

Alloreactive Memory T Cells Are Responsible for the Persistence of Graft-versus-Host Disease¹

Yi Zhang, Gerard Joe, Elizabeth Hexner, Jiang Zhu, and Stephen G. Emerson²

Graft-vs-host disease (GVHD) is caused by a donor T cell anti-host reaction that evolves over several weeks to months, suggesting a requirement for persistent alloreactive T cells. Using the C3H.SW anti-C57BL/6 (B6) mouse model of human GVHD directed against minor histocompatibility Ags, we found that donor CD8⁺ T cells secreting high levels of IFN- γ in GVHD B6 mice receiving C3H.SW naive CD8⁺ T cells peaked by day 14, declined by day 28 after transplantation, and persisted thereafter, corresponding to the kinetics of a memory T cell response. Donor CD8⁺ T cells recovered on day 42 after allogeneic bone marrow transplantation expressed the phenotype of CD44^{high}CD122^{high}CD25^{low}, were able to homeostatically survive in response to IL-2, IL-7, and IL-15 and rapidly proliferated upon restimulation with host dendritic cells. Both allogeneic effector memory (CD44^{high}CD62L^{low}) and central memory (CD44^{high}CD62L^{high}) CD8⁺ T cells were identified in B6 mice with ongoing GVHD, with effector memory CD8⁺ T cells as the dominant (>80%) population. Administration of these allogeneic memory CD8⁺ T cells into secondary B6 recipients caused virulent GVHD. A similar allogeneic memory CD4⁺ T cell population with the ability to mediate persistent GVHD was also identified in BALB/b mice receiving minor histocompatibility Ag-mismatched B6 T cell-replete bone marrow transplantation. These results indicate that allogeneic memory T cells are generated *in vivo* during GVH reactions and are able to cause GVHD, resulting in persistent host tissue injury. Thus, *in vivo* blockade of both alloreactive effector and memory T cell-mediated host tissue injury may prove to be valuable for GVHD prevention and treatment. *The Journal of Immunology*, 2005, 174: 3051–3058.

Acute graft-vs-host disease (GVHD)³ occurs when donor T cells are transferred into hosts expressing histocompatibility differences (1–6). Patients who receive HLA-matched sibling or unrelated donor transplants may develop acute GVHD within the first 100 days after transplantation. Clinical acute GVHD syndromes in these patients may persist over several months to years after allogeneic bone marrow transplantation (allo-BMT), and some proceed to chronic GVHD (1–6). In experimental GVHD models, lethal GVHD directed against minor histocompatibility (miHAs) may also evolve over several weeks to months (2–9). Although it is well known that the persistence of alloreactive T cells is necessary for host tissue damage and subsequent GVHD, previous studies have shown that massive apoptotic death of alloreactive effector T cells occurs in GVHD recipients of T cell-replete allo-BMT (10–12). Thus, which alloreactive T cell subsets are responsible for GVHD persistence is still not well understood.

Typical T cell responses to pathogen (i.e., virus) consist of three characteristic phases (13–17). During the first phase of clonal expansion, pathogen-specific T cells are activated and expanded,

leading to the acquisition of effector cell functions. During the second phase of T cell death, the effector T cell pool contracts after the elimination of pathogen, with >95% of effector T cells dying through apoptosis (15, 17). The final phase is characterized by the generation of a small number of memory T cells that can survive *in vivo* in the absence of pathogen and are able to proliferate as well as to produce inflammatory cytokines and cytotoxicity more rapidly upon secondary Ag encounter than naive T cells. In the case of GVHD, host Ags causing GVHD persist *in vivo* in recipients after allo-BMT (2, 4–6). Although both the expansion and contraction of host Ag-specific donor T cells have been observed in the recipients of allo-BMT (10–12), it is not known whether the persistence of host miHAs affects the generation of functional alloreactive memory T cells responsible for GVHD.

Interestingly, recent studies suggest that T cells with a memory phenotype isolated from normal donors are much less efficient than donor T cells with a naive phenotype at causing acute GVHD (12, 18–21). Because these memory T cells may have developed *in vivo* in normal donors in response to third-party environmental Ags, they may not be able to mediate GVH reactions after adoptive transfer into recipients expressing allogeneic Ags (12, 18–21). Surprisingly, however, we found that even *ex vivo* activated donor CD44^{high}CD8⁺ T cells with effector/effector memory phenotype that were induced *in vitro* by host dendritic cells (DCs) for 5 days failed to mediate acute GVHD, partially due to their enhanced apoptotic depletion *in vivo*. In contrast, host DC-induced donor CD44^{low}CD8⁺ T cells with a naive phenotype are extremely virulent inducers of GVHD (12). Taken together, these data would seem to indicate, paradoxically, that the role of memory T cells in GVHD is problematic and leave open the question of which T cell subsets are, in fact, required for persistent GVHD responses *in vivo*. Given the potential importance of such T cell subsets as targets for prevention and treatment, identifying which alloreactive T cell subsets are responsible for the persistence of host tissue injury of GVHD is clearly important.

Departments of Medicine and Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Received for publication October 4, 2004. Accepted for publication December 1, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by a Specialized Center for Research from the Leukemia and Lymphoma Society of America and by National Institutes of Health Grant RO1CA102464-01.

² Address correspondence and reprint requests to Dr. Stephen G. Emerson, Division of Hematology and Oncology, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. E-mail address: emersons@mail.med.upenn.edu

³ Abbreviations used in this paper: GVHD, graft-vs-host disease; allo-BMT, allogeneic bone marrow transplantation; DC, dendritic cell; miHA, minor histocompatibility Ag; T⁻BM, T cell-depleted BM.

Using miHA-mismatched mouse GVHD models of human allo-BMT, we report that both donor CD8⁺ and CD4⁺ T cells can differentiate into allogeneic memory T cells mediating GVHD persistence. These *in vivo*-generated alloreactive memory T cells expressed CD44^{high}CD122^{high}CD25^{low} memory phenotype, were able to respond to secondary stimulation of host DCs more rapidly than donor naive and effector T cells and caused persistent host tissue injury. We also found that the persistence of host miHAs was critical for functional expansion of these alloreactive memory T cells. Thus, prevention and treatment of GVHD directed against miHAs should target both alloreactive effector and memory T cell populations.

Materials and Methods

Mice

B6/SJL (H-2D^b, CD45.1⁺), C3H.SW (H-2D^b, CD45.2⁺, and Ly9.1⁺), and BALB/b (H-2D^b, CD45.2⁺) mice were purchased from The Jackson Laboratory. Drinking water of BMT recipients was supplemented with neomycin sulfate and polymyxin B (Sigma-Aldrich) as we previously described (12, 22, 23).

Abs, cell lines, cytokines, and flow cytometric analysis

Biotinylated anti-mouse CD44 Ab and all other Abs used for immunofluorescence staining were obtained from BD Pharmingen. Microbead-conjugated Abs and streptavidin were purchased from Miltenyi-Biotec. B6 mouse-derived EL-4 leukemic cells (T-ALL and H-2D^b) and BALB/c mouse-derived NS-1 cells (myeloma and H-2D^d) were obtained from American Type Culture Collection and grown in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% FBS. All the recombinant cytokines including IL-2, IL-4, IL-7, IL-15, GM-CSF, stem cell factor, and TNF- α , were purchased from R&D Systems. H60/MHC-I tetramer (H2K^b) was prepared by the National Institute of Allergy and Infectious Diseases Tetramer Facility. Immunofluorescence analyses of cell surface phenotypes and intracellular cytokines were performed by FACScan (BD Biosciences) as previously described (12, 22, 24, 25).

