Memory CD4\({}^+\) T cells do not induce graft-versus-host disease

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Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality in allogeneic stem cell transplantation (alloSCT). Donor T cells that accompany stem cell grafts cause GVHD by attacking recipient tissues; therefore, all patients receive GVHD prophylaxis by depletion of T cells from the allograft or through immunosuppressant drugs. In addition to providing a graft-versus-leukemia effect, donor T cells are critical for reconstituting T cell–mediated immunity. Ideally, immunity to infectious agents would be transferred from donor to host without GVHD. Most donors have been exposed to common pathogens and have an increased precursor frequency of memory T cells against pathogenic antigens. We therefore asked whether memory CD62L–CD44+ CD4\({}^+\) T cells would induce less GVHD than unfractionated or naive CD4\({}^+\) T cells. Strikingly, we found that memory CD4 cells induced neither clinical nor histologic GVHD. This effect was not due to the increased number of CD4\({}^+\)CD25+ regulatory T cells found in the CD62L–CD44+ fraction because memory T cells depletion of these cells did not cause GVHD. Memory CD4 cells engrafted and responded to antigen both in vivo and in vitro. If these murine results are applicable to human alloSCT, selective administration of memory T cells could greatly improve post-transplant immune reconstitution.

already have an increased precursor frequency of memory T cells that can respond quickly when rechallenged with antigen. There are reasons to think that memory T cells would be more potent at inducing GVHD than naïve T cells, however. When challenged by antigen, memory T cells enter the cell cycle and produce cytokines more rapidly than do naïve T cells (15, 16, 19). Memory cells may also have less stringent APC requirements, and thus allospecific memory T cells that do not contact professional APCs might also expand and participate in GVHD reactions (20). Memory T cells also undergo more rapid homeostatic proliferation in irradiated recipients than do naïve T cells (21). Memory T cells might have a skewed T cell receptor repertoire, which could either increase or decrease GVHD. On the other hand, naïve cells could be more potent at GVHD induction because they uniformly express CD62L and CCR7, which promotes T cell trafficking into secondary lymphoid tissues where they can encounter APCs presenting alloantigens. A potentially more diverse repertoire in naïve cells may also enhance the ability of this subset to cause GVHD. If either naïve or memory cells were less potent in GVHD induction, then this could open an avenue for mediating T cell–immunopathologic situations, including GVHD and donor male 8- to 12-week-old B10.D2 (H-2d) mice and rest 3 weeks prior to transplant. Mice were housed in microisolator cages and fed nonautoclaved food and acidified water containing sulfatrim for 2 weeks after transplant.

**GVHD model.** Recipient BALB/c mice received 700–850 cGy from a cesium irradiator (see legends for individual experiments) and were reconstituted with 8 × 10^6 B10.D2 T cell–depleted bone marrow with spleen cells or purified naïve or memory CD4+ T cells as described below.

**Bone marrow T cell depletion.** Bone marrow (BM) cells were collected by flushing femurs and tibias from B10.D2 donor mice into MACS buffer (1× PBS, 5 mM EDTA, 3% calf serum). After red blood cell lysis, BM cells were incubated with biotinylated anti-Thy1.2 (30H12) mAb for 20 minutes on ice, washed once, then incubated with streptavidin-conjugated microbeads (SA-beads; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) for 20 minutes at 4°C. Cells were depleted of Thy1.2-positive cells using an AutoMACS (Miltenyi Biotech GmbH). Remaining Thy1.2-positive cells were routinely less than 0.5% of BM cells. Cells were resuspended in injection buffer (1× PBS, 10 mM HEPES, 2.5% acid citrate dextrose anticoagulant, 0.5% penicillin-streptomycin) prior to transplant.

**T cell purifications.** B10.D2 spleens were crushed through 70-μm screens in MACS buffer. After red blood cell lysis, spleen cells were resuspended in injection buffer prior to transplant or underwent further cell purifications.

