Suppression of B-Cell Development as a Result of Selective Expansion of Donor T Cells During the Minor H Antigen Graft-Versus-Host Reaction

By Beth A. Garvy, Jeanne M. Elia, Brian L. Hamilton, and Richard L. Riley

A murine model of bone marrow (BM) transplantation in which donor (B10.D2) and recipient (BALB/c) mice were major histocompatibility complex (MHC) (H-2d) and Mls-1 identical, but incompatible at multiple non-MHC minor histocompatibility (H) antigens, and at Mls-2,3 was used to examine regeneration of B-cell development during the minor H antigen graft-versus-host reaction (GVHR). Mice that received T-cell–depleted allogeneic BM regained significant pre-B cells (sIg-14.8+*) in their BM. Mice undergoing GVHR after transplantation with allogeneic BM + T cells had less than 2% pre-B cells in their BM at day 7 and only 12% to 14% pre-B cells at days 21 and 28 compared with greater than 20% pre-B cells in the allogeneic controls. After partial recovery, the pre-B cells in the BM of GVH mice again decreased to less than 3% by day 42. This abnormal pattern of pre-B cell development in mice undergoing GVHR was associated with a reduced response to interleukin-7 (IL-7) in vitro. The delay in B-lineage cell reconstitution in mice with GVHR correlated with the expansion of donor V$\beta$3+ T cells in both the spleen and BM. BM T cells from mice with GVHR as well as isolated V$\beta$3+ T cells inhibited IL-7 colony-forming units from normal BM in coculture assays. This inhibition could be reversed with anti-interferon $\gamma$ (IFN$\gamma$) antibody. These data suggest that the delay in appearance and the reduction in proportion and number of pre-B cells observed early during the GVH reaction in this model is caused, in part, by the inhibitory actions of IFN$\gamma$ derived from donor V$\beta$3+ T cells on B-lineage cell development.

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ABNORMAL B-CELL DEVELOPMENT DURING THE GVHR

Fig 1. BM cellularity through 6 weeks posttransplant. Total numbers of viable nucleated cells were determined from the femurs and tibias of mice receiving syngeneic BM (B10.D2 into B10.D2), allogeneic BM without T cells, or allogeneic BM with added lymph node T cells (GVH). Ranges for normal B10.D2 donors and BALB/c recipients are shown at day 7. Data are expressed as the mean ± SD. n = 6 to 14 mice per time point. (A) B10.D2; (C) BALB/c; (S) syngeneic; (●) allogeneic; (△) GVH.

Tonic ammonium chloride buffer for 2 minutes. Cells were washed by centrifugation and viable and nonviable cells enumerated using trypan blue exclusion.

Immunofluorescence labeling and fluorescence-activated cell sorter (FACS) analysis. RBC depleted BM and splenocytes were labeled using MoAbs to detect pro-B, pre-B, and B cells in the BM and Vβ3+ T cells in both spleen and BM. Aliquots (10⁶) of BM or spleen cells were incubated with biotinylated antimouse Ig (Boer-

Fig 2. Proportion of pre-B (slg<sup>−</sup> 14.8<sup>+</sup>) cells in the BM over 6 weeks posttransplant. BM was labeled for slg and B220 (14.8) and analyzed using flow cytometry. Both pro- and pre-B cells are slg<sup>−</sup> 14.8<sup>+</sup> and labeled as pre-B cells here. Proportions of pre-B cells found in normal B10.D2 and BALB/c mice are also shown. Data are expressed as the mean ± SD. n = 6 to 14 mice per time point. (A) B10.D2; (C) BALB/c; (S) syngeneic; (●) allogeneic; (△) GVH.

