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Involvement of CD40 Ligand-CD40 and CTLA4-B7 Pathways in Murine Acute Graft-Versus-Host Disease Induced by Allogeneic T Cells Lacking CD28

Kiyoshi Saito,*† Jinkyo Sakurai,* Junko Ohata,* Takao Kohsaka,* Hiroshi Hashimoto,† Ko Okumura,‡ Ryo Abe,§ and Miyuki Azuma*‡*

The blockade of B7, using B7 antagonists such as anti-CD80 and/or -CD86 mAbs or CTLA4Ig in vivo, has been shown to induce an efficient suppression of T cell-mediated immune responses in allograft, allergy, and autoimmune models. However, this treatment does not result in complete tolerance. In this study, we examined CD28-B7-independent activation pathways in the pathogenesis of graft-vs-host disease (GVHD) using allogeneic T cells from CD28-deficient mice. Acute GVHD was induced in the absence of CD28 on donor T cells and its manifestations were obvious in the lymphoid tissues. The CD28-independent GVHD was significantly improved by treatment with anti-CD40 ligand (CD40L) mAb. In contrast, treatment with anti-CD80 plus anti-CD86 mAbs exacerbated the clinical manifestations of GVHD and increased the T cell response against host alloantigen, resulting in the expression of CTLA4, CD40L, and CD25 on splenic T cells. These data suggested that the CD40L-CD40 pathway significantly contributed to the CD28-independent pathogenesis of acute GVHD, whereas the CTLA4-B7 pathway acted protectively in the development of GVHD. These results imply that selectively blocking CD28, instead of disrupting both CD28 and CTLA4, would be a better therapeutic strategy for GVHD. Additionally, the simultaneous use of CD40 antagonists may be advantageous. The Journal of Immunology, 1998, 160: 4225–4231.

Successful T cell activation requires the engagement of the TCR with Ag/MHC, as well as the engagement of appropriate costimulatory molecules (1). In particular, signals through the CD28 costimulatory pathway play a critical role in the primary activation of Ag-specific T cells (2). In contrast, accumulating evidence suggests that CTLA4, a homologue of CD28, which also binds B7 ligands (CD80 and CD86), may inhibit T cell activation (3–5). In vivo treatment with CTLA4Ig fusion protein (6–11) or mAbs against CD80 and/or CD86 (12–17) efficiently affect a variety of T cell-mediated immune responses. However, the relative contributions of CD28 and CTLA4 have not been determined in the studies using CTLA4Ig or anti-CD80 plus anti-CD86 (anti-CD80 +86) mAbs. The mechanism whereby CTLA4 regulates the immune response is still largely unknown. In addition to the CD28/CTLA4 costimulatory signals, CD40 ligand (CD40L) also plays an important role in the interaction between Th cells and APCs. The CD40L-CD40 interaction induces the B7 family of molecules on APC, which may be essential for T cell activation (18–20). Recent findings also suggested the existence of CD28-B7-independent costimulatory activity induced by CD40 ligation (21, 22).

In a murine acute GVHD model, we previously reported that treatment with anti-CD80 +86 mAbs efficiently prevented lethality but did not ameliorate hematologic and histologic abnormalities (16). To investigate the CD28-independent activation mechanism in the development of GVHD, we established an acute GVHD model by transferring allogeneic splenocytes from CD28-deficient mice and investigated alternative activation pathways of alloreactive T cells. In this report, we demonstrate that the CD40L-CD40 pathway may be critical for the pathogenesis of GVHD in the absence of CD28-mediated activation signals.

Materials and Methods

Mice

CD28-deficient (CD28(−/−)) mice were generated as previously described (23) and were backcrossed onto a C57BL/6 (B6, H-2b) background for five generations. Female CD28(−/−)B6 mice were used as T cell donors. Wild-type female B6 mice (CD28(+/+)) and (BALB/c × C57BL/6)F1 (CBF1, H-2b) recipient mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Donors were 6 to 8 wk old and recipients were 8 to 10 wk old at the time of bone marrow transplantation (BMT). For in vitro experiments, female 6-wk-old BALB/c (H-2b) mice were also obtained from SLC.

