Naive and Memory T Cells Induce Different Types of Graft-versus-Host Disease[1]

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The goal of this study was to compare the ability of donor naive and alloantigen-primed effector memory T cells to induce graft-vs-host disease after bone marrow transplantation in MHC-mismatched irradiated host mice. Purified $CD4^+$ naive ($CD62L^{high}CD44^{low}$) T cells and $CD4^+$ effector memory ($CD62L^{low}CD44^{high}$) T cells obtained from unprimed donors and donors primed to host alloantigens, respectively, were injected into host mice, and the rapidity, severity, and pattern of tissue injury of graft-vs-host disease was assessed. Unexpectedly, the naive T cells induced a more acute and severe colitis than the primed memory cells. Whereas the naive T cells expressing CD62L and CCR7 lymph node homing receptors vigorously expanded in mesenteric lymph nodes and colon by day 6 after transplantation, the primed memory T cells without these receptors had 20- to 100-fold lower accumulation at this early time point. These differences were reflected in the significantly more rapid decline in survival and weight loss induced by naive T cells. The primed memory T cells had a greater capacity to induce chronic colitis and liver injury and secrete IL-2 and IFN-γ in response to alloantigenic stimulation compared with memory T cells from unprimed donors. Nevertheless, the expected increase in potency as compared with naive T cells was not observed due to differences in the pattern and kinetics of tissue injury. *The Journal of Immunology,* **2007, 179: 6547–6554.**

Graft-vs-host disease (GVHD)[3] is a major complication of allogeneic hemopoietic cell transplantation that is caused by mature donor T cells contained in the graft from untreated normal donors (1). The contribution of different subsets of donor T cells to the pathogenesis of GVHD has been studied extensively. Donor $CD4^+$ T cells induce injurious immune responses to alloantigens presented by MHC class II molecules on host APCs, and donor $CD8^+$ T cells induces injurious responses to alloantigens presented by MHC class I molecules (2, 3). Donor $CD4^+$ and $CD8^+$ T cell responses may predominate in mouse models of GVHD depending on the donor and host strain combinations involving major and/or minor histocompatibility Ag differences (2). Subsets of naturally occurring regulatory T cells contained in the graft, such as NK T cells and $CD4^+CD25^+$ T cells, suppress GVHD induced by $CD4^+$ and $CD8^+$ T cells (4, 5).

The role of naive and effector memory donor T cell subsets in the induction of GVHD has been studied recently using untreated normal donor mice (6–9). These subsets can be separated according to the expression of the cell-trafficking molecule CD62L (L-selectin), and the T cell activation molecule, CD44 (10). Naive T cells express the $CD62L^{high}$ $CD44^{low}$ phenotype, whereas the effector memory T cells express the $CD62L^{low}CD44^{high}$ phenotype (10, 11). The differences in the patterns of receptor expression are due to the down-regulation of CD62L and the up-regulation of CD44 on the naive T cell surface after antigenic exposure (10, 11). In normal untreated mice, it is thought that $CD4^+$ and $CD8^+$ T cells with the effector memory phenotype are derived from naive T cells that have been exposed to environmental Ags (12, 13) or by homeostatic proliferation of naive $CD4^+$ T cells (14). It is not clear whether environmental Ags in the mouse cross-react with mouse MHC and/or minor histocompatibility Ags.

Although highly purified naive T cells from untreated normal donors induced acute lethal GVHD, purified effector memory T cells from the same donors failed to induce GVHD in irradiated MHC-mismatched or matched murine hosts used for investigation (6–9). The failure of the latter subset to induce GVHD may reflect important differences in the biology of naive and effector memory T cells, or may instead reflect the lack of cross-reactivity between environmental Ags and host histocompatibility Ags. In the absence of cross-reactivity, effector memory T cells from untreated normal donors would be directed to "third-party Ags" and would fail to specifically recognize host alloantigens unless the donors had been primed to the alloantigens. In a previous study using an MHC-matched model, effector memory donor $CD4^+$ T cells isolated from mice undergoing chronic GVHD induced potent GVHD in secondary recipients (15). However, the potency of primed effector memory cells an unprimed effector memory cells was not compared, nor was the distribution and kinetics of tissue injury induced by primed $CD4^+$ memory T cells compared with purified donor $CD4^+$ naive T cells. Naive $CD4^+$ T cells failed to induce lethal GVHD in the dose range tested in the MHC-matched strain combination chosen for the latter study (15). Thus, the $CD4^+$ T memory cells were considerably more potent than naive $CD4^+$ cells (15).

To make a direct comparison of naive and memory T cells in a setting in which naive T cells induce vigorous GVHD, we studied

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[3] Abbreviations used in this paper: GVHD, graft-vs-host disease; TCD, T cell depleted; BM, bone marrow; LP, lamina propria.

the ability of purified naive and effector memory $CD4^+$ T cells from untreated C57BL/6 (H-2^b) donors and from C57BL/6 donors primed to BALB/c (H-2^d) alloantigens for their capacity to induce lethal GVHD in MHC-mismatched irradiated BALB/c hosts. We have previously shown that acute lethal GVHD induced by $CD4^+$ T cells in this strain combination is associated with acute colitis that is dependent upon early migration of the $CD4^+$ T cells to the mesenteric lymph nodes facilitated by the cell trafficking molecules CD62L and $\alpha_4\beta_7$ integrin (16). Subsequently, the donor $CD4^+$ T cells migrate to the colon, and induce severe injury to the colonic crypts, associated with diarrhea and death.

In the current study, we found that $CD4^+$ effector memory T cells from unprimed donors failed to induce lethal GVHD, but that the same cell subset from host alloantigen-primed donors induced uniformly lethal GVHD. Surprisingly, the naive T cells induced a more severe and rapid type of GVHD with acute colitis than the primed memory T cells.

Materials and Methods

Animals

Wild-type C57BL/6 (H-2^b) male mice, 5–8 wk old, and male BALB/c (H-2^d) mice, 5–10 wk old, were purchased from the breeding facility of the Department of Comparative Medicine (Stanford University, Stanford, CA). All mice were housed in a specific pathogen-free facility. Care of all experimental animals was in accordance with institutional and National Institutes of Health guidelines.

