Introduction

Graft-versus-host disease (GVHD) is a life-threatening complication of allogeneic hematopoietic cell transplantation (HCT). GVHD occurs when mature T cells in the donor bone marrow (BM) graft respond to host tissues expressing incompatible histocompatibility antigens, represented by either MHC antigens or minor histocompatibility antigens (miHAs). In an MHC-matched donor/recipient combination, miHAs are derived from translated polymorphic protein-degradation products that can be presented in the context of either MHC class I or class II molecules, and consequently both CD8+ and CD4+ T cells can participate in the development of miHA-driven GVHD (1, 2). The ensuing inflammatory response is characterized acutely by alloreactive T cell–dependent injury to the epithelial tissues of the skin, liver, and gut (3).

The development of lethal GVHD has been found to depend on T cell recognition of alloantigens presented by host professional APCs (4). In this regard, recipients that lack MHC class I expression on cells of the hematopoietic compartment, while expressing normal levels on all other tissues (i.e., epithelium, endothelium, parenchyma, etc.), do not develop GVHD following injection of MHC-matched, miHA-disparate allogeneic CD8+ T cells (4). However, it is still unclear whether alloantigen expression by the host hematopoietic compartment alone is sufficient to elicit lethal GVHD. An understanding of the implications of alloreponses directed against hematopoietic cells for the development of GVHD will also positively impact the understanding of the beneficial graft-versus-leukemia (GVL) response. As has been suggested (5, 6), GVH reactions directed against the hematopoietic compartment alone may preferentially drive a GVL response with reduced risk of direct GVHD target-tissue injury. Similarly, following donor leukocyte infusion, allogeneic T cell responses directed against the host hematopoietic compartment have been observed to generate an effective GVL response (7).

In an effort to better understand the role of T cell responses directed against antigens presented by the hematopoietic compartment in the development of GVHD, our previous studies utilized the MHC-matched, miHA-disparate BM chimera transplantation model B10.BR → CBA (H2k). The results demonstrated that miHA expression on host APCs alone was insufficient to elicit the CD4-independent, CD8-mediated...
lethal GVHD (8). Surprisingly, however, in murine BM chimeric recipients with allogeneic MHC class I and II expression restricted exclusively to the hematopoietic compartment, Teshima et al. (9) demonstrated that both CD8+ and CD4+ T cells, respectively, could mediate lethal GVHD. In light of the apparent discrepancy in the role of the hematopoietic compartment in GVHD between these two studies, we have now undertaken to examine the CD4+ T cell responses in a miHA-mismatched BM chimera transplantation model. Here, using the C57BL/6→BALB.B (both H2b) system, we show that hematopoietic expression of miHAs is not enough to drive a CD4-mediated lethal GVHD response, thus implicating nonhematopoietic host cells as critical targets in acute GVHD in a miHA-disparate transplantation setting.

**Methods**

**Mice.** C.B10-H2b/LiMc dyst (BALB.B) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) or raised in our breeding colony from breeder pairs purchased from The Jackson Laboratory. C57BL/6NCR (B6) mice were purchased from the National Cancer Institute Laboratory Animal Production Program (Frederick, Maryland, USA). For all experiments, sex-matched mice between the ages of 6 and 14 weeks were used as donors and recipients. Mice were housed in a pathogen-free environment in autoclaved microisolator cages and were provided with autoclaved water and food ad libitum. All experiments were performed with approval of the Thomas Jefferson University Institutional Animal Care and Use Committee (Philadelphia, Pennsylvania, USA).

**Monoclonal antibodies.** Ascites fluid containing anti-Thy-1.2 (J1j, rat IgM) (10), anti-CD8 (3.168, rat IgM) (11) mAb’s were used along with guinea pig complement (C′; Rockland, Boyertown, Pennsylvania, USA) for cell subset depletions. Affinity-purified goat anti-mouse IgG antibody (Cappel-Organon Teknika Corp., West Chester, Pennsylvania, USA) was used for B cell panning. For donor chimerism analysis and phenotypic analysis of donor T cell grafts, FITC- and/or phycoerythrin-labeled mAb’s specific for the following determinants were used: irrelevant isotype control, Ly9.1, CD3ε, CD4, CD8ε, B220, and CD11c (all from Pharmingen, San Diego, California, USA).

