A Donor-Derived Asialo-GM1+ Cell Induces Depression of B-Cell Genesis During Systemic Graft-Versus-Host Disease

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The nature of the effector cell(s) responsible for the depression of B-cell genesis in the bone marrow of mice undergoing systemic graft-versus-host disease (GVHD) has been examined. Donor C57BL/6 (B6) mice were treated in vivo with either a single injection of anti-asialo GM1 antibody (anti-ASGM1) to eliminate naturally occurring (endogenous) ASGM1+ cells or B6xAFl (B6AF1) lymphoid cells followed by anti-ASGM1 to eliminate both endogenous and "induced" ASGM1+ cells. Lymphoid cells from donor mice after the elimination of endogenous ASGM1+ cells produced severe GVHD and concomitant depression of B-cell genesis when injected into B6AF1 recipients. In contrast, cells from donors depleted of both the endogenous and inducible ASGM1+ populations did not cause GVHD or depletion of B lineage cells in B6AF1 recipients but did depress B-cell genesis in B6C3F1 mice. The "induced" ASGM1+ cells were Thy 1+, but their elimination did not significantly alter either overall T-cell function or specific cytotoxic T-cell (CTL) reactivity against the sensitizing (B6AF1) strain. The results suggest that the effector cell responsible for the depression of B-cell genesis during systemic GVHD can be induced to express ASGM1, is strain-specific and Thy 1+; but is not a conventional CTL.

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SYSTEMIC graft-versus-host disease (GVHD) induced in mice by injecting parenteral-strain lymphoid cells into unirradiated F2 hybrid recipients can lead to the destruction of the host's immune system and severe immunosuppression. Studies of the mechanisms underlying the loss of cellular and humoral immune responsiveness during GVHD have demonstrated two phases of immunosuppression. The early phase is mediated by a variety of suppressor cells, while in the later phase histopathologic lesions appear in the absence of suppressor cell activity.

During the early phase of GVHD, defects of the humoral immune response include suppression of B lymphocyte responses to thymus-dependent and thymus-independent antigens and bacterial lipopolysaccharide (LPS). Recent studies have attributed the lack of B-cell function during the later phase of GVHD to the near-total depletion of mature B cells in the peripheral immune system, due to both a direct effect of GVHD on the recirculating B-cell population and a failure of B-cell production in the bone marrow. Bone marrow transfer into irradiated recipients and the evaluation of precursors by immunofluorescence labeling have shown that stem cells committed to the B lineage, as well as early B-cell precursors (before μ chain expression) and pre-B cells (expressing cytoplasmic μ chains) are all deleted from the bone marrow. Thus, B lineage cells in both the bone marrow and the periphery appear to be targeted during GVHD.

The underlying mechanisms responsible for the depletion of B lineage cells in the F2 host remain largely unknown. It has been generally assumed that specific donor anti-host cytotoxic T lymphocytes (CTLs) are the effector cells. However, little direct evidence is available to support this view. In fact, studies have suggested that CTLs may not be essential for the pathogenesis of GVHD. Recent results have suggested that natural killer (NK)-like cells of donor origin play a crucial role in the development of GVH-associated tissue damage and the suppression of both T- and B-cell immune responses. These donor-derived NK-like cells can be activated and induced by allogenic stimulation to become ASGM1-sensitive ("induced" ASGM1+ cells), display NK cell activity, and induce specific pathologic injury. Furthermore, these cells appear to be distinct from the T cells that are responsible for anti-host mixed lymphocyte reactions (MLR) and CTL activities in vitro and the endogenous NK cell. However, it has been unclear whether this donor-derived ASGM1+ inducible population might also be implicated in the depletion of B lineage cells and failure of B-cell production in the bone marrow during graft-versus-host reaction (GVHR).

This study examines the nature of the effector cells responsible for the GVH-associated depletion of B lineage cells and consequent depression of B-cell responsiveness in the host. Donor B6 mice were treated with B6AF1 cells to induce ASGM1+ cells followed by anti-ASGM1 treatment to eliminate the ASGM1+ NK-like cells. Our results suggest that an allotriggered ASGM1+ effector cell population exhibiting specificity for the sensitizing alloantigens, but distinct from T helper or T killer cells, exerts a crucial role in the depletion of B lineage cells in mice undergoing systemic GVHD.