Fig 3. Light scatter and two-color fluorescent profiles of days 7, 21, and 42 posttransplant. BM was labeled for slg and B220 (14.8) and analyzed using flow cytometry. (A) Allogeneic control BM; (B) BM from mice with GVHR. Light scatter dot-plots show the position of lymphocytes in the box marked a. Fluorescence dot-plots show slg<sup>−</sup> B220<sup>+</sup> B cells in box b and slg<sup>−</sup> B220<sup>+</sup> pre-B cells in box c. The proportions of B or pre-B cells in whole BM are shown with the proportions of B or pre-B cells in the lymphocyte gates (box a) in parentheses. Individuals are representative of four separate experiments.
hinger Mannheim, Indianapolis, IN), biotinylated antimouse CD43 (S7 MoAb; PharMingen, San Diego, CA) or antimouse Thy1.2 (PharMingen) for 20 minutes on ice. After washing by centrifugation in HBSS with 0.1% bovine serum albumin (BSA) and NaN₃, cells were incubated with either anti-B220 (14.8 culture supernatant (American Type Culture Collection [ATCC], Rockville, MD), fluorescein isothiocyanate (FITC)-conjugated antihuman B220 (RA3.6B2; Pharmingen), or FITC-conjugated antimouse Vβ3 MoAb (Pharmingen). Streptavidin-conjugated phycoerythrin (Jackson Immunoresearch, West Grove, PA) was used to fluorescently label biotinylated antibodies and FITC-conjugated mouse antirat Ig (Boehringer Mannheim) was used to fluorescently label the 14.8 MoAb. After washing, cells were suspended at 10⁶ cells/mL and analyzed using a FACScan (Becton Dickinson, Mountain View, CA). Five thousand to 10,000 events were routinely acquired. In addition to binding to surface B220, the 14.8 MoAb may also react with a minor population of peripheral T cells. In contrast, the RA3.6B2 MoAb reacts exclusively with B-lineage cells. No difference in results was observed when either antibody was used.

Sorting Vβ3⁺ T cells from mice undergoing the GVHR. Spleen cells from mice receiving allogeneic BM and T cells day 7 to 12 after transplant were labeled as described above under sterile conditions and in the absence of NaN₃ in the wash buffer. Vβ3⁺ T cells were sorted from spleens using a FACStar Plus (Becton Dickinson) with a purity routinely greater than 80%.

Interleukin-7 (IL-7) colony-forming unit (CFU) assay. BM cells were cultured in semisolid media containing 0.9% methylcellulose as previously described. Briefly, mixtures of BM cells, recombinant (r) IL-7 (Biosource International, Camarillo, CA) and 0.9% methylcellulose in α-MEM with 30% FCS and 0.1 mmol/L 2-mercaptoethanol (2-ME) were vortexed and aliquoted into triplicate wells of 12-well plates. Each well contained 2 × 10⁶ BM cells and 50 U murine rIL-7 in 1 mL of medium. In some wells, murine rIFNγ (Biosource International) or anti-IFNγ MoAb (PharMingen) was also added. Cells were cultured for 7 days under 5% CO₂ at 37°C and colonies of 10 or more cells scored using an inverted microscope. In coculture experiments, BM cells (2 × 10⁶) from experimental groups (or 2 × 10⁴ sorted Vβ3⁺ T cells) were mixed with 2 × 10⁵ BALB/c or B10.D2 BM cells and incubated for 2 hours in RPMI supplemented with 5% fetal bovine serum (FBS) at 37°C before being cultured in semisolid media as above.

RESULTS

BM cellularity is unchanged in mice undergoing the GVHR compared with controls. The number of nucleated BM cells in mice undergoing the GVHR (B10.D2 BM + T
ABNORMAL B-CELL DEVELOPMENT DURING THE GVHR

Reconstituted more than 50% of those in allogeneic control mice (Fig 4B). Regeneration of small pre-B cells lagged behind the appearance of large pre-B cells by a week in the allogeneic control mice (Fig 4). This was not surprising because large pre-B cells are the immediate precursors of small pre-B cells.17-20 Though delayed, this same pattern of regeneration was observed through day 21 in the mice with GVHR (Fig 4). The gradual decrease of the large pre-B cells in mice with GVHR at day 28 was accompanied by a decrease in small pre-B cells (Fig 4). Both large and small pre-B cells were less than 2% of nucleated BM cells in mice with GVHR by 42 days posttransplant.

