ALLOSUPPRESSOR AND ALLOHELPER T CELLS IN ACUTE AND CHRONIC GRAFT-VS.-HOST DISEASE

I. Alloreactive Suppressor Cells Rather Than Killer T Cells Appear to Be the Decisive Effector Cells in Lethal Graft-Vs.-Host Disease

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The graft-vs.-host reaction (GVHR) is initiated by immune responses of donor T lymphocytes to allogeneic histocompatibility antigens of the recipient. These initial reactions cause a chain of complex and sometimes antagonistic events that can result in various pathological symptoms. One possible outcome of the GVHR is acute GVH disease (GVHD). It is caused by donor T cells and produces thymic involution (1) and severe hypoplasia of the lympho-hemopoietic tissue (1, 4), including depletion of plasma cells in the gut (5). The resulting clinical symptoms of acute GVHD comprise aplastic anemia (1), severe immunodeficiency, including hypogammaglobulinemia (1, 6), and, finally, sepsis. Often, acute GVHD terminates as lethal GVH (LGVHD). A different possible outcome of the GVHR is chronic GVHD. This is a pleiotropic syndrome, which can manifest itself by lymphoid hyperplasia, hypergammaglobulinemia, autoantibody formation, and/or symptoms of collagen disease (4, 7–9). The mechanisms that determine whether donor T cells will induce acute or chronic GVHD and the pathological symptoms associated with them are poorly understood. Alloreactive T cells are known to consist of functionally distinct subsets that can be separated, for instance, on the basis of differences in their Lyt phenotypes (10, 11). Conceivably, the different and sometimes diametrically opposed pathological symptoms of GVHR are due to differences in subpopulations of the alloreactive donor T cells.
T cells administered to the recipients. Thus far, however, there is only little experimental data supporting this concept (12-15). Several authors have implicated T killer (TK) cells of the donor as the main effector cells that kill the recipients in LGVHD (16-19). This straightforward explanation has recently been questioned, however, and it has been suggested that alloreactive T suppressor (TS) cells are the main effector cells causing LGVHD (13, 14).

In the present study, a novel experimental approach was chosen for preparing two inocula of syngeneic T cells that were enriched for allosuppressor and allohelper cells, respectively. T cells from C57Bl/10ScSn (B10) donors were first activated for different times, either 5 or 6 d, in the spleens of irradiated (B10 × DBA/2)F1 (1° F1) mice, and then assayed for their anti-F1 activities by transferring them to secondary F1 (2° F1) mice or testing them in vitro. Although donor T cells activated for 5 d were found to be enriched for alloreactive TS cells, T cells activated for 6 d were enriched for alloreactive T helper (TH) cells. Although both day-5- and day-6-activated T cells were equally capable of lysing F1 target cells in vitro, nevertheless, upon transfer to nonirradiated 2° F1 mice, only day-5-activated T cells induced LGVHD. Day-6-activated T cells, on the other hand, induced symptoms of chronic GVHD upon transfer to nonirradiated 2° F1 mice.

Materials and Methods

**Mice.** B10 mice and (B10 × DBA/2)F1 hybrids were purchased from the TNO Laboratory (Zeist, The Netherlands). The B10 donors were males or females, 6-12 wk old. The 1° F1 recipients (Fig. 1) were males or females 10-12 wk old, and the 2° F1 recipient mice were males or females 8-12 wk old. In a given experiment, all mice were of the same sex.

**Irradiation of Mice.** Mice were subjected to 750 rad total body irradiation by applying 662 KeV gamma rays, emitted from a 137Cs source (Gammator, model 381, Isomedic) at a dose of 375 rad/min. Within the next 3 h, the mice were injected with the cells indicated.

**Preparation and Testing of Day-5- and Day-6-Activated Anti-F1 T Cells.** Single-cell suspensions of spleen cells were prepared as described (13). Spleen cells enriched for T cells were obtained by passage over nylon wool (20). The basic experimental design for preparing day-5- and day-6-activated parental T cells and for testing their capacities to induce LGVHD is depicted in Fig. 1. In experiments in which the effects of the activated T cells on erythropoiesis or on antibody formation to levan were studied, smaller numbers of activated cells were transferred to irradiated 2° recipients of F1 or B10 origin (other than shown in Fig. 1).

**Complement (C)-dependent Cytotoxicity Test.** The trypan-blue exclusion method was used. A 1 in 1,000 dilution of the monoclonal anti-Thy-1.2 antibody was used for the determination of T cells. Cells of F1 origin were identified with a 1 in 10 dilution of a B10 anti-DBA/2 and a DBA/2 anti-B10 alloantiserum, respectively, which were prepared by repeated injections of lymphoid cells. The Lyt phenotypes of activated B10 cells were determined with anti-Lyt-1.2 and anti-Lyt-2.2 sera, respectively, at a dilution of 1 in 10. As a source of C we used a 1 in 12 dilution of serum obtained from selected rabbits.

**Cell-mediated Lymphocytotoxicity (CML) Test.** Two different experimental protocols were applied to assess the 4-h CML activity of day-5- and day-6-activated B10 T cells. In the first protocol, the activated T cells obtained from the spleens of the 1° F1 mice were cultured for 18 h without the addition of stimulator cells and then tested by CML. In the second protocol, 1.2 × 10^7 day-5- or day-6-activated cells were first restimulated by mixing them with 1.2 × 10^7 F1 spleen cells irradiated with 3,300 rad. The cell mixtures were cultured for 5 d at a 5-ml volume in tissue-culture flasks (3013; Falcon Labware, Div. of Becton, Dickenson & Co., Oxnard, CA). The restimulated cells were then tested by CML. The culture conditions, determination of 4-h CML activity, and inhibition of CML activity by unlabeled target cells were the same as described elsewhere (13).
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Fig. 1. Experimental design for the preparation and testing of day-5- and day-6-activated B10 T cells. On day zero, $5 \times 10^6$ splenic T cells from B10 donors were injected into H-2-incompatible irradiated (B10 × DBA/2)F1 hybrids, termed primary F1 (1° F1) mice. Cells were recovered from the spleens of 1° F1 mice, either on day 5 or day 6; they were termed day-5-activated and day-6-activated B10 T cells, respectively. For assessment of the functional capacities of day-5- and day-6-activated T cells, cells of either population were transferred to secondary (2°) recipients. When the capacities of the two populations of activated parental T cells for induction of LGVHD were to be studied, $3 \times 10^7$ live cells of either population were transferred into nonirradiated F1 (2° F1) recipients (see figure). In experiments in which the effects of day-5 and day-6 cells on erythropoiesis or antibody production were studied, smaller numbers of day-5 and day-6 cells, along with other cells, were transferred into irradiated 2° recipients, as described in the text. Alternatively, different numbers of activated T cells were studied in vitro.