Abs and FACS

The following reagents were used for flow cytometric analysis: unconjugated anti-CD16/32 (2.4G2), anti-CD4 FITC (RM4-5), anti-TCR-β allophycocyanin (H57-597), anti-CD62L allophycocyanin, anti-CD62L biotin (Mel-14), anti-CD44 PE (IM7), anti-LPAM-1 PE ($\alpha_4\beta_7$ integrin complex) (DATK32), anti-H-$2K^b$ FITC (AF6-88.5), and anti-CD4 Cy7 allophycocyanin (GK1.5) mAbs. The latter were purchased from BD Pharmingen. Anti-CCR7 biotin (4B12) was purchased from eBioscience. All stainings were performed in PBS/1% calf serum in the presence of purified anti-CD16/32 at saturation to block unspecific staining via FcRII/III. Propidium iodide (Sigma-Aldrich) was added before analysis to exclude dead cells. All sorts and analyses were done on a modified dual-laser FACSVantage (BD Biosciences) or on the six-color dual-laser FACS LSR in the Shared FACS Facility, Center for Molecular and Genetic Medicine (Stanford University), using FlowJo software (Tree Star) for data analysis.

Cell preparations

Single-cell suspensions were prepared from spleens, washed twice, and filtered through a fine nitex membrane. The samples were then enriched for $CD4^+$ cells with anti-CD4 magnetic microbeads using the MidiMACS system (Miltenyi Biotec). After staining with anti-CD4 FITC, anti-CD62L allophycocyanin, and anti-CD44 PE, cells were sorted into $CD4^+$ $CD62L^{high}CD44^{low}$ and $CD4^+CD62L^{low}CD44^{high}$ populations on a modified dual-laser FACSVantage (BD Biosciences). For preparation of T cell-depleted bone marrow (TCD BM), BM cells were obtained from the femur and tibia, and single-cell suspensions were filtered through nitex membranes. Suspensions were stained with anti-Thy1.2 biotin mAb (5a-8; Caltag Laboratories) and streptavidin-magnetic beads (Miltenyi Biotec), and passed over two consecutive MACS LS-separation columns (Miltenyi Biotec). TCD BM contained <0.01% T cells, as determined by staining with anti-TCR-β allophycocyanin. Thy1.2-depleted splenocytes were used as allogeneic stimulator cells.

Immunization

Five-week-old male C57BL/6 mice were immunized by i.p. injection of 50×10^6 splenocytes from age matched male BALB/c mice followed by a dose of 10×10^6 splenocytes 1 wk later. Four weeks after immunization, splenocytes from the primed C57BL/6 mice were used for further studies.

GVHD model

Acute GVHD was induced as described previously (5). In brief, BALB/c hosts were lethally irradiated (800 cGy) from a 200 Kv x-ray source and injected with donor cells via tail vein within 24 h. All mice received 2×10^6 TCD BM cells for reconstitution with or without $CD4^+$ T cells as indicated in the text and figures. Mice were kept on antibiotic water (25 μg/ml neomycin/0.3 U/ml polymyxin B; Sigma-Aldrich) for the first 28 days. Survival and the signs of GVHD, hair loss, hunched back, swollen faces, and diarrhea were monitored daily and body weight was measured weekly.

Histopathology and pathologic scoring for GVHD severity

Histopathological specimens from the liver and large intestines of hosts were obtained at 7 and 72 days after transplantation and fixed in formalin before embedding into paraffin blocks. Tissue sections of 4- to 5-μm thickness were stained with H&E. Microscopic images were obtained using an Eclipse E1000M microscope (Nikon) with SPOT RT digital camera and acquisition software (Diagnostic Instruments) with a final magnification of $\times$300 for all images. Image processing was performed with Photoshop CS (Adobe) with standard adjustments of brightness, contrast, and color balance to the entire image. Histologic assessment of liver and colonic GVHD was performed in a blinded fashion using the histopathological scoring system described by Kaplan et al. (17). The mean GVHD score represents the mean $\pm$ SD among five animals per group.

Cell distribution studies

Cell preparation and acute GVHD induction were performed as described above. For day 6 analysis, lymphocytes from tissues of individual mice were used for flow cytometric analysis. Single-cell suspensions from mesenteric lymph nodes, peripheral lymph nodes (bilateral axillary and inguinal nodes were pooled), and spleen were filtered through fine nitex membranes to remove aggregates. Mononuclear cells from the liver were isolated according to the method by Lan et al. (18). Lamina propria (LP) lymphocytes were purified as described (19).

Cytokine assay

Cytokine production after 60 h of allogeneic stimulation was assessed in supernatants of cultures of sorted naive and effector memory $CD4^+$ T (1×10^5 cells) from primed and unprimed C57BL/6 donors as responders and irradiated (3000 cGy) Thy1.2-depleted BALB/c splenocytes as allogeneic stimulator cells (5×10^5 cells). Five different cytokines were analyzed in a multiplex assay system using fluorescently labeled microsphere beads (Beadlyte Mouse Multi Cytokine Detection system; Upstate Biotechnology) and cytokine levels were quantitated using the Luminex 100 system.

Statistical analysis

Kaplan-Meier survival curves were made using Prism (GraphPad Software). Statistical differences in animal survival were analyzed by log-rank test. Differences in donor type T cell recovery in tissues of hosts and cytokine production were analyzed using the two-tailed Student *t* test. For all tests, $p \leq 0.05$ was considered significant.

Results

Expression of chemokine receptors and activation molecules on naive and effector memory $CD4^+$ T cells

Naive and effector memory $CD4^+$T cells can be separated according to the expression of CD62L and CD44 surface markers, because the former express the $CD62L^{high}CD44^{low}$ pattern and the latter express the $CD62L^{low}CD44^{high}$ pattern (10). Fig. 1*A* shows the flow cytometric analysis of CD62L vs CD44 on gated $CD4^+$ T cells from the spleen of untreated C57BL/6 mice (*top panel*). Approximately 70% were $CD62L^{high}CD44^{low}$ and 18% were $CD62L^{low}CD44^{high}$ (*left upper* and *right lower quadrants*). These two populations of cells were sorted and reanalyzed for purity. The sorted cells were ~99% pure naive or effector memory type according to the patterns shown in the two *lower panels* of Fig. 1*A*.