**Flow cytometry.** Between 0.06 and 0.125 µg of each mAb was incubated with 1 × 10⁶ cells or fewer, in PBS containing 1% BSA and 0.01% NaN₃ (wash buffer) for 25 minutes at 4°C. Following multiple washes in wash buffer, either fluorescence analysis was performed immediately or cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) for 15 minutes at 4°C, followed by a final wash. Fluorescence analysis was performed on a Beckman Coulter XL-MCL analytic cytometer (Beckman Coulter Inc., Miami, Florida, USA). Flow cytometric gates were established by exclusion of background isotype-control antibody binding. In the case of two-color staining, individual populations of control cells were stained with positive-control phycoerythrin- and FITC-labeled antibodies, respectively, to assure no background overlap in fluorescence emission between the two fluorochromes.

**Preparation of donor cells.** PBS (BioWhittaker Inc., Walkersville, Maryland, USA) supplemented with 0.1% BSA (Sigma-Aldrich, St. Louis, Missouri, USA) was used for all preparative manipulations of the donor BM and lymphocytes. Antibody–T cell–depleted BM (ATBM) was prepared by flushing of BM cells from the femurs and tibiae of donor mice, followed by incubation with J1j mAb (1:50 dilution) and C′ (1:12 dilution) for 45 minutes at 37°C. T cell–enriched donor cells were prepared from pooled spleen and lymph node cell suspensions from donor mice as previously described (12). Briefly, following red blood cell lysis with Gay’s balanced salt solution containing 0.7% NH₄Cl, B cells were removed by panning of the cell suspension over goat anti-mouse IgG-coated plastic Petri dishes for 1 hour at 4°C. Nonadherent lymphocytes were depleted of CD8+ T cells by incubation with 3.168 mAb (1:50 dilution) and C′ (1:12 dilution) for 45 minutes at 37°C. Donor T cells were more than 97% enriched for the CD4+ subset, as determined by flow cytometry. Cells were injected i.v. in PBS alone.

**Preparation of BM chimeras.** BM chimeras were prepared by transfer of 1 × 10⁶ ATBM cells i.v. into mice exposed 4–6 hours earlier to 13 Gy, split dose (6.5 Gy at 1.36 Gy/min), from a 137Cs Mark-1 model 68 gamma irradiator (J.L. Shepherd and Associates, San Fernando, California, USA). Mice were allowed to reconstitute for 14–17 weeks, at which time a minimum of three mice of representative weight were sacrificed to determine the percentage of donor hematopoietic chimerism by two-color flow cytometric analysis.

**In vivo analysis for GVHD.** BM transplantation was performed as previously described, with some modifications (12). At 14–17 weeks after reconstitution, BM chimeric recipients (indicated in brackets) were reirradiated with 10–13 Gy (split dose) and were injected i.v. 4–6 hours later with a B6 donor inoculum of 2 × 10⁶ ATBM cells, in combination with 1.5 × 10⁶ to 2 × 10⁶ CD4+ T cells. Animals were monitored for morbidity and mortality and weighed regularly until the termination of the experiment. Mid-survival times (MSTs) were calculated as the interpolated 50% survival point of a linear regression through all of the death data points, including zero. Statistical comparisons between experimental groups were based on individual days of death and were performed by the nonparametric Wilcoxon signed rank test. Weight data were expressed as the mean percentage of initial body weight during sequential 1-week periods following transplantation. Significance for weight comparisons between groups was determined by the Student’s t test at particular time points.

**Histology.** Sequential ear biopsies were collected from mice at specific time points, or upon sacrifice of the animal at the conclusion of the experiment, and prepared for routine histological analysis, as previously described.
Figure 1
Minor histocompatibility antigen expression by host hematopoietic tissues alone does not elicit lethal GVHD. Established [BALB.B→BALB.B], [BALB.B→B6], and [B6→B6] BM chimeras were reirradiated (10 Gy, split dose) and injected with 2×10^6 B6 ATBM cells in combination with 1.5×10^7 B6 CD4+ T cells. (a) Survival of transplanted recipients. (b) Body weights for each group normalized as the mean percentage initial body weight ± SEM over sequential 1-week periods. n = 5 for B6→[BALB.B→BALB.B] and [BALB.B→B6], and n = 6 for B6→[B6→B6].