Quantitation of Mouse IgG in the Supernatants of Cultured Spleen Cells and in the Serum. This assay was performed as described (13).

Determination of Hemopoiesis by Incorporation of $^{59}$Fe. This assay was performed as described (21).

Anti-Levan Plaque-forming Cell (PFC) Response. Irradiated 2° recipient mice were repopulated intravenously with $3 \times 10^7$ T cell-depleted spleen cells and various numbers of day-5- or day-6-activated B10 cells. 2 d later, the mice were injected intravenously with 10 µg levan. 5 d after injection of the T cell-independent antigen levan, the spleens of the mice were removed, and
the direct PFC responses of individual mice were determined by applying a Jerne-plaque assay with levan-coupled sheep erythrocytes (SRBC) (22).

Ig Allotype. Antisera specific for the Ig-1\(^b\) allotype of B10 and the Ig-1\(^c\) allotype of DBA/2, respectively, were prepared as described (23). The anti-Ig-1\(^c\) serum was used at a constant dilution of 1 in 3 and the anti-Ig-1\(^b\) serum at a constant dilution of 1 in 9. The relative concentrations of Ig allotypes in the sera of 2\(^{o}\) F\(_1\) recipients of day-6 cells were determined by double-immunodiffusion in agar gel. The sera of 2\(^{o}\) F\(_1\) mice were tested at doubling dilutions starting at 1 in 8. The titer of the highest serum dilution that gave a precipitation line was recorded.

Pathological Examination. At autopsy, organs were removed and fixed in 8% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and cut at 5 \(\mu\)m. Sections stained with hematoxylin-eosin or Giemsa stain were prepared.

Immunofluorescence and Immunoperoxidase Examination. Indirect immunofluorescence staining was performed on frozen sections of lymph nodes and spleens of the 2\(^{o}\) F\(_1\) recipients. For the determination of T cells, a suitably absorbed rabbit-anti-mouse brain serum was used in combination with a horse-anti-rabbit IgG labeled with tetramethylrhodamine isothiocyanate (17-2T2; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam). Direct immunofluorescence staining was used for the determination of B cells and plasma cells, applying a rabbit-anti-mouse Ig serum labeled with fluorescein isothiocyanate (KM 16-11; CLB, Amsterdam).

Immunoperoxidase studies for the detection of intracytoplasmic Ig in plasma cells and plasma-cell precursors in the lamina propria of the upper part of the small bowel (duodenum and jejunum) were performed in paraffin sections, pretreated with protease (type VII; Sigma Chemical Co., St. Louis, MO) (24). Rabbit antisera against mouse IgG, IgA, and IgM (Miles Laboratories, Elkhart, IN) were diluted 1 in 20 (IgG) and 1 in 10 (IgA and IgM), respectively. Swine-anti-rabbit serum (Dakopatts, Copenhagen, Denmark) was used in a dilution of 1 in 40, and the peroxidase-antiperoxidase complex produced in swine (Dakopatts) was diluted 1 in 50. All dilutions were made in normal swine serum (Dakopatts).

Primary PFC Responses against SRBC In Vitro. 3 \(\times\) 10\(^5\) normal spleen or T cell-depleted spleen cells were cultured in volumes of 300 \(\mu\)l together with various numbers of day-5- or day-6-activated B10 cells or “activated” T cells of F\(_1\) origin. The latter were nylon wool-passaged F\(_1\) spleen cells injected into 1\(^{o}\) F\(_1\) mice and recovered from their spleens on day 5 or day 6. Cultures were performed in Falcon multi-well tissue-culture plates (3008; Falcon Labware). The culture medium consisted of sodium-bicarbonate-buffered RPMI 1640 supplemented with l-glutamine (2 mm), 2-mercaptoethanol (2 \(\times\) 10\(^{-5}\) M), streptomycin (50 U/ml), penicillin (50 U/ml), and 10% fetal calf serum (FCS). The cultures were immunized with 2.5 \(\times\) 10\(^3\) SRBC. On day 4, the numbers of direct PFC were determined in a Jerne-plaque assay. Triplicate cultures were set up for each determination.

Preparation of T Cell-depleted Spleen Cells (B Cells). B cells were prepared by incubating spleen cells with monoclonal anti-Thy-1.2 (clone F7D5; Olac 1976 Ltd., Bicester, United Kingdom) and C, as previously described (13). Thereafter, the cells were washed three times and viable T cells determined by indirect immunofluorescence with an appropriately absorbed rabbit anti-mouse-brain serum in combination with fluorescein isothiocyanate (FITC)-labeled swine-anti-rabbit IgG (F2190, Dakopatts). The proportion of T cells never exceeded 1%.

Statistical Significance. Student’s t test was applied.

Results

Serological Characterization of Day-5- and Day-6-activated Cells. The absolute numbers of live cells recovered from the spleens of 1\(^{o}\) F\(_1\) mice on day 5 did not differ from those recovered on day 6. On both days, the recovery per spleen varied from 1.4 \(\times\) 10\(^7\) to 2.1 \(\times\) 10\(^7\) cells in different experiments. About 90% of the live cells recovered on both days 5 and 6 were T cells of B10 origin (Table I). Viable cells of F\(_1\) origin, as determined by a cytotoxic antiserum against DBA/2, were undetectable among both day-5 and day-6 cells. Therefore, the two cell populations were termed day-5- and
Table I

**Day-5- and Day-6-Activated Cells Are B10 T Cells with the Same Lyt Phenotypes**

<table>
<thead>
<tr>
<th>Spleen cells of 1° F1 recipients obtained on</th>
<th>Percentages of cells killed with antiserum and C*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Thy-1:2</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>mean ± SE</td>
</tr>
<tr>
<td>Day 5</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>Day 6</td>
<td>92 ± 4</td>
</tr>
</tbody>
</table>

* Results of three individual experiments, each of which was performed with pooled spleen cells from 5 to 15 primary F1 mice.