We analyzed the gated naive and effector memory cells from untreated (unprimed) and alloimmunized (primed) C57BL/6 mice for the expression of CCR7, $\alpha_4\beta_7$, CD25, CD69, and CXCR3 surface markers as judged by flow cytometry in Fig. 1*B*. Alloimmunized mice were given 50×10^6 BALB/c spleen cells i.p. followed by a booster injection of 10×10^6 spleen cells 1 wk later. The spleen cells from alloimmunized C57BL/6 mice were harvested 4 wk later.

Both the unprimed and primed mice showed a narrow peak of intense staining of the chemokine receptor CCR7 among gated

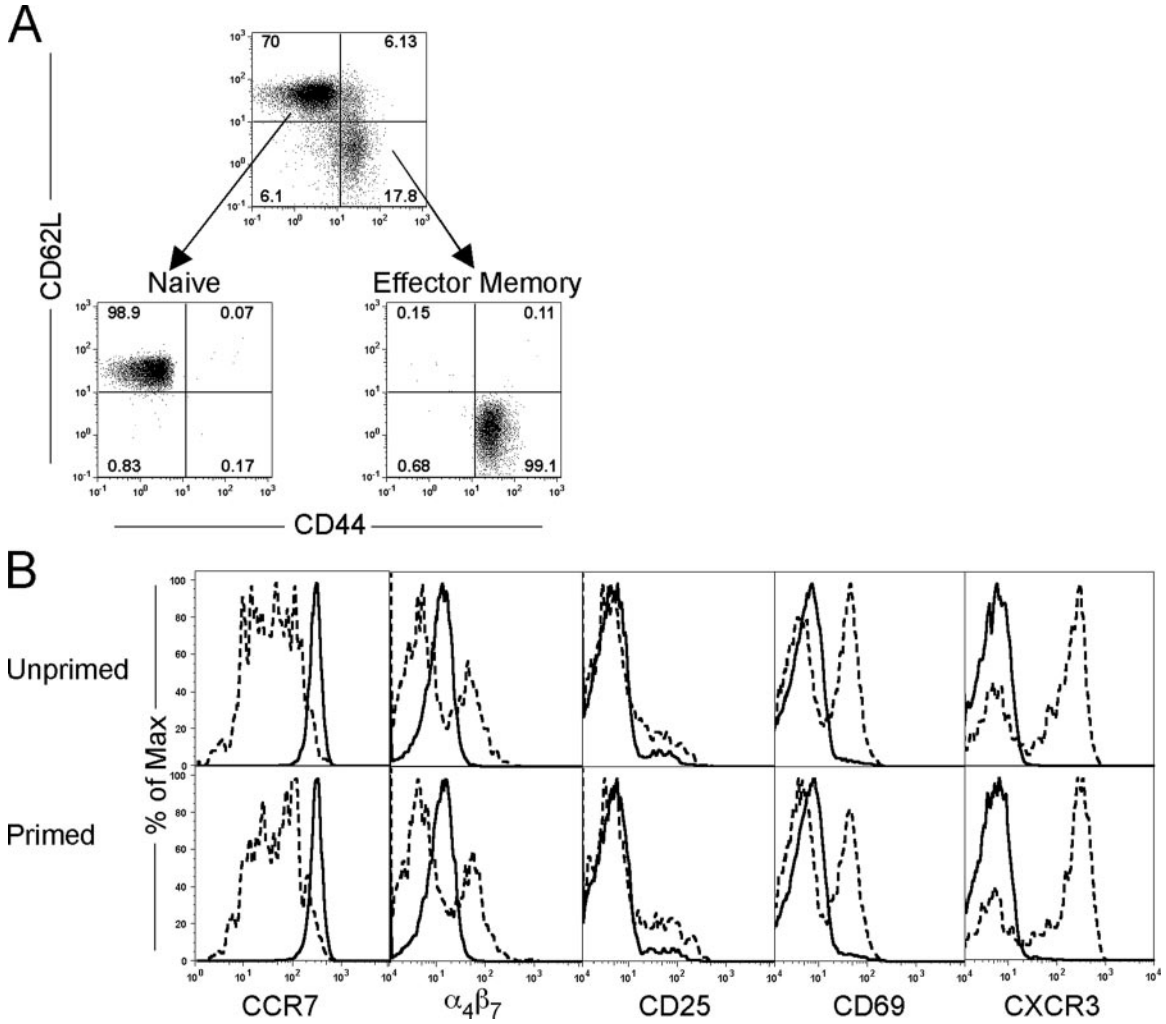

FIGURE 1. Unprimed and primed CD62L^{high}CD44^{low} and CD62L^{low}CD44^{high} CD4$^+$ T cells express comparable levels of different surface markers. *A*, Splenocytes from C57BL/6 donors were sorted into CD62L^{high}CD44^{low} (naive) and CD62L^{low}CD44^{high} (effector memory) CD4$^+$ T cell populations. *B*, C57BL/6 splenocytes from primed and unprimed animals were stained with anti-CD4, anti-CD44, and anti-CD62L mAbs. Gated CD62L^{high}CD44^{low} and CD62L^{low}CD44^{high} CD4$^+$ T were analyzed for CCR7, $\alpha_4\beta_7$, CD25, CD69, and CXCR3 expression. Histogram plots of the respective markers are overlaid for CD62L^{high}CD44^{low} CD4$^+$ T cells (bold line) and CD62L^{low}CD44^{high} CD4$^+$ T (broken line).

naive CD4$^+$ T cells as compared with the broader peak of less intense staining of effector memory cells (Fig. 1*B*). In contrast, the effector memory T cells from primed and unprimed mice showed both a dull and bright peak of staining for the activation marker CD69, and the naive cells showed only a dull peak (Fig. 1*B*). A similar bimodal pattern was observed in effector memory T cells, and a single dull or negative peak in naive T cells for the $\alpha_4\beta_7$ integrin and CXCR3 chemokine receptors (Fig. 1*B*). In addition, there was a slight increase in the bright staining of CD25 among the effector memory (11.5%) as compared with naive (4%) T cells. In general, the staining patterns were similar using both primed and unprimed mice. The results indicate that CCR7 is down-regulated and $\alpha_4\beta_7$, CD69, and CXCR3 are up-regulated on effector memory as compared with naive T cells.