Cell preparations

BM cells were prepared from donor mice as previously described (12, 22, 24, 25). T cell-depleted BM (T⁻BM) cells were isolated with anti-CD4 and anti-CD8 Ab-conjugated microbeads. CD8⁺ and CD4⁺ T cells from

spleens and lymph nodes of mice incubated with anti-CD8 and anti-CD4 Ab-conjugated microbeads, respectively, followed by positive selection using MACS (MiniMACS; Miltenyi Biotec). CD11c⁺ cells were magnetically removed by anti-CD11c Ab conjugated with microbeads before purifying donor T cells. To further separate CD44⁻CD8⁺, CD62L^{high}CD8⁺, or CD62L^{low}CD8⁺ T cells, purified CD8⁺ T cells were stained with allophycocyanin-conjugated anti-CD44 Ab or with PE-conjugated anti-CD62L Ab and sorted into CD44⁻CD8⁺, CD62L^{high}CD8⁺, and CD62L^{low}CD8⁺ T cells by FACS (MoFlow; DakoCytomation). The purity of each sorted T cell subset was consistently >95%. CFSE labeling of donor CD8⁺ T cells was performed as previously described (22).

Mature DCs were prepared from B6 BM as previously described (24–26). Briefly, B6 BM were stained with biotinylated anti-c-Kit Ab, followed by streptavidin-conjugated microbeads (Miltenyi Biotec). The c-Kit⁺ hemopoietic progenitor cells were magnetically sorted and incubated for 6 days in IMDM (Invitrogen Life Technologies) supplemented with 10% FBS in the presence of GM-CSF, stem cell factor, and IL-4. CD11c⁺ immature DCs were magnetically sorted from this 6-day culture using anti-CD11c Ab-conjugated microbeads and stimulated with GM-CSF and TNF- α for an additional 2 days to induce their maturation.

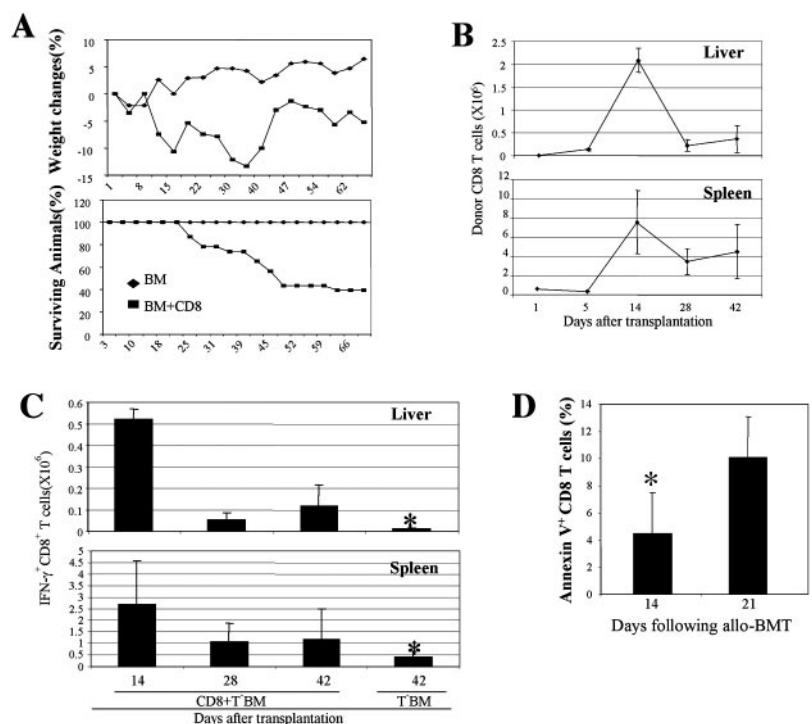
GVHD induction

Mice underwent allo-BMT as previously described (12, 22, 23). Briefly, for C3H.SW anti-B6 mouse GVHD, B6 recipients were irradiated with 10.0 Gy, administered in two fractions from a ¹³⁷Cs source. C3H.SW T⁻BM (5 × 10⁶), mixed with or without C3H.SW CD8⁺ T cells, were transplanted into B6 recipients via tail vein injection (four to eight mice per group per experiment) immediately after irradiation. In the B6 anti-BALB/b mouse GVHD model, BALB/b recipients were irradiated with 8.5 Gy in two separate fractions. B6 T⁻BM cells (5 × 10⁶), mixed with or without B6 CD4⁺ and CD8⁺ T cells, were transplanted into BALB/b recipients via tail vein injection. Recipient mice were weighed twice weekly and monitored for the clinical signs of acute GVHD and survival. The clinical grading criteria for the cutaneous inflammation and clinical score of acute GVHD were followed as established previously (27). Mice were killed, and specimens of liver, skin, and intestine were taken for histopathologic assessment of GVHD as previously described (28).

Ex vivo stimulation of CD8⁺ T cells

Donor CD44⁻CD8⁺ T cells from normal C3H.SW mice or donor CD8⁺ T cells recovered from B6 mice receiving C3H.SW T⁻BM plus CD8⁺ T cells were cultured in IMDM containing 10% FBS with or without addition of IL-2 (5 ng/ml), IL-7(3 ng/ml), and IL-15 (3 ng/ml) in a 96-well plate,

FIGURE 1. Persistence of alloreactive donor CD8⁺ T cells in recipient mice. *A*, Weight changes and survival rate of lethally irradiated (10.0 Gy) mice. B6 mice were injected with 5 × 10⁶ C3H.SW T⁻BM, mixed with or without 2 × 10⁶ C3H.SW CD8⁺ T cells. *B* and *C*, Mononuclear cells were recovered from the spleens and livers of B6 mice (CD45.1) receiving C3H.SW T⁻BM and CD8⁺ T cells (CD45.2), numerated, and stained with anti-CD45.2 and anti-CD8. The number of donor CD8⁺ T cells was calculated after flow cytometric analysis (*B*). Donor lymphocytes that were separately recovered from the spleens and livers of B6 mice receiving C3H.SW T⁻BM and CD8⁺ T cells were cultured in the presence of anti-CD3 Ab for 12 h to measure the production of IFN- γ by intracellular cytokine staining. The number of alloreactive CD8⁺ T cells secreting high levels of IFN- γ was calculated after flow cytometric analysis (*C*). All values represent the mean \pm SD cell number in each group. Data shown in this study are pooled from five separate experiments. *D*, Lymphocytes were recovered from the spleens and livers of B6 mice receiving C3H.SW T⁻BM and CD8⁺ T cells on days 14 and 21, respectively, after transplantation, pooled, and stained with anti-CD45.2, anti-CD8 Abs, and FITC-annexin V for flow cytometric analysis. All values represent the mean \pm SD cell percentage from three mice for each group, which represents two independent experiments.



with or without addition of B6 BM-derived mature DCs at a DC:T cell ratio of 1:1.

Cytolytic assay

CFSE-based cytolytic assay was performed to determine the cytolytic ability of T cells as previously described (29). Donor CD8⁺ T cells were either freshly isolated from naive C3H.SW mice (unstimulated CD8⁺ T cells) or recovered from the spleens and livers of irradiated B6 mice (CD45.1) receiving B6 CD8⁺ T cells by MACS or FACS. The cytolytic activity was directly assessed without any additional ex vivo stimulation of these CD8⁺ T cells.

Statistical analysis

Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of χ^2 . A value of $p < 0.05$ was considered significant.