Monoclonal Abs

Hybridomas producing anti-mouse CD80 (RM80, rat IgG2a) and CD86 (PO3, rat IgG2b) mAbs were generated as described previously (14, 15). A hybridoma producing anti-mouse CD40L mAb (MR1, hamster IgG1) (24) was obtained from the American Type Culture Collection (Rockville, MD). These mAbs were purified from ascites as described (15). The pyrogen

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level was <0.01 ng/µg protein, as determined by a Limulus amebocyte lysate assay. mAbs against the following Ags were used for immunofluorescence analysis: CD3 (145-2C11, hamster IgG), CD4 (RM4-5, rat IgG2a), CD8α (53-6.7, rat IgG2a), CD45RB/B220 (RA3-6B2, rat IgG2a), CTLA-4 (UC10-4F10, hamster IgG), CD40L (MR1), CD25 (7D4, rat IgG), CD69 (H1.2F3, hamster IgG), and H-2Kd (SF1-1.1, mouse IgG2a). All FITC-, PE-, eFluor, or biotin-conjugated mAbs were obtained from Pharmingen (San Diego, CA). For staining with biotinylated mAbs, fluorescence was visualized by R-PE-streptavidin (Dako-Japan, Kyoto, Japan). Immunofluorescent staining, flow cytometry, and data analysis were performed as described (25) using FACSsort and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Bone marrow transplantation
Recipient CBF1 mice were lethally irradiated with 9.0 Gy using a 60Co irradiator (MBR 1505 R, Hitachi, Tokyo, Japan). Bone marrow (BM) cells were flushed from the shafts of femurs and tibias of B6(+/+) mice, then treated with anti-Thy-1.2 (30-H12, rat IgG2b) mAb (Pharmingen) and rabbit complement and used as T cell-depleted (TCD)-BM cells. Single-cell suspensions of splenocytes from either CD28(+/+)B6 or CD28(−/−)B6 mice were used as the source of GVHD-causing T cells. Recipients received 2.5 × 10^7 TCD-BM cells and 2.5 × 10^7 splenocytes obtained from either CD28(+/+)B6 or CD28(−/−)B6 mice. Control mice received TCD-BM cells alone. The day of BMT was designated as day 0.

Treatment regimen
Recipients receiving TCD-BM and splenocytes from CD28(−/−)B6 mice were randomly divided into groups of 8 to 10 mice and treated with either control reagents, anti-CD80 + 86 (RM80 + PO3) mAbs, or anti-CD40L (MR1) mAb. As control reagents, either 100 µg of normal rat Ig (Sigma, St. Louis, MO) or hamster IgG (Pharmingen) was administered. One hundred micrograms each of anti-CD80 and anti-CD86 mAb or 200 µg of anti-CD40L mAb per mouse was injected i.p. on day −1, day 0, and then every other day until day 21 post-BMT.

Hematologic and pathologic examination
Peripheral blood was obtained by retro-orbital venipuncture. The total number of lymphocytes, white blood cells, and RBCs were counted by an automated cell counter (Toa Medical Electronics, Tokyo, Japan), and the hemogram was assessed by Wright-Giemsa staining. For pathologic examination, formalin-fixed, paraflin-embedded tissue sections were stained with hematoxylin-eosin.

Measurement of anti-host proliferative responses and cytotoxicity
Splenocytes were obtained from three to four spleens in each group of mice, and T cells were purified as described previously (16). For measurement of T cell proliferative responses against host alloantigen, purified splenic T cells (2 × 10^6/well) were cocultured with irradiated (20 Gy) BALB/c splenocytes (4 × 10^5/well) in 96-well flat-bottom plates. Cultures were pulsed with [3H]thymidine (1 µCi/well) (New England Nuclear, Boston, MA) for 18 h and harvested at day 5. To generate allospecific CTL, purified splenic T cells (2 × 10^6/ml) were cocultured with the same number of irradiated (20 Gy) BALB/c splenocytes in the presence of rIL-2 (40 U/ml) in 24-well plates. After 6 days of culture, viable cells were isolated by Ficoll-Hypaque gradient centrifugation and used as effector cells for cytotoxic assay. Cytotoxicity against A20 (H-2d) target cells was determined microtiter plates. Cytotoxicity against A20 (H-2d) target cells was determined microtiter plates.