Ability of naive and effector memory CD4$^+$ T cells from primed and unprimed donors to induce GVHD

Previous studies have shown that purified naive T cells from unprimed donors induce lethal GVHD in irradiated allogeneic hosts and that purified effector memory cells do not (6–9). In the current study, we compared the ability of purified effector memory CD4$^+$ T cells from both unprimed C57BL/6 donors and C57BL/6 donors primed to host alloantigens for their capacity to induce lethal GVHD in MHC- and minor Ag-mismatched BALB/c hosts. The hosts were conditioned with a single dose of myeloablative total body radiation and injected i.v. with 2×10^6 TCD BM from unprimed donors and equal numbers (0.125×10^6) of sorted naive or effector memory CD4$^+$ T cells from primed and unprimed donors 24 h later. The latter cell dose had been previously determined to be on a sensitive portion of the dose-response curve for inducing lethal GVHD (16). The sorted cells were collected by first gating on CD4$^+$ cells then using the thresholds shown in Fig. 1*A* to separate CD62L^{high}CD44^{low} (naive) and CD62L^{low}CD44^{high} (effector memory cells). As shown in Fig. 2, naive CD4$^+$ T cells from unprimed donors induce severe GVHD, and all the hosts died by day 65 with clinical signs of GVHD including diarrhea, loss of fur, and weight loss. Hosts given TCD BM cells uniformly survived >100 days, and their body weights returned to pretransplant levels within one month.

In contrast, effector memory CD4$^+$ T cells from unprimed donors induced less severe GVHD and 80% hosts survived for at least 100 days (Fig. 2*A*). The difference in survival of hosts given unprimed naive or effector memory cells was significantly different ($p < 0.0001$). The attenuated GVHD induced by effector memory

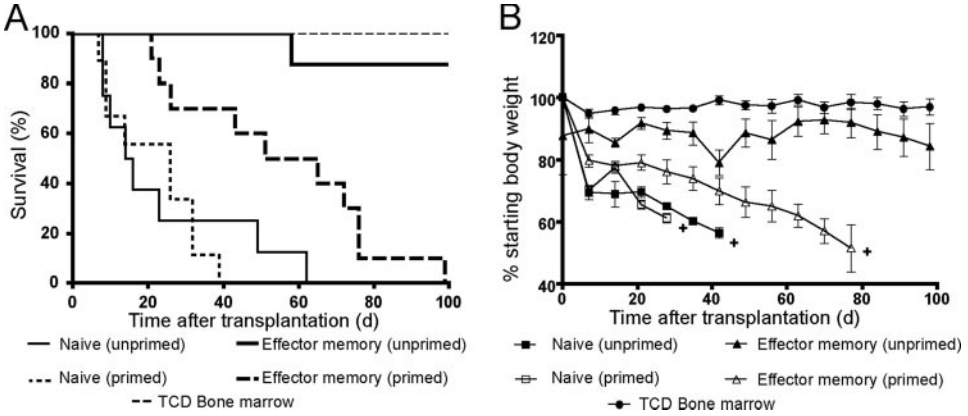

FIGURE 2. Naive $CD4^+$ T induce a more rapid and severe type of GVHD than primed $CD4^+$ effector memory T cells. Lethally irradiated BALB/c host mice were given i.v. injections of 2×10^6 TCD BM cells from unprimed C57BL/6 donors with or without $CD62L^{high}CD44^{low}$ or $CD62L^{low}CD44^{high}$ $CD4^+$ T cells from primed or unprimed donors. The data were pooled from two independent experiments. There were 8–10 hosts in each group. *A*, Survival of irradiated hosts given TCD BM cells from unprimed donors with or without 0.125×10^6 sorted naive and effector memory $CD4^+$ T cells from primed and unprimed donors. *B*, Percentage of starting body weight of host mice given TCD BM with or without sorted naive and effector memory $CD4^+$ T cells as in *A*. Brackets show SEs of the mean. +, Analysis was stopped for a given group when there were two hosts remaining positive.

T cells was reflected in the weight loss of hosts which was significantly less severe ($p < 0.0001$) as compared with the group given naive T cells (Fig. 2*B*).

BALB/c hosts given TCD BM and sorted naive T cells from primed donors developed severe GVHD with marked weight loss, and all hosts died by day 40 (Fig. 2*A*). There were no significant differences ($p > 0.742$) in survival and weight loss, in the groups given primed or unprimed sorted naive T cells. However, there were highly significant differences in survival ($p < 0.0004$) and weight loss ($p < 0.0002$) in the groups given primed or unprimed sorted effector memory T cells (Fig. 2). Whereas 80% of the hosts given unprimed effector memory T cells survived at least 100 days, all hosts given primed effector memory T cells died by 100 days (Fig. 2*A*). In addition, the serial body weights of hosts given unprimed effector memory cells were constant over 100 days and similar to pretransplant values, but body weights of hosts given primed effector memory T cells gradually fell over a period of at least 80 days (Fig. 2*B*). The difference of body weights was significant ($p < 0.0002$). All recipients had complete donor chimerism (>95%) as detected by staining with anti $H\text{-}2D^b$ mAb (data not shown).

The marked early weight loss and acute GVHD in hosts given unprimed naive T cells was reflected in the microscopic changes in the tissue sections of the colon 7 days after transplantation. Fig. 3*C* shows that the colon from hosts given naive T cells from unprimed donors had a marked dropout of crypts, loss of crypt goblet cells, and a marked mononuclear cell infiltrate between crypts. In contrast, the colon of hosts given unprimed effector memory T cells showed no crypt dropout, retention of goblet cells, and little or no mononuclear cell infiltrate (Fig. 3*D*). The colon of hosts given naive T cells from primed donors showed a pattern of severe crypt injury (Fig. 3*A*). Interestingly, the colon of hosts given effector memory T cells from primed donors showed minimal injury at day 7 with a mild infiltrate and occasional abscesses at the crypt bases (Fig. 3*B*). The normal appearance of colon sections from control hosts given irradiation and no transplant (Fig. 3*E*), and from control hosts given irradiation and TCD BM alone (Fig. 3*F*) are shown for comparison. Sections of the liver and skin of all hosts were normal at day 7 (data not shown).