Fig. 2. Different capacities of day-5- and day-6-activated B10 T cells for induction of LGVHD. Nonirradiated 2° F1 mice received either 3 × 10⁷ day-5-activated or 3 × 10⁷ day-6-activated T cells. The results represent the cumulative data from four subsequent experiments.

Table II

**Effects of Day-5- and Day-6-activated B10 T Cells upon the Body Weight, Hematocrit Value, and Serum-IgG Concentration of 2° Recipients**

<table>
<thead>
<tr>
<th>Time of activation (in 1° F1) of B10 T cells</th>
<th>Strain</th>
<th>2° recipients*</th>
<th>Mean body weight ± SEM</th>
<th>Mean hematocrit ± SEM</th>
<th>Mean serum-IgG concentration ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (B10 × DBA/2)F1</td>
<td>15.0  ± 2.6</td>
<td>14.8 ± 3.9</td>
<td>38 ± 10‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (B10 × DBA/2)F1</td>
<td>20.0  ± 0.8</td>
<td>48.8 ± 2.1</td>
<td>276 ± 60‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 B10</td>
<td>20.3  ± 0.9</td>
<td>48.3 ± 0.3</td>
<td>105 ± 11§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 B10</td>
<td>21.0  ± 0.4</td>
<td>50.7 ± 0.9</td>
<td>99 ± 9§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each group of recipients consisted of three to five mice. All mice were tested 20 d after the injection of activated T cells.

‡ P < 0.05 when compared with the 100% values of noninjected F1 mice.
§ P > 0.2 when compared with the 100% values of noninjected B10 mice.

day-6-activated B10 T cells, respectively, although minor contaminations by non-T cells and/or F1 cells might have remained undetected (21). The percentages of Lyt-1⁺Lyt-2⁻ cells and Lyt-1⁻Lyt-2⁺ cells among day-6-activated T cells were not significantly different from those found among day-5-activated T cells (Table I).

**Different Capacities for Induction of LGVHD.** Groups of nonirradiated 2° F1 mice were injected with either 3 × 10⁷ day-5-activated or 3 × 10⁷ day-6-activated B10 T cells. Of the 2° F1 recipients of day-5-activated T cells, 92% died from GVHD (Fig. 2).
### Table III

Weights of, and Cell Numbers in, the Spleens of 2° F₁ Mice at 14 d after the Injection of Day-5 or Day-6 Cells

<table>
<thead>
<tr>
<th>2° F₁ mice* injected with</th>
<th>Mean spleen weight (mg) ± 1 SEM</th>
<th>Mean number of nucleated cells per spleen (× 10⁷) ± 1 SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nothing</td>
<td>127 ± 4</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Day-5 cells</td>
<td>137 ± 24</td>
<td>2.0 ± 0.25§</td>
</tr>
<tr>
<td>Day-6 cells</td>
<td>273 ± 49</td>
<td>7.3 ± 1.1¶</td>
</tr>
</tbody>
</table>

* Each group consisted of six F₁ mice.

§ Significantly different (P < 0.005) from the other two groups.

¶ Not significantly different (P > 0.1) from the noninjected group.

Such 2° F₁ recipients showed characteristic symptoms of acute GVHD, such as reduced body weight and hematocrit, a decreased level of serum IgG, and a decreased number of cells in their spleens (Tables II and III). In marked contrast, none of the 2° F₁ recipients of day-6-activated T cells died from or showed clinical symptoms of acute GVHD (Fig. 2, Table II). However, at 2 wk after the transfer of day-6-activated T cells, the 2° F₁ recipients showed splenomegaly and a slight increase in the number of cells in their spleens (Table III). Typing of these cells in a C-dependent cytotoxicity test, applying an anti-B10 and an anti-DBA/2 serum, showed that at least 90% were of F₁ origin (data not shown). Furthermore, the injection of day-6 cells caused a considerable increase in the serum IgG level of 2° F₁ recipients (Table II). By applying an anti-Ig-1b and an Ig-1c serum for immunoprecipitation in agar gel, it was demonstrated that the levels of both Ig-1 allotypes were increased in the sera of 2° F₁ recipients of day-6 cells (data not shown). This indicated that at least a major portion of the increased serum IgG level in these mice was produced by B cells of the 2° F₁ mice, which were Ig-1b/c, and not by B cells of B10 origin (Ig-1b/b). When syngeneic (B10) mice instead of F₁ mice were used as 2° recipients, neither day-5 nor day-6 cells induced clinical abnormalities (Table II).

#### Morphological Findings in the Lympho-Hemopoietic Tissues of 2° Recipients.

Inoculation of day-5-activated T cells into 2° F₁ recipients caused a progressive hypoplasia of the peripheral lymphoid tissues and the thymus. Immunofluorescence studies of T and B cells in the spleens and lymph nodes of such 2° F₁ recipients showed that virtually all of the few lymphoid cells present were T cells. In contrast, inoculation of day-6-activated T cells into 2° F₁ mice caused stimulation of the peripheral lymphoid tissues and no involution of the thymus. The lymphoid stimulation induced by day-6 cells was already present 10 d after the cell transfer and, in five out of six 2° F₁ recipients studied, persisted for at least 3 mo. Immunofluorescence studies of the stimulated spleens and lymph nodes of 2° F₁ recipients of day-6 cells showed that the T cell areas and, especially, the B cell areas, were stimulated.

In control experiments, syngeneic (B10) mice, instead of F₁ mice, served as 2° recipients of day-5 and day-6 cells, respectively. Neither lymphoid hypoplasia nor thymus involution were observed in these recipients; however, both day-6 and day-5
cells induced a moderate and transient lymphoid stimulation, which had disappeared at day 40 after the cell transfer. There are at least three possible, mutually not exclusive explanations for this transient lymphoid stimulation: (a) small numbers of F$_1$ cells might have been co-transferred from the 1$^°$ F$_1$ animals to the 2$^°$ B10 mice and might thus have provided an alloantigenic stimulus; (b) the alloactivated B10 T cells themselves might have picked F$_1$ antigens (25) that then elicited an immune response in the 2$^°$ B10 mice; (c) the 2$^°$ B10 recipients might have responded to idiotypic receptors on the alloactivated syngeneic T cells (26).