Results

Generation of allogeneic memory T cell response in recipients with ongoing GVHD

We first asked whether the allogeneic memory T cell responses develop in CD8⁺ T cell-dependent C3H.SW anti-B6 mouse model following the kinetics of a classical Ag-specific T cell expansion, apoptotic contraction, and survival of memory T cells. C3H.SW CD8⁺ T cells (2×10^6) were i.v. injected into lethally irradiated B6 mice together with C3H.SW T⁻BM (5×10^6). Most of B6 mice receiving C3H.SW T⁻BM and CD8⁺ T cells developed

acute GVHD and died on day 50 after transplantation, whereas some survived free of clinical GVHD for >100 days (Fig. 1A). In parallel to GVHD development, donor (CD45.2⁺) CD8⁺ T cells and CD8⁺ T cells secreting high levels of IFN- γ peaked in the livers of these B6 mice (CD45.1⁺) by day 14 ($2.1 \pm 0.27 \times 10^6$ and $0.52 \pm 0.048 \times 10^6$, respectively), considerably declined by day 28 ($0.22 \pm 0.13 \times 10^6$ and $0.055 \pm 0.03 \times 10^6$, respectively), increased again by day 42 ($0.36 \pm 0.29 \times 10^6$ and $0.12 \pm 0.1 \times 10^6$, respectively) after transplantation, and persisted thereafter (Fig. 1, B and C). Similar biphasic kinetics of donor CD8⁺ T cells and CD8⁺ T cells producing high levels of IFN- γ were also observed in the spleens of these B6 recipients (Fig. 1, B and C). The decline in donor CD8⁺ T cells in these B6 mice was accompanied with increased annexin V-positive donor CD8⁺ T cells in vivo ($10.1 \pm 3.1\%$ on day 21 vs $4.5 \pm 3.0\%$ on day 14; $p < 0.05$; Fig. 1D). In contrast, B6 mice receiving C3H.SW T⁻BM alone did not develop any signs of GVHD and had 3- to 10-fold less donor CD8⁺ T cells that produced IFN- γ by day 42 after transplantation compared with B6 recipients of donor CD8⁺ T cell-replete BMT (Fig. 1C). These results suggest that a typical memory T cell response, as manifested by T cell expansion, contraction, and re-expansion, develops in recipients during the GVHD process, and a small population of alloreactive CD8⁺ T cells survives after the death phase of effector cells.

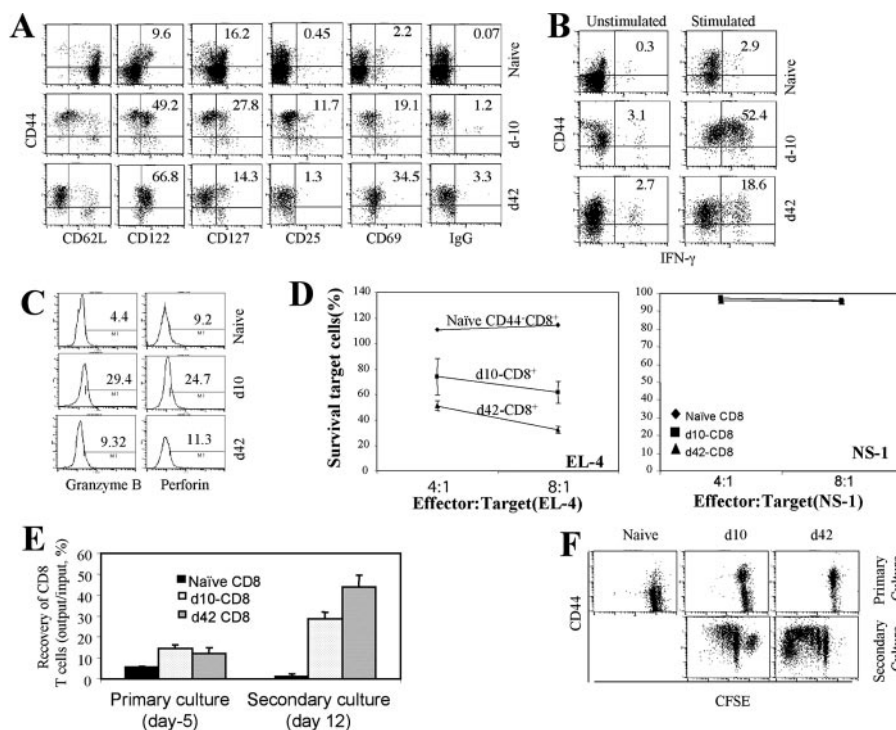
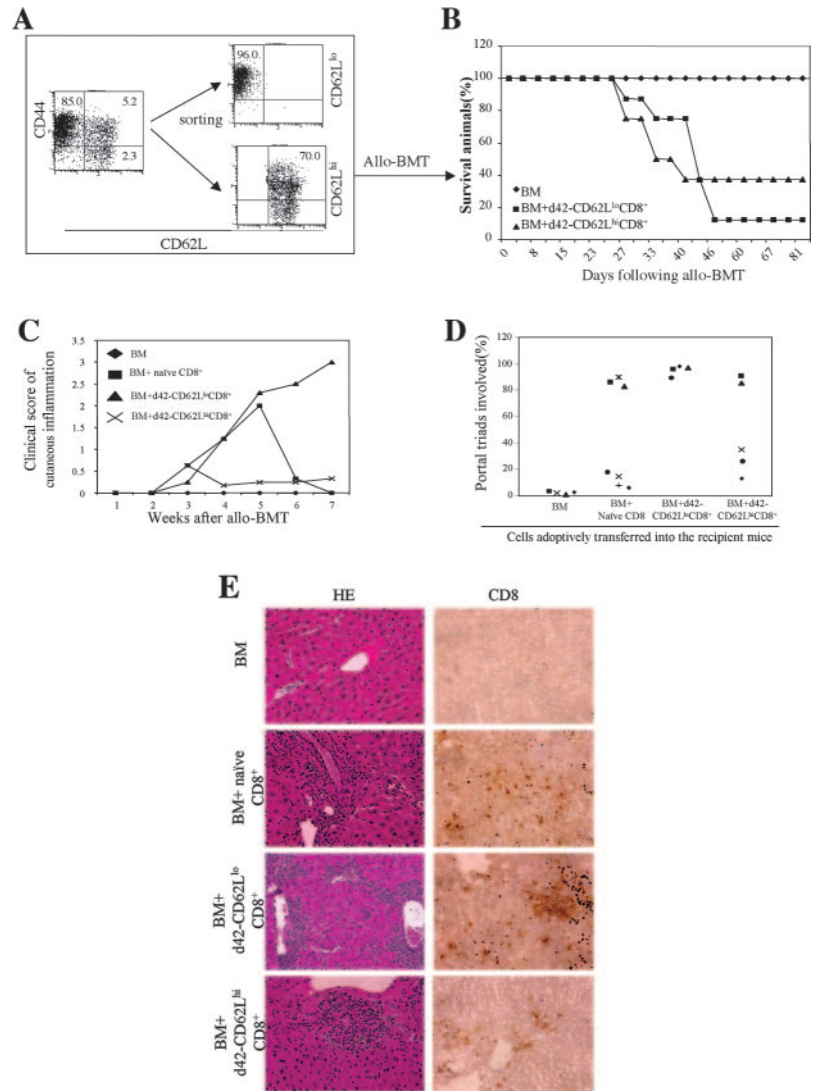