To compare late changes in the histopathology of the liver and colon in hosts given effector memory T cells from unprimed and primed donors, tissues were harvested from groups of hosts at 72 days after transplantation. Fig. 4*A* shows that liver from a representative host given effector memory cells from primed donors had a portal infiltrate consisting of lymphocytes, macrophages, and fewer neutrophils. This infiltrate is centered primarily on bile ducts with some clusters adjacent to portal veins. Fig. 4*B* shows a representative example of a subset of portal tracts that is invested by small numbers of mixed inflammatory cells centered primarily on the bile ducts that was observed in hosts given unprimed effector memory T cells. The liver of hosts given TCD BM cells showed very mild infiltrates of lymphocytes, plasma cells, and neutrophils centered on small bile ductules (Fig. 4*C*). In Fig. 4*D*, the colon of a representative host given primed effector memory cells, showed a hyperplastic mucosa with dilated crypts that contain necrotic cell debris. Few mucin containing goblet cells remained. The LP was moderately expanded with neutrophils, lymphocytes, and macrophages. This inflammation extends into the submucosa. In Fig. 4*E*, the LP of a representative host given unprimed effector memory T cells is mildly expanded by lymphocytes, plasma cells, and neutrophils. Goblet cells are retained.

Fig. 4*F* shows the colon of host given only TCD BM cells. The colon had a mild diffuse infiltrate of lymphocytes, plasma cells, and neutrophils in the deep LP. Goblet cells were retained in hosts given unprimed effector memory T cells or no T cells. The mean GVHD histopathologic scores of colon was significantly increased ($p = 0.02$) in the group given primed effector memory T cells as compared with groups that received unprimed effector memory T cells or no T cells (Fig. 4*G*). The mean histopathologic score of the liver was higher in the group given primed effector memory cells as compared with the groups given no T cells or unprimed effector memory T cells (Fig. 4*G*). However, the difference was at the border of statistical significance ($p = 0.056$). Hosts with primed or unprimed effector memory T cells had no microscopic abnormalities of GVHD in the skin (data not shown).

Accumulation of naive and effector memory $CD4^+$ T cells from primed and unprimed donors in host tissues at day 6

Our previous studies have shown that the acute colitis associated with lethal GVHD in BALB/c hosts given C57BL/6 TCD BM and sorted $CD4^+$ T cells was dependent on the ability of the donor T cells to migrate to the mesenteric lymph nodes by day 2 after transplantation (16). The rapid nodal migration preceded the

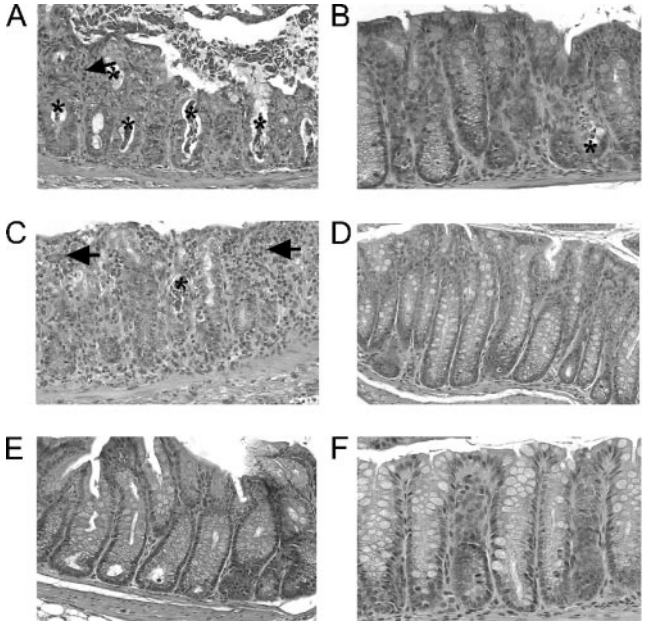

FIGURE 3. Histopathologic changes of acute severe colitis induced with $CD4^+$ T naive T cells but not $CD4^+$ T effector memory T cells. Representative tissue sections were obtained from hosts that received 0.125×10^6 $CD62L^{high}CD44^{low}$ or $CD62L^{low}CD44^{high}$ $CD4^+$ T cells from primed or unprimed donors and 2×10^6 TCD BM cells from unprimed donors. *A*, Colon of a host given naive $CD4^+$ T cells from primed donors. Severe acute graft vs host disease is present with numerous crypt abscesses (∗) and erosion with exudate on surface of mucosa. Arrow highlights the accompanying mononuclear cell infiltrate. *B*, Colon of a host given primed effector memory $CD4^+$ T cells. A mild inflammatory infiltrate separates crypts which are lined by goblet cells with abundant mucin. A focal abscess (∗) is shown in one crypt. *C*, Colon of a host given unprimed naive $CD4^+$ T cells. Crypts are shortened and separated by a prominent inflammatory cell infiltrate composed of lymphocytes and plasma cells (arrows). ∗, Crypt abscess and there are decreased numbers of goblet cells lining crypts. *D*, Colon of a host given unprimed effector memory $CD4^+$ T cells. Goblet cells are retained in crypt walls with normal architecture and minimal inflammatory cell infiltrate. *E*, Colon of an irradiated control host showing normal appearing colonic mucosa and scant inflammatory infiltrate. *F*, Colon of a control host given only TCD BM cells showing unremarkable colon with back-to-back crypts containing numerous goblet cells. Inflammatory infiltrate is minimal. Tissue sections were stained with H&E. Each panel is representative of three hosts examined.

