Inability of memory T cells to induce graft-versus-host disease is a result of an abortive alloreponse

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Several groups, including our own, have independently demonstrated that effector memory T cells from non–alloantigen-primed donors do not cause graft-versus-host disease (GVHD). In the current study, we further investigated whether this approach could be extended to all memory T cells, and we studied the underlying mechanisms. Neither total memory T cells nor purified central memory T cells were able to induce GVHD. Memory T cells were at least 3-log less potent than bulk T cells in mediating GVHD. As expected, memory T cells failed to elicit cytotoxicity and proliferated poorly against alloantigens in standard 5-day mixed-lymphocyte cultures. However, the proliferative responses of memory T cells were more comparable with those of bulk and naive T cells when the culture time was shortened. Moreover, the frequencies of IL-2–secreting cells measured by 42-hour enzyme-linked immunosorbent spot (ELISPOT) assay were similar among naive, memory, and bulk T cells. These data indicated that memory T cells are able to respond to alloantigens initially but fail to develop to full potential. The abortive immune response, which was mediated by non–alloantigen-specific memory T cells in response to alloantigens, may explain why memory T cells from unprimed and non–alloantigen-primed donors could not induce GVHD. (Blood. 2007;109:3115-3123)

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Introduction

Allogeneic stem cell transplantation offers the hope of cure for a wide variety of otherwise lethal malignant and nonmalignant diseases.1 However, this procedure is associated with substantial risks, including graft-versus-host disease (GVHD), infection, and disease relapse.1 These complications are intricately related to the function of mature T cells contained in the graft.1,2 T cells mediate GVHD,3 a life-threatening adverse effect that can occur after allogeneic stem cell transplantation.1 Depletion of T cells from the graft is effective in preventing GVHD but is associated with increased risk for graft failure, leukemia relapse, and opportunistic infection.2 Thus, the major challenge in allogeneic stem cell transplantation is how to transfer the T cell–mediated beneficial effects without causing GVHD.

Because the antigen specificity of each T lymphocyte is determined before its contact with antigen by random gene rearrangements in the thymus,4 mature T cells can be divided into naive and memory subsets based on whether they have encountered their corresponding antigens.5 Naive and memory T cells can be distinguished by cell surface markers.5-7 We hypothesized that, if a donor has never encountered the host alloantigens, all host alloantigen-specific T cells are exclusively contained in the naive T-cell compartment and that memory T cells from these animals would not cause GVHD. Based on this hypothesis, we have demonstrated that CD62L- T cells (effector memory T cells [T EM]) from unprimed or non–host alloantigen–primed donors do not induce GVHD.8 Similar results have been reported by several other groups using different animal systems.8-12 Based on the expression of homing molecules, such as CD62L, memory T cells can be further divided into central memory (T CM; all CD62L+ memory T cells) and effector memory (T EM; all CD62L- T cells) subsets.8,14 We asked whether the findings with T EM could be extended to all memory T cells. If our hypothesis is correct, all memory T cells, including T CM and T EM, should be unable to induce GVHD. To answer this question directly, we tested the ability of all memory T cells to induce GVHD. Moreover, we explored the mechanisms by which memory T cells failed to cause GVHD.

Material and methods

Mice

BALB/c (H2d), C57BL/6 (H2b), and C3H/HeJ (H2b) were purchased from The Jackson Laboratories (Bar Harbor, ME). C57BL/Ka, CD45.1, Thy1.1 mice (gifts from Dr Jos Domen, Duke University, Durham, NC), which are congenic to C57BL/6, were bred at Duke University. Female mice were primarily used in this study. Most of the unprimed donor mice were between 10 and 30 weeks of age. Some of the repeated experiments were performed using male or older donor mice (up to 20 months of age) or C57BL/Ka, CD45.1, Thy1.1 mice. No differences were observed when male, older, or C57BL/Ka, CD45.1, Thy1.1 donor mice were used (data not shown). Recipient mice were 7 to 12 weeks old when they underwent transplantation. The mice were housed in a specific pathogen-free facility throughout the study.