FIGURE 2. Generation of allogeneic memory CD8⁺ T cells in recipient mice with ongoing GVHD. Mononuclear cells were separately recovered from the spleens and lymph nodes of normal C3H.SW mice or from the spleens of GVHD B6 mice (CD45.1) injected with 5×10^6 C3H.SW T⁻BM and 1×10^6 CD44⁻CD8⁺ T cells (CD45.2) at 10 days (day 10-CD8⁺) or 42 days (day 42-CD8⁺) after allo-BMT. Lymphocytes from the spleens and lymph nodes of normal C3H.SW mice were used as controls (naive CD8⁺). Cells were stained with anti-CD45.2, anti-CD8, anti-CD62L, anti-CD122, anti-CD25, or IgG control for flow cytometric analysis (A). Cells were also stimulated with anti-CD3 Ab for 12 h for measuring the production of IFN- γ by intracellular cytokine staining, followed by flow cytometric analysis (B). Dot plots shown are gated CD8⁺ T cells. The values represent percentages of donor CD8⁺ T cells in each cell fraction. C, The expression of cytotoxic molecules in naive CD44⁻CD8⁺, day 10-CD8⁺, and day 42-CD8⁺ T cells were intracellularly stained with anti-granzyme B and anti-perforin Abs for flow cytometric analysis. Data are shown as a histogram by gating on CD8⁺ T cells. Values represent the percentage of CD8⁺ T cells expressing the tested molecule. D, The ability of naive CD44⁻CD8⁺, day 10-CD8⁺, and day 42-CD8⁺ T cells to kill EL-4 cells and NS-1 cells ex vivo was examined by a CFSE-labeling method. E and F, Magnetically sorted, naive CD44⁻CD8⁺, day 10-CD8⁺, and day 42-CD8⁺ T cells were labeled with CFSE and separately cultured in medium containing a low dose of IL-2, IL-15, and IL-7 for 5 days. Cells were then harvested, enumerated (E), and stained with anti-CD44 and anti-CD8 Abs for flow cytometric analysis (F). Data shown are representative of three independent experiments.

FIGURE 3. Allogeneic memory CD8⁺ T cells are virulent GVHD inducers. **A**, Donor CD8⁺ T cells were magnetically separated from mononuclear cells recovered from the spleens and livers of GVHD B6 mice receiving C3H.SW T⁻BM and CD8 T cells 42 days after allo-BMT. After staining with anti-CD62L Ab, day 42-CD62L^{low} and day 42-CD62L^{high} CD8⁺ T cells were sorted, mixed with C3H.SW T⁻BM, and transplanted into lethally irradiated secondary B6 recipient mice. **B**, Survival rate of B6 mice injected with alloreactive day 42-CD62L^{low} and day 42-CD62L^{high} CD8⁺ memory T cells. Data are pooled from two separate experiments, and each group contains eight recipient mice. **C–E**, The clinical score of cutaneous inflammation in lethally irradiated B6 mice receiving alloreactive day 42-CD62L^{low} and day 42-CD62L^{high} CD8⁺ memory T cells, as mentioned in **A**, was monitored and determined twice a week for 7 wk after allo-BMT (**C**). The liver sections of B6 mice receiving C3H.SW T⁻BM, B6 mice receiving C3H.SW T⁻BM and CD8⁺ naive T cells, B6 mice receiving alloreactive day 42-CD62L^{low}CD8⁺ T cells, or B6 mice receiving day 42-CD62L^{high}CD8⁺ T cells were stained with H&E or immunohistochemically stained with anti-CD8 Ab (**E**). Histologic assessment of hepatic GVHD was performed by calculating the percentage of portal triads involved based on HE-stained liver sections from B6 recipients (**D**).



Alloreactive CD8⁺ T cells that survive the contraction phase are memory T cells

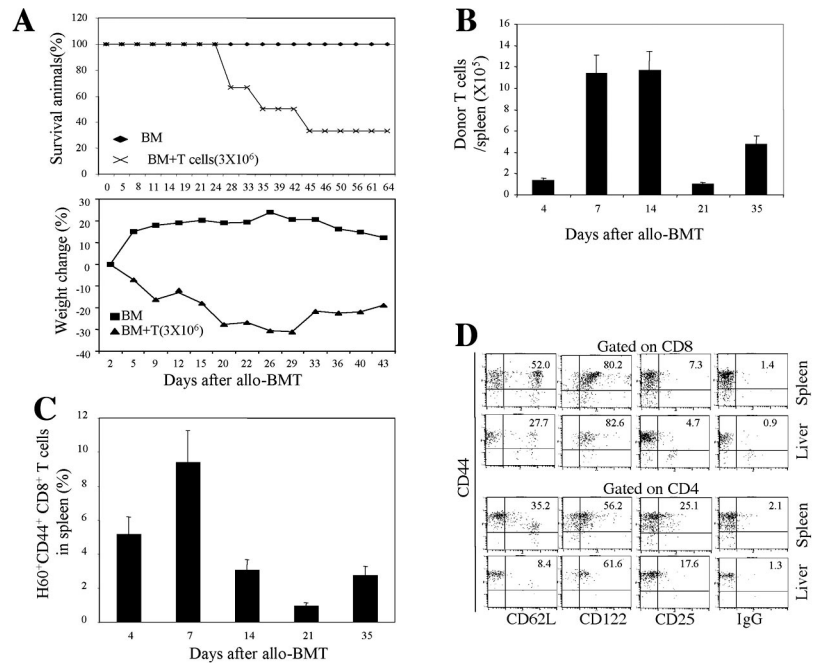
Previous studies of classical immune responses after Ag administration *in vivo* have demonstrated that functional memory CD8⁺ T cells have definitively developed *in vivo* by day 40 after immunization (13–15, 30). Memory CD8⁺ T cells express a CD44^{high}CD122^{high}CD25^{low} phenotype, exhibit homeostatic survival in the presence of cytokines such as IL-15 and IL-7 and can respond to secondary antigenic stimulation more rapidly than naive T cells (15–17, 31). To characterize *in vitro* and *in vivo* whether donor CD8⁺ T cells that persist in GVHD B6 mice have similar memory T cell phenotype and functions, these cells were recovered from the spleens and livers of B6 mice with ongoing GVHD on day 42 after transplantation of C3H.SW T⁻BM⁺CD44⁻CD8⁺ T cells (termed day 42-CD8⁺). Donor CD8⁺ effector T cells were also isolated from B6 mice receiving C3H.SW T⁻BM⁺CD44⁻CD8⁺ T cells 10 days after allo-BMT (named day 10-CD8⁺), at the peak of effector CD8⁺ T cell expansion. Host residual hemopoietic cells (CD45.1⁺) were magnetically depleted before isolation of donor T cells (CD45.2⁺) from these B6 recipient mice (CD45.1⁺), unless otherwise mentioned. Donor naive CD8⁺ T cells from normal C3H.SW mice (termed naive CD8⁺) were prepared as controls.

Naive CD44⁻CD8⁺ T cells from normal C3H.SW mice were CD44⁻CD25⁻CD122⁻ and did not produce IFN- γ (Fig. 2, *A* and *B*). They expressed lower levels of the cytotoxic molecules granzyme B and perforin compared with day 10-CD8⁺ effector T cells and were not cytolytic killers (Fig. 2, *C* and *D*). By contrast, both donor day 10-CD8⁺ and day 42-CD8⁺ T cells expressed CD44^{high}CD122^{high} and produced high levels of IFN- γ upon *ex vivo* stimulation (Fig. 2, *A* and *B*). These donor day 10-CD8⁺ and day 42-CD8⁺ T cells were cytolytic killers, with day 42-CD8⁺ T cells showing more potent ability than day 10-CD8⁺ T cells to kill B6 mouse-

derived EL-4 leukemic cells, but not unrelated BALB/c mouse-derived NS-1 cells (Fig. 2*D*). Interestingly, compared with day 10-CD8⁺ effector cells, day 42 CD8⁺ T cells did not express the effector T cell marker CD25 (1.3% of day 42-CD8⁺ vs 11.7% of day 10-CD8⁺) and showed reduced levels of cytotoxic molecules of granzyme B and perforin (Fig. 2, *A* and *C*). These results suggest that donor day 42-CD8⁺ T cells predominantly express the phenotype of a memory T cells and contain a higher frequency of cytolytic killer cells than do day 10-CD8⁺ effector T cells.