For quantitation of dyskeratotic epidermal cells, skin samples were evaluated for features of apoptotic keratinocytes (contracted hypereosinophilic cytoplasm, pyknotic nuclei) by light microscopy (13). Data are based on counts from at least 10 linear millimeters (Lmm) of epidermis per sample and are reported as the dyskeratotic index (mean ± SEM number of apoptotic keratinocytes per Lmm; ref 14). Measurements of dermal thickness were obtained, using an ocular grid micrometer, from the dermal/epidermal junction to the deepest extent of the reticular dermis and are based on ten random measurements per sample, arbitrarily separated by 1 Lmm. To avoid nonspecific variability in dermal thickness, all measurements were taken from biopsies harvested from the same region of the ear. All observations were made in a double-blind manner by an experienced dermatopathologist.

Results
In the B6→BALB.B lethal-irradiation GVHD model, donor and recipient mice differ from one another by at least 29 different miHA loci (15). To determine the relevance of miHA expression by host hematopoietic or nonhematopoietic tissues to the development of lethal GVHD across this multiple-miHA barrier, [BALB.B→B6] BM chimeric recipients were established such that the only source of alloantigen was the hematopoietic compartment. To this end, B6 mice were lethally irradiated (13 Gy, split dose) and injected with 1×10^7 BALB.B ATBM cells. BALB.B chimerism was determined 3–4 months later by cyometric staining of splenocytes for the Ly9.1 cell-surface glycoprotein, a lymphoid marker detected on more than 98% of spleen and lymph node cells (16), with differential expression between BALB.B (Ly9.1+) and B6 (Ly9.1-) strains of mice. Within the representative [BALB.B→B6] chimeras analyzed, at least 87% of the T cell compartment and at least 98% of the B cell compartment were BALB.B-derived, as measured by anti-CD3 and anti-B220 staining, respectively. Most importantly, more than 90% of large, CD11c+ cells were BALB.B-derived, likely representing DCs of lymphoid origin (17–19).

Following confirmation of BALB.B hematopoietic engraftment, [BALB.B→B6] BM chimeras were reirradiated (10 Gy, split dose) and injected with 2×10^6 B6 ATBM cells plus 1.5×10^7 B6 CD4+ T cells. Identically transplanted [BALB.B→BALB.B] and [B6→B6] BM chimeras were used as GVHD positive and negative controls, respectively. All [BALB.B→BALB.B] chimeric recipients succumbed to acute GVHD, with an MST of 26.2 days, whereas the [B6→B6] CD4 recipients experienced no GVHD lethality (MST >75 days; Figure 1a; Table 1). Importantly, the injection of 1.5×10^7 B6 CD4+ T cells into [BALB.B→B6] chimeric recipients did not result in GVHD lethality (MST >75 days). Transient cachexia was noted in this group, however (P ≤ 0.024 vs. [B6→B6] syngeneic control; Figure 1b), during weeks 2 and 3 after HCT, indicative of an initial B6 CD4+ T cell response against miHAs expressed by the BALB.B hematopoietic compartment. A replicate experiment using identical irradiation doses, but with the injection of 2×10^6 B6 CD4+ T cells, likewise failed to produce lethality in the [BALB.B→B6] chimeras (MST >90 days; Table 1).

Table 1
Summary of data from transplants with miHAs restricted to the hematopoietic compartment