Pro-B cells, identified as CD43+ B220+,21 represented approximately 10% of total allogeneic control BM at day 7 (Fig 5). Conversely, less than 1% of total BM were CD43+ B220+ cells in the mice with GVHR at day 7, indicating that B-cell development was impaired at the level of pro-B cells or earlier in the B-lymphopoietic pathway. In preliminary experiments, we have also found that a population of early pre-B cells, 6C3+ CD43+ B220+,21 were virtually undetected in mice undergoing the GVHR (data not shown). As expected, CD43+ B220+ pre-B cells were substantially depleted in mice with GVHR when compared to allogeneic controls (Fig 5). It should be noted that B220+ pre-B cells were not found in the BM of mice undergoing the GVHR at day 7 regardless of whether either the 14.8 or RA3.6B2 MoAb was used (Figs 3 and 5).

The regeneration of slg+ 14.8+ B cells followed a pattern similar to that of pre-B cells in that very few B cells were found in the BM of mice with GVHR until days 21 and 28 after transplant (box b, Fig 3). These B cells were of donor origin (IgM type) (data not shown). As seen with small pre-B cells, the reconstitution of B cells was impaired in the BM of mice with GVHR compared to the BM of allogeneic control mice (Fig 3).

BM response to murine IL-7 is impaired in mice undergoing the GVHR. The ability of large pro-/pre-B cells to proliferate in the presence of murine rIL-722,23 was impaired in BM cells from mice with GVHR (Table 1). IL-7 CFU

Table 1. IL-7 CFU Kinetics Three Weeks After BM Transplant

<table>
<thead>
<tr>
<th>Day</th>
<th>Allogeneic</th>
<th>GVH</th>
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<tbody>
<tr>
<td>7</td>
<td>61 ± 15</td>
<td>7 ± 4 (11%)*</td>
</tr>
<tr>
<td>14</td>
<td>76 ± 35</td>
<td>3 ± 2 (4%)</td>
</tr>
<tr>
<td>21</td>
<td>548 ± 28</td>
<td>131 ± 43 (24%)</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± SEM of triplicate wells from one representative experiment. Data are representative of four separate kinetics experiments.

* GVH colonies expressed as a percent of allogeneic control colony counts.

cells into BALB/c did not differ from that of mice receiving allogeneic BM depleted of T cells (allogeneic controls) at any time up to 6 weeks postransplant. However, the group receiving syngeneic BM and T cells (B10.D2 BM + T cells into B10.D2) consistently had a higher number of BM cells than either of the other two groups (Fig 1). The disparity of BM cellularity between B10.D2 and BALB/c recipients was consistent with the cellularity of normal, untreated B10.D2 mice and did not represent a difference in engraftment (Figs 1 and 2).

B-cell precursors in the BM of mice undergoing GVHR are inhibited through day 14 after transplant. The regeneration of B-lineage cells was examined using MoAbs to slg and B220 (14.8). B cells were slg+ 14.8+ and B-cell precursors (including both pro- and pre-B cells) were slg+ 14.8+ and are referred to herein collectively as pre-B cells. FACs light scatter profiles indicated that there were greater numbers of lymphocytes in the BM of allogeneic control mice compared to mice with GVHR at day 7 after transplant (box a of Fig 3). There was also a significantly higher proportion of slg+ 14.8+ pre-B cells (9.0%) in the BM of allogeneic control mice than in mice with GVHR (2.4%) at day 7 (box c of Fig 3). When the kinetics of B-lineage cell regeneration was examined over 6 weeks, there was a delay of about 2 weeks before significant numbers of pre-B cells appeared in the BM of mice with GVHR. Pre-B cells became maximal at day 28 in mice with GVHR, albeit at reduced levels, and began to decrease again by day 35 (Figs 2 and 3). However, both the syngeneic and allogeneic control mice developed normal percentages of pre-B cells by day 14 and their numbers and percentages remained constant over the study period (Fig 2). Although the proportion of pre-B cells in both the syngeneic and allogeneic control mice were virtually identical, the absolute number of pre-B cells in the allogeneic control mice was about 20% lower than in the syngeneic controls because of the lower number of total BM cells in the latter.

