor mice received either G-CSF (Amgen, Thousand Oaks, CA) given as s.c. injections daily \(5\) d (10 \(\mu\)g/mouse/day) or plerixafor (Genzyme, Cambridge, MA) given as a single s.c. injection (100 \(\mu\)g/mouse) to mobilize PBSCs. Mobilized PBSCs were collected from spleens harvested 6 h after the last injection for both groups. Spleens were also harvested from B10.d2 donor mice received saline (HBSS) injections as controls.

Flow cytometry. The phenotype of mobilized cells obtained from spleens (6 h after last injection) was evaluated after staining with fluorochrome-conjugated anti-CD3, CD4, CD8, CD19, DX5, Gr1, sca-1, c-Kit, CD25, and CD62L (BD Pharmingen) for 10 min at room temperature before incubation with rat anti-mouse CD16/CD32 (BD Pharmingen) and 7/4-PE (Cedarlane, Hornby, ON). For all flow cytometric staining assays, cells were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen) and 7/4-PE (Cedarlane, Hornby, ON). All flow cytometric staining profiles were performed according to manufacturer’s instructions (eBioscience, San Diego, CA). Regulatory T cells (Tregs) were determined by positive staining for CD4 and Foxp3. To measure lymphoid and myeloid chimerism, we stained peripheral blood collected after transplantation with Ly9.1-FITC, CD3-PerCP, and CD19-allophycocyanin (BD Pharmingen) and 7/4-PE (Cedarlane, Hornby, ON). For all flow cytometric staining assays, cells were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen) for 10 min at room temperature before incubation with specific Abs. Cells were acquired on FACSCalibur (BD Biosciences) and analyzed using Summit Software v3.3 (Dako Cytomation, Glostrup, Denmark). FCS export software (DeNovo, Thornhill, ON).

Proliferation and suppression assays. CD4\(^+\) T cells were selected using Miltenyi immunomagnetic beads from the spleens of mobilized animals and then cocultured (50,000/well) with MHC-mismatched irradiated (50 Gy) Con A blasts (range 8000–500,000 cells) from C57BL/6 mice in 96-well plates in DMEM supplemented with 10% FCS, 2-ME (Invitrogen, Life Technologies, Carlsbad, CA). After a 72- to 96-h culture, cells were pulsed with 1 \(\mu\)Ci/well [\(\text{H}\)]thymidine for 16 h and then were harvested onto solid filters and measured in a microbeta scintillation counter (Perkin Elmer, Wellesley, MA). To examine whether plerixafor mobilization altered the cytokine polarization of T cells, we analyzed cytokine gene expression profiles using a Th1-Th2-Th3 RT-PCR plate in CD3\(^+\) T cells collected from subjects mobilized with a single injection of plerixafor or 2 h after the fifth dose of G-CSF.

Results

Mobilization with plerixafor in healthy subjects

Apheresis products were collected from eight healthy subjects mobilized with a single 240 \(\mu\)g/kg injection of plerixafor. Relative to the weight of the subjects mobilized, apheresis collections after plerixafor mobilization (median 19.6 l apheresed; range 15–22 l) contained a median 81 \(\times\) 10\(^6\) CD19\(^+\) B cells/kg, a median 274 \(\times\) 10\(^6\) CD3\(^+\) T cells/kg, and a median 1.6 \(\times\) 10\(^5\) CD34\(^+\) cells/kg (Table I). Plerixafor preferentially mobilized CD34\(^+\) cells followed by monocytes and lymphocytes (Fig. 1A). Within the lymphocyte compartment, B cells were preferentially mobilized followed by T cells and NK cells. Among CD19\(^+\) B cells, CD20, k, and \(\lambda\) expression did not change from baseline, although the percentage of B cells expressing CD27 declined significantly in seven of eight donors, consistent with plerixafor preferentially mobilizing naive type B cells; the median percentage of CD27\(^+\)CD19\(^+\) B cells was 35.1% at baseline and 19% after plerixafor mobilization \((p = 0.011)\). The total WBC count and the absolute numbers of blood neutrophils, monocytes, lymphocytes, and CD34\(^+\) cells increased significantly from baseline after plerixafor administration (Fig. 1B–F). A detailed phenotypic analysis using six-color flow cytometry of CD4\(^+\) and CD8\(^+\) lymphocyte subsets at baseline and 6 h after a single injection of plerixafor or 2 h after the fifth dose of G-CSF is shown in Table II. No significant change from baseline was observed after mobilization with plerixafor in the percentage of CD4\(^+\) and CD8\(^+\) T cells expressing the majority of surface markers analyzed including CD45RA, CD45RO, CD34, CD56, CD57, CD27, CD71, and CD62L. Although the phenotype also did not change after G-CSF mobilization in most CD4\(^+\) and CD8\(^+\) T cell populations, there was a significant decline in the percentage of CD4 and CD8 T cells that expressed CD62L and in CD8 T cells that expressed CD27 (Table II).