<table>
<thead>
<tr>
<th>Dose of B6 CD4+ T cells</th>
<th>Reirradiated chimeras</th>
<th>Dead/Total</th>
<th>MST</th>
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<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
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<tr>
<td>1.5×10^7</td>
<td>BALB.B→13 Gy BALB.B</td>
<td>5/5</td>
<td>26.2</td>
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<tr>
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<td>B6→13 Gy B6</td>
<td>0/5</td>
<td>&gt;75</td>
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<tr>
<td>1.5×10^7</td>
<td>BALB.B→13 Gy B6</td>
<td>0/5</td>
<td>&gt;75</td>
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<td><strong>Experiment 2</strong></td>
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<tr>
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<td>BALB.B→13 Gy BALB.B</td>
<td>3/3</td>
<td>13.3</td>
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<td>0/4</td>
<td>&gt;90</td>
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<tr>
<td>2×10^7</td>
<td>BALB.B→13 Gy B6</td>
<td>0/5</td>
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Chimeras were prepared by transfer of 1×10^7 ATBM cells into irradiated (13 Gy) mice and then left for 14–17 weeks. Chimeras were then reirradiated (10 Gy) and injected with 1.5×10^7 to 2×10^8 B6 CD4+ T cells along with 2×10^6 B6 ATBM cells.
before, all [BALB.B→BALB.B] recipients succumbed to GVHD (MST 13.3 days), whereas lethality was not noted in the [B6→B6] group (MST >90 days).

Cutaneous manifestations of acute GVHD are an accurate indicator of clinical disease severity, with the extent of apoptotic injury to skin epidermal cells representing a reliable parameter of disease (14). Ear biopsies harvested on day 8 after HCT from [BALB.B→BALB.B], [BALB.B→B6], and [B6→B6] chimeric recipients of 1.5×10⁷ B6 CD4+ T cells revealed similar numbers of dyskeratotic cells, likely reflecting irradiation-related injury at this early time point (P ≥ 0.23, all groups vs. [B6→B6] recipients; Figure 2d). This early injury was subsequently resolved in [B6→B6] recipients; however, the level of epidermal apoptosis remained elevated in the skin of [BALB.B→BALB.B] recipients, peaking by day 21 after HCT (Figure 2, a and d). In contrast, in [BALB.B→B6] recipients, the extent of apoptotic injury was similar to [B6→B6] syngeneic-control levels (P ≥ 0.71, days 21 and 32 vs. [B6→B6] recipients; Figure 2, b–d).

Despite the absence of acute GVHD in [BALB.B→B6] chimeric recipients, evaluation of ear samples at the conclusion of the experiment (day 90) revealed dermal sclerosis consistent with chronic GVHD in this group (20). This unexpected finding involved dermal thickening (Figure 3c; P = 0.007 vs. [B6→B6] control) by randomly oriented and tightly compacted bundles of collagen that differed qualitatively and quantitatively from the skin of [B6→B6] syngeneic-control recipients (Figure 3, a–d).

To determine the potential of nonhematopoietically derived miHAs to elicit lethal GVHD, [B6→BALB.B] BM chimeras were established by injection of lethally irradiated (13 Gy, split dose) BALB.B mice with 1×10⁷ B6 ATBM cells. Following 3–5 months of hematopoietic reconstitution, the nearly sole source of allogeneic BALB.B miHAs was radioreistant tissues, such as the

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**Figure 2**
Reduced epithelial injury over time in [BALB.B→B6] chimeric recipients. (a–c) Representative histopathological changes in ear biopsies taken on day 21 after HCT. Note the hyperplastic epithelial cell layer and epidermal cell apoptosis (arrows) in skin from [BALB.B→BALB.B] recipients (a), compared with normal-looking skin from [BALB.B→B6] and [B6→B6] recipients (b and c, respectively). Original magnification: ×200. (d) Sequentially analyzed dyskeratotic index following HCT. Apoptotic-cell counts were taken from more than 10 Lmm of epidermis per sample. Four animals were analyzed per group for all time points, except that only two animals from the [BALB.B→BALB.B] group were analyzed on days 21 and 32; SEM could not be calculated for these latter values (asterisk), but their mean was more than five times the SEM of the experimental and control groups.