We next determined whether day 42-CD8⁺ T cells had acquired memory T cell functions, e.g., the ability to homeostatically survive in the presence of a low dose of IL-2, IL-7, and IL-15 and the ability to rapidly proliferate in response to host mHAs. Donor naive CD44⁻CD8⁺, day 10-CD8⁺, and day 42-CD8⁺ T cells were separately labeled with CFSE and cultured in the presence of IL-2, IL-7, and IL-15. Five days later, 2- to 3-fold more donor CD8⁺ T cells were recovered from day 10-CD8⁺ and day 42-CD8⁺ T cell cultures compared with naive CD44⁻CD8⁺ T cell cultures (Fig. 2*E*). Flow cytometric analysis showed that although both donor day 42-CD8⁺ and day 10-CD8⁺ T cells remained CD44^{high}, neither population had undergone division in this primary culture (Fig. 2*F*). Interestingly, when these surviving donor CD8⁺ T cells were harvested and recultured in the presence of B6 DCs combined with IL-2, IL-7, and IL-15 for an additional 5 days, both day 42-CD8⁺ and day 10-CD8⁺ T cells proliferated and expanded, with day 42-CD8⁺ T cells dividing more vigorously than day 10-CD8⁺ T cells (Fig. 2*F*). Consequently, there were more donor CD8⁺ T cells recovered from day 42-CD8⁺ T cell cultures than from day 10-CD8⁺ T cell cultures (Fig. 2*E*). By contrast, donor naive CD8⁺ T cells had diminished by day 5 in the secondary culture despite host B6 DC stimulation (Fig. 2*E*). Furthermore, when donor day 42-CD8⁺ T cells from B6 mice with ongoing GVHD were further sorted into

FIGURE 4. Persistence of alloreactive T cells in the B anti-BALB/b mouse GVHD model. *A*, Weight changes and survival rate of lethally irradiated (10.0 Gy) BALB/b mice (CD45.2) injected with 5×10^6 B6 T⁻BM together with or without 3×10^6 B6 T cells (CD45.1, containing equal numbers of CD4⁺ and CD8⁺ T cells). *B*, Mononuclear cells were recovered from the spleens of BALB/b mice (CD45.2) receiving B6 T⁻BM and T cells (CD45.1), enumerated, and stained with anti-CD45.1 and anti-CD8 Abs. The number of donor CD8⁺ T cells was calculated after flow cytometric analysis. All values represent the mean \pm SD cell number in each group. *C*, Lymphocytes recovered from the spleens, livers, and BM of BALB/b mice receiving B6 T⁻BM and T cells were stained with anti-CD45.1, anti-CD8, anti-CD44, and PE-conjugated H60/MHC-I tetramer. The percentage of alloreactive H60⁺CD44^{high}CD8⁺ T cells specific to host miHA H60 was analyzed by flow cytometry. All values represent the mean \pm SD of cell percentage in each group. *D*, Donor T cells were recovered from the spleens and livers of BALB/b mice receiving B6 T cells 28 days or more after allo-BMT, stained with Abs as indicated, and analyzed by flow cytometry. Dot plots show the cells gated on donor-derived CD8⁺ or CD4⁺ T cells. The values represent the percentages of indicated cells in each cell fraction. Data shown represent three independent experiments.



CD62L^{low} (2×10^6 cells/recipient) and CD62L^{high} (0.3×10^6 cells/recipient) T cell subsets that predominantly contained effector memory (CD44^{high}CD62L^{low}, >95%) and central memory (CD44^{high}CD62L^{high}, >70%) T cells, respectively, and transplanted together with C3H.SW T⁻BM into lethally irradiated secondary B6 mice (Fig. 3A), both populations caused lethal GVHD with cutaneous and hepatic inflammation (Fig. 3, B–E).

Taken together, these results indicate that infused donor CD8⁺ T cells can differentiate in GVHD recipients into functionally allogeneic memory T cells with the ability to homeostatically survive and to rapidly proliferate in response to host miHAs, leading to persistent GVHD.

In vivo generation of allogeneic CD4⁺ memory T cells

Although CD8⁺ T cells are the most potent effectors mediating GVHD, both CD8⁺ and CD4⁺ T cells may contribute to the induction and effector phases of GVHD (7, 32–34). We therefore asked whether allogeneic memory CD4⁺ T cells are also responsible for persistent host tissue injury in recipients. Using an established miHA-mismatched CD4⁺ T cell-dependent B6 anti-BALB/b mouse GVHD model (33, 34), donor (CD45.1⁺) B6 T⁻BM (5×10^6), mixed with or without B6 T cells (1.5×10^6 CD4⁺ + 1.5×10^6 CD8⁺ T cells), were transplanted into lethally irradiated BALB/b recipient mice (CD45.2⁺). BALB/b mice receiving B6 T⁻BM+T cells, but not B6 T⁻BM, developed severe acute GVHD, with ~70% of mice dying of GVHD by day 50 after allo-BMT (Fig. 4A). Host-reactive donor T cells (CD45.1⁺), including both CD4⁺ and CD8⁺ T cells, peaked between days 7 and 14, declined by day 21, and increased again by day 35 after transplantation (Fig. 4B). Staining of donor T cells with H60/MHC-I tetramers that specifically bind to alloreactive CD8⁺ T cells against H60 peptide also confirmed that the biphasic kinetics of donor CD8⁺ T cells specific to host miHA H60 were identified in these BALB/b recipients (Fig. 4C). Flow cytometric analysis showed that most of alloreactive CD4⁺ and CD8⁺ T cells recovered from BALB/b mice with ongoing GVHD on day 28 or later after allo-BMT were CD44^{high}CD62L^{low}CD122^{high} (Fig. 4D), similar to an effector memory T cell phenotype observed in CD8⁺ T cell-dependent GVHD in B6 recipients of C3H.SW T cells (Fig. 2A). This suggests that allogeneic memory CD4⁺ T cells developed in BALB/b during the GVHD process.

We then tested whether these allogeneic CD4⁺ T memory cells are able to induce host tissue injury. Host-reactive donor memory CD4⁺ T cells (CD45.1⁺) were separated from the spleens and livers of primary BALB/b mice (CD45.2⁺) with ongoing GVHD on day 28 or later after infusion of B6 T cells and T⁻BM after magnetically depleting CD45.1⁺ host residual cells and were transplanted together with B6 T⁻BM into lethally irradiated secondary BALB/b mice. Purified naive CD4⁺ and CD8⁺ T cells isolated from normal B6 mice were transplanted into BALB/b mice as controls. All BALB/b mice receiving 5×10^6 B6 naive T cells (containing equal numbers of CD4⁺ and CD8⁺) and B6 T⁻BM developed lethally acute GVHD (Fig. 5A), whereas all BALB/b mice receiving 3×10^6 or 4×10^5 naive B6 CD4⁺ T cells survived >100 days after allo-BMT, without developing any signs of acute GVHD (Fig. 5A). In contrast, as few as 4×10^5 donor memory CD4⁺ T cells recovered from primary GVHD BALB/b recipients of B6 T cells caused severe GVHD in secondary BALB/b recipients, with 100% lethality (Fig. 5A). Cutaneous GVHD with lichenoid-like lesions was a prominent clinical sign of these secondary BALB/b mice receiving alloreactive memory CD4⁺ T cells (Fig. 5, B and C). Thus, *in vivo*-generated allogeneic memory CD4⁺ T cells from primary GVHD BALB/b mice are also potent inducers of GVHD.