Impact of plerixafor on cytokine gene expression profiles in T cells

To examine whether plerixafor mobilization altered the cytokine polarization of T cells, we analyzed cytokine gene expression profiles using a Th1-Th2-Th3 RT-PCR plate in CD3\(^+\) T cells collected from subjects mobilized with a single injection of plerixafor versus CD34\(^+\) cells collected from subjects mobilized with five daily

<table>
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<th>Age (y)</th>
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<th>Weight (kg)</th>
<th>Apheresis Volume (l)</th>
<th>Total T Cells/kg (10(^6))</th>
<th>Total B Cells/kg (10(^6))</th>
<th>Total CD34(^+) Cells/kg (10(^5))</th>
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doses of G-CSF alone. None of the 84 cytokine genes examined was significantly altered from baseline in CD3+ cells mobilized with plerixafor. In contrast, 16 of 84 (19%) cytokine-related genes were significantly altered from baseline in CD3+ cells after G-CSF mobilization (Fig. 2).

Impact of plerixafor on T cell alloreactivity
The impact of plerixafor mobilization on T cell alloreactivity was assessed by two different MLR methods. Compared with baseline, plerixafor mobilized mononuclear cells had slightly decreased alloreactivity against third-party cells using a standard 5-d ¹H MLR assay (Fig. 3A). Alloreactivity assessed using a CFSE MLR proliferation assay gated on CD3+ T cells showed plerixafor mobilized T cells had a statistically nonsignificant decrease in precursor frequency against third-party allogeneic stimulators as compared with T cells at baseline (Fig. 3B). A similar decline in alloreactivity against third-party allogeneic cells was observed in mononuclear cells after G-CSF mobilization as assessed by the 5-d ¹H MLR assay (Fig. 3C) or CFSE (Fig. 3D).

Impact of plerixafor on Foxp3 gene expression in CD4+ T cells
In murine transplant studies, Tregs transplanted with allogeneic PBPCs appear to reduce the risk for GVHD. In humans undergoing allogeneic transplantation, an inverse correlation between GVHD and Foxp3 mRNA expression has been reported (29). Therefore, we analyzed FOXP3 expression by RT-PCR in CD4 cells isolated from healthy subjects after mobilization with plerixafor versus G-CSF. Compared with baseline, no statistically significant changes in Foxp3 gene expression were observed in CD4+ T cells mobilized with either plerixafor or G-CSF (Fig. 4).

Mobilization of murine lymphocyte subsets after plerixafor versus G-CSF administration
Mobilized PBSCs were collected from spleens of mice harvested 6 h after the last injection of G-CSF versus plerixafor. Compared with HBSS (saline) controls, there was a trend toward an increase in the absolute number of monocytes (p = 0.09) and lymphocytes (p = 0.09) in the blood after plerixafor mobilization (Fig. 5A). The absolute number of circulating lymphocytes in the blood after G-CSF mobilization was similar to plerixafor mobilization (p = 0.68), although there was a trend toward a higher number of monocytes being mobilized with plerixafor compared with G-CSF (p = 0.07). Both mobilizing agents increased absolute circulating granulocyte numbers, but granulocyte numbers in the blood after G-CSF mobilization were significantly higher compared with saline controls (p = 0.02) and plerixafor mobilized mice (p = 0.007). Similarly, absolute numbers of c-Kit+/Lin-/sca-1+ (KLS) cells were highest in spleens of G-CSF–treated mice. Spleens of G-CSF–treated mice contained a 3.6-fold and a 1.7-fold higher number of KLS cells compared with HBSS controls (p = 0.002) and plerixafor-treated mice (p = 0.07), respectively. Spleens from plerixafor–treated mice contained 2.2-fold higher numbers of KLS cells compared with HBSS controls (p = 0.02).