After the injection of day-5 cells, hemopoiesis in the bone marrow of 2$^°$ F$_1$ recipients was moderately suppressed after 10 d (Fig. 3 A) and was no longer detectable after 20 d. However, a conspicuous depletion in the numbers of the reticular fibroblasts in the marrow was undetectable in the 2$^°$ F$_1$ recipients of day-5 cells (nor were such changes detectable in any other group). In all 2$^°$ F$_1$ mice injected with day-6 cells, by contrast, the bone marrow showed fully developed hemopoiesis at days 10, 20, 30, and 90 (Fig. 3 B). No hypoplastic changes were detectable in the bone marrow of syngeneic (B10) recipients of either day-5 or day-6 cells.

Morphological Findings in the Gut and Other Organs of 2$^°$ Recipients. Whereas in the 2$^°$ F$_1$ recipients of day-5 cells there was a progressive disappearance of plasma cells and their precursors in the lamina propria of the small bowel, no such changes were found in the 2$^°$ F$_1$ recipients of day-6 cells. In both groups, however, small foci of lymphocytes, obviously invading the epithelium, were found in the crypts. In addition, villous edema and focal superficial epithelial necrosis were present in these areas. In contrast, the injection of neither day-5 nor day-6 cells changed the number of plasma cells or caused epithelial lesions in the intestinal villi of syngeneic (B10) recipients.

The above-mentioned histological findings were confirmed by the results of immunoperoxidase staining. After the injection of day-5 cells, the 2$^°$ F$_1$ recipients showed a progressive depletion of IgG-, IgM-, and IgA-containing plasma cells and plasma-cell precursors in the lamina propria of the gut (Fig. 4 A). In contrast, at all times after the transfer of day-6 cells, the guts of 2$^°$ F$_1$ animals contained numerous cells cytoplasmically positive for IgA, IgM, or IgG (Fig. 4 B). Both day-5 and day-6 cells failed to change the number of isotypes of Ig-producing cells in the gut of syngeneic (B10) recipients. 20 d after the cell transfer, the 2$^°$ F$_1$ recipients of day-5 cells but not those of day-6 cells showed histological signs of sepsis in that bacterial colonies without inflammatory reaction were found in the liver and other parenchymal organs.

Lack of Difference in Anti-F$_1$ TK Cell Activity between Day-5- and Day-6-activated Cells. Both day-5- and day-6-activated B10 T cells lysed F$_1$ target cells in vitro but failed to lyse syngeneic (B10) target cells (Fig. 5). When day-5 and day-6 cells were restimulated for 5 d with irradiated F$_1$ spleen cells in vitro before being tested by CML, they had retained their cytotoxic anti-F$_1$ activity, but again we did not find that the anti-F$_1$ TK cell activity of day-6 cells was inferior to that of day-5 cells (data not shown). Cold-target inhibition studies indicated that the anti-F$_1$ TK cell activity of both day-5 and day-6-activated B10 (H-2$^{b/b}$) T cells was specific for the H-2$^d$ antigens of (B10 × DBA/2)$F_1$ (H-2$^{b/d}$) mice. Specificity for non-H-2 antigens was not observed (data not shown). As far as the anti-H-2$^d$ specificity of day-5-activated B10 TK cells is concerned, the results of cold-target inhibition studies were reported elsewhere (13).

Differences in Allogeneic Helper and Suppressor Effects of Day-5- and Day-6-activated T Cells
FIG. 3. Bone marrow of 3-8 F mice 10 d after cell transfer. (A) Moderate depletion of the reticular fibroblasts, no conspicuous depletion of the hemopoietic cells after injection of day-5-activated B10 T cells; no conspicuous depletion of the reticular fibroblasts, no conspicuous depletion of the hemopoietic cells after injection of day-6-activated B10 T cells. H and E staining.
Fig. 4. Small bowel of 2° F1 recipients 20 d after cell transfer. Immunoperoxidase staining of IgA-containing plasma cells in the mucosa. (A) almost complete absence of IgA-containing cells after injection of day-5-activated B10 T cells. (B) normal numbers of IgA-containing plasma cells after injection of day-6-activated B10 T cells. Hematoxylin counterstain.
Fig. 5. Lack of difference in cytotoxic anti-F1 activity between day-5- and day-6-activated T cells. After activated B10 T cells had been recovered from two groups of 1° Fl mice, they were kept in culture for 18 h. Thereafter, they were simultaneously tested by CML on Con-A-activated, 51Cr-labeled (B10 X DBA/2)F1 spleen cells (© and ■) or B10 spleen cells (O and □).

Fig. 6. Opposite effects of day-5 and day-6-activated T cells upon the IgG production by spleen cells of 2° Fl recipients. Nonirradiated 2° Fl animals received either 3 × 10⁷ day-5-activated or 3 × 10⁷ day-6-activated T cells. After 14 d, the spleens of 2° Fl mice were excised, and 2.5 × 10⁶ cells of each mouse were cultured overnight. The IgG concentrations in the culture supernatants were determined and expressed as the percentage of IgG concentration found in cultures containing spleen cells of noninjected Fl mice. (The 100% value corresponds to 11 250 ng IgG). Each group consisted of five Fl mice. Columns represent the arithmetic mean; vertical bars represent 1 SEM.

on the IgG Production by Spleen Cells of 2° Fl Mice. Groups of nonirradiated 2° Fl mice were injected with 3 × 10⁷ day-5- or day-6-activated cells. After 14 d, the spleen cells of the 2° Fl recipients were assayed in vitro for the amount of IgG released into the culture supernatant. Spleen cells obtained from day-5-injected 2° Fl recipients produced only about one-fourteenth of the IgG produced by the same number of cells obtained from day-6-injected 2° Fl recipients (Fig. 6). However, the absolute number of cells in the spleen of 2° Fl mice injected with day-6 cells was more than three times higher as the number of cells found in 2° Fl recipients of day-5 cells (Table III). Therefore, when the IgG production was calculated on the basis of whole spleens, the difference in IgG production between those 2° Fl mice injected with day-5 cells and those injected with day-6 cells was even 40- to 50-fold. These data are consistent with the serum IgG levels of 2° Fl mice injected with day-5 and day-6 cells, as shown in Table II.