Discussion

Although it is well understood that donor-derived CTL are the effector cells responsible for GVHD directed against host miHAs (1–5), little is known about the role of host-reactive donor effector- vs-memory T cells in mediating persistent host tissue injury. Our studies demonstrate that a small proportion of alloreactive T cells responsible for acute GVHD survived after the massive apoptotic death of host-reactive donor effector cells in recipient mice. These alloreactive T cells possessed memory T cell features, persisted for the lifetime of mice with ongoing GVHD, and were able to cause virulent acute GVHD when adoptively transferred into secondary recipient mice. Thus, allogeneic memory T cells generated during the development of acute GVHD against miHAs are responsible

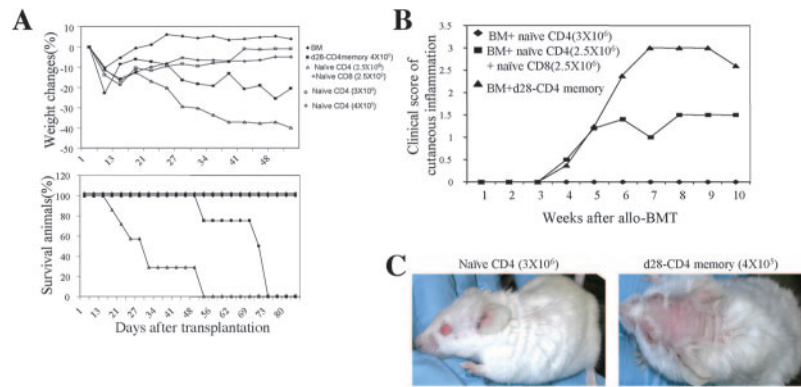


FIGURE 5. Allogeneic memory T cells from GVHD BALB/b recipients of B6 T cells are also virulent GVHD inducers. Donor CD4⁺ T cells were magnetically separated from GVHD BALB/b mice receiving B6 T cells and T⁻BM on day 28 or later after allo-BMT. CD4 memory T cells (4×10^5) were transplanted together with 5×10^6 B6 T⁻BM into lethally irradiated BALB/b mice. BALB/b mice receiving B6 T⁻BM, B6 T⁻BM, and 3×10^6 naive CD4⁺ T cells; B6 T⁻BM and 4×10^5 naive CD4⁺ T cells; or B6 T⁻BM and 2.5×10^6 naive CD4⁺ T cells and 2.5×10^6 naive CD8⁺ T cells were used as controls. **A**, Weight changes and survival rate of BALB/b. **B**, Clinical scores of cutaneous inflammation of BALB/b mice receiving B6 T⁻BM, B6 T⁻BM and alloreactive CD4⁺ memory T cells, or B6 T⁻BM and alloreactive CD4⁺ memory T cells and CD8⁺ memory T cells. **C**, Lichenoid cutaneous inflammation developed in BALB/b mice receiving 4×10^5 alloreactive CD4 memory T cells as mentioned in **A**, but not in BALB/b recipient mice of B6 T⁻BM and 3×10^6 naive CD4⁺ T cells. Data shown in this study represent two independent experiments, and each group contained four to 12 mice.

for persistent host tissue injury; whereas host-reactive donor effector T cells are short-lived cells, the majority of which diminish during the GVHD process.

There is a sharp contrast between memory T cells generated in recipients with GVHD and memory T cells derived from normal donors in their ability to induce GVHD (12, 18–21). Such a discrepancy could be explained by the hypothesis that normal donor-derived memory T cells are induced after exposure to environmental Ags (12, 18, 20, 21), whereas allogeneic memory T cells in our experiments are specifically induced by host miHAs. However, this would not explain why ex vivo host DC-induced donor CD44^{high}CD8⁺ T cells with enriched host-reactive T cells failed to mediate virulent GVHD in our previous study (12). In that study we demonstrated that the in vivo expansion of host DC-induced donor CD44^{high}CD8⁺ T cells was significantly impaired due to increased apoptotic deletion compared with CD44^{low}CD8⁺ T cells (12). Although such apoptotic death of allogeneic effector T cells was also observed in the recipients of donor naive T cells in our present study, alloreactive memory T cells survived effector cell contraction and persisted throughout the life of recipients with ongoing GVHD. Because the development of memory T cells evolves over a period of 3–4 wk (13–16, 35, 36), it is possible that ex vivo host DC-induced CD44^{high}CD8⁺ T cells are susceptible to apoptotic deletion, but not to effector memory T cell differentiation, leading to their inability to induce GVHD (12). In contrast, in vivo generated alloreactive memory T cells in recipients with ongoing GVHD are able to persist in vivo longer than effector T cells, leading to GVHD persistence.

Our findings suggest that the alloreactive memory T cell response is an important characteristic of acute GVHD progression (10, 11, 37–40). Previous studies show that after a brisk expansion of donor T cells in the host, massive activation-induced cell death of alloreactive T cells follows (10, 11). In these studies (10, 11), both donor CD4⁺ and CD8⁺ T cells demonstrated virtually identical in vivo kinetics in recipient mice: rapid expansion by day 7 and considerable decline by day 21 after transplantation. However, these studies did not further characterize the allogeneic T cells that survived this contraction (10, 11). We found that donor T cells that survived the contraction were allogeneic memory T cells specific for host miHAs. These allogeneic memory T cells have the ability to proliferate more rapidly upon secondary host miHA stimulation

and contained a higher frequency of cytotoxic killers against host cells compared with donor naive or effector T cells. Interestingly, we observed that this rapid proliferation of alloreactive donor memory T cells in secondary recipients did not result in the expansion of more effector cells than naive T cells early after transplantation (data not shown), suggesting that effector cells derived from memory T cells are as susceptible as those from naive T cells to activation-induced cell death. It is possible that GVHD in secondary recipients of alloreactive memory T cells, like the primary recipients of donor naive T cells, also requires de novo-generated donor memory T cells. This may explain why in vivo administration of alloreactive memory T cells in secondary recipients did not dramatically hasten the onset of GVHD. Thus, generation of allogeneic memory T cells determines the fate of GVHD progression or resolution, an important prognostic marker for GVHD prevention and treatment.

It is intriguing to consider the implications of allogeneic memory CD8⁺ T cells generated in GVHD, which is unusual among immune responses in that the stimulating Ag is never completely cleared. Previous studies indicate that memory CD8⁺ T cells develop after Ag clearance and death of short-lived effector cells (14–17, 31, 41). However, it is unclear whether the persistence of host miHAs affects the development of functional alloreactive memory CD8⁺ T cells. We found that in vivo-generated allogeneic memory CD8⁺ T cells in recipient mice with ongoing GVHD acquired the ability to rapidly proliferate and expand in response to host DCs in the presence of IL-2, IL-7, and IL-15. By contrast, in the absence of B6 DCs, stimulation of allogeneic memory T cells with the cytokine combination of IL-2, IL-7, and IL-15 alone merely induced their homeostatic survival. Interestingly, these allogeneic memory CD8⁺ T cells survived when adoptively transferred into secondary B6 mice, resulting in severe GVHD, but not when transferred into β_2 -microglobulin gene-deficient ($\beta_{2\mu}^{-/-}$) B6 mice (data not shown). All these data suggest that in vivo generation and maintenance of allogeneic memory T cells against host miHAs require the persistence of host Ags and functional APCs during the GVHD process. This is consistent with a recent report that donor APCs are required for maximal GVHD (42), in which de novo-generated donor APCs present host miHAs to amplify allogeneic memory T cell response. Thus, blocking the constant stimulation of host-reactive allogeneic memory T cells by host miHAs could ameliorate GVHD.