Compared with saline controls, spleen sizes appeared grossly similar after plerixafor mobilization and were larger after G-CSF mobilization (Supplemental Fig. 1A). There was an ~2-fold increase in the number of cells contained within the spleen after mobilization with G-CSF compared with saline controls (p < 0.001). Splenocyte cell numbers after plerixafor also increased significantly (p = 0.04) compared with saline controls, although this increase was smaller than that observed with G-CSF (Fig. 5A). The higher number of splenocytes observed in G-CSF mobilized spleens was mostly due to an increase in the number of splenic granulocytes.

To identify cellular phenotypic differences that might impact GVHD and graft-versus-leukemia, we harvested and stained mobilized splenocytes with cell-specific differentiation markers. The percentages of splenocytes expressing T cell, NK cell, B cell, and granulocyte markers after plerixafor mobilization were similar to saline control–injected animals (Table III). In contrast, animals mobilized with G-CSF had a significantly higher percentage of splenic granulocytes, a significantly lower percentage of CD4+ T cells and B cells, and a similar number of CD8+ T cells compared with plerixafor–injected animals. There was no difference in the percentage of regulatory CD4+Foxp3+ T cells mobilized between any of the groups (Table III). Furthermore, CD4+ T cells mobilized with plerixafor had similar proliferation against MHC-mismatched cells as was observed with CD4+ T cells mobilized...
with G-CSF or CD4+ T cells obtained from saline controls (Fig. 5B). Similar to human T cells, a decline in CD62L expression was observed on murine T cells after mobilization with G-CSF (Supplemental Fig. 1B).

Comparison of transplant outcome in a murine MHC-matched transplantation model using allografts mobilized with plerixafor versus G-CSF

BALB/c recipient mice were conditioned with total body irradiation and then were transplanted i.v. with $10^6$ splenocytes after mobilization with G-CSF versus plerixafor. To compensate for the higher CD4+ T cell numbers in the spleens of plerixafor mobilized mice, we also conducted experiments in which transplant recipients had the lymphocyte dose adjusted to $2 \times 10^6$ CD4+ T cells combined with $13 \times 10^6$ CD8+ splenocytes mobilized with either G-CSF or plerixafor.

Lymphoid and myeloid chimerism assessed 30 d after transplantation by flow cytometry was >90% donor in origin in BALB/c recipients of G-CSF ($n = 6$) and plerixafor mobilized splenocytes ($n = 6$). The incidence of skin GVHD in recipients of plerixafor and saline mobilized splenocytes was comparable. In contrast, recipients of G-CSF mobilized splenocytes had a reduced incidence of skin GVHD compared with recipients of either plerixafor or saline mobilized splenocytes ($p = 0.02$, Fisher test; $p < 0.05$, log-rank test; Fig. 5C). Furthermore, recipients of G-CSF mobilized splenocytes had significantly less weight loss on day 15 post-transplantation compared with mice transplanted with HBSS

![FIGURE 2.](image)