CD4 cells were enriched from total spleen cells using BioMag (Qiagen, Hilden, Germany) cell separation as follows. Cells were incubated with the following Ab supernatants for 30 minutes on ice, followed by two washes in MACS buffer: anti–CD8-α (TIB105), anti-B220 (RA3-6B2), anti–Mac-1 (M1/70), and anti–FcR (24G.2). Cells were incubated with prepared BioMag goat anti-rat magnetic beads for 30 minutes on ice in a T75 or T125 flask, which was then placed next to a strong magnet. Cells not bound to magnetic particles were collected and were typically 70–80% CD4+ without contaminating CD8+, B220+, CD11b+, or FcR+ cells. For initial separations of naïve and memory cells, enriched CD4+ T cells were incubated with mAb’s anti–CD4-APC/Cy7 (GK1.5; Caltag Laboratories Inc., Burlingame, California, USA), anti–CD62L-FITC (Mel-14; lab prepared), anti–CD44-APC (Pgp-1; BD Pharmingen, San Diego, California, USA) for 20 minutes on ice, washed once in MACS buffer, and resuspended in sort buffer (1× PBS, 5 mM EDTA, 0.5% calf serum, and 1× gentamicin). Cells were separated into CD62L−CD44− naive and CD62L+CD44+ memory populations using a FACSVantage cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Two procedures were used for the purification of CD25− naïve and memory cells. In the first, spleen cells enriched for CD4+ T cells were stained with anti–CD4–APC/Cy7, anti–CD62L–FITC, anti–CD44–APC, and biotin anti–CD25 (7D4; BD Pharmingen) for 20 minutes on ice, washed once in MACS buffer, and incubated with streptavidin-phycocerythrin (streptavidin-PE)
(Intergen Co., Purchase, New York, USA) for 15 minutes on ice, washed again, and resuspended in sort buffer. Gated CD4+CD25− cells were separated into CD62L−CD44− naive and CD62L−CD44+ memory subsets using a FACSVantage.

In the second approach, separation of CD25+ and CD25− naive and memory CD4+ T cells was achieved by first incubating BioMag-enriched CD4+ T cells with biotinylated anti-CD62L and biotinylated anti-CD25 cells for 30 minutes on ice. Cells were washed once in MACS buffer and then incubated with SA-beads for 25 minutes at 4°C. Cells were separated on the AutoMACs into CD62L−CD25− and CD62L−CD25+ fractions. The negative fraction (CD62L−CD25−) was used as memory cells. The positive fraction, containing CD62L−CD25+ cells, was incubated with anti–CD4-APC-Cy7, anti–CD62L-PE, anti–CD25-APC, and anti–CD25-PE for 20 minutes on ice, washed once in MACS buffer, and resuspended in sort buffer. Naïve CD4+CD25−CD62L− cells were then isolated on a FACStar cell sorter (Becton Dickinson Immunocytometry Systems).

**Immunizations.** Donor B10.D2 mice were immunized by intraperitoneal injection with 50 µg chicken γ globulin (CGG; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) in CFA (Sigma-Aldrich, St. Louis, Missouri, USA) 3 weeks prior to transplantation. Adult thymectomized (ATX) BALB/c mice underwent a GVHD-inducing transplant as described above, using CGG-immunized B10.D2 mice as BM and CD4+ T cell donors. Mice received (a) no CD4 cells, (b) CD25− memory CD4 cells, or (c) unfractionated CD4 cells. Thirty-seven days after transplant, recipients were immunized with either 50 µg CGG or pigeon cytochrome c (PCC; an irrelevant control antigen) in CFA by foot-pad and tail-base subcutaneous injections. Unmanipulated ATX mice were also immunized as a control.