The proportion (and absolute number) of BM large pre-B cells (determined by forward light scatter) in mice with GVHR recovered to levels approaching those of mice receiving allogeneic control transplants by day 21 before decreasing to less than 2% of BM cells at day 42 (Fig 4A). In contrast, small pre-B cells in the BM of mice with GVHR were not observed before 2 weeks postransplant and never reconstituted more than 50% of those in allogeneic control mice (Fig 4B).
Fig 7. Vβ3+ T cells in BM and spleen day 7 posttransplant. FACS dot-plots of BM (C and D) and spleen (A and B) cells from mice with GVHR (B and D) and allogeneic control (A and C) mice labeled for Thy1.2 and Vβ3 day 7 posttransplant are shown. Dot-plots are gated to include only cells within the lymphocyte region that are Thy1.2+ T cells. Vβ3+ T cells are found in box b and all other T cells are found in box a. Data are expressed as percentage of total BM cells (percentage of lymphocytes that are Vβ3+ is in parentheses) and are representative of four separate experiments.

Donor Vβ3+ T cells are expanded in mice with GVHR. Two-color flow cytometry showed that Vβ3+ T cells contributed disproportionately to the T cells present in spleens and BM of mice with GVHR at day 7 (Fig 7). Vβ3+ cells represented 40% to 60% of T cells in the spleen and 60% to 70% of T cells in the BM of mice with GVHR at day 7 and decreased to levels similar to those in allogeneic control mice by day 14 (Figs 7 and 8). Vβ3+ T cells have been shown to be Mls 2+3+ reactive and are normally deleted intrathymically in BALB/c mice, therefore it is likely that expansion of Vβ3+ cells in GVH recipients was caused by activation of donor B10.D2 Vβ3+ T cells. Interestingly, the absolute number of Vβ3+ T cells did not change significantly in the mice with GVHR (approximately $7 \times 10^4$ per femur/tibia pair) through day 42 posttransplant, indicating that Vβ3+ T cells increased in number after day 7 (from $4 \times 10^4$ at day 7 to $5 \times 10^5$ at day 14).
ABNORMAL B-CELL DEVELOPMENT DURING THE GVHR

BM T cells and isolated Vß3+ T cells from mice with GVHR inhibit IL-7 CFU. BM cells from mice with GVHR were cocultured with normal BALB/c or B10.D2 BM cells to determine if GVH BM contained cells or produced cytokines that impaired pre-B cell responses to IL-7. Figure 9A shows the IL-7 CFU response of either BALB/c or B10.D2 BM cells when cultured with equal numbers of BM cells from mice with GVHR. BM from mice with GVHR at day 7 posttransplant caused a greater than 60% reduction in IL-7 CFU response of normal BM (Fig 9A). BM from syngeneic and allogeneic control mice either had no effect or enhanced the response of normal BM to IL-7 (data not shown), indicating that the inhibition was specific for BM from mice with GVHR and not due to overcrowding of the wells. BM from mice with GVHR more than 14 days posttransplant did not inhibit the generation of IL-7 CFU from normal BM (data not shown).

BM cells from mice with GVHR were treated with anti-Thy1.2 and C to remove T cells before coculture with normal BM in IL-7 CFU assays. Removal of T cells caused a complete reversal of the inhibition of IL-7 CFU by BM cells from mice with GVHR in two separate experiments and partial reversal in a third experiment (Fig 9A), indicating that the T cells in the BM of mice with GVHR played a major role in inhibiting IL-7 CFU in these experiments. Because Vß3+ T cells were expanded in the BM of mice with GVHR early after transplant, Vß3+ Thy1.2+ T cells were sorted from the spleens of mice with GVHR at either day 7 or day 12 posttransplant and added to normal BM cells in IL-7 CFU cultures. Figure 9B shows that Vß3+ T cells were able to inhibit the IL-7 CFU response of normal BM by ≥50% whereas Vß3+ T cells from the spleens of mice with GVHR also inhibited IL-7 CFU in 3 of 6 experiments. As few as 5 × 10² Vß3+ T cells were able to inhibit IL-7 CFU formation by 50% (data not shown). Because inhibitory activity was found in the Vß3+ T-cell cocultures in some experiments (Fig 9B), T cells other than those expressing Vß3+ also may have inhibitory properties.