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**Figure 3**
Minor histocompatibility antigen expression by the hematopoietic compartment results in cutaneous chronic GVHD. (a–d) Sclerotic dermis of skin from [BALB.B→B6] chimeric recipients, harvested day 90 after HCT, was distinguished by thickened dermis (vertical line, a) containing randomly oriented and tightly compacted bundles of collagen (c). In contrast, skin from [B6→B6] syngeneic controls had a normal dermal thickness (vertical line, b) and contained mostly horizontally oriented, loosely packed bundles of collagen (d). Original magnification: a and b, ×200; c and d, ×400. (e) Mean dermal thickness ± SEM, in millimeters. n = 5 for both groups.
epithelium, endothelium, and related nonhematopoietic mesenchymal elements. In contrast to the transient weight loss seen when the hematopoietic compartment was the exclusive source of miHAs, when [B6→BALB.B] chimeras were reirradiated (13 Gy, split dose) and transplanted with $2 \times 10^7$ B6 CD4+ T cells, all recipient chimeras succumbed to lethal GVHD (MST 20.6 days; Figure 4). Identically transplanted [B6→B6] chimeras did not experience GVHD lethality (MST >52 days). The miHAs responsible for driving the GVHD response in the [B6→BALB.B] recipients likely originated from non-hematopoietic tissue, as cytometric analysis of representative chimeras shortly before allogeneic HCT indicated that at least 96% of the T cell compartment, at least 99% of the B cell compartment, and at least 90% of large CD11c+ lymphoid DCs were of B6 origin. The low number of residual DCs of BALB.B origin by themselves were unlikely to account for fully developed GVHD, although they may have played a role in its initiation, considering that significantly greater numbers of these cells, as demonstrated above, were ineffective at causing lethality in the [BALB.B→B6] chimeric mice. Rather, the combined results suggest that CD4-mediated GVHD across this multiple-miHA barrier depended on miHA expression by nonhematopoietic tissues. Since these [B6→BALB.B] chimeras succumbed to lethal GVHD before day 90, it was not possible to determine whether they also developed chronic GVHD.

Discussion

Using reirradiated [BALB.B→B6] BM chimeras, we have demonstrated significantly diminished B6 CD4+ T cell–mediated GVHD development when recipient BALB.B miHAs were exclusively derived from cells of the hematopoietic compartment. BALB.B APCs might capture and process the source proteins from dead or dying hematopoietic cells following lethal irradiation, and present them as MHC class II–restricted miHAs. In addition, direct MHC class II loading of cytosolic miHAs for presentation by BALB.B APCs, an endogenous pathway that has been demonstrated in other systems (21, 22), might also have occurred. In either case, the data presented here suggest that multiple miHAs expressed by hematopoietic tissues alone is insufficient to drive fully developed lethal, CD4+ T cell–mediated GVHD in the B6→BALB.B model.

The observations made here agree with previous studies involving the CD8+ T cell–mediated B10. BR→CBA miHA-disparate GVHD model. Expression of recipient CBA miHAs exclusively by cells of the hematopoietic compartment in [CBA→B10. BR] chimeras significantly diminished the development of B10. BR CD8+ T cell–mediated GVHD (8). Moreover, Shlomchik et al. recently utilized high-level irradiation BM chimeras in the CD8-mediated C3H.SW→B6 GVHD miHA-disparate strain combination and found that C3H.SW CD8+ T cells were unable to mediate disease in [C3H.SW→B6] chimeras. This suggested that neither donor nor host DC cross-presentation of miHAs alone played a significant role in the pathogenesis of CD8-mediated lethal GVHD (4). However, the role of cross-presentation may still depend on the immunogenicity of the antigens being presented in the particular strain combination (23).

Collectively, the current results are in sharp contrast to the work of Teshima et al., who demonstrated in the MHC class II–disparate bm12→[B6→B6.MHC II–/–] BM chimeric model that alloantigen expression by the hematopoietic compartment alone was sufficient to obtain lethal GVHD (9). Clearly there are differences in the allogeneic T cell response directed across MHC versus miHA barriers, most notably the involvement of a much higher alloreactive CD4+ T cell precursor frequency in the former situation (24, 25). In bm12→B6 (9) and similar models (26–30), such a vigorous T cell response can rapidly generate high levels of proinflammatory cytokines responsible for initiating acute tissue damage in the early post-HCT period. Early injury to the gut epithelium allows for the transmission of LPS into the systemic circulation and peripheral tissues, with subsequent activation of macrophages, and release of high levels IL-1 and TNF-α (31, 32). Both of these inflammatory cytokines have established roles in the pathogenesis of lethal GVHD (26, 30, 33–37). The finding that MHC class II expression by host nonhematopoietic target tissues in the [B6→B6.MHC II–/–] chimera was not essential for the cascade of inflammatory events that ultimately lead to GVHD and lethality (9) suggested that, in such models, either large amounts of systemic cytokines alone are sufficient to cause severe tissue injury, or resident allogeneic B6 APCs in the tissues are adequate to activate infiltrating T cells to mediate injury via localized cytokine production. In this model, allogeneic B6 APCs would still be able to process and present MHC class II molecules bearing antigens derived from their own self-proteins, but even more so from the surrounding nonhematopoietic cells of B6 origin, which merely lack MHC class II expres-