**Lymphocyte proliferation assay.** Draining LN cells were collected from transplanted and unmanipulated ATX BALB/c mice 14 days after immunization. Prior to the LN collection, ATX recipient mice were inspected for the absence of a thymus to ensure complete thymectomy. Cells from each experimental group were pooled, and residual recipient cells from transplanted groups were depleted using MACS depletion with recipient-specific biotinylated anti-Ly9.1 (clone 30C7; BD PharMingen). Residual contaminating Ly9.1+ cells were less than 1% of total. Cells were resuspended in complete Click’s media (Irvine Scientific, Irvine, California, USA) with 5% FCS (Gemini Bio-Products, Woodward, California, USA), 100 U/ml penicillin G, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 2 mM l-glutamine, and 0.0568 mM 2-mercaptoethanol. Conventional T lymphocyte proliferation assays were performed (106 cells/well); all assays were set up with triplicate samples and incubated with media only (no antigen), CGG titrations (12.5 µg, 25 µg, or 50 µg), or 15 µg PPD (Mycobacterium tuberculosis H37 RA; Difco Laboratories, Detroit, Michigan, USA) for 72 hours. Lymphocyte proliferation was assayed by [3H]-thymidine incorporation (1 µCi/well; ICN Radiochemicals Inc., Irvine, California, USA) during the last 16 hours of culture. Sample wells were harvested onto filters, and incorporated radioactivity was counted in a Betaplate liquid scintillation counter (LKB/Wallac, Gaithersburg, Maryland, USA).

**GVHD clinical-scoring system.** Animals were weighed every 3 days following BM transplantation and scored for skin manifestations of GVHD beginning on day 18. The scoring system was as follows: skin ulcers with alopecia less than 1 cm2 in area = 1; skin ulcers with alopecia 1–2 cm2 in area = 2; skin ulcers with alopecia greater than 2 cm2 in area = 3; mice lacking skin ulcers and alopecia received a score of 0. Additionally, animals were assigned 0.3 point each for skin disease ( ulcers or scaling) on ears, tails, and paws. Thus, the minimum score was 0, and the maximum score was 3.9. Incidence is graphed as percentage of mice that have never achieved a clinical score of 0.6 or greater. Once a mouse has had a score of at least 0.6, it is always considered affected, even if GVHD subsides to a score of less than 0.6. We also plotted disease severity of an experimental group, which is the mean clinical score of all mice in that group that were affected by GVHD (i.e., have had a score of 0.6 or greater). When mice either died or were euthanized for humane reasons, their disease severity scores at time of death remained included in subsequent mean scores.

**Pathologic scoring.** Shaved skin from the interscapular region (approximately 2 cm2) was fixed in 10% formalin, embedded in paraffin, sectioned, slide mounted, and stained with hematoxylin and eosin. Slides were scored by a dermatopathologist (J. McNiff; blinded to experimental groups) on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop-out (0–2 for each category).

**Statistical methods.** The significance of differences in GVHD incidence was calculated as a χ2 at the last observation day as no events occurred in the memory T cell recipients. The significance of differences between clinical scores and pathology scores were calculated by Mann-Whitney. Significance of differences in proliferation were calculated by an unpaired t test.

**Results**

**Memory CD4 cells do not cause GVHD.** To compare the GVHD-inducing potency of naive and memory T cells, we used the B10.D2 (H-2d) → BALB/c (H-2d) model of chronic GVHD. In this model CD4 cells are both required and sufficient for GVHD, whereas, in contrast, CD8 cells alone are incapable of causing GVHD (27, 28). The syndrome induced with purified CD4 cells is indistinguishable from that induced by unfractionated splenocytes. In this model, GVHD is primarily cutaneous, manifested by alopecia, erythema, ulceration, and fibrosis.

Naïve and memory phenotype CD4 cells were purified by magnetically depleting B cells and granulocytes from spleens, followed by FACS sorting. Naive CD4 cells were defined as CD62L−CD44−, whereas memory cells were CD62L−CD44+ (Figure 1). BALB/c recipients were irradiated and reconstituted with B10.D2 T
cell–depleted BM along with no T cells, 10⁷ unfractionated spleen cells containing 1.2 × 10⁶ CD4 cells, 10⁶ memory CD4 cells, or 10⁶ naive CD4 cells.