IFNy secreted from BM cells and isolated Vß3+ T cells from mice with GVHR inhibit IL-7 CFU. It has been previously shown that IFNy is a potent inhibitor of the IL-7 CFU response in BALB/c BM15 (M. Kruger and R. Riley, unpublished results, October 1991, and Table 2). When an anti-IFNy neutralizing antibody (see Table 2) was placed in IL-7 CFU cocultures with BALB/c BM, complete reversal of inhibition by BM cells from mice with GVHR was observed; partial to complete reversal of the inhibition caused by Vß3+ T cells was also seen (Fig 9C). These data suggested that IFNy was responsible for the inhibition of IL-7 CFU by BM from mice with GVHR.

DISCUSSION

B-cell development proceeds through a series of stages distinguishable by cell surface phenotype (Fig 10).15,17-21 The experiments reported herein define two phases of inhibition of this B-cell developmental pathway during the minor H antigen GVHR. The initial inhibitory phase is manifested as a 2-week delay in the development of pro-B and pre-B cells. After a brief recovery period, the second inhibitory phase of B-cell development appears as a gradual decrease in large B-cell precursors along with a decrease in small pre-B cells. Although large B-cell precursors initially approached levels found in the allogeneic control mice, the small pre-B cells never recovered to more than 50% of those found in the allogeneic control BM. Large B-cell precursor recovery previous to that of small pre-B cells was expected as a result of their precursor-progeny relationship.5,10,17-20 It is apparent that the second phase of inhibition of B-cell development began before small pre-B cells were able to completely recover from the first wave of inhibition.

Responsiveness to IL-7 was depressed at early time points after BMT in mice with GVHR. This was most likely due to the low numbers of IL-7 responsive pro- and large pre-B cells present in the BM of mice with GVHR through day 14. Although there was a trend toward recovery of IL-7 responses after the second week, this was inconsistent among individual kinetics experiments.

This inhibition of B-cell development during the early stages of minor antigen-induced GVHR is consistent with earlier reports in which early B-lineage cells expressing TdT and B220 decreased rapidly after completely MHC mismatched allogeneic lymphocyte transplantation into non-irradiated recipients.9,10 These reports indicated that the earliest B-lineage cells detectable were affected first. Consistent with this view, in our studies pro-B cells (CD43+ B220+)

21 were also very low at early time points after transplantation.

In earlier studies,9,10 the loss of B-cell precursors may have been caused by an allo-reaction by donor T cells against recipient B-lineage cells. This was not the case in the minor H antigen GVHR presented herein because host BM B-lineage cells were deleted by irradiation before BM transplant. In contrast to other protocols,9,10 our model closely parallels clinical BMT in which the host immune system is compromised by either whole-body irradiation or chemotherapy followed by reconstitution with MHC matched allogeneic
donor BM. In our model system we found that reconstitution of the B-lineage by donor BM was temporally inhibited in minor H antigen mismatched BM transplant recipients. Our data suggest that the delay in B-cell development was either at the pro-B cell level or before because pro-B cells were also quite low during the first 2 weeks after transplant. It may be that hematopoietic stem cells are also affected because, using a similar minor H antigen mismatched murine GVH model, Van Dijken et al found that BM from GVH mice had impaired CFU-s compared with allogeneic control mice.

The initial inhibition of B-lineage cell regeneration correlated with the expansion of Vβ3+ T cells at day 7 posttransplant in both the spleen and BM of mice with GVHR. The inhibition of IL-7 CFU by BM T cells from mice with GVHR and isolated Vβ3+ T cells was clearly mediated by IFNγ. IFNγ is secreted by activated T cells. A large proportion of Vβ3+ T cells found in the spleen at day 7 were also shown to be large in size, actively cycling, and expressed

Table 2. IL-7 CFU Responses of Normal BALB/c BM in the Presence of IFNγ With or Without Anti-IFNγ

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IFNγ Concentration</th>
<th>None</th>
<th>Anti-IFNγ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>566 ± 179</td>
<td>581 ± 77</td>
<td></td>
</tr>
<tr>
<td>10 U/mL</td>
<td>10 ± 1</td>
<td>430 ± 108</td>
<td></td>
</tr>
<tr>
<td>20 U/mL</td>
<td>7 ± 1</td>
<td>449 ± 33</td>
<td></td>
</tr>
<tr>
<td>50 U/mL</td>
<td>5 ± 1</td>
<td>322 ± 53</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM of triplicate wells from a single experiment. Data are representative of two separate experiments.