MATERIALS AND METHODS

Animals. Male and female mice of the inbred strains C57BL/6 (B6; H-2b) and A (H-2a), as well as the B6xAFl (B6AF1; H-2b), B6xB6AF1; (B6CBAF1; H-2a) and B6x3H/HeJ (B6C3F1; H-2a) hybrids were used. The C3H/HeJ (C3) and SJL mice were purchased from Jackson Laboratories (Bar Harbour, ME). All other animals were bred and maintained in our animal colony under standard conditions. Both the donors and recipients were sex-matched and 15 to 20 weeks of age at the start of the experiments.

Anti-ASGM1 and polyinosinic:polycytidylic acid. Rabbit anti-ASGM1 was purchased from Wako Chemicals (Dallas, TX) and reconstituted with 1 mL of distilled water. Strain A or B6 donor
mice were injected intravenously (IV) with 75 to 100 µL of anti-ASGM1 in 0.2 mL phosphate-buffered saline (PBS; GIBCO, Grand Island, NY). The gammaglobulin fraction of normal rabbit serum (NRS) obtained by ammonium sulfate precipitation was used as a control nonimmune serum antibody. Polyclonins:polycytidylic acid (poly I:C; Sigma Chemical Co, St Louis, MO) was dissolved in Hank’s balanced salt solution (HBSS; GIBCO). B6 donors were injected intraperitoneally (IP) with 100 µg of poly I:C in a volume of 0.5 mL.

**Induction and elimination of specific and nonspecific anti-ASGM1-sensitive cells from the B6 or A donor mice.** The protocol used for the elimination of specific anti-ASGM1-sensitive cells from the donor inoculum has been described in detail previously. Briefly, B6 or A donor mice were injected IV with 15 × 10⁸ B6AF; spleen and lymph node cells to induce specific B6–anti-B6AF, or A–anti-B6AF, anti-ASGM1+ cells. To eliminate the specifically “induced” anti-ASGM1+ cells in the B6 donor mice, anti-ASGM1 (75 to 100 µL in a volume of 0.2 mL) was administered IV either 30 minutes before or 30 minutes after the injection of B6AF, lymphoid cells. To non-specifically activate anti-ASGM1+ cells, B6 donor mice were injected IP with poly I:C (100 µg in a volume of 0.5 mL). To eliminate the non-specific anti-ASGM1+ cells in donor B6 mice, anti-ASGM1 (100 µL in a volume of 0.2 mL) was administered together with the poly I:C. Parental strain mice were killed 44 to 48 hours after treatment, and their lymphoid cells were used to induce GVH reactions. We have previously shown that treatment with anti-ASGM1 eliminates virtually all the endogenous NK cell lytic activity in B6 donors treated with B6AF1 cells or poly I:C.⁹

**Depletion of T cells from donor cell suspensions.** Spleen and lymph node cell suspensions from normal B6 donors were incubated with hybridoma supernatants containing anti-Thy 1 monoclonal antibody (MoAb; clone AT83A; IgM) for 60 minutes at 4°C, washed, and then incubated with agarose-absorbed guinea pig complement (C³; GIBCO) for 45 minutes at 37°C. Cell suspensions depleted of Thy 1+ cells typically showed Con A and phytohemagglutinin (PHA) responses less than 2% of control values, but normal LPS responses.**

**Mitogen assays.** The mitogen assays were performed as previously described.²¹ Data are presented as the average net counts per minute ± SE of cultured cells from individual mice for each experimental group. The net counts per minute for each mouse was calculated from triplicate cultures using the following formula:

Net cpm = cpm Values From Cultures With Mitogen

− cpm Values From Cultures Without Mitogen

**Assay for interleukin-2 (IL-2) activity.** IL-2 activity in Con A-stimulated spleen cell culture supernatants was measured by the ability of the culture supernatants to support the proliferation of IL-2–dependent CTLL cells, as previously described.²² IL-2 activity was assessed by the degree of H-thymidine incorporation (cpm ± SE) in triplicate cultures of CTLL cells containing serial twofold dilutions of the supernatants.

**Generation and assay of specific CTLs.** Specific CTLs were generated and assayed in vitro as previously described.²⁶ To assay the CTL activity, various effector:target cell ratios (50:1 to 0.8:1) were plated in quadruplicate cultures.³¹ Cr-labeled Con A-induced blasts were used as targets. The percent cytotoxicity was calculated as follows:

\[
\% \text{ Specific Lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100
\]

The data shown are from the 25:1 effector to target cell ratio that fell on the exponential portion of the lysis curve obtained with the various effector to target ratios.