Figure 4
Minor histocompatibility antigen expression by nonhematopoietic tissues stimulates lethal GVHD. Established [B6→BALB.B] and [B6→B6] BM chimeras were reirradiated (13 Gy, split dose) and injected with $2 \times 10^6$ B6 ATBM cells in combination with $2 \times 10^7$ B6 CD4+ T cells. Survival of transplanted recipients. Results are expressed as survival of transplanted recipients; $n = 5$ for B6→[B6→BALB.B], and $n = 4$ for B6→[B6→B6].
sion by APCs within target tissues for reactivation of effector alloreactive bm12 CD4+ T cells. The comparatively vastly reduced alloactive B6 donor CD4+ T cell frequency involved in the specific response to BALB.B miHAs would be expected to result in a lower level of T cell expansion in the recipient lymphoid compartment (24, 25), thus providing a lower level of early cytokine release. T cell activation by miHAs expressed within the hematopoietic compartment likely occurs in the [BALB.B→B6] chimeras and is evidenced by the induction of an early, transitory cachexia response. Overall, the less aggressive nature of the early miHA-driven GVHD response is underscored by comparison of the 26-day MST for [BALB.B→BALB.B] chimeric recipients of 15 × 10^6 B6 CD4+ T cells, with the 100% mortality observed by day 5 after HCT in the [B6→B6] chimeric recipients of 4 × 10^6 bm12 T cells, in the Teshima et al. study (9).

The slower kinetics of miHA-elicited GVHD is common in several other CD4+ and CD8+ T cell–mediated models (e.g., B10.D2→DBA and C3H.SW→B6 combinations, respectively). MST values in these models often range from 30 to 40 days, depending on the number of donor T cells infused, while the degree of tissue injury peaks between 21 and 28 days after HCT, increasing concomitantly with the clinical severity of disease (38, 39). The delayed disease kinetics in these models suggests that continued T cell recognition of alloantigen, particularly in recipient peripheral tissue sites, is required to reach threshold levels of immunopathological target-cell injury, rather than a mechanism dependent on an early and profound inflammatory cytokine flux. In addition, the notion of a required sustained donor T cell involvement during miHA-elicited GVHD is supported by data demonstrating amelioration of disease progression by effective disruption of alloreactive T cell responses at later time points (10–18 days) after HCT (40–42).

In conjunction with potential differences in the mechanistic basis of pathological tissue injury between an MHC class II–disparate model and our miHA chimeric model, GVHD progression may be limited by the models often range from 30 to 40 days, depending on the number of donor T cells infused, while the degree of tissue injury peaks between 21 and 28 days after HCT, increasing concomitantly with the clinical severity of disease (38, 39). The delayed disease kinetics in these models suggests that continued T cell recognition of alloantigen, particularly in recipient peripheral tissue sites, is required to reach threshold levels of immunopathological target-cell injury, rather than a mechanism dependent on an early and profound inflammatory cytokine flux. In addition, the notion of a required sustained donor T cell involvement during miHA-elicited GVHD is supported by data demonstrating amelioration of disease progression by effective disruption of alloreactive T cell responses at later time points (10–18 days) after HCT (40–42).

In conclusion, in the B6→BALB.B model studied here, donor CD4+ T cell–alloresponses directed against multiple-miHa differences derived exclusively from the recipient hematopoietic compartment results in a limited form of GVHD characterized by early, transitory cachexia, with minimal lasting tissue injury. In contrast, T cell responses directed against miHAs derived from nonhematopoietic tissues result in severe, lethal GVHD. Ultimately, studies investigating the identity of GVHD-causing class II–restricted miHAs will permit elucidation of their tissue-expression patterns and thus facilitate understanding of their individual role in development of GVHD immunopathology.

Acknowledgments

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