T-cell depletion from bone marrow

T cells were depleted from bone marrow using anti–Thy1.2 antibody and complement according to a published protocol from our laboratory.15
Enzyme-linked immunosorbent spot assay

Enzyme-linked immunosorbent spot (ELISPOT) assay was performed using ELISPOT kits (R&D Systems) according to the manufacturer’s protocol. Graded numbers of C57BL/6 responder cells were incubated with 5 × 10^5/well irradiated (20 Gy) BALB/c spleen cells at 37°C and 5% CO₂ for 42 hours in the microplates. Wells were washed 3 times before the detection anti-cytokine antibody was added. Microplates were then incubated at 4°C overnight. At the end of incubation, the plates were washed 3 times again, then 100μL streptavidin-AP was added to each well. After 2 hours of incubation at room temperature, the microplates were washed 3 times, and the substrate solution (BCIP/NBT chromogen) was added. Plates were further incubated in the dark at room temperature for 1 hour. The chromogen solution was then discarded from the microplates, and the microplates were rinsed with distilled water. After the microplates were dry, the ELISPOTS, which represented the number of cytokine-secreting cells, were scored using a KS ELISPOT reader (Carl Zeiss Vision, Hallbergmoos, Germany). Triplicate cultures were set up for every cell population tested.

Histopathological analysis of GVHD

Biopsy samples were taken from skin, tongue, lung, intestine, liver, and spleen and were stored in buffered formalin. Specimens were then embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin-eosin. Slides were examined under a light microscope (Zeiss Standard 25; Carl Zeiss, Thornwood, NY) by B.J.C. in a blinded manner and further verified independently by N.T.L. (blinded to experimental groups). To detect subclinical acute and chronic GVHD, we scored histological GVHD using a semiquantitative scoring system adapted from systems previously published by other groups, with additional measurements of histological changes of chronic GVHD. Slides were scored on the basis of inflammation, tissue damage, and fibrosis. The scoring system for each category measured the extent and the severity of the histological changes: 0, normal; 0.5, focal and rare; 1, focal and mild; 2, diffuse and mild; 3, diffuse and moderate; 4, diffuse and severe. Scores were subsequently added to provide a total score for each specimen. Minimum score was 0, and maximum score was 12 for each target organ. A score greater than 0.5 in any category or a total score more than 1.5 was considered positive for GVHD. Specific changes examined included inflammatory infiltrates, apoptosis of keratinocytes, separation of dermo-epidermal junction, and formation of cleft, follicular dropout, and fibrosis in the skin; inflammation, apoptosis of bile duct epithelial cells, apoptosis of hepatocytes, cholestasis, fibrosis, and parenchyma in the liver; and lamina propria inflammatory cell infiltrate, crypt regeneration, crypt epithelial cell apoptosis, crypt loss, mucosal ulceration, and fibrosis in the intestine. Only skin, liver, and intestine slides were scored. Slides from other tissues (tongue, lung, and spleen) were examined but not scored because the criteria for GVHD in these tissues have not yet been established. The histologic pictures were taken under an Olympus Vanox-S light microscope (Olympus, Center Valley, PA) using an Olympus DP11C camera. We used a 40×/0.7 NA objective lens. The total magnification of the picture was ×100 (objective, ×40; photolens, ×2.5). The original, unmodified pictures were used in this paper.
Immunization

Each donor mouse was injected intraperitoneally with 50 µg KLH emulsified in complete Freund adjuvant (Pierce). Donor animals were used more than 4 weeks after immunization.

Coculture assay

The assay was performed as previously described. Briefly, T cells with putative regulatory activity were added to the mixed lymphocyte cultures. Cocultured cells were added at different ratios to the responder cells. Bulk T cells and irradiated (20 Gy) responder spleen cells were included as controls. Experiments were performed in 96-well, flat-bottom culture plates with a total volume of 200 µL/well. Proliferation was measured as described after 112-hour culture.