**Induction of GVHR.** GVHR were induced as described previously.⁸ Single cell suspensions were prepared from pooled spleen and lymph nodes from each group of B6 donor mice that received the treatments indicated above. Each F₁ hybrid recipient was injected IV with 60 × 10⁶ viable lymphoid cells from donor B6 or A mice. In some experiments 60 × 10⁶ lymphoid cells from two different donor groups were mixed, and a total of 120 × 10⁶ cells were injected. Cell viability was determined by trypan blue dye exclusion. Control F₁ hybrids received no lymphoid cell inoculum.

Seven different groups of donor mice were tested for their ability to induce GVHR in B6AF₁ and B6C3F₁ hybrids. The donor groups were as follows: (1) untreated, (2) treated with B6AF₁, lymphoid cells alone; (3) treated with anti-ASGM1 alone, (4) treated with both B6AF₁, lymphoid cells and anti-ASGM1, (5) treated with poly I:C alone, (6) treated with both poly I:C and anti-ASGM1, and (7) normal B6 donor cells treated with anti-Thy 1 and C³ in vitro.

**Assessment of the GVH-induced depression of B-cell production.** The numbers of pre-B and B cells in the bone marrow, the number of B cells in the spleen, and the proliferative response of B cells in the spleen were assessed in at least two independent experiments, as described previously.⁴ In each experiment three to seven animals per group were tested, and each experiment gave consistent results. Data from one representative experiment are shown in each case. The number of B lineage cells in the bone marrow and spleen were determined by epifluorescence microscopy using pooled cells from each experimental group or by flow cytometry using cells from individual mice. Splenic B cell mitogen responses were determined on individual mice.

**Spleen and bone marrow cell samples.** Single cell suspensions of spleen and femoral bone marrow from the recipient mice were prepared as previously described.⁸

**Antisera.** To label B lineage cells, fluorescein isothiocyanate (FITC)-conjugated and TRITC-conjugated affinity-purified goat antibody to mouse μ heavy chains (anti-μ FITC, and anti-μ TRITC, respectively; Southern Biotechnology Associates, Birmingham, AL) were used. All affinity-purified antibodies were centrifuged (122,000g for 30 minutes) in an Airfuge (Beckman Instruments, Palo Alto, CA) to remove aggregates just before use. CD4⁻ and CD8⁻ T cells were labeled using PE-conjugated anti-CD4 MoAbs (clone GK1.5) or FITC-conjugated anti-CD8 MoAbs (clone 53-6.7), purchased from Becton Dickinson (Mountain View, CA).

**Immunofluorescence labeling.** T cell subsets were labeled using direct immunofluorescent staining as previously described.²⁵ Details of the procedure used to label pre-B and B cells are fully described elsewhere.²⁴ Briefly, the spleen and bone marrow cell suspensions were incubated with anti-μ FITC antibodies to label surface μ chains (µs). The cells were then either analyzed by flow cytometry to determine the incidence of µ⁺ B cells or spun onto glass slides, fixed, stained with anti-μ TRITC antibodies to label the cytoplasmic μ chains (cµ), and analyzed using epifluorescence microscopy. Individual nucleated cells were scored for the presence of rhodamine fluorescence alone (cµ⁺; pre-B cells) and for double-labeling with fluorescein and rhodamine (µ⁺ cµ⁺; B cells). At least 100 cells of each phenotype were examined, or when the incidence of positive cells was low, at least 2,000 nucleated cells were counted. From the incidence of pre-B and B cells and the total cellularity of the spleen and femurs the absolute numbers of pre-B and B cells were calculated.

**Flow cytometry analysis.** Multicolor fluorescence analysis was performed using a FACScan (Becton Dickinson) flow cytometer. Selective acquisition gates were set to analyze all viable nucleated cells. Forward and side light scatter were used to exclude cellular debris and erythrocytes; damaged and dead cells were excluded using...
propidium iodide (0.5 μg/mL; Sigma). Fluorescence data from 5,000 viable cells were collected for each cell sample using logarithmic amplification. The labeling procedures used allowed for the labeled cell populations to be clearly distinguished. The enumeration of B cells in normal and GVH-reactive mice by flow cytometry resulted in values that were consistent with the values derived from microscopic analysis.

Direct plaque-forming cell assay. The direct plaque-forming cell (PFC) response of spleen cells to sheep red blood cells (SRBC) was assayed as described previously.