Given that allogeneic memory T cells persist in recipients with ongoing GVHD, one could ask whether allogeneic memory T cells generated during the acute GVHD process contribute to the development of chronic GVHD. Previous studies have demonstrated that up to 65% of patients with chronic GVHD have had prior acute GVHD (6). Chronic GVHD may evolve directly from the progression of acute GVHD or after a period of resolution of acute GVHD (6). Although the role of allogeneic memory T cells in mediating chronic GVHD is not yet known, current data suggest that infiltration of allogeneic memory T cells in GVHD-involved host tissues occurs in chronic GVHD (37, 38, 40). Data from our experiments using the CD4⁺ T cell-dependent B6 anti-BALB/b mouse model of GVHD against miHAs demonstrates that donor B6 CD44^{high}CD62L^{low} CD4⁺ effector memory cells were the predominant CD4⁺ T cell population in BALB/b mice with ongoing acute GVHD on day 28 after allo-BMT. Interestingly, administration of these allogeneic memory CD4⁺ T cells at a dose 10 times less than naive donor CD4⁺ T cells caused severe GVHD in secondary BALB/b recipient mice accompanied by lichenoid cutaneous inflammation, a clinical sign also seen in recipients with chronic GVHD (6). These data suggest that alloreactive memory CD4⁺ T cells contain a highly enriched host-reactive donor T cell population and are responsible for persistent GVHD with clinical signs similar to chronic GVHD. Further investigation of the possible role of allogeneic memory T cells in chronic GVHD induction will prove valuable for understanding the pathophysiology of as well as establishing strategies for chronic GVHD prevention and treatment.

The data presented in this study indicate that alloreactive memory T cells recovered from mice undergoing GVHD reactions are more virulent than naive T cells from normal donors in mediating the disease. The difference may be accounted for by the fact that memory T cells were recovered from primary recipients with GVHD and were enriched in host-reactive T cells. Indeed, these alloreactive memory T cells continually expressed the activation marker CD69 and were able to directly kill host cells without ex vivo restimulation with host miHAs, indicating their recent TCR activation *in vivo* (15–17, 36). Interestingly, we found that although ~60–70% of the primary recipients of unmanipulated donor T cells developed severe GVHD and died by day 50 after allo-BMT, others survived >100 days without significant clinical and histological signs of GVHD (Fig. 1A and Fig. 3, C–E). Furthermore, donor CD8⁺ T cells recovered from these primary recipients without GVHD 60 days after allo-BMT contained many fewer donor CD44^{high}CD62L^{low} effector memory T cells and T cells producing IFN- γ (data not shown). Although it remains to be determined whether CD4⁺CD25⁺ regulatory T cells are responsible for the resolution of GVH responses in these primary recipients (43–47), we have recently demonstrated that unmanipulated CD8⁺ T cells caused much less severe GVHD than purified CD44^{low}CD8⁺ T cells (12), suggesting that this heterogeneous cell population contains cells with the ability to modulate GVHD *in vivo*. It will be interesting to further investigate whether memory T cells are more refractory to the inhibitory effect of regulatory T cells than naive T cells.

In summary, we demonstrate that allogeneic memory T cell responses develop *in vivo* in recipients with ongoing GVHD and the generation of alloreactive memory T cells against miHAs requires the persistent stimulation of host miHAs. These alloreactive memory T cells account for the persistent host tissue injury. Although current GVHD prophylaxis is focusing on effector T cell-mediated host tissue damage, our findings suggest that effective GVHD prevention and treatment can only be achieved by targeting both effector and memory T cell tissue injury.

Disclosures

The authors have no financial conflict of interest.

References

- Blazar, B. R., R. Korngold, and D. A. Valleria. 1997. Recent advances in graft-versus-host disease (GVHD) prevention. *Immunol. Rev.* 157:79.
- Ferrara, J. L., K. R. Cooke, and T. Teshima. 2003. The pathophysiology of acute graft-versus-host disease. *Int. J. Hematol.* 78:181.
- Ferrara, J. L., R. Levy, and N. J. Chao. 1999. Pathophysiologic mechanisms of acute graft-versus-host disease. *Biol. Blood Marrow Transplant.* 5:347.
- Goker, H., I. C. Haznedaroglu, and N. J. Chao. 2001. Acute graft-versus-host disease: pathobiology and management. *Exp. Hematol.* 29:259.
- Ho, V. T., and R. J. Soiffer. 2001. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood* 98:3192.
- Higman, M. A., and G. B. Vogelsang. 2004. Chronic graft versus host disease. *Br. J. Haematol.* 125:435.
- Korngold, R., and J. Sprent. 1981. H-2 restriction of T cells causing lethal graft-versus-host disease across minor histocompatibility barriers in mice. *Transplant. Proc.* 13:1217.
- Kosaka, H., C. D. Surh, and J. Sprent. 1992. Stimulation of mature unprimed CD8⁺ T cells by semiprofessional antigen-presenting cells *in vivo*. *J. Exp. Med.* 176:1291.
- den Haan, J. M., N. E. Sherman, E. Blokland, E. Huczko, F. Koning, J. W. Drijfhout, J. Skipper, J. Shabanowitz, D. F. Hunt, V. H. Engelhard, et al. 1995. Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* 268:1476.
- Brochu, S., B. Rioux-Masse, J. Roy, D. C. Roy, and C. Perreault. 1999. Massive activation-induced cell death of alloreactive T cells with apoptosis of bystander postthymic T cells prevents immune reconstitution in mice with graft-versus-host disease. *Blood* 94:390.
- Choi, E. Y., G. J. Christianson, Y. Yoshimura, N. Jung, T. J. Sproule, S. Malarkannan, S. Joyce, and D. C. Roopenian. 2002. Real-time T-cell profiling identifies H60 as a major minor histocompatibility antigen in murine graft-versus-host disease. *Blood* 100:4259.
- Zhang, Y., G. Joe, J. Zhu, R. Carroll, B. Levine, E. Hexner, C. June, and S. G. Emerson. 2004. Dendritic cell-activated CD44^{hi}CD8⁺ T cells are defective in mediating acute graft-versus-host disease but retain graft-versus-leukemia activity. *Blood* 103:3970.
- Dutton, R. W., L. M. Bradley, and S. L. Swain. 1998. T cell memory. *Annu. Rev. Immunol.* 16:201.
- Homann, D., L. Teyton, and M. B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8⁺ but declining CD4⁺ T-cell memory. *Nat. Med.* 7:913.
- Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* 111:837.
- Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225.
- Masopust, D., and R. Ahmed. 2004. Reflections on CD8 T-cell activation and memory. *Immunol. Res.* 29:151.
- Giver, C. R., J. M. Li, M. S. Hossain, S. Lonial, and E. K. Waller. 2004. Reconstructing immunity after allogeneic transplantation. *Immunol. Res.* 29:269.
- Foster, A. E., M. Marangolo, M. M. Sartor, S. I. Alexander, M. Hu, K. F. Bradstock, and D. J. Gottlieb. 2004. Human CD62L⁻ Memory T cells are less responsive to alloantigen stimulation than CD62L⁺ naive T cells: potential for adoptive immunotherapy. *Blood*.
- Chen, B. J., X. Cui, G. D. Sempowski, C. Liu, and N. J. Chao. 2004. Transfer of allogeneic CD62L⁻ memory T cells without graft-versus-host disease. *Blood* 103:1534.
- Anderson, B. E., J. McNiff, J. Yan, H. Doyle, M. Mamula, M. J. Shlomchik, and W. D. Shlomchik. 2003. Memory CD4⁺ T cells do not induce graft-versus-host disease. *J. Clin. Invest.* 112:101.
- Zhang, Y., J. P. Louboutin, J. Zhu, A. J. Rivera, and S. G. Emerson. 2002. Preterminal host dendritic cells in irradiated mice prime CD8⁺ T cell-mediated acute graft-versus-host disease. *J. Clin. Invest.* 109:1335.
- Zhang, Y., W. D. Shlomchik, G. Joe, J. P. Louboutin, J. Zhu, A. Rivera, D. Giannola, and S. G. Emerson. 2002. APCs in the liver and spleen recruit activated allogeneic CD8⁺ T cells to elicit hepatic graft-versus-host disease. *J. Immunol.* 169:7111.
- Zhang, Y., Y. Y. Zhang, M. Ogata, P. Chen, A. Harada, S. Hashimoto, and K. Matsushima. 1999. Transforming growth factor- β polarizes murine hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells through a monocyte/macrophage differentiation pathway. *Blood* 93:1208.
- Zhang, Y., A. Harada, J. B. Wang, Y. Y. Zhang, S. Hashimoto, M. Naito, and K. Matsushima. 1998. Bifurcated dendritic cell differentiation *in vitro* from murine lineage phenotype-negative c-kit⁺ bone marrow hematopoietic progenitor cells. *Blood* 92:118.
- Zhang, Y., N. Mukaida, J. Wang, A. Harada, M. Akiyama, and K. Matsushima. 1997. Induction of dendritic cell differentiation by granulocyte-macrophage colony-stimulating factor, stem cell factor, and tumor necrosis factor α *in vitro* from lineage phenotypes-negative c-kit⁺ murine hematopoietic progenitor cells. *Blood* 90:4842.
- Cooke, K. R., L. Kobzik, T. R. Martin, J. Brewer, J. Delmonte, Jr., J. M. Crawford, and J. L. Ferrara. 1996. An experimental model of idiopathic