Statistical analysis

Data are represented as mean ± SD or as mean ± SD. Comparison between groups was performed by analysis of variance (ANOVA) because all the experiments involved more than 2 groups in this study. When a difference was detected by ANOVA among the groups, the Fisher protected least significant difference test was used to determine the difference between 2 groups. Survival data were analyzed by log rank test. All statistical analyses were performed using StatView software (SAS Institution, Cary, NC). P values below .05 were considered significant.

Results

Memory T cells from unprimed mice respond poorly to alloantigens in 5-day mixed-lymphocyte cultures

Others and we have demonstrated that TEM cannot cause GVHD. In this study, we further explored whether these observations could be extended to all memory T cells. To test this, we sorted T cells into naive and memory (defined as all T cells except naive T cells, including TEM and TCM) populations (Figure 1A-B) and first evaluated their ability to respond to alloantigens using optimal 5-day proliferation and CTL assays. As demonstrated in 2 different systems (Figure 1C), memory T cells proliferated poorly against alloantigens compared with naive and bulk T-cell controls. Similar results were observed in 2 other animal systems (C57BL/6-anti-C3H/HeJ, BALB/c-anti-C3H/HeJ; data not shown). Consistent with the results in the proliferation assays, memory T cells were unable to elicit cytotoxicity in response to alloantigens, whereas the cytotoxicity elicited by naive and bulk T cells was readily
detectable (Figure 1D). It is important to point out that, unlike FVB/N mice, as previously reported,24 young (8-12 weeks old) and old (9-10 months old) C57BL/6 mice responded similarly against BALB/c and C3H/HeJ antigens in proliferation assays (data not shown).

**Memory T cells from unprimed donors were unable to induce GVHD**

We then tested whether memory T cells could induce GVHD in several different systems (C57BL/6→BALB/c, C57BL/6→C3H/HeJ, BALB/c→C57BL/6). T cells (1×10⁶/mouse) were transplanted into lethally irradiated recipients together with 1×10⁷ T-cell–depleted bone marrow (TCD BM) cells. GVHD did not develop in any of the recipients of memory T cells. In contrast, lethal GVHD developed in almost all recipients of naive and bulk T cells. As a result, almost all memory T-cell recipients survived more than 100 days after transplantation, whereas all naive and almost all bulk T-cell recipients died of GVHD within 80 days of transplantation (Figure 2A). Body weights in some of the memory T-cell recipients were lower (though not statistically significant) than in the recipients of T-cell–depleted bone marrow, but body weights on day 98 were not different from those on day 0 (Figure 2A). Moreover, no histological evidence was found in recipients of memory T cells (Figure 2B; Table 1). These data demonstrate that memory T cells from unprimed mice are unable to induce GVHD in major histocompatibility complex (MHC)–mismatched recipients.

To quantify the degree of GVHD reduction after depletion of naïve T cells, we titrated the cell dose down in the control bulk T-cell group but did not increase the memory T-cell dose because it was technically difficult to sort greater numbers of cells than 1×10⁶ cells/mouse for transplantation. As demonstrated in Figure 2C, purified bulk T cells induced GVHD in a dose-dependent manner. As expected, lethal GVHD developed in all recipients of 1×10⁶ T cells, and they died within 1 month of transplantation. GVHD developed in 4 of 5 mice in the 1×10⁵ T cell group, and 1 died on day 98. GVHD developed in 3 of 5 of the recipients of 1×10⁴ T cells, and 2 of them died within 200 days of transplantation. GVHD developed in 2 of 5 recipients of 1×10³ T cells, and 1 of them died on day 162. Clinical data were further confirmed by histological examination (data not shown). These data suggest that...
memory T cells are at least 3-log less potent at inducing GVHD than bulk T cells because GVHD did not develop in any of the memory T-cell recipients receiving $1 \times 10^6$ T cells (Figure 2A-B; Table 1).