Statistics. The P values were determined using the Student’s t test.

RESULTS

Our initial experiments evaluated the T-cell content and immune function of lymphoid cells derived from B6 and A strain donor mice after various in vivo treatments. Subsequently, we examined the capacity of lymphoid cells derived from treated donors to deplete B lineage cells and suppress mitogen responses in B6AF1 recipients.

CD8+ and CD4+ cells in spleen and lymph nodes of B6 and A strain donor mice treated with B6AF1 cells and/or anti-ASGM1. The treatment of B6 and A strain donor mice with either anti-ASGM1 alone or anti-ASGM1 and B6AF1 cells together, decreased the incidence of CD8+ T cells in the spleen and lymph nodes by approximately one third of normal levels but had little effect on the incidence of CD4+ T cells (Table 1). When donor mice were treated with B6AF1 cells alone, no significant changes in the incidence of the CD8+ or CD4+ cells were seen. Flow cytometric analysis demonstrated that the proportion of CD8+ and CD4+ cells binding anti-ASGM1 (28% and 8%, respectively) corresponded closely with the proportion of CD8+ and CD4+ cells depleted by the anti-ASGM1 treatment (≈34% and 10%, respectively; Table 1) in donor mice. The various donor treatments did not change the total cellularity of the spleen and lymph nodes (data not shown).

Immune functions of spleen and lymph node cells from donor mice treated with B6AF1 cells and/or anti-ASGM1.

The in vivo treatment of B6 donors with B6AF1 cells, anti-ASGM1 alone, or B6AF1 cells and anti-ASGM1 together had no effect on the in vitro proliferative responses of T cells to Con A or PHA (Fig 1). Furthermore, spleen and lymph node cells derived from the B6 donors after the various treatments showed normal levels of IL-2 production when stimulated with Con A in vitro (Table 2). The T cell mitogen responses and the amount of IL-2 produced from cells derived from A strain donors that received the various treatments were also normal (data not shown). Lymphoid cells derived from all three treated B6 donor groups generated B6-anti-B6AF1 (recipient) and B6-anti-SJL (third-party) CTL responses in vitro, as shown in Table 3. However, lymphoid cells derived from B6 donors treated either with B6AF1 cells alone or with B6AF1 cells and anti-ASGM1 together showed some reduction (≈10% to 25%) in specific CTL activity against B6AF1 and SJL target blasts. The reasons for this reduced activity are not clear. The B6-anti-SJL CTLs could crossreact against B6AF1 targets; however,
little or no crossreactivity was observed between the B6–
anti-B6AF1 CTLs and SJL target blasts (Table 3).

Collectively, these results suggest that despite some deple-
tion of the CD8+ cell population in mice pretreated with
anti-ASGM1, the T-cell mitogen responses, production of
IL-2, MLC proliferative responses to alloantigens, and the
generation of specific CTL responses against the recipient
by the donor cells were near normal.

Ability of lymphoid cells from donor mice treated with
B6AF1 cells and/or anti-ASGM1 to induce GVH-associated
depression of primary B-cell production. Mice undergoing
GVHD characteristically show a marked depression of the
central genesis of B cells in the bone marrow associated
with a virtual elimination of B lineage precursors and B
cells in the bone marrow and of B cells in the spleen.6,11 In
agreement with previous observations, B6AF1 mice injected
2 to 4 weeks previously with normal B6 or A strain donor
cells showed markedly reduced numbers of pre-B cells and
B cells in the bone marrow and reduced B-cell numbers and
an absence of B-cell proliferative responses to LPS in the
spleen (Fig 2, group 1; Fig 3, group 1). Treating the donors
with B6AF1 cells to induce specific anti-B6AF1-reactive
cells (Fig 2, group 2; Fig 3, group 2) or treating with anti-
ASGM1 alone (Fig 2, group 3; Fig 3, group 3) to eliminate
the endogenous NK cell activity from the donor inoculum
before inducing GVH reactions16 did not prevent the GVH-
associated depression of pre-B and B cells from the bone
marrow or B cells from the spleen. However, B6AF1 recipi-
ents injected with cells from donor mice treated with both
B6AF1 cells and anti-ASGM1 showed near-normal numbers
of pre-B and B cells in the bone marrow, normal number of
B cells in the spleen, and normal mitogen responses (Figs 2
and 3, group 4). These results suggest that the cells of donor
origin that are required in the inoculum to induce the GVH-
associated depression of B-cell production are ASGM1–
 cells which become ASGM1+ after exposure to the alloanti-
gens of the host.