Different Effects of Day-5- and Day-6-activated T Cells on the Anti-Levan Response of Fl Spleen Cells. The effects of activated B10 T cells on the PFC response to levan were
studied in irradiated 2° F1 mice. In the first experiment (Fig. 7), all seven groups of 2° F1 mice were repopulated with a standard number of 3 × 10⁷ nonirradiated spleen cells from syngeneic F1 donors. Six groups received graded numbers of day-5- or day-6-activated T cells in addition. All three groups of the repopulated 2° F1 mice that had received day-5 cells exhibited a dose-dependent suppression of the PFC response. In these three groups, the PFC responses were significantly lower (P < 0.002) than those of 2° F1 control mice. In contrast, day-6 cells, at doses of 1.25 × 10⁶ and 5 × 10⁶ cells, did not induce a significant suppression (P > 0.15). Although the PFC response

Fig. 7. Different effects of day-5-activated and day-6-activated T cells upon the anti-levan response of F1 spleen cells. All seven groups of irradiated 2° F1 mice were repopulated with 3 × 10⁷ F1 spleen cells. In addition, the mice of six groups received graded numbers of activated B10 T cells, as indicated. On day 2, all animals were immunized with levan; on day 7, the anti-levan PFC responses were determined. Each group consisted of five F1 mice. The anti-levan response is expressed as the percentage of PFC found in 2° mice that were repopulated with 3 × 10⁷ spleen cells alone. (The 100% value corresponds to 3,524 PFC.) Columns represent the arithmetic means; vertical bars represent 1 SEM.

Fig. 8. Suppressive effect of both day-5- and day-6-activated B10 T cells upon the anti-levan response of syngeneic B10 B cells in an F1 environment. All seven groups of irradiated 2° F1 mice were repopulated with 3 × 10⁷ B10 spleen cells, which had been treated with anti-Thy-1.2 and complement. In addition, the animals of six groups received graded numbers of activated B10 T cells, as indicated. On day 2, the animals were immunized with levan; on day 7, the anti-levan responses in their spleens were determined. Each group consisted of five F1 mice. The anti-levan response is expressed as described for Fig. 7. (The 100% value in this experiment corresponds to 2,230 PFC.)
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Fig. 9. Comparison of the amount of allohelper activity present among day-5-activated and day-6-activated T cells. The effects of these cells upon a primary anti-SRBC response by T cell-depleted F1 spleen cells were studied. A standard number of 3 × 10^6 F1 spleen cells, which had been treated with anti-Thy-1.2 and complement, were cultured together with SRBC and graded numbers of activated B10 T cells (○ and □) or F1 T cells (△). Direct anti-SRBC PFC responses were determined on day 4. The arithmetic means of triplicate cultures are shown; vertical bars indicate 1 SEM.

Fig. 10. Comparison of the amount of allosuppressor activity present among day-5-activated and day-6-activated T cells. The effects of these cells upon a primary anti-SRBC response by normal F1 (○ and □) or B10 (□ and □) spleen cells were studied. A standard number of 3 × 10^6 spleen cells were cultured together with SRBC and graded numbers of activated B10 T cells, as indicated. The numbers of PFC were determined and expressed as described for Fig. 8. At each point, the value of 1 SEM was <8%. (The 100% value corresponded to 850 PFC per culture.)

of 2° F1 recipients of 2.5 × 10^6 day-6 cells was significantly lower (P < 0.01) than that of the control mice, it was still significantly higher (P < 0.01) than the response of the group injected with day-5 cells.

Next, we investigated whether day-5 cells were also able to suppress the anti-levan response of syngeneic (B10) cells in an F1 environment. Irradiated 2° F1 mice were therefore repopulated with a standard number of 3 × 10^7 spleen cells of B10 origin. To prevent GVH effects by the repopulating B10 spleen cells themselves, we used B10 spleen cells that were depleted of T cells (“B” cells). In addition to receiving B10 B cells, 6 groups of the 2° F1 mice received graded numbers of day-5- or day-6-activated B10 T cells. In the environment of the irradiated 2° F1 recipients, day-5-activated
B10 T cells were capable of suppressing the PFC response of B10 B cells (Fig. 8). Surprisingly, however, even day-6-activated T cells at doses of 2.5 and 5 × 10^6 cells caused a significant \((P < 0.05)\) suppression of syngeneic B cells in 2° F1 recipients.

The Different Capacities of Day-5- and Day-6-activated B10 T Cells for Induction of Positive and Negative Allogeneic Effects In Vitro. Helper effects of activated B10 T cells upon the primary anti-SRBC response of F1 B cells ("allohelp") were assayed by culturing graded numbers of day-5 or day-6 cells with \(3 \times 10^6\) unprimed F1 B cells and SRBC. The help supplied by day-6 cells was considerably higher than that induced by day-5 cells. However, day-5 cells, too, were able to induce allohelp because many fewer day-5-activated B10 T cells than syngeneic T cells were needed for providing help to the F1 B cells. Moreover, day-6 cells at doses of \(2 \times 10^6\) and higher were also able to suppress F1 B cells, when compared with syngeneic T cells (Fig. 9).

In another experiment (Fig. 10), the suppressor effect of activated B10 T cells upon the primary anti-SRBC response of F1 spleen cells ("allosuppression") was tested. To this end, \(3 \times 10^6\) unprimed F1 spleen cells (instead of the F1 B cells as used in the previous experiment) were cultured with graded numbers of day-5 or day-6 cells and SRBC. Addition of very small numbers of day-5 cells already induced a distinct suppression of the PFC response by F1 spleen cells: the addition of only \(10^5\) day-5 cells suppressed almost 50% of the PFC response. This suppression of the response became more profound when higher numbers of day-5 cells were added to the cultures. Although day-6 cells, too, were able to suppress the response, this occurred only at cell numbers of \(32 \times 10^3\) and higher, and then the extent of suppression was significantly lower \((P < 0.03)\) than that induced by day-5 cells. No suppression was observed when day-5- or day-6-activated B10 T cells were added to syngeneic B10 spleen cells (Fig. 9). This indicates that, for suppression to occur, allore cognition by the activated T cells had to be possible. The finding that addition of 0.25 to \(64 \times 10^3\) "activated" F1