Memory T cells from KLH-primed donors are unable to induce GVHD

To be certain that the true functional memory T cells are unable to induce GVHD, we repeated the experiments using T cells from KLH-primed donors. Although memory T cells from KLH-primed mice proliferated vigorously against KLH (Figure 3A), proliferation against alloantigens remained low (Figure 3B). In contrast, naive T cells responded vigorously to alloantigens (Figure 3B) but not to KLH (Figure 3A). These data demonstrate that the memory phenotype T cells used in the experiments were true functional memory T cells. We next tested their ability to induce GVHD in vivo. Similar to those from unprimed donors (Figure 2A-B), memory T cells from KLH-primed donors were unable to induce GVHD (Figure 3C-D). In contrast, naive and bulk T cells from KLH-primed donors induced lethal GVHD (Figure 3C-D). Histological analyses were consistent with the clinical observations (data not shown), indicating that functional memory T cells from non–alloantigen-primed animals were unable to induce GVHD.

Early alloresponses mediated by memory T cells from unprimed mice

Our next question was why memory T cells lack the ability to induce GVHD. Although proliferative responses in the memory T-cell group were very low (Figure 1C), we observed some counts in the standard optimal 5-day MLR assays (eg, 11 364 cpm for $2.5 \times 10^5$/well in the C57BL/6–anti-BALB/c model). Moreover, microscopy revealed that memory T cells did expand initially on challenge with alloantigens. However, when ob-
CD4+ memory T cells do not cause GVHD

Although proliferation mediated by memory T cells was low, it was detectable in 5-day culture (Figure 1C). However, CTL activity was completely undetectable (Figure 1D). These observations might suggest that the lack of capability of memory T cells to induce GVHD was the result of defective CTLs. To exclude this possibility, we tested the effect of purified CD4+ memory T cells on GVHD. CD8+ CTL cells were not involved. We isolated CD4+ memory T cells by cell sorting and tested them in the C57BL/6→BALB/c model. Sorted CD4+ memory T cells (1 × 10^6/mouse) were transplanted into lethally irradiated BALB/c recipients along with T-cell–depleted bone marrow. Similar to T-cell–depleted bone marrow alone and TCM recipients, TCM recipients did not develop GVHD, and all the mice in this group survived more than 100 days after transplantation (Figure 5A-B). In contrast, all naive T-cell recipients developed lethal GVHD and died within 40 days of transplantation (Figure 5A-B). These clinical observations were further confirmed by histological analyses (data not shown). Our data definitively demonstrated that TCM cells are unable to induce GVHD, as has been found with T EM cells.

CD4+ memory T cells are unable to induce GVHD

Figure 6. CD4+ memory T cells do not cause GVHD. Sorted CD4+ T-cell subsets were transplanted into lethally irradiated recipients along with T-cell–depleted BM cells. Mice were monitored for the development of GVHD. (A) Survival. P < .05, CD4+ memory versus bulk or CD4+ naive; P < .05, bulk versus CD4+ naive. (B) Weight curve. P < .05, CD4+ memory versus TCD BM only after day 42.

GVHD because as few as 1 × 10^6 bulk T cells are able to induce GVHD in this model (Figure 2C). To determine the role of TCM in GVHD more definitively, we isolated TCM cells by cell sorting (Figure 1A-B) and tested their ability to induce GVHD in vivo. C57BL/6 splenic TCM cells (1 × 10^6/mouse) were transplanted into lethally irradiated BALB/c recipients along with T-cell–depleted bone marrow. Similar to T-cell–depleted bone marrow alone and TCM recipients, TCM recipients did not develop GVHD, and all the mice in this group survived more than 100 days after transplantation (Figure 5A-B). To determine the role of TCM in GVHD more definitively, we isolated TCM cells by cell sorting (Figure 1A-B) and tested their ability to induce GVHD in vivo. C57BL/6 splenic TCM cells (1 × 10^6/mouse) were transplanted into lethally irradiated BALB/c recipients along with T-cell–depleted bone marrow. Similar to T-cell–depleted bone marrow alone and TCM recipients, TCM recipients did not develop GVHD, and all the mice in this group survived more than 100 days after transplantation (Figure 5A-B). In contrast, all naive T-cell recipients developed lethal GVHD and died within 40 days of transplantation (Figure 5A-B). These clinical observations were further confirmed by histological analyses (data not shown). Our data definitively demonstrated that TCM cells are unable to induce GVHD, as has been found with T EM cells.