The T-cell–dependent B-cell responses as measured by
the PFC responses to SRBC were partially suppressed in
B6AF1 mice that received cells from donor mice treated
with both B6AF1 lymphoid cells and anti-ASGM1 (Table 4).
In addition, this group of recipients demonstrated significant
splenomegaly (data not shown). These results indicate the
presence of functional donor cells capable of suppressing
PFC responses and inducing splenomegaly but not causing
depression of B-cell genesis.

Table 2. Production of IL-2 by Lymphoid Cells From B6 Donor Mice Treated With B6AF1 Cells and/or Anti-ASGM1

<table>
<thead>
<tr>
<th>Treatment Before GVH Induction</th>
<th>Dilution of IL-2 Containing Supernatants*</th>
<th>Dilution of Control Supernatants†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>None</td>
<td>107.7 ± 3.4</td>
<td>43.3 ± 1.7</td>
</tr>
<tr>
<td>B6AF1</td>
<td>115.4 ± 8.1</td>
<td>39.5 ± 3.0</td>
</tr>
<tr>
<td>αASGM1</td>
<td>97.8 ± 11.9</td>
<td>35.3 ± 3.3</td>
</tr>
<tr>
<td>B6AF1 + αASGM1</td>
<td>95.8 ± 13.4</td>
<td>44.2 ± 2.4</td>
</tr>
</tbody>
</table>

Treated animals were injected with B6AF1 cells and/or anti-ASGM1 44 to 48 hrs before testing. Supernatants were derived from cultures of pooled spleen and lymph node cells from three to seven animals per experimental group and then added to 2 x 10⁴ CTLL cells. Values for [3H]-thymidine incorporation from triplicate cultures are shown (mean ± SE).

* Supernatants derived from donor cells cultured with 5 μg/ml Con A.
† Supernatants derived from donor cells cultured without Con A.

Table 3. Spleen and Lymph Node Cells From B6 Donor Mice Treated With B6AF1 Cells and Anti-ASGM1

<table>
<thead>
<tr>
<th>Treatment Before GVH Induction</th>
<th>Stimulators B6</th>
<th>B6AF1</th>
<th>SJL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B6AF1</td>
<td>-1.2 ± 0.7</td>
<td>60.1 ± 1.1</td>
</tr>
<tr>
<td>None</td>
<td>B6AF1</td>
<td>ND</td>
<td>44.9 ± 1.0</td>
</tr>
<tr>
<td>αASGM1</td>
<td>B6AF1</td>
<td>1.4 ± 1.1</td>
<td>58.4 ± 1.6</td>
</tr>
<tr>
<td>B6AF1 + αASGM1</td>
<td>B6AF1</td>
<td>ND</td>
<td>48.3 ± 1.4</td>
</tr>
<tr>
<td>None</td>
<td>SJL</td>
<td>1.5 ± 0.6</td>
<td>37.8 ± 0.6</td>
</tr>
<tr>
<td>B6AF1</td>
<td>SJL</td>
<td>ND</td>
<td>17.5 ± 4.4</td>
</tr>
<tr>
<td>αASGM1</td>
<td>SJL</td>
<td>-1.7 ± 1.9</td>
<td>46.9 ± 2.3</td>
</tr>
<tr>
<td>B6AF1 + αASGM1</td>
<td>SJL</td>
<td>ND</td>
<td>19.1 ± 4.8</td>
</tr>
</tbody>
</table>

Treated animals were injected with B6AF1 cells and/or anti-ASGM1 44-48 hrs prior to testing. Spleen and lymph node cells were pooled from 3-4 animals/group and tested. The data are presented as the percent specific lysis (mean ± SE) of quadruplicate cultures containing effectors and targets (effector: target, 25:1).

Abbreviation: ND, not done.
Fig 2. Donor pretreatment with both B6AF1 cells and anti-ASGM1 before GVHD induction prevents depression of B cell production in F1 hosts. Cells derived from B6 (A) or A (B) donor mice that were untreated (1), treated with F1 cells (2), treated with anti-ASGM1 (3), or treated with both anti-ASGM1 and F1 cells (4) were injected into groups of 4-7 BSAF1 mice to induce GVHD. The number of B cells in the bone marrow and spleen and the splenic LPS mitogen response (net counts per minute) of individual mice (mean ± SE) were determined 27 days (A) or 14 days (B) after GVH induction. P values were calculated for means derived from WAF1 mice injected with donor cells (1-4) compared with the mean derived from normal WAF1 mice (5; ***, P < .0001; **, .001 < P < .01 < P < .05.