- pneumonia syndrome after bone marrow transplantation. I. The roles of minor H antigens and endotoxin. *Blood* 88:3230.
28. del Rosario, M. L., J. R. Zucali, and K. J. Kao. 1999. Prevention of graft-versus-host disease by induction of immune tolerance with ultraviolet B-irradiated leukocytes in H-2 disparate bone marrow donor. *Blood* 93:3558.
 29. Jedema, I., N. M. van der Werff, R. M. Barge, R. Willemze, and J. H. Falkenburg. 2004. New CFSE-based assay to determine susceptibility to lysis by cytotoxic T cells of leukemic precursor cells within a heterogeneous target cell population. *Blood* 103:2677.
 30. Masopust, D., V. Vezyz, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291:2413.
 31. Swain, S. L., R. W. Dutton, and D. L. Woodland. 2004. T cell responses to influenza virus infection: effector and memory cells. *Viral. Immunol.* 17:197.
 32. Friedman, T. M., S. C. Jones, D. Statton, G. F. Murphy, and R. Korngold. 2004. Evolution of responding CD4⁺ and CD8⁺ T-cell repertoires during the development of graft-versus-host disease directed to minor histocompatibility antigens. *Biol. Blood Marrow Transplant.* 10:224.
 33. Berger, M., P. J. Wettstein, and R. Korngold. 1994. T cell subsets involved in lethal graft-versus-host disease directed to immunodominant minor histocompatibility antigens. *Transplantation* 57:1095.
 34. Berger, M. A., and R. Korngold. 1997. Immunodominant CD4⁺ T cell receptor V β repertoires involved in graft-versus-host disease responses to minor histocompatibility antigens. *J. Immunol.* 159:77.
 35. Murali-Krishna, K., J. D. Altman, M. Suresh, D. Sourdive, A. Zajac, and R. Ahmed. 1998. In vivo dynamics of anti-viral CD8 T cell responses to different epitopes: an evaluation of bystander activation in primary and secondary responses to viral infection. *Adv. Exp. Med. Biol.* 452:123.
 36. Jameson, S. C. 2002. Maintaining the norm: T-cell homeostasis. *Nat. Rev. Immunol.* 2:547.
 37. Diamond, D. J., K. L. Chang, K. A. Jenkins, and S. J. Forman. 1995. Immunohistochemical analysis of T cell phenotypes in patients with graft-versus-host disease following allogeneic bone marrow transplantation. *Transplantation* 59:1436.
 38. Favre, A., A. Cerri, A. Bacigalupo, E. Lanino, E. Berti, and C. E. Grossi. 1997. Immunohistochemical study of skin lesions in acute and chronic graft versus host disease following bone marrow transplantation. *Am. J. Surg. Pathol.* 21:23.
 39. Theobald, M., and D. Bunjes. 1993. Pretransplant detection of human minor histocompatibility antigen-specific naive and memory interleukin-2-secreting T cells within class I major histocompatibility complex (MHC)-restricted CD8⁺ and class II MHC-restricted CD4⁺ T-cell subsets. *Blood* 82:298.
 40. Yamashita, K., U. Choi, P. C. Woltz, S. F. Foster, M. C. Sneller, F. T. Hakim, D. H. Fowler, M. R. Bishop, S. Z. Pavletic, M. Tamari, et al. 2004. Severe chronic graft-versus-host disease is characterized by a preponderance of CD4⁺ effector memory cells relative to central memory cells. *Blood* 103:3986.
 41. Sondel, P. M., I. N. Buhtoiarov, and K. DeSantes. 2003. Pleasant memories: remembering immune protection while forgetting about graft-versus-host disease. *J. Clin. Invest.* 112:25.
 42. Maris, M., M. Boeckh, B. Storer, M. Dawson, K. White, M. Keng, B. Sandmaier, D. Maloney, R. Storb, and J. Storek. 2003. Immunologic recovery after hematopoietic cell transplantation with nonmyeloablative conditioning. *Exp. Hematol.* 31:941.
 43. Hoffmann, P., J. Ermann, M. Etinger, C. G. Fathman, and S. Strober. 2002. Donor-type CD4⁺CD25⁺ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J. Exp. Med.* 196:389.
 44. Johnson, B. D., M. C. Konkol, and R. L. Truitt. 2002. CD25⁺ immunoregulatory T-cells of donor origin suppress alloreactivity after BMT. *Biol. Blood Marrow Transplant.* 8:525.
 45. Chen, Z. M., M. J. O'Shaughnessy, I. Gramaglia, A. Panoskaltis-Mortari, W. J. Murphy, S. Narula, M. G. Roncarolo, and B. R. Blazar. 2003. IL-10 and TGF- β induce alloreactive CD4⁺CD25⁻ T cells to acquire regulatory cell function. *Blood* 101:5076.
 46. Etinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4⁺CD25⁺ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9:1144.
 47. Trenado, A., F. Charlotte, S. Fisson, M. Yagello, D. Klatzmann, B. L. Salomon, and J. L. Cohen. 2003. Recipient-type specific CD4⁺CD25⁺ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J. Clin. Invest.* 112:1688.