immunity against viruses compared with CD8+ TCM.14 Thus, it was of interest to test whether TCM cells, which have the capacity to home to secondary lymphoid organs and are more efficient in eliciting protective immunity,14 could induce GVHD. Data presented in Figure 2A-B demonstrate that 1 × 10^6 total memory T cells, which contain approximately 20% (2 × 10^5) TCM and 80% (8 × 10^5) T EM (Figure 1A-B), do not cause GVHD. These data strongly suggest that TCM cells also lack the ability to induce

Figure 4. Early alloresponses mediated by memory T cells from unprimed mice. Sorted T-cell subsets were cultured with irradiated allogeneic stimulators. All experiments were repeated at least twice and yielded similar results. Results represent mean ± SD of triplicate wells. (A) Kinetics of proliferative response. Cells were harvested at different times after the initiation of culture. Proliferation was determined by 3H-thymidine incorporation. Proliferation rates in the syngeneic controls were less than 10%, 5%, and 1% of the experimental groups for 2-, 3-, and 4-day cultures, respectively. (B) IL-2 production. Frequencies of IL-2–producing cells were determined by ELISPOT assay. IL-2 indicates interleukin-2; ELISPOT, enzyme-linked immunosorbent spot assay. * P < .05, memory versus other groups; # P < .05, memory versus naive.
Table 2. Recipients of CD4+ memory T cells do not have histological GVHD

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Tissues were harvested from representative mice shown in Figure 6. Tissues from recipients of CD4+ naive T cells were harvested between day 8 and day 9. Tissues from recipients of TCD BM only and memory T cells were harvested after recipients were electively killed on day 182. Shown are individual scores of maximum scores of 12 for each target organ.

TCD BM indicates T-cell-depleted BM; ND, not determined.

Discussion

Several groups, including our own, have independently demonstrated that T_{EM} cells are unable to induce GVHD in different animal models. The first goal of the current study was to determine whether the observations on T_{EM} could be extended to all memory T cells. Our in vivo data clearly demonstrated that the GVHD-inducing potential was exclusively contained in naive phenotype T cells and that memory phenotype T cells did not cause GVHD in unprimed (Figure 2A-B; Table 1) and non–alloantigen-primed mice (Figure 3). Moreover, sorted T_{CM} (Figure 5) and CD4+ memory T (Figure 6; Table 2) cells were unable to induce GVHD. Because it is technically difficult to sort more cells than 1 x 10^6 cells/mouse for transplantation, we titrated the cell dose down in the control bulk T-cell group rather than increase the cell dose of memory T cells to quantify the degree of GVHD reduction. Titration data (Figure 2C) demonstrated that the ability of memory T cells to induce GVHD is at least 3-log decreased because as few as 1 x 10^3 bulk T cells were able to induce GVHD whereas as many as 1 x 10^6 memory T cells were unable to cause GVHD (Figure 2A-B; Table 1).

Such dramatic decrease in the ability to induce GVHD may allow the transfer of higher number of T cells than is currently possible, leading to stronger immune effects. Thus, this strategy may have a positive impact on the safety and broader application of allogeneic stem cell transplantation.

Another major goal of the current study was to understand why memory T cells are unable to induce GVHD. Our working hypothesis was that memory T cells from unprimed and non–alloantigen-primed animals do not contain alloantigen-specific T cells and, therefore, are unable to induce GVHD. However, even if our hypothesis is correct, the question remains: why is it that non–alloantigen-specific memory T cells, which likely contain T cells cross-reacting with MHC antigens, do not induce GVHD in MHC-mismatched recipients? Low responses mediated by memory T cells in 5-day MLR and CTL assays (Figure 1) seem to corroborate our hypothesis. However, when cultured for shorter periods of time, the responses mediated by memory T cells in response to alloantigens were not different from those mediated by naïve and bulk T cells (Figure 4). These data indicated that memory T cells are able to respond to alloantigens initially but are unable to maintain the proliferative response. These data further suggest that unprimed memory T cells do contain “alloreactive” T cells defined by in vitro proliferation. However, these cells do not appear to be alloantigen specific because the responses mediated by these cells are different from those mediated by true alloantigen-specific memory T cells (Table 1). Even though we do not fully understand the mechanism(s), we speculate that these are non–alloantigen-specific memory T cells cross-reacting with alloantigens at a level that does not lead to full activation. If they are reflecting non–alloantigen-specific memory T cells cross-reacting with alloantigens, these observations may explain why the proliferative alloresponses mediated by unprimed memory T cells are so different between different models because different cross-reactive T cells are likely involved in different systems. Thus, the inability of memory T cells from unprimed donors to maintain the responses on challenge with alloantigens (Figure 4) may explain why they are unable to induce GVHD.