Strikingly, in two independent experiments, memory CD4 cells did not induce clinical or histologic GVHD, while naive CD4 cells caused more severe GVHD than unfractionated splenocytes containing a greater number of CD4 cells (Figure 1, d–f). Between the two experiments, a total of ten mice received memory cells, and none developed GVHD. In contrast, 19 of 20 recipients of naive cells and 23 of 25 spleen cell recipients developed GVHD (P < 0.0001 for both memory versus spleen and memory versus naive). Affected naive CD4 recipients had a mean clinical score of 2.5 compared with 1.5 in spleen cell recipients (P < 0.05 for time points 1–3; P < 0.01 for time points 4–6). Memory cell recipients had a score of 0 (P < 0.05 for time point 1 and P < 0.001 for time points 2–10, memory versus spleen; P < 0.0001 for all time points, memory versus naive). Histologically, memory cell recipients had scores indistinguishable from hosts that received no T cells. In contrast, recipients of spleen cells and naive T cells had mean scores of 5.2 and 5.9, respectively (P < 0.0005, memory versus spleen; P < 0.0004, memory versus naive cells). Thus, GVHD in this model is induced only by naive and not memory CD4 cells. The differences in clinical scores between naive and spleen cell recipients were not reflected by histologic analysis of affected skin as the clinical score measures the extent of skin involvement. The nature of the lesions in affected skin was quantitatively and qualitatively similar in the total spleen cell and naive groups.

Memory CD4 cells depleted of regulatory CD4⁺CD25⁻ cells do not cause GVHD. Differential GVHD-inducing capacity could be an intrinsic property of naive and memory T cells. Alternatively, the CD4⁺CD25⁻ population that contains memory T cells might also contain a regulatory cell that suppresses GVHD that is not present among the naive cell population. Potential candidate regulatory cells include CD4⁺CD25⁻ T regulatory cells (Treg), which have been shown to suppress GVHD in MHC-incompatible GVHD models (22–24). We therefore enumerated the percentage of CD4⁺CD25⁻ cells present in B10.D2 naive and memory CD4 cells. We found that 5.5% of naive cells and 33% of memory CD4 cells were CD25⁻ (Figure 2). Thus, memory cells could have been less efficacious at inducing GVHD due to the large number of putative Treg cells present.

To evaluate this possibility, we performed GVHD experiments with naive and memory CD4⁺ T cells that were depleted of CD4⁺CD25⁻ cells (see Methods). In two independent experiments (Figures 3 and 4), 10⁶ memory CD4 cells depleted of CD4⁺CD25⁻ cells did not cause GVHD, whereas an equal number (Figure 3) or only 250,000 naive CD4 cells (Figure 4) caused severe GVHD.

In the first experiment, naive and memory CD25⁻ cells were isolated by FACs sorting (Figure 3, a–d). Irradiated BALB/c hosts received 8 × 10⁶ B10.D2 T cell–depleted BM with no T cells, 2 × 10⁶ unfractionated CD4 cells, and 10⁶ naive or 10⁶ memory CD4 cells. As in our experiments with CD25-replete naive and memory cells, CD25⁻ memory CD4 cells did not cause GVHD, whereas naive CD25⁻ cells caused more severe GVHD than did an equal number of unfractionated CD4 cells (Figure 3, e–g). Differences in GVHD incidence between recipients of memory versus naive (P < 0.0005) and memory versus total CD4 cells (P < 0.008) were highly significant.