![FIGURE 3.](image)

| Table II. Effect of plerixafor or G-CSF mobilization on circulating T cell subsets |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Baseline | After Plerixafor | $p^*$ | Baseline | After G-CSF | $p^*$ |
| CD3+CD4+ T cells |          |                 |       |          |             |       |
| CD45RA+         | 45.0 ± 8.2 | 50.8 ± 12.4 | 0.1   | 41.8 ± 10.5 | 31.0 ± 4.5 | 0.06 |
| CD45RO+         | 49.4 ± 9.3 | 45.3 ± 10.3 | 0.06  | 57.0 ± 14.3 | 58.4 ± 5.8 | 0.26 |
| CD57+           | 3.3 ± 3.3  | 2.4 ± 1.9  | 0.28  | 5.7 ± 4.8  | 6.5 ± 9.8  | 0.50 |
| CD27+           | 87.7 ± 6.8 | 81.6 ± 17.6 | 0.42  | 70.5 ± 25.9 | 57.3 ± 31.4| 0.20 |
| CD25+           | 16.2 ± 11.1| 11.7 ± 10.0 | 0.1   | 23.9 ± 19.1| 10.6 ± 7.1 | 0.20 |
| CD62L+          | 34.1 ± 32.4| 27.4 ± 32.7 | 0.1   | 67.8 ± 15.2| 29.5 ± 22.9| 0.03 |
| CD71+           | 1.4 ± 0.9  | 1.8 ± 1.5  | 0.55  | 0.6 ± 0.4  | 1.2 ± 0.8  | 0.31 |
| HLA-DR+         | 5.3 ± 1.6  | 3.8 ± 1.2  | 0.1   | 5.8 ± 1.9  | 4.4 ± 2.1  | 0.40 |
| CD3+CD8+ T cells |          |                 |       |          |             |       |
| CD45RA+         | 58.2 ± 24.5| 65.5 ± 23.9 | 0.11  | 55.2 ± 12.2| 51.4 ± 3.2 | 0.79 |
| CD45RO+         | 43.0 ± 21.0| 34.9 ± 17.8 | 0.09  | 49.3 ± 13.3| 46.6 ± 4.4 | 0.13 |
| CD57+           | 7.0 ± 3.3  | 5.0 ± 1.7  | 0.09  | 22.0 ± 17.4| 21.9 ± 14.0| 0.41 |
| CD27+           | 86.4 ± 10.4| 83.8 ± 22.2 | 0.71  | 65.0 ± 9.7 | 50.2 ± 13.0| 0.03 |
| CD25+           | 4.0 ± 3.2  | 1.3 ± 1.1  | 0.12  | 5.5 ± 6.5  | 4.6 ± 3.7 | 0.58 |
| CD62L+          | 32.0 ± 33.7| 29.6 ± 37.1 | 0.39  | 47.1 ± 14.1| 16.4 ± 12.4| 0.01 |
| CD71+           | 0.7 ± 0.4  | 0.7 ± 0.36 | 0.88  | 0.5 ± 0.3  | 0.8 ± 0.9 | 0.65 |
| HLA-DR+         | 8.2 ± 3.3  | 4.9 ± 2.1  | 0.08  | 14.9 ± 11.3| 8.6 ± 6.3 | 0.27 |

Values are reported as percent of total CD4+ or CD8+ T cells.

*Comparison of baseline and postplerixafor or G-CSF on T cell subsets. The $p$ values were calculated as paired $t$ test.
mobilized splenocytes ($p = 0.002$) and mice transplanted with plerixafor mobilized splenocytes ($p = 0.04$). When the number of transplanted CD4$^+$ T cells was normalized to $2 \times 10^6$ cells across all 3 groups, recipients of G-CSF mobilized splenocytes still had a significantly lower incidence (7/15 recipients developed GVHD within day +60; $p < 0.05$) of skin GVHD compared with plerixafor (16/17 recipients developed GVHD within day +60) or saline mobilized mice (16/17 recipients developed GVHD within day +60). Furthermore, total clinical GVHD scores were significantly lower ($p < 0.05$) in recipients of G-CSF mobilized splenocytes ($1.6 \pm 0.3$) compared with recipients of plerixafor ($2.9 \pm 0.5$) or saline mobilized splenocytes ($2.7 \pm 0.4$).

**Discussion**

Although G-CSF mobilization is known to alter the phenotype and cytokine polarization of transplanted T cells, the effects of plerixafor mobilization on T cells have not been well characterized. This study provides, to our knowledge, the first detailed analysis of both phenotypic and functional properties of lymphocytes mobilized with plerixafor. In this study, we show that alterations in T cell phenotype and cytokine gene expression profiles characteristic of G-CSF mobilization do not occur after mobilization with plerixafor.