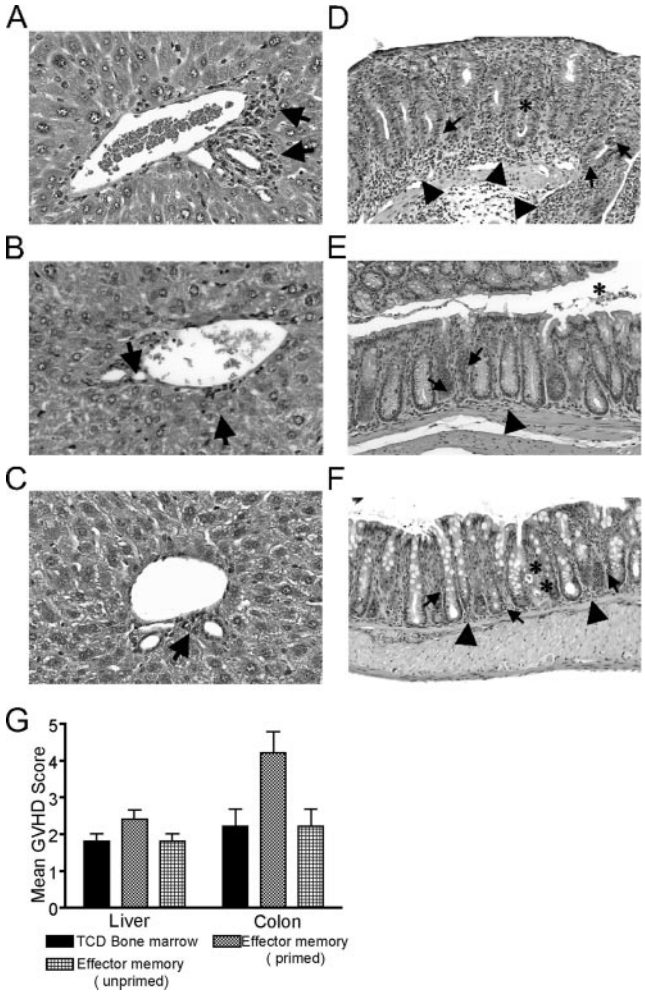

FIGURE 4. Histopathologic changes of tissue injury are increased 72 days after transplantation of primed vs unprimed $CD4^+$ effector memory T cells. Representative tissue sections were obtained from hosts that received 0.125×10^6 $CD62L^{low}CD44^{high}$ $CD4^+$ T cells from primed or unprimed donors and 2×10^6 TCD BM cells from unprimed donors. *A*, Liver of host given primed effector memory $CD4^+$ T cells. There is a portal infiltrate (arrows). *B*, Liver of host given unprimed effector memory $CD4^+$ T cells. The arrows indicate mild portal infiltrates. *C*, Liver of host given TCD BM. Occasional small clusters of inflammatory cells surround small bile ductules (arrow). *D*, Colon of host given primed effector memory $CD4^+$ T cells. The mucosa is hyperplastic, and LP has heavy cell infiltrate (arrowheads). Numerous crypts are dilated and contain necrotic cell debris (∗). Apoptotic cells are frequent (arrows). *E*, Colon of host given unprimed effector memory cells. The LP is mildly infiltrated (arrowheads) The arrows indicate apoptotic cells; ∗, degenerate cell debris. Goblet cells are retained. *F*, Colon of host given TCD BM cells. The LP has mild infiltrate (arrowheads). Crypts contain some necrotic cell debris (∗). Occasional apoptotic cells are found (arrows). Light microscopy, H&E stain. Each panel is representative of five hosts examined. *G*, Mean (±SD) of histopathologic GVHD scores of liver and colon from the three groups ($n = 5$) per group.

accumulation of donor T cells in the colon on day 6. Targeted inactivation of genes encoding both CD62L and $\alpha_4\beta_7$ trafficking molecules of donor T cells prevented nodal and colonic accumulation (16). Sorted effector memory $CD4^+$ T cells have reduced expression of both CD62L and CCR7 and increased expression of $\alpha_4\beta_7$ receptors as compared with sorted naive $CD4^+$ T cells (Fig. 1).

Because it is unclear how these changes affect migration to the mesenteric lymph nodes and colon, we compared the accumulation of the sorted $CD4^+$ T cells from primed and unprimed donors in the host tissues at day 6. Fig. 5 compares the accumulation of total $CD4^+$ T cells to that of the sorted naive and effector memory $CD4^+$ T cells from untreated donors. Accumulation of the total $CD4^+$ T cells was not significantly different ($p = 0.05–0.3$) from that of sorted naive $CD4^+$ T cells from primed or unprimed donors in spleen, peripheral lymph nodes, mesenteric lymph nodes, colon, and liver (Fig. 5). In contrast, the accumulation of sorted unprimed effector memory $CD4^+$ T cells was ~20-fold reduced in the spleen ($p = 0.01$) and >100-fold reduced in the peripheral and mesenteric lymph nodes, colon, and liver as compared with total $CD4^+$ T cells ($p = 0.01–0.001$) (Fig. 5). Interestingly, the accumulation of primed effector memory $CD4^+$ T cells was not significantly different ($p = 0.07–0.78$) from that of unprimed effector memory $CD4^+$ T cells except in the liver. In the liver, the accumulation of primed cells was ~100-fold higher than that of unprimed cells ($p = 0.02$).

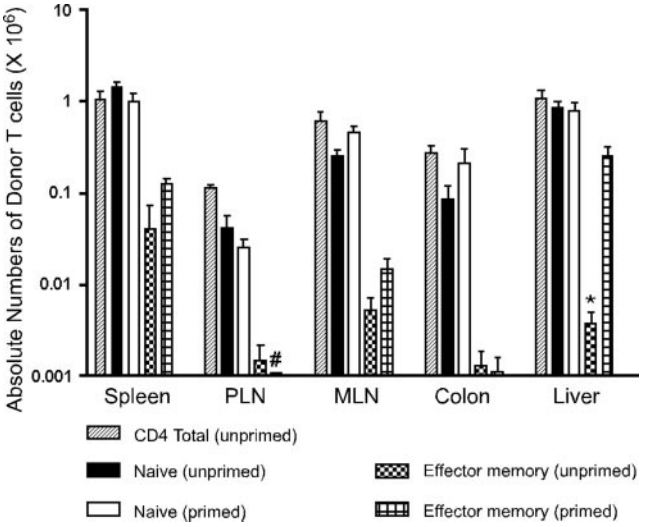

FIGURE 5. Absolute number of donor T cells in the spleen, mesenteric lymph nodes, peripheral lymph nodes, liver, and colon of irradiated hosts day 6 after the injection of 0.5×10^6 total $CD4^+$ T cells or $CD62L^{high}CD44^{low}CD4^+$ T cells or $CD62L^{low}CD44^{high}CD4^+$ T cells. Bars show the means of the absolute number of donor T cells, and brackets show SEs of groups of mice given T cells from primed or unprimed donors. Means are from two separate experiments, with three mice in each experiment. #, The absolute number of donor T cells was $<0.001 \times 10^6$. *, Statistically significant difference between primed and unprimed group ($p \leq 0.05$). All hosts received 2×10^6 wild-type (WT) TCD BM cells.