Fig 9. T cells from mice with GVHR suppress IL-7 CFU via secreted IFNγ. BM from mice with GVHR or sorted T-cell populations were cultured with normal control BM (B10.D2 or BALB/c) and 50U/mL IL-7 and colonies enumerated 7 days later. Data are expressed as percentage of colonies compared with normal BM alone. (A) Coculture of equal numbers of control BM and day 7 BM from mice with GVHR with or without T-cell removal; (B) coculture of FACS sorted Vβ3+ or Vβ3− T cells with normal BM (2 x 10^6 sorted cells were cultured with 2 x 10^6 BM cells); (C) coculture of normal BM with sorted Vβ3+ T cells or BM from mice with GVHR ± 10 μg/mL anti-IFNγ neutralizing antibody.

Fig 10. Schematic diagram of the phenotypic stages of normal BM B-cell development.
high levels of Ly-6 antigen (R. Levy, B. Garvey, unpublished observations, May 1992), suggesting they were activated, presumably by encountering the Mls2* antigen in the host mice.\textsuperscript{1,12,14} It is possible that T cells other than the $\text{Vp3}^+$ subset also play important roles in the development of the GVHR. FACs sorted V$\beta$3$^+$ Thyl.2$^+$ cells in several experiments inhibited IL-7 CFU to some extent, indicating the complexity of interactions taking place during the GVHR.

Because relatively few V$\beta$3$^+$ donor T cells were detected in BM from mice with GVHR (<2% of nucleated cells) it is possible that IFN$\gamma$ acts via effects on accessory cells in the BM milieu. It has been reported that IFN$\gamma$ inhibits BM stromal cell proliferation\textsuperscript{24} and the ability to support pre-B cell growth in vitro (M. Kruger and R. Riley, unpublished observations, October 1991). Alternatively, IFN$\gamma$ may have affected the ability of stromal cells to support lymphopoiesis because it has been reported that IFN$\gamma$ inhibits BM stromal cell proliferation\textsuperscript{24} and the ability to support pre-B cell growth in vitro (M. Kruger and R. Riley, unpublished observations, October 1991).

It is interesting to note that although the proportion of T cells that were V$\beta$3$^+$ was greater than 50% in BM from mice with GVHR at day 7 and decreased to less than 10% thereafter, the absolute number of V$\beta$3$^+$ T cells in BM from mice with GVHR were estimated to remain relatively constant over the 6-week experimental period. This is in contrast to splenic V$\beta$3$^+$ T cells that peak in number around day 7 and are very low by day 14.\textsuperscript{13} It has been reported that T cells which are stimulated by superantigens, including Mls, are either deleted or become anergic after initial clonal expansion.\textsuperscript{14} Although the absolute number of V$\beta$3$^+$ T cells in the BM is unchanged through day 42, these cells may be anergic after day 14, because BM from mice with GVHR has no inhibitory activity in IL-7 CFU cocultures at later time points (data not shown).

It is clear from the data presented herein that the expansion of V$\beta$3$^+$ T cells correlates with the initial delay in B-cell regeneration during the minor H antigen GVHR. However, causes for the second phase of inhibition after day 35 in GVH mice remain to be identified. By day 21, the V$\beta$3$^+$ T cells in the BM no longer have inhibitory activity and have virtually disappeared from the spleen. Although elaboration of cytokines may also inhibit B-cell development in the late stages of GVHR, it is also possible that this second wave of inhibition results, in part, from stress-related hormones such as glucocorticoids that accompany clinical disease in GVH mice.\textsuperscript{29,30} In any event, our data suggest that allogeneic T-cell activation produces lymphokines antagonistic to pre-B cell development during BMT, thereby contributing to the immunodeficiency in GVHR.

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