G-CSF and plerixafor mobilized a similar number of T cells into circulation. As reported by others, we observed the expression of CD62L on T cells to decline after G-CSF mobilization (30). In contrast, we observed no change in T cell phenotypic markers including CD62L expression after plerixafor mobilization. Normally, CD62L$^+$ T cells are effector memory cells; in contrast with CD62L$^-$ T cells, transplantation of CD62L$^-$ T cells does not appear to cause GVHD in murine models (31). The impact of transplanting higher numbers of CD62L$^+$ T cells using allogeneic grafts is unknown. Although animal models might suggest the incidence of GVHD could increase, it is important to consider that CD62L expression on CD3$^+$ T cells alone may not reflect the true subtype of T cells. Studies have also shown that serum-soluble 1-selectin levels are increased after G-CSF mobilization (32) and that some CD62L loss can be reversed with in vitro incubation (30). Based on this observation, it is possible that the lower levels of CD62L found on G-CSF mobilized T cells occur as a consequence of shedding, rather than reflecting preferential mobilization of an effector type T cell. Although no kinetic studies on CD62L status were performed in our study, CD62L shedding after G-CSF treatment has previously been established to be a temporary phenomenon. Importantly, after mobilization with either plerixafor versus G-CSF, no differences in the percentage of T cells expressing CD45RA or CCR7, markers of naive (both) and central memory (CCR7) cells, were observed. Although the expression of CD27 on plerixafor mobilized T cells did not change, a significant

**FIGURE 4.** The impact of mobilization on CD4$^+$ Foxp3 expression. Foxp3 expression after mobilization compared with baseline in CD4$^+$ cells mobilized with plerixafor or G-CSF showed no fold-change in expression from baseline in healthy subjects mobilized with either plerixafor or G-CSF alone. Line represents the median change from baseline in 13 samples obtained after plerixafor mobilization and 7 samples obtained after G-CSF mobilization.

**FIGURE 5.** Animal transplant model. Donor B10.d2 mice were injected s.c. with either 10 μg G-CSF daily for 5 d, one injection of 100 μg plerixafor, or saline control (HBSS). (A) Number of splenocytes and complete blood counts were obtained from G-CSF and plerixafor mobilized donors. (B) The alloreactivity of donor B10.d2 splenocytes in response to Con A blasts derived from C57BL/6 donors was assessed by MLR and showed no significant differences between the three groups. (C) Incidence of skin GVHD in recipients receiving $15 \times 10^6$ mobilized total unfractionated splenocytes ($p < 0.05$, G-CSF compared with HBSS controls).
significantly in CD3+ T cells after G-CSF mobilization, although

tiations. In our analysis, GATA3 levels in T cells did not change

Th2-type phenotype characterized by increased expression of IL-4

we did observe a small reduction in responsiveness to allogeneic

(T cells mobilized with plerixafor did not display any changes in

incidence of GVHD observed in this cohort.

G-CSF altered a number of cytokine-related genes in mobilized

CD8+, but not CD4+, T cells. The functional consequence of

ne with higher numbers of CD27−/CD8 T cells using allogeneic grafts is

unknown. CD27 binds CD70 and is required for generation and

long-term maintenance of T cell immunity, and was recently shown
to augment CD8+ T cell activation (33, 34). One might therefore

speculate that allografting of G-CSF mobilized CD8+ T cells

results in reduced GVHD.

G-CSF mobilization has been shown to skew T cells toward a

Th2-type phenotype characterized by increased expression of IL-4

and IL-10, and decreased IFN-γ production. Th2-type T cells are

thought to be associated with a lower risk for causing acute GVHD

but may play a part in contributing to the increased incidence of

chronic GVHD, which occurs with the use of G-CSF mobilized

allografts compared with bone marrow transplants. We found that

T cells mobilized with plerixafor did not display any changes in

Th1- and Th2-type cytokines after nonspecific mitogen stimulation

(data not shown). However, similar to G-CSF mobilized T cells, we

did observe a small reduction in responsiveness to allogeneic

stimuli in vitro by [3H]thymidine uptake MLR in plerixafor mo-

bilized T cells. Only minor changes in serum levels of IL-4, IL-10,

and IFN-γ were found in mice receiving G-CSF compared with

HBSS-treated controls. However, we did observe significant de-

crease in serum levels of IL-8 in donors mobilized with G-CSF

(data not shown). Similar to this observation, investigators have

previously reported that IL-8 levels decline in patients with esoph-
ageal cancer after treatment with G-CSF (35). rIL-8 is known to