TABLE IV

Erythropoietic Activities of Various Cell Populations Separately Injected into Irradiated 2° F1 Mice

<table>
<thead>
<tr>
<th>Origin and number of cells transferred (\times 10^6)</th>
<th>(^{59})Fe incorporation in the spleen of 2° F1 recipients* (\text{mean cpm } \pm SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day-5 cells</td>
<td>Day-6 cells</td>
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</table>

* Each group consisted of five irradiated F1 mice.
† No cells injected.
Fig. 11. Opposite effects of day-5-activated and day-6-activated T cells on the hemopoietic activity of F1 BMC. On day 0, irradiated 2° F1 mice were repopulated with 2 × 10⁶ BMC from normal F1 mice and graded numbers of activated B10 T cells, as indicated. On day 7, the amount of ⁵⁹Fe uptake in the spleens of the repopulated mice was determined. Each group consisted of five F1 mice. The data are expressed as mean percentages of the ⁵⁹Fe uptake found in those 2° mice that had been repopulated with BMC alone. Vertical bars indicate 1 SEM.

Fig. 12. Suppressive effects of both day-5-activated and day-6-activated B10 T cells on the hemopoietic activity of B10 BMC in an F1 environment. On day 0, irradiated 2° F1 mice were repopulated with 2 × 10⁷ BMC from normal B10 mice and graded numbers of activated B10 T cells, as indicated. On day 7, the amount of ⁵⁹Fe uptake in the spleens of the repopulated mice was determined. Each group consisted of five F1 mice. Results are expressed as described for Fig. 11.

T cells to the F1 spleen cells likewise failed to suppress the PFC response (data not shown) is consistent with this conclusion.

The Different Capacities of Day-5- and Day-6-activated B10 T Cells for Suppression of Erythropoietic F1 Cells. Before the effects of day-5- and day-6-activated parental T cells upon the hemopoietic activity of BMC could be studied, we had to exclude the possibility that day-5 and day-6 cells themselves had a different erythropoietic activity. Therefore, equal numbers (0.6 × 10⁶ to 2.4 × 10⁶) of day-5 or day-6 cells were injected into groups of irradiated 2° F1 recipients, who did not receive any other cells. 7 d later, the ⁵⁹Fe incorporation into the spleen of the 2° F1 mice was determined. Neither the 2° F1 recipients of day-5 cells nor those of day-6 cells contained a significantly higher erythropoietic activity than unrepopulated 2° F1 mice (Table IV). Table IV
also shows the different repopulating capacities of F1 BMC and B10 BMC. The number of B10 BMC that was required to give a good 59Fe uptake in the spleens of the irradiated 2° F1 recipients, was 10 times as great as the number of F1 BMC. The large number of B10 BMC was needed to overcome the F1 hybrid resistance of the irradiated F1 mice to hemopoietic cells of B10 origin (27).

We then studied the effects of the two kinds of activated parental T cells on the hemopoietic activity of the BMC taken to repopulate irradiated 2° F1 recipients. Graded numbers of activated T cells (0.6 × 10^6 to 2.4 × 10^6) were injected into the following kinds of irradiated 2° recipients: (a) F1 mice repopulated with 2 × 10^6 F1 BMC (Fig. 11); (b) F1 mice repopulated with 20 × 10^6 B10 BMC (Fig. 12), and (c) B10 mice repopulated with 2 × 10^6 B10 BMC. As can be seen in Fig. 11, day-5- and day-6-activated T cells exerted opposite effects on the repopulation by F1 BMC of irradiated F1 (2° F1) mice. To test whether day-5-activated B10 T cells would also suppress syngeneic B10 BMC in an F1 environment, protocol (b) was used. All doses of day-5 cells markedly depressed the 59Fe uptake by syngeneic BMC (Fig. 12). By contrast, day-5-activated B10 T cells failed to suppress, but stimulated the 59Fe uptake of B10 BMC in the completely syngeneic environment described under (c) (data not shown). In summary, the results of experiments (b) and (c) indicate that the nonspecific suppression by day-5 cells of syngeneic B10 BMC depended on the triggering of day-5 cells by F1 alloantigens.

A surprising finding was that day-6-activated B10 T cells, too, although they failed to suppress erythropoietic cells of F1 origin (Fig. 11), did suppress the erythropoietic activity of syngeneic BMC in an F1 environment (Fig. 12). In the absence of F1 antigens, however, neither day-5- nor day-6-activated B10 T cells suppressed the erythropoiesis of B10 BMC, but rather stimulated it (data not shown).

Discussion

We prepared two functionally different subpopulations of alloactivated parental T lymphocytes: day-5 cells, which were enriched for T cells inducing LGVHD, and day-6 cells, which were unable to induce LGVHD (Fig. 2). In addition, upon transfer to the nonirradiated 2° F1 mice, day-5-activated T cells suppressed the IgG production by the recipients' spleen cells, whereas day-6-activated T cells increased it (Fig. 6). Furthermore, upon transfer to nonirradiated 2° F1 mice, day-5 cells caused progressive thymic atrophy and cellular depletion, especially of plasma cells, in the lympho-hemopoietic tissues (Fig. 3 A). Concomitant clinical symptoms were aplastic anemia and hypogammaglobulinemia (Table II). An important finding in the 2° F1 recipients of day-5 cells was the virtually complete depletion of plasma cells and plasma-cell precursors of the three major Ig classes in the lamina propria of the small intestine (Fig. 4 A). This, in combination with the observed epithelial lesions in the small intestine, was the most likely cause of LGVHD and sepsis caused by day-5 cells. Sprent and Miller (28) observed that a substantial portion of alloactivated parental T cells eventually home to the intestinal villi of 2° F1 recipient mice. This observation suggests that the depletion of plasma cells and plasma-cell precursors in the 2° F1 recipients of day-5 cells was because of a local immune response by the day-5-activated T cells. Van Bekkum et al. (29) have demonstrated that the quality of the intestinal microflora can decisively influence the mortality rate of mice undergoing GVHR. It seems fair to conclude from the combined findings that the primary cause of GVH
mortality lies in the T cell-induced pathological damage in several tissues, including the gut. Thereafter, pathogenic microorganisms, invading from the damaged intestine and infecting an immunodeficient organism, can play an important secondary role.