In the second experiment, naive and memory CD25⁻ cells were isolated by MACS depletion to obtain memory cells, followed by FACs sorting to obtain naive cells

![Figure 1](image-url)
Engrafted memory CD4 cells respond to antigen in vivo. For donor memory T cell infusions to be effective in immune reconstituting alloSCT recipients, they must engraft and be able to respond to antigen. We could not ask this in the experiments described above because memory CD4 cell recipients also had donor BM-derived T cells. To evaluate memory donor CD4 cell engraftment and function, we therefore used ATX BALB/c mice as recipients. Because donor BM-derived cells cannot differentiate into T cells due to the absence of a thymus, the only donor-derived T cells in these mice are derived from those infused at the time of transplant. Donor- and host-derived cells can be distinguished by expression of the recipient-specific Ly9.1 isoform.

To mimic the situation of a recipient responding to an antigen against which the donor has already been exposed, we used B10.D2 mice that were immunized 21 days prior to transplant with CGG as CD4+ T cell and BM donors. ATX BALB/c mice were irradiated and reconstituted with T cell–depleted B10.D2 BM, with (a) no CD4 cells, (b) unfractionated CD4 cells, or (c) CD25– memory CD4+ T cells. To test functional in vivo memory of donor T cells, recipients and unmanipulated ATX mice were immunized on day 37 after transplant with CGG or the
irrelevant control antigen PCC. Fourteen days later, mice were sacrificed and draining lymph node cells were harvested. Residual recipient host cells were depleted (less than 1% contaminating host cells), and the remaining cells were restimulated in vitro with CGG. Proliferation was measured by [3H]-thymidine incorporation.

As in previous experiments, memory T cell recipients did not develop clinical GVHD (Figure 6a), whereas most recipients of unfractionated CD4 cells did. Donor memory T cells engrafted, with 75% of LN CD4 cells being Ly9.1– and therefore derived from the mature donor T cells given at the time of transplant. These memory cells made strong proliferative recall responses when challenged in vivo. If translatable to humans, these results could have an important clinical impact. AlloSCT recipients could potentially receive substantial numbers of donor memory T cells, leading to immune reconstitution with a lower risk of GVHD. These cells might also be capable of mediating antitumor effects, an issue that will have to be addressed in future studies.

There are several potential explanations for why memory T cells failed to induce GVHD. In general, the differences we observed could be due to differences in T cell receptor (TCR) repertoire, intrinsic properties of memory T cells, or both. It is presumed that T cells with a memory phenotype have responded to environmental antigens. Thus, the TCR repertoire could be altered such that the immunodominant miHAs targeted by naive T cells are not recognized by memory cells. It is also possible that the primary stimulation that created these memory cells polarized them toward a T1 or T2 response, and if these phenotypes were preserved on restimulation (29), this could account for the decreased GVHD. Using a number of approaches,
however, we have not seen evidence that either T1 or T2 cells are exclusively required for GVHD in this model (our unpublished observations). In addition, memory T cells might not traffic efficiently to secondary lymphoid organs. At least some memory T cells did traffic to lymph nodes, however, because memory CD4 cell recipients mounted proliferative responses to CGG. This is consistent with data on the responsiveness of memory CD4 cells transferred in nontransplant settings (30). Finally, the population that includes memory T cells could contain a regulatory T cell population other than CD4+CD25+ cells. These possibilities will need to be addressed in a series of future experiments that will require unique genetics to distinguish donor-engrafted mature T cells from donor marrow–derived newly generated T cells.

While memory cells are clearly less potent at inducing GVHD, they may still be capable of doing so under certain conditions. In other GVHD models, immunizing T cell donors with recipient antigens reduces the number of T cells required for GVHD induction (28). In these cases, however, the precursor frequency of alloreactive cells would be greatly increased. Thus, even though memory T cells from immune mice mediate GVHD, they may be less effective at inducing GVHD than naive T cells bearing the same TCRs on a per cell basis. Furthermore, GVHD in these experiments may have been caused by persistent activated effectors rather than memory cells, since donor cell purifications were not performed.