directly suppress the spontaneous production of IL-4 by CD4+ T

cells (36). Taken together, these data suggest that G-CSF–mediated

reductions in serum levels of IL-8 may lead to a shift toward a Th2

phenotype in CD4+ cells potentially accounting for the reduced

incidence of GVHD observed in this cohort.

Remarkably, the cytokine gene expression profiles related to

Th1/Th2/Th3 pathways differed significantly in T cells mobilized

with these two different agents. Real-time PCR assays showed

t cells mobilized with plerixafor had cytokine gene expression

patterns that were similar to nonmobilized T cells. In contrast,

G-CSF altered a number of cytokine-related genes in mobilized

CD3+ T cells including both TH1 and TH2 cytokine-related

genes, transcription factors, and genes regulating T cell activation.

Numerous prior studies have also reported that G-CSF induces

alterations in cytokine-related genes. G-CSF mobilized allografts

contain more than a log higher T cell dose than bone marrow

allografts but are not associated with an increased incidence of

acute GVHD. This has led investigators to speculate that G-CSF–

induced alterations in cytokines inhibit T cells from causing acute

GVHD. Franzke et al. (37) showed G-CSF mobilization increased
gene expression of the Th2-related gene GATA3 in CD4+ T cells,

with this effect being less apparent in nonsorted CD3+ cell

fractions. In our analysis, GATA3 levels in T cells did not change

significantly in CD3+ T cells after G-CSF mobilization, although

our genotype study did not include an analysis that was specific

for the CD4+ T cell subset. Recent data suggest reductions in Th17

cells may be partially responsible for the avoidance of acute GVHD

in G-CSF mobilized allografts (38). However, using a Luminex

array analysis, we observed no changes in serum IL-17 levels

after G-CSF mobilization. Instead, serum levels of G-CSF, IL-

1RA, IL-4, and MIP-1β increased significantly from baseline after

G-CSF mobilization. In contrast with these findings, we observed

no significant changes in any of these or other serum cytokines

or chemokines measured after plerixafor mobilization (data not

shown).

Increasing data suggest Tregs play an important role in regu-

lating GVHD after allogeneic hematopoietic cell transplantation

(a-HCT). Transplanting PBSC allografts that contain higher doses

of Tregs has been associated with a reduced incidence of GVHD

(39–41). In preclinical murine models of a-HCT, injection of

CD4+CD25+ Tregs was shown to suppress the early expansion of

alloreactive donor T cells and their capacity to induce GVHD

without abrogating graft-versus-tumor effector function (42). Fur-

thermore, data in humans have shown that supplementation of

allografts with donor Tregs reduces the risk for acute GVHD after

haploidentical a-HCT (43). Consistent with these observations,

a number of studies have recently reported that Treg numbers are

decreased in patients who develop chronic GVHD (29, 44).

Therefore, we measured the percentage of Tregs mobilized in

mice and also measured Foxp3 gene expression in human CD4+

T cells after plerixafor versus G-CSF administration. In mice,

there was no significant difference in the percentage of regulatory

CD4+Foxp3+ T cells mobilized with either G-CSF or plerixafor.

Furthermore, HBSS, G-CSF, or plerixafor mobilized murine Tregs

did not differ in their suppressive activity of anti-CD3–stimulated

T cells in [3H]thymidine proliferation assays (data not shown).

Furthermore, in humans, Foxp3 gene expression levels did not

change significantly from baseline and were similar in G-CSF and

plerixafor mobilized CD4+ T cells. Based on these data, one

would expect any impact that Tregs will have on GVHD would

not differ significantly between allografts mobilized with pleri-

xafor versus G-CSF.