To examine the possible role of donor-derived NK cells and the activation requirements of the inducible ASGM1+ effector cells, B6 donor mice were treated either with poly I:C, an interferon inducer, to boost endogenous NK cell activity (Fig 4, group 2) or with both poly I:C and anti-ASGM1 together, a treatment that we have previously shown eliminates the poly I:C-activated NK cell lytic activity (Fig 4, group 3), before inducing GVH reactions.20 The elimination of the poly I:C-activated NK cell activity from the donor inocula did not prevent the depletion of B lineage cells in B6AF1 recipients (Fig 4). Thus, the effector population that is induced by semiallogeneic stimulation to become sensitive to anti-ASGM1 in donor mice appears to be distinct from either endogenous or poly I:C-activated ASGM1+ NK cell populations.

Reactive specificity of the "induced" ASGM1+ effector population. If the pretreatment of B6 donors with B6AF1 cells and anti-ASGM1 selectively induces and eliminates cells that recognize A strain alloantigens, the lymphoid cells from these donors should retain the ability to induce the GVH-associated depression of B-cell genesis in another B6F1 hybrid combination (B6C3F1). Figure 3 demonstrates that the injection of lymphoid cells from B6 donors treated with both B6AF1 cells and anti-ASGM1 did not significantly reduce the B lineage cells in B6AF1 recipients. In contrast, injection of the same donor inoculum into B6C3F1 recipients resulted in a marked depletion of B lineage cells in the bone marrow and spleen and an absence of B-cell proliferative function in the spleen. B6CBAF1 recipients injected with lymphoid cells derived from treated B6 donors also showed a similar depression of B-cell genesis (data not shown).

The "induced" ASGM1+ effector cells express Thy 1 antigen. The selective specificity of induced cells after antigen stimulation suggests that these cells express a specific antigen receptor. To further characterize these cells we examined whether they expressed the Thy 1 antigen. Our results show that combining two populations of B6 donor cells in which one was depleted of the induced ASGM1+ cells and the other of Thy 1+ cells failed to reconstitute GVHD activity, suggesting that at least the precursor of this ASGM1+ inducible cell was Thy 1+ (Fig 5, group 4). Treatment of B6 mice with B6AF1 cells and anti-ASGM1 did not appear to depress effector cell function indirectly by inducing some form of suppressor activity, as the addition of cells from these donors to cells from untreated B6 donors did not prevent the depression of B-cell genesis (Fig 5, group 3).

DISCUSSION

Our results demonstrate that a donor-derived effector cell population plays a necessary role in the depression of B-cell genesis in the bone marrow of GVH-reactive mice. This study shows that the effector cells can be induced and activated by allostimulation ("specific induction") in vivo to become anti-ASGM1-sensitive, but they appear to be distinct
from either endogenous or poly I:C-activated ("nonspecific induction") ASGM1+ cells. Furthermore, allostimulation followed by anti-ASGM1 treatment selectively depletes effectors that recognize the sensitizing alloantigens because the treated inoculum retains the capacity to induce GVHD in recipients expressing different alloantigens. The effector cells and their precursors are rapidly "induced" and rendered sensitive to treatment with anti-ASGM1. Just one single dose of anti-ASGM1 after semiallogeneic stimulation with F1 cells is effective in eliminating them from either B6 or A strain donors after a 2-day period. In contrast, the elimination of either endogenous or non-specifically (poly I:C)-activated anti-ASGM1-sensitive cells from the donor inoculum does not prevent the development of GVH-associated B lineage depletion.

Our results suggest that conventional NK cells present in the donor inoculum are not essential for the development of the GVH-associated depletion of B lineage cells. Previously, we have shown that donor mice treated with poly I:C develop an elevated NK cell lytic activity against YAC targets which can be eliminated by anti-ASGM1 treatment.25 Our observation that the elimination of either endogenous or poly I:C-activated NK cell activity from the donor does not prevent systemic GVHD is in agreement with other studies in which NK cell activity has been eliminated from donor cells either in vivo or in vitro.26-28 Furthermore, any effector function for Thy 1+ donor-derived cell populations (eg, Thy 1+ NK cells, B cells, myeloid cells) was excluded by the results of the experiments where Thy 1+ -depleted donor cells were added to the treated inoculum before induction of GVHD (Fig 5). A majority of the NK lytic activity present in the spleen of normal animals is resistant to treatment with anti-Thy 1 and C' and presumably remains in our Thy 1-depleted population, which did not reconstitute the effector population.