Naive T cells, with or without CD25+ cells, were more potent than unFractionated cells and caused more severe GVHD. Approximately 10% of unFractionated CD4 cells are CD25+ as compared with 5.5% of naive CD4 cells. It is therefore possible that a reduction in CD25+ cells from 10% to 5.5% that accompanies naive cell purification accounts for this difference. Alternatively, differences in regulatory activity among subsets of CD4+CD25+ cells could account for this. CD4+CD25+CD62L+CD44+ cells could be less efficacious in suppressing GVHD than are CD4+CD25+CD62L−CD44+ cells. Against this idea, CD62L+ and CD62L−CD4+CD25+ cells were equally efficacious in suppressing T cell proliferation in vitro (31), though to our knowledge they have not yet been compared in vivo. Finally, as mentioned above, a regulatory cell that is CD25+ may also be among cells with a CD62L−CD44+ phenotype, and the exclusion of this cell from the naive population could account for its greater potency compared with unFractionated cells. We think it is unlikely that naive cells are resistant to the activity of CD4+CD25+ regulatory cells because it appears that most, if not all, of the GVHD-inducing activity in this model resides in the naive population, and depletion of CD25+ cells from CD4 cells results in more severe GVHD (our unpublished observations).

If memory CD4 cells cause less GVHD than unFractionated CD4 cells in human alloSCT recipients, selective administration of memory T cells could greatly improve immune reconstitution, as was the case in our murine model. The importance of donor immunity in reconstituting host immunity in human alloSCT is dramatically demonstrated by the increased incidence

Figure 5
Representative histology. Representative skin histology from BALB/c recipients of B10.D2 T cell–depleted BM alone (a), with memory cells (b), unFractionated CD4 cells (c), or naive CD4 cells (d). Note thickening of keratinocyte layer, interface dermatitis, and ulcerations (c and d) not present in a and b.

Figure 6
Donor memory cells engraft and respond to antigenic challenge. B10.D2 mice were immunized intraperitoneally with CGG in CFA and used 3 weeks later as CD4+ cell and BM donors. ATX BALB/c mice were irradiated and reconstituted with 8 × 10^6 T cell–depleted BM cells with no CD4 cells (thin dashed line, n = 9), 1.5 × 10^6 unFractionated CD4 cells (thick solid line, n = 17), or 10^6 CD4+CD25− memory cells (thick line, n = 14). Incidence of GVHD (a). P < 0.001 for GVHD incidence in recipients of CD25− memory cells versus total CD4 cells. Transplanted memory cells respond to CGG (b). Thirty-seven days after the transplant, recipients and unmanipulated ATX BALB/c mice were immunized with CGG or PCC in CFA. Two weeks later, draining LN cells were collected, depleted of residual recipient cells, and rechallenged with 50 μg CGG in vitro in a standard proliferation assay. Cells were pooled from all animals (n = 3–7) of an experimental group: untransplanted ATX control, BM alone, BM plus unFractionated CD4 cells, BM plus CD25−CD4+ memory cells. Background counts (no antigen) were subtracted from plotted data. P = 0.0002 for proliferation to CGG for BM plus memory cells versus BM plus unFractionated CD4 cells. P < 0.0001 for BM plus memory cells versus BM alone. Error bars indicate standard deviation of samples run in triplicate.
of EBV-related post-transplant lymphoproliferative disorders (PTLDs) in recipients of T cell–depleted allografts as compared with recipients of T cell–replete grafts. Infusions of donor T cells from EBV-immune donors induce regressions of PTLDs (32). Adaptive immunotherapy with ex vivo–generated donor T cells specific for CMV or EBV have been effectively used to reconstitute anti-CMV immunity and treat PTLD (29, 33–36). Such a practice is not likely to become widespread due to the cost and technical difficulties in generating these cells. Transfer of a smaller number of naturally occurring donor memory cells might be a more widely applicable approach. Not only would the precursor frequency of cells that can respond to infectious pathogens be increased, immunosuppressive therapy that restricts their function might also be reduced, if not eliminated. Such an approach would be particularly important for adult patients, in which the majority of T cell reconstitution is mediated by donor T cells rather than by thymically derived T cells (1), especially in those patients with GVHD (37).

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