In marked contrast with day-5 cells, transfer of day-6 cells to 2° F1 mice caused splenomegaly, lymphoid stimulation (especially of the F1 B cells), and hypergamma-globulinemia (Tables II and III). An intriguing observation was that the lymphoid tissue of almost all the 2° F1 recipients of day-6 cells remained stimulated throughout the 3-mo observation period. Thus, the lymphoid stimulation induced by day-6-activated B10 T cells did not rapidly return to normal or revert to the lymphoid hypoplasia and pancytopenia that is characteristic of the acute GVHD induced in identical F1 mice by the injection of nonactivated, unselected B10 spleen cells (14). These results indicate that day-6-activated B10 T cells, in contrast to unselected B10 spleen cells and day-5-activated B10 T cells, had undergone an almost irreversible reduction in those T cells that induce the suppressive pathological phenomena characteristic of acute GVHD. These impressive functional differences between day-5 and day-6 cells cannot be ascribed to differences in either the number of parental T cells or the Lyt phenotypes expressed by them (Table I).

Mode of Elicitation and Specificity of Allosuppression and Allohelp. Apart from suppressing the IgG production of nonirradiated 2° F1 recipients, day-5-activated T cells also suppressed the anti-levan response (Figs. 7 and 8) and erythropoiesis (Figs. 11 and 12) of irradiated and repopulated 2° F1 recipients. In addition, they suppressed the anti-SRBC PFC response of F1 spleen cells cultured in vitro (Fig. 10). By contrast, day-6-activated T cells, in spite of an identical capacity for killing F1 target cells, failed to suppress (or only weakly suppressed) the anti-SRBC response of F1 spleen cells in vitro (Fig. 10) and the anti-levan response and erythropoiesis of irradiated 2° F1 mice repopulated with F1 cells (Figs. 7 and 11). Correspondingly, we assume that the decrease in erythropoiesis and antibody formation caused by day-5-activated B10 cells was the result of suppressive rather than cytotoxic activity. This assumption is based on the observation that day-5 cells were able to reduce the erythropoiesis and antibody formation not only of F1 cells (Figs. 7, 10 and 11) but also, at least to some extent, that of syngeneic B10 cells exposed to an F1 environment (Figs. 8 and 12). A reduction in the functional activities of syngeneic cells cannot be ascribed to cytotoxicity (Fig. 5). The extremely low number of 0.5 × 10⁵ day-5 cells that effectively reduced the anti-SRBC response of 3 × 10⁶ F1 spleen cells (Fig. 10) also tends to support the notion that this effect was because of suppression rather than cytotoxicity.

The elicitation of eventually nonspecific suppressor activity depended on the allore cognition by the activated parental T cells of F1 antigens. Whereas day-5-activated B10 cells did suppress the erythropoietic activity of B10 cells in irradiated 2° F1 (Fig. 12), they completely failed to do so in the absence of F1 antigens. Further, day-5-activated B10 cells did not suppress the primary anti-SRBC response of B10 spleen cells, whereas they suppressed the response of F1 spleen cells (Fig. 10). Once it was elicited, however, the suppressor activity could affect syngeneic B10 cells as well (Figs. 8 and 12). Comparable suppressor activities, triggered by specific T cell allore cognition and nonspecifically affecting bystander cells, have previously been reported by others (6, 30, 31). The suppression exerted by day-5 cells, nevertheless, seemed to have a stronger effect on F1 cells than on syngeneic B10 cells (Figs. 7 and 11 vs. 8 and 12). One possible explanation for this phenomenon is that nonspecific as
well as F1-restricted TS cells or suppressor factors operate in our model. Schwartz et al. (32) described the induction of both restricted and unrestricted TS cells during a primary MLR, and it has been reported (33, 34) that both restricted and unrestricted suppressor factors preventing cell proliferation were produced by or under the influence of alloactivated T cells. Hence, it is conceivable that more than one type of TS cell (or factor) was present among the day-5-activated T cells, one type that acted specifically on F1 cells and another that acted nonspecifically in that it also suppressed B10 cells. Another explanation for the stronger suppression of F1 cells could be that the F1 cells, taken to repopulate the irradiated 2° F1 mice, provided a quantitatively or qualitatively better antigenic stimulus for triggering the suppression by day-5 cells.

As far as the activity of day-6-activated T cells in irradiated 2°F1 mice is concerned, different results were found, depending on whether the antibody-forming and erythropoietic progenitor cells were of F1 or B10 origin. Whereas day-6 cells failed to suppress or only marginally suppressed the antibody-forming and erythropoietic activity of F1 cells (Figs. 7 and 11), they did suppress the antibody-forming and erythropoietic activity of B10 cells (Figs. 8 and 12). At least two mechanisms, which are mutually not exclusive, might account for this discrepancy. First, it is conceivable that day-6 cells produced a helper factor that was specific for F1 cells but did not act on B10 cells. Hence, the suppressive effect also exerted by day-6 cells might have been neutralized by an F1-specific helper factor (35) in those experiments where cells of F1 origin were used, but not in those where B10 cells were used. An F1-restricted helper effect (35, 36) might have neutralized the suppression by day-6 cells of both F1 B cells and F1 erythropoietic progenitor cells because there is evidence that activated TH cells release factor(s) stimulating both kinds of cells (37, 38). Second, cells from B10 (H-2b/b) donors are especially susceptible to F1 hybrid resistance in H-2b/d recipients (27, and Table IV). Although high numbers of B10 BMC were used to correct for this, the susceptibility of B10 BMC to the combined effect of F1 hybrid resistance and allo-suppression might have been greater than the susceptibility of small numbers of F1 BMC to allo-suppression alone.