Table 4. PFC Response to SRBC of B6AF1 Mice Injected With Lymphoid Cells From B6 or A Donor Cells Treated With B6AF1 Cells and/or Anti-ASGM1

<table>
<thead>
<tr>
<th>Treatment Before GVH Induction</th>
<th>No. PFC ± SE/10^6 Spleen Cells From B6AF1 Mice After GVH Induction (% of normal response)</th>
<th>A Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.2 ± 0.7 (0.2)</td>
<td>3.1 ± 2.1 (0.2)</td>
</tr>
<tr>
<td>B6AF1</td>
<td>6.9 ± 5.2 (0.6)</td>
<td>0 ± 0 (0)</td>
</tr>
<tr>
<td>αASGM1</td>
<td>24.9 ± 23.8 (2.4)</td>
<td>0 ± 0 (0)</td>
</tr>
<tr>
<td>B6AF1, + αASGM1</td>
<td>108.8 ± 83.7 (10.4)</td>
<td>341.6 ± 140.1 (27.2)</td>
</tr>
</tbody>
</table>

Cells from B6 or A donors were injected into B6AF1 mice to induce GVHD, and the PFC responses were measured on days 27 and 14, respectively. Data are presented as means ± SE and are derived from the same mice shown in Fig 2. The PFC responses of normal B6AF1 controls were 1,045.4 ± 218.1 for the group that received B6 cells and 1,256.9 ± 119.8 for the group that received A cells.
Fig 4. Elimination of poly I:C-induced NK cell activity in donors before GVHD induction does not prevent the depression of B-cell production in F₁ hosts. Cells derived from B6 donor mice that were untreated (1, 2), treated with poly I:C (2, 3), or treated with both anti-ASGM1 and poly I:C (3, 4), were injected into groups of three to six B6AF₁ mice to induce GVHD (group 4, normal F₁). The number of pre-B and B cells in the bone marrow and the number of B cells in the spleen were determined using pooled cell suspensions, and the splenic LPS mitogen responses (net cpm; mean ± SE) were determined individually 20 days after GVHD induction.

necessary for the development of the GVH-associated depression of B-cell genesis.²⁹ However, the effector population may display NK-like lytic function as we have described a population of cells with NK lytic activity against YAC targets that can be induced by allostimulation in the donors and eliminated by anti-ASGM1.¹⁹²⁰ Recently, MacDonald and Gartner³¹ have described a donor-derived NK-like population of cells that expresses ASGM1 and Thy 1 but not CD 8 or CD 4 and that is able to lyse both NK-sensitive YAC and resistant P815 target cells, with peak lytic activity in the lymphoid tissues of the host coinciding with the onset of histopathologic lesions during acute GVHD. Mononuclear infiltrates at sites of epithelial injury during GVHD have recently been shown by Ferrara et al³¹ to be donor-derived large granular lymphocytes with similar surface markers (Thy 1⁺, ASGM1⁺, Mac-1⁺, CD8⁻, and Ia⁻). These NK-like cells may in part be contributing to the allosuppression of B cells observed in vitro and previously believed to be effected solely by conventional CTLs.³²

It is unlikely that the prevention of GVHD by treatment of donors results from a failure of engraftment in the F₁ host. Evidence provided in other studies and our results have
shown that F₁ mice that received cells depleted of the inducible ASGM1+ cell also displayed donor cell-mediated suppression of the PFC response to SRBC and splenomelyg.

In addition, by labeling with FITC as described previously we observed that the depletion of the ASGM1+ inducible cell from the graft does not decrease the recovery of donor cells but conversely results in a greater recovery of donor cells in the recipient 2 days after GVH induction (Agopian et al., manuscript in preparation). Thus, it appears that the depletion of the inducible ASGM1+ cell does not impair early engraftment. Furthermore, these results suggest a dissociation between the donor cells responsible for humoral immunosuppression and splenomelyg and those responsible for pathologic lesions during GVHD.