Comparison of immune responses of sorted donor naive and effector memory cells to host allostimulation in vitro

The potency of donor T cell induction of GVHD in vivo is dependent upon both the magnitude of the immune response to host alloantigens, and the ability of the donor T cells to migrate to host tissues to encounter alloantigens and migrate to subsequent sites of tissue injury. To examine the magnitude of the alloresponse of donor T cells to host stimulation in absence of the cell migration, we incubated the sorted responder $CD4^+$ T cell subsets from primed and unprimed donors with host stimulator cells in the MLR (16). In all instances, a constant number of sorted C57BL/6 donor cells (1×10^5) were incubated with a constant number of irradiated BALB/c host splenocytes (5×10^5), and the concentrations of IL-2 and IFN- γ were measured in the supernatant 60 h later. Control cultures contained syngeneic donor stimulator cells.

Fig. 6 shows that sorted naive $CD4^+$ T cells from unprimed and primed donors secreted mean concentrations of IL-2 that were ~400–800 pg/ml after stimulation with host cells. Stimulation of the sorted donor naive cells with syngeneic donor stimulator cells resulted in the secretion of <50 pg/ml IL-2, and differences in the mean IL-2 concentrations between the allogeneic and syngeneic cultures were significant ($p = 0.01$). In contrast, the mean concentration of IL-2 secreted by sorted $CD4^+$ effector memory T cells from unprimed donors was below 50 pg/ml in both allogeneic and syngeneic cultures (Fig. 6).

Mean IL-2 secretion by sorted effector memory $CD4^+$ T cells from primed donors was ~46-fold higher ($p = 0.002$) than that of unprimed effector memory $CD4^+$ T cells. The differences between allogeneic and syngeneic primed effector memory T cell cultures were significant ($p = 0.02$). It is of interest that IL-2 secretion of primed effector memory T cells in syngeneic cultures was markedly increased ($p = 0.01$) as compared with the unprimed effector memory T cells. This result suggests residual T cell activation after priming. The pattern of IL-2 secretion was similar to that observed with day 5 [^{3}H]thymidine incorporation of the different responder cell subsets and the level of IL-2 and IFN-γ produced by alloantigen-primed effector memory cells in the MLR decreased over time after immunization (data not shown).

Cultures were also assayed for the secretion of IFN-γ at 60 h. Fig. 6 shows the only allogeneic cultures with sorted effector memory cells $CD4^+$ T cells from primed donors vigorously secreted IFN-γ (3500 pg/ml). The concentration of IFN-γ in the these cultures was significantly increased as compared with the primed effector memory syngeneic cultures ($p = 0.02$), and as compared with allogeneic cultures with naive T cells from unprimed and primed donors ($p = 0.02$). Although all cultures were assayed for IL-4 and IL-10 in the supernatants, these cytokines were not detected in multiplex cytokine assays (data not shown).

Discussion

Previous studies of untreated (unprimed) donor mice showed that sorted T cells expressing the naive ($CD62L^{high}CD44^{low}$) phenotype vigorously induced lethal GVHD in MHC-mismatched irradiated hosts, and that sorted T cells expressing the effector memory phenotype ($CD62L^{low}CD44^{high}$) did not (6–9). Although the result was interpreted as a failure of effector memory T cells to induce GVHD, it is possible that the environmental Ags or endogenous Ags that generated these memory T cells did not cross-react with alloantigens of the host. Thus, "third-party" memory T cells may have been studied rather than host alloantigen-specific memory T cells. In the current study, naive and effector memory $CD4^+$ T cells from unprimed C57BL/6 donors and from C57BL/6 donors primed to BALB/c host alloantigens were tested for their capacity

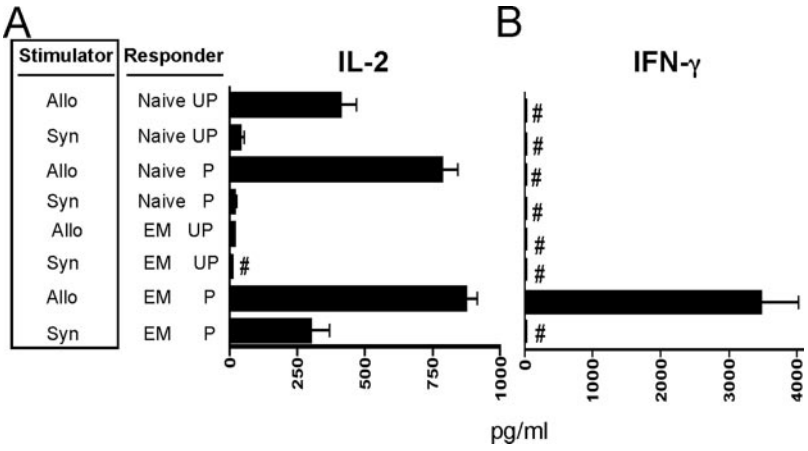

FIGURE 6. Cytokine responses of unprimed and primed C57BL/6 donor naive or effector memory $CD4^+$ T cells to host stimulator cells in the MLR. *A*, Mean ± SEM concentrations of IL-2; *B*, mean ± SEM concentrations of IFN-γ in the supernatants of triplicate MLR cultures at 60 h. #, The concentration of cytokine was <50 pg/ml. Results are representative of at least three MLR cultures. EM, UP, and P denote effector memory, unprimed, and primed, respectively. Allo and Syn denote BALB/c and C57BL/6 stimulator cells, respectively.

Table I. *Differences in naive CD4$^+$ T cell and host alloantigen-primed effector memory CD4$^+$ T cell-induced GVHD*

GVHD Parameters	Naive Cell GVHD	Memory Cell GVHD
Accumulation of donor cells in mesenteric lymph nodes and colon	Rapid	Slow
Colitis	Acute[a]	Chronic[b]
Accumulation of donor cells in liver	Rapid	Rapid
Portal tract and biliary injury	None early	Late onset
Cytokine secretion by donor cells in MLR	IL-2	IL-2 and IFN-γ
Kinetics of host weight loss and death	Rapid	Slow

[a] Early severe diarrhea and early crypt injury.
[b] No early diarrhea and late onset of crypt injury.

to induce lethal GVHD in irradiated MHC- and minor Ag-mismatched BALB/c hosts.