It was proposed that the lack of capability of T_{EM} cells to induce GVHD is the result of their inability to home to lymph nodes and Peyer patches. Our data using purified T_{CM} cells...
which are able to home to lymph nodes and Peyer patches, suggest that other mechanisms are involved. In fact, defects in homing cannot explain why the in vitro responses were dramatically decreased (Figures 1C-D, 7C). It was noted that TCM cells contain fewer CD4+ T cells than other T-cell subsets (Figure 1B), which may contribute to the lack of GVHD in TCM recipients (Figure 5) because CD8+ T cells have less ability to induce GVHD than do CD4+ T cells in this model. However, the decreased percentage of CD4+ T cells cannot completely explain why TCM recipients are free of GVHD (Figure 5) because TCM recipients do contain sufficient numbers of CD4+ T cells, which have been demonstrated to be able to induce lethal GVHD in this model.32,33 However, the decreased percentage of CD4+ T cells cannot completely explain why TCM recipients are free of GVHD (Figure 5) because TCM recipients do contain sufficient numbers of CD4+ T cells, which have been demonstrated to be able to induce lethal GVHD in this model.32,33 Decreased CTL activity in memory T cells (Figure 1D) may result in reduced GVHD. However, our data, presented in Figure 6 and Table 2, strongly suggest that defective CTLs did not play a pivotal role because purified CD4+ memory T cells, which do not contain CD8+ CTLs, were also unable to induce GVHD. Lack of GVHD could be attributed to increased regulatory T-cell activity in the graft.35 However, memory T cells depleted of CD4+CD25+ regulatory T cells remained low responsive against alloantigens (Figure 7C-D). Moreover, the coculture assay failed to detect any regulatory activity in memory T cells (Figure 7E). These data indicate that regulatory T cells are not responsible for the inability of memory phenotype T cells to induce GVHD.

In conclusion, we have demonstrated that all memory T cells, including T EM and T CM, are unable to induce GVHD and that GVHD-inducing T cells exclusively express the naive phenotype in unprimed donors. The lack of capability of memory T cells from unprimed donors to induce GVHD may be a result of “abortive” alloresponses mediated by non–alloantigen-specific memory T cells. Regulatory T cells do not play a major role in this process. This novel approach may allow the transfer of recall T-cell immunity without causing GVHD. The better understanding of memory T-cell responses in GVHD will facilitate the clinical translation of this approach to improve the safety, broaden the scope, and enhance the effectiveness of allogeneic stem cell transplantation.

Acknowledgments

This work was supported by National Institutes of Health grants P01-HL67314 and P01-CA47741.

We thank Patti McDermott and Danielle King from the Duke Human Vaccine Institute Flow Cytometry Core Facility (supported
by National Institute of Health grant AI-051445) for cell sorting and Dr Gwynn Long for critical reading of the manuscript.

**Authorship**

Contribution: B.J.C. designed the research, performed the research, analyzed the data, and wrote the paper. D.D. performed the research. X.C. designed the research, performed the research, and analyzed the data. N.T.L. performed the research and analyzed the data. J.S. designed the research, performed the research, and analyzed the data. J.W.F. designed the research, performed the research, and analyzed the data. N.J.C. provided advice on experimental design and manuscript preparation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**References**

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