Possible Biological Mechanism Underlying the Enrichment of Alloreactive TS Cells on Day 5 and of TH Cells on Day 6. The switch from allo-suppression to allo-help from day 5 to day 6 was highly reproducible. Conceivably, the time dependency of this switch was because of time differences in the splenic sojourn and recirculation of subsets of donor T cells in the 1° F1 mice. Supposing that significant numbers of donor TS cells left the spleens of 1° F1 mice between days 5 and 6, they might have left behind a population of alloreactive donor cells enriched in TH cells. Sprent and Miller (28, 39) previously studied the functional capacities of parental strain T cells that were activated against the H-2 alloantigens of irradiated F1 animals. The overall conclusion from their investigations is that donor T cells first home to the peripheral lymphoid organs, including the spleen, where they proliferate and differentiate in response to the alloantigens of the recipients. After a lag period of 3.5 days, the alloactivated T cells start re-entering the circulation. Sprent and Miller (28) found that T cells that were recovered from the circulation at 4–6 d after the injection into irradiated F1 mice had lost their specific ability to induce GVH splenomegaly when transferred to nonirradiated 2° F1 mice. They further noticed that the same activated parental T cells were strongly cytotoxic for F1 target cells in vitro. This finding of but a partial anti-F1 reactivity of the recirculating parental T cells indicates that, indeed, only
selected subpopulations of the alloactivated donor T cells start to re-enter the circulation of the F1 recipient around day 5. As far as the inability to induce GVH splenomegaly and the capacity for anti-F1 cytotoxicity is concerned, the recirculating parental T cells described by Sprent and Miller (28) resemble the day-5-activated splenic T cells described in the present paper. However, an important difference was the reported inability of the recirculating parental T cells to induce LGVHD or acute GVHD upon transfer to 2° F1 mice (28). The reason for this difference is not known at present. In the light of the T cell circuit (10), it is conceivable that the induction of severe allosuppression and eventually LGVHD requires at least two subsets of alloactivated parental T cells: (a) an effector cell population mediating allosuppression that might have been present among both day-5 cells and the recirculating T cells, and (b) an additional helper cell population that keeps inducing (10) the suppressor effector cells. Possibly, this second subset of parental T cells does not recirculate and thus was present only among the day-5 cells obtained from the spleens of the 1° F1 recipients. In any event, the early observation (28) that anti-F1 cytotoxicity of the activated parental T cells is not sufficient for induction of acute GVHD and LGVHD has long been overlooked by investigators in the field (16–19). This important observation has been confirmed and extended by the data of the present study, as discussed below.

**Lack of a Distinct Role of TK Cells in the Pathogenesis of LGVHD.** Interestingly, day-5-activated T cells and day-6-activated T cells had equal capacity to lyse F1 target cells in vitro (Fig. 5). The good anti-F1 cytotoxicity of day-6 cells, which were incapable of inducing LGVHD, indicated that donor TK cells are insufficient for induction of LGVHD. Our concept that LGVHD is caused not by TK but by alloreactive TS cells is further supported by theoretical considerations. In view of the tremendous regenerative capacity of the lympho-hemopoietic tissue, it has been argued that a general suppression of cell proliferation by TS cells would be much more efficient than cytotoxicity to cause cellular depletion in that tissue (13). A general suppression of cell proliferation should cause a depletion of most of the lympho-hemopoietic bone marrow cells because they are short-lived, but not of the neighboring reticular fibroblasts that are long-lived (40). This is precisely what we observed in the 2° F1 recipients of day-5 cells (Fig. 3 A). Although fibroblasts can be killed by alloreactive TK cells in vitro (41), they were not visibly damaged or reduced in numbers by the cytotoxic T cells present among day-5 and day-6 cells. In conclusion, both experimental evidence and theoretical reasoning indicate that donor TK cells alone are incapable of inducing LGVHD. On the other hand, no donor-cell population that is devoid of cytotoxic anti-F1 activity while maintaining the capacity for induction of LGVHD is now available. Hence, on the basis of the available data, we cannot exclude a limited participation of donor TK cells in the pathogenesis of LGVHD. A possible place of TK cell activity was the small intestine, where lymphocytes invading the epithelium were noticed. This, however, was found in the 2° F1 recipients of both day-5 and day-6 cells. Hence, lymphocytic invasion of the intestinal epithelium cannot account, or cannot account alone, for the sepsis and LGVHD exclusively induced by day-5 cells.

Possibly, in the GVHR, alloreactive TK cells of the donor are readily blocked by the large excess of alloantigen-bearing cells of the recipient. Whatever the explanation, we were impressed by the fact that in two other models of T cell reactivity in vivo,
too, no functional role of TK cells could be detected, although such cells were readily
demonstrable in vitro (42, 43). It appears that cytotoxicity is an exceptional T cell
function because it is performed by T cells themselves. Instead, the main function of
T lymphocytes as a class appears to be an indirect one in that T cell subsets act by
regulating, mainly through the release of factors, the function of other cells, such as
lymphocytes, macrophages, and the progenitors of erythrocytes, monocytes, and
myeloid cells (10, 37, 38). The results of the present investigation fit into this general
concept. Conceivably, a better understanding of stimulatory GVH mechanisms that
cause the hypergammaglobulinemia and lymphadenopathy, on the one hand, and
the suppressive mechanisms that cause the hypogammaglobulinemia, aplastic anemia,
and pancytopenia of LGVHD, on the other hand, will eventually improve our
understanding of the same pathological conditions that develop after viral infections
or exposure to sensitizing drugs. There is reason to assume that GVH-like reactions to
"altered-self" structures on lympho-hemopoietic cells can underly all these conditions
(9).

Summary
Splenic T cells from B10 donors were injected into irradiated (B10 × DBA/2)F1
mice. Either 5 or 6 d later, activated donor T cells were recovered from the spleens of
these primary F1 (1° F1) recipients and transferred to groups of nonirradiated
syngeneic F1 (2° F1) recipients. Whereas day-5-activated parental T cells induced the
characteristic symptoms of acute graft-vs.-host disease (GVHD) and eventually lethal
GVHD, day-6-activated B10 T cells failed to induce acute GVHD but induced
symptoms of chronic GVHD. Interestingly, the inability of day-6-activated T cells to
induce lethal GVHD could not be ascribed to a lack in anti-F1 T killer cells.

The combined results of functional studies indicated that day-6 cells were enriched
for alloreactive helper T cells, whereas day-5 cells were enriched for alloreactive
suppressor cells. Hence, our findings indicate that acute GVHD and lethal GVHD
are caused by alloreactive donor T suppressor but not T killer cells, and that symptoms
of chronic GVHD are caused by alloreactive donor T helper cells.

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