The mechanisms by which the donor cells injure the host's lymphohematopoietic system and epithelial tissues remain unknown. Our previous data demonstrating a rapid decline of B- and pre-B-cell numbers in the bone marrow and subsequent near complete depletion of the long-lived peripheral B-cell population suggest that effector mechanisms can act directly to eliminate B lineage cells. Alternatively, the effectors could impair the hematopoietic inductive microenvironment of the bone marrow either by injury to stromal cells or by an augmented production of cytokines such as interferon-γ (IFNγ) and transforming growth factor β (TGFβ), which have been reported to inhibit bone marrow stromal cell proliferation and B-cell lymphopoiesis. In irradiated F₁ recipients given minor histocompatibility antigen mismatched donor cells Garvy et al. have demonstrated a delay in the reconstitution of the B lineage during the early phase of GVHD that appeared to be mediated by donor-derived IFNγ-producing T cells present in the bone marrow. However, during the later phase of GVHD when B lineage cells were depleted, no inhibitory cells could be identified. The inducible ASGM1-sensitive donor-derived effector population described by us appears to exert both an early and a late effect on B-cell development, where the late effect was associated with the development of histopathologic lesions in the thymus, pancreas, and liver and the suppression of T-cell proliferative functions. Genetic analysis of the strains used in this study suggests that either the H-2D or a closely related region of the H-2 complex may serve as a potent stimulus for the activation/induction of the anti-ASGM1-sensitive effector cell population, as described by us previously. Thus, the initiation of tissue injury to nonlymphoid stromal elements, particularly in the thymic epithelium and possibly also in the bone marrow, appears to play a pivotal role in the pathogenesis of acute GVHD, leading to the development of the severe long-term suppression and dysregulation of the host immune response.

The precise precursors of the effector population remain unknown. However, our results demonstrating the immune function of the treated donor inocula permit us to exclude certain cell types. Pretreatment of the donors with anti-ASGM1 has previously been shown to eliminate both endogenous and poly I:C-activated NK cell lytic function but not to influence the course of GVHD. In the present study, cells from all treated donor groups showed a normal incidence of CD 4+ cells, whereas donor groups treated with either anti-ASGM1 or B6AF₁ and anti-ASGM1 showed some reduction in the incidence of CD 8+ cells, but only the group treated with F₁ cells and anti-ASGM1 failed to induce GVHR. The T-cell mitogen responses and the production of IL-2 by cells from all three treated groups were normal. We have previously shown that lymphoid cells derived from similarly treated B6 donors retain the ability to proliferate normally in response to B6AF₁ and B6C3F₁ alloantigens in MLC. Anti-host CTL activity was generated by in vitro MLC from all donor groups. Cells derived from B6 donors treated with B6AF₁ cells generated slightly reduced levels of CTL activity against B6AF₁, and additional treatment of donors with anti-ASGM1 did not further depress the level of CTL activity. The reasons for the reduced generation of B6-anti-B6AF₁ and B6-anti-SJL CTL activity from cells derived from donors treated with B6AF₁ cells are not clear. It is possible that the injected B6AF₁ cells may exert veto activity against B6 CTL precursors reacting against A or SJL strain alloantigens. However, the precursor frequency of anti-host specific CTLs in donors after treatment has been shown to be normal by limiting dilution analysis (T. Ghayur, unpublished data). Collectively, these data suggest that the donor pretreatments do not eliminate functional T cells that can proliferate in response to mitogens, produce IL-2, and generate specific anti-host CTL and MLC responses in vitro. The fact that the various in vitro T-cell functions fail to predict accurately the capacity of the donor inoculum to cause acute GVH-associated depression of B-cell production and tissue damage argues that the GVH effector cell may be distinct from the endogenous or poly I:C-activated NK cells, T-helper, and CTL cell populations. We have recently proposed that T cells that express the CD3/γδ receptors (γδ TcR) can function as effector cells in GVHR. Our suggestion that γδ TcR+ cells are involved in GVHD is supported by the recent demonstration that lymphoid cells from TcR β-chain transgenic mice, which do not rearrange their endogenous γ-chain genes, fail to induce GVHD when injected into normal or lethally irradiated F₁ recipients. Further studies are necessary and are currently underway to delineate the precise role of γδ TcR+ cells in the pathogenesis of acute GVHD.

Acknowledgment

We thank Ailsa Lee Loy, Rosmarie Siegrist-Johnstone, Jenny Woon Lun Eng, Lyne Bourbonnière, and Michel Emond for excellent technical assistance.

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