The experimental data confirm the results of the previous report, and show that sorted naive CD4$^+$ T cells from unprimed donors induce lethal GVHD whereas sorted effector memory CD4$^+$ T cells from unprimed donors failed to induce severe GVHD, as measured by either skin changes, progressive weight loss, or death. Whereas sorted unprimed naive CD4$^+$ T cells induced severe acute colitis that was apparent by histopathologic examination by day 6 after transplantation, the unprimed sorted effector memory CD4$^+$ T cells induced minimal or no microscopic injury to colonic crypts at the same time point. In addition, the hosts that received unprimed effector memory T cells had no significant increase in GVHD scores in the liver and colon at day 72 as compared with hosts given no donor T cells.

Although the sorted effector memory CD4$^+$ T cells from primed donors failed to induce acute colitis by day 6, these donor T cells induced lethal GVHD and all hosts died by 100 days with progressive weight loss. The tempo of morbidity and mortality in the latter hosts was considerably slower than the severe GVHD with acute colitis induced by naive T cells from primed or unprimed donors. The slower progressive weight loss induced by the primed effector memory T cells was associated with increased injury to the portal tracts, biliary tree, and colon at day 72, as compared with that in hosts given unprimed memory T cells or no T cells. However, acute or chronic diarrhea was not apparent in these hosts. It was surprising that the naive T cells induced a more rapid and severe form of GVHD as compared with the primed memory T cells because adoptively transferred alloantigen-primed memory T cells reject tissue allografts more rapidly than naive T cells (20).

In an MHC-matched model, donor effector memory CD4$^+$ T cells exposed to host alloantigens during chronic GVHD transferred GVHD to irradiated adoptive hosts while the similar numbers of naive CD4$^+$ T cells from unprimed mice did not induce GVHD (15). Thus, the memory cells were more potent inducers of GVHD than naive cells. The MHC-matched model did not address the issue of acute gut injury that is a central feature of acute GVHD induced by naive donor T cells. In our model system, the severe intestinal injury and death occurred within 2–3 wk. However, in both model systems, primed memory CD4$^+$ T cells induced a disease with more chronic intestinal injury and most deaths occurred $>$40 days after transplantation.

The failure of primed effector memory cells to induce acute colitis characterized by early severe diarrhea, rapid weight loss, and death, and early microscopic injury of colonic crypts as compared with naive T cells in the current study was reflected in the differences in the accumulation of the donor T cells in the host mesenteric lymph nodes and colon by day 6. Approximately 20- to 100-fold more naive T cells accumulated in the latter tissues as compared with the primed effector memory T cells. In contrast, no significant difference in accumulation was observed between naive and primed effector memory T cells in the liver and spleen at day 6. It is of interest that 50-fold more primed than unprimed memory T cells accumulated in the liver at day 6. The marked increase in accumulation of the primed memory cells may be linked to the more severe liver injury observed with primed as compared with unprimed memory T cells at day 72.

The markedly reduced expression of both of the lymph node homing receptors, CD62L and CCR7, and reduced capacity for expansion can account for the differences in the ability of naive and effector memory CD4$^+$ T cells to induce acute colitis. Our previous study showed that rapid migration of donor T cells to the mesenteric lymph nodes was required to induce acute colitis (16). The reduced expression of both CD62L and CCR7 on effector memory as compared with naive T cells may have impaired the migration of the primed and unprimed effector memory cells into the mesenteric lymph nodes. CD62L is required for binding of T cells to the peripheral lymph node addressin molecule on the high endothelial cells in the lymph nodes, and the interaction facilitates passage through these endothelial cells and into the nodes (21). The interaction between the chemokine receptor, CCR7, and the chemokines CCL19 and CCL21 produced by the lymph nodes also facilitates the transit of T cells from the blood into the nodes by mediating lymphocyte arrest (22). Although effector memory phenotype cells from 8-mo-old mice have been shown to be alloreactive in vitro, they failed to induce GVHD (8). This may be attributed to their lack of homing receptors to access secondary lymphoid organs and reduced capacity to expand during the initiation phase of GVHD.

Interestingly, the primed effector memory T cells rapidly accumulated in the liver despite the deficits in the lymph node trafficking receptors. A possible explanation is that priming of memory T cells up-regulates the tissue homing chemokine receptor CXCR3 (23). Up-regulation of CXCR3 may facilitate migration of T cells to the tissues such as the liver that produce the chemokine ligands CXCL9, CXCL10, and CXCL11 (24). The priming procedure is likely to have not only increased the clonal frequency of alloreactive CD4$^+$ T cells within the sorted effector memory T cells, but also to have increased CXCR3 expression on these newly generated host alloreactive T cells. Both of these increases may have contributed to increased accumulation in the liver.

Priming increased the reactivity of sorted effector memory T cells to host alloantigens, because primed memory T cells secreted significantly more IL-2 and IFN-γ in response to host alloantigens than the memory T cells from unprimed donors in the MLR at 60 h. The primed memory T cell vigorous secretion of IFN- γ likely contributed to host tissue injury, because IFN-γ secretion activates tissue macrophages to secrete TNF-α and to injure intestine, skin, and liver (25–27). The vigorous IL-2 response of naive T cells in the MLR without an IFN-γ response is consistent with

the Th0 cytokine pattern reported previously for naive T cells (28, 29). Secretion of IFN-γ with IL-2 is characteristic of Th1-biased memory T cells induced after alloantigenic priming.

Table I summarizes the differences in GVHD induced by naive and alloantigen-primed memory $CD4^+$ T cells. Naive cell GVHD resulted in acute colitis characterized by severe early diarrhea, rapid host weight loss, and death, and early microscopic injury of colonic crypts whereas memory cell GVHD had a more chronic pattern of liver and colon injury characterized by no early diarrhea, slow weight loss, and late appearance of histopathological injuries of colon and liver. In conclusion, the paradoxical ability of naive $CD4^+$ T to induce a more rapid and severe type of GVHD than primed $CD4^+$ T effector memory T cells is related to the different trafficking patterns and expansion capacity of the two T cell subsets, and the requirement of early homing to the mesenteric lymph nodes in the development of acute colitis. The results may provide some insights into the different patterns of tissue injury in acute and chronic GVHD.

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Disclosures

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