Because plerixafor does not alter the phenotype and cytokine

polarization of T lymphocytes, one might postulate the T cell–

mediated immune sequelae of allogeneic transplantation may
differ when allografts are mobilized with plerixafor compared

with G-CSF. To better interpret the clinical relevance of trans-

planting T cells with these differences, we explored a murine

model of PBSC transplantation in which transplant recipients re-

ceived a T cell replete plerixafor versus G-CSF mobilized allo-

graft from MHC-matched, minor histocompatibility–mismatched

donors. A single injection of plerixafor resulted in the rapid mo-

bilization of KLS hematopoietic stem cells into the peripheral

blood of B10.d2 donor mice. The phenotype and MLR reactivity
to alloantigens of T cells mobilized into the spleen with plerixafor

was similar to T cells mobilized with G-CSF and plerixafor com-
pared with HBSS. In contrast with these findings, we observed a

mRNA increase in CD27 expression was observed in G-CSF mobilized

CD8+, but not CD4+, T cells. The functional consequence of

higher numbers of CD27−/CD8 T cells using allogeneic grafts is

unknown. CD27 binds CD70 and is required for generation and

long-term maintenance of T cell immunity, and was recently shown
to augment CD8+ T cell activation (33, 34). One might therefore

speculate that allografting of G-CSF mobilized CD8+ T cells

results in reduced GVHD.

Table III. Phenotype of mobilized splenic lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>HBSS</th>
<th>G-CSF</th>
<th>Plerixafor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 T cells</td>
<td>21.2</td>
<td>15.9</td>
<td>20.3</td>
</tr>
<tr>
<td>CD4 T cells</td>
<td>12.1</td>
<td>8.9</td>
<td>14.2</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>6.9</td>
<td>5.2</td>
<td>6.4</td>
</tr>
<tr>
<td>Treg cells</td>
<td>1.7</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>NK cells</td>
<td>3.0</td>
<td>2.5</td>
<td>3.1</td>
</tr>
<tr>
<td>B cells</td>
<td>52.5</td>
<td>39.2</td>
<td>51.5</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>6.1</td>
<td>20.8</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Values are represented as percentage ± SD from 13–18 donors in each group. *p < 0.05 compared with HBSS and plerixafor mobilization, **p < 0.05 compared with HBSS controls.
bilation could potentially account for the higher incidence of acute GVHD observed in this analysis with plerixafor mobilized allografts. Because CXCR4 is expressed on many different hematopoietic cells, changes in the homing capacity of leukocytes mobilized with plerixafor or G-CSF might also have an impact on GVHD induction. To the best of our knowledge, no study has investigated whether CXCR4 antagonists impact the risk for GVHD by altering leukocyte migratory patterns. However, the magnitude and clinical significance of this effect with plerixafor mobilized lymphocytes would likely be low given the rapidly reversible nature of CXCR4 inhibition with this agent.

In conclusion, our results show important differences in the cellular composition of products mobilized with plerixafor compared with G-CSF. Based on these data, one could hypothesize that immune reconstitution, graft–versus-tumor effects, and acute and chronic GVHD might differ in recipients receiving PBSC transplants mobilized with plerixafor compared with G-CSF. A pilot trial evaluating plerixafor for the mobilization and transplantation of HLA-matched sibling donor hematopoietic stem cells was recently reported. Although preliminary results from this trial have reported a similar incidence of acute and chronic GVHD and relapse rates as observed with historical controls receiving allografts mobilized with G-CSF (45), larger patient numbers will be needed to evaluate the true impact of plerixafor mobilization on engraftment, immune reconstitution, and other immune-mediated events. Finally, because it is possible that plerixafor administered concurrently with G-CSF may be used for allogeneic stem cell donors who fail to mobilize sufficient CD34+ cells with G-CSF alone, the effect of combining both mobilizing agents on T cell function will also need to be analyzed.

Disclosures

G.C. is formerly of AnorMED, a subsidiary of Sanofi; he has no financial interests. The other authors have no financial conflicts of interest.

References