Statistical analyses
Significant differences between experimental groups were analyzed by Student’s t test.

Results
Allogeneic T cells lacking CD28 induce acute GVHD
Previously, we and others have shown that the in vivo administration of CTLA4Ig (8, 9) or anti-CD80 + 86 mAbs (16) could prevent the lethality of acute GVHD, but did not completely ameliorate hematologic or histologic abnormalities. To investigate the reason for the incomplete protection against GVHD, we focused on the CD28-independent activation pathways in the development of GVHD. We established a CD28-independent acute GVHD model by transferring allogeneic splenocytes from CD28(−/−) mice. Splenocytes (2.5 × 10^7) from either CD28(+/+)B6 or CD28(−/−)B6 mice were coinjected with 2.5 × 10^7 TCD-BM cells from wild-type B6 mice into lethally irradiated CBF1 recipient mice. As shown in Figure 1, CBF1 mice receiving TCD-BM cells alone (referred to as BM mice) survived more than 150 days, with a gradual increase in body weight, and did not exhibit visible signs of GVHD. In contrast, all of the CBF1 mice that received BM mice with either CD28(+/+)B6 or CD28(−/−)B6 mice also exhibited a smaller increase in body weight as compared with the BM mice. The spleens from CD28(−/−)BMS mice at 30 days post-BMT were smaller, and the spleen weight was clearly reduced compared with BM mice. The histopathology of the spleens from CD28(−/−)BMS mice showed severe tissue destruction, although it was slightly milder than in CD28(+/+)BMS mice (Fig. 2). However, in the other target organs such as the liver, the intestine, and the skin, the manifestations of acute GVHD in the CD28(−/−)BMS mice were much milder than in the CD28(+/+)BMS mice (not shown). This result suggests that the manifestation of GVHD that developed in the CD28(−/−)BMS mice was preferentially ameliorated in the lymphoid organs.

Consistent with our previous report (16), reductions in the absolute count for leukocytes and lymphocytes (white blood cells and absolute lymphocyte count) were observed in the CD28(+/+)BMS mice at 21 days post-BMT (Table 1). The peripheral blood in the CD28(−/−)BMS mice also exhibited a
a (GVHD manifestation. The spleens were surgically excised from BM mice than in CD28(1). Interestingly, T cells from CD28(−/−)BMS mice expressed these Ags at an intermediate level. We have not observed any differences in CD25 and CD69 expression between the three groups (not shown). These data suggest a possible involvement of CTLA4 and CD40L in the development of GVHD.

To directly investigate the involvement of the CTLA4-B7 or CD40L-CD40 pathway in the development of GVHD, we examined the effects of in vivo treatment with either anti-CD40L mAb or anti-CD80+86 mAbs in CD28(−/−)BMS mice. As shown in Figure 3, the anti-CD40L mAb-treated mice showed an increase in body weight similar to BM mice. To the contrary, in the anti-CD80+86 mAb-treated mice, one mouse died at 9 days, and the recovery of body weight after BMT was clearly delayed. This was further indicated by analyzing the hematologic reconstitution of the peripheral blood (Fig. 4). The administration of anti-CD40L mAb significantly augmented the number of lymphocytes above the level found in BM mice, reaching the highest level at day 60. In the anti-CD80+86 mAb-treated mice, the recovery of lymphocytes was clearly delayed compared with the untreated CD28(−/−)BMS mice. The RBC count at 90 days post-BMT, which is a good indicator for assessment of late phase GVHD, was ameliorated by the anti-CD40L mAb treatment, whereas it was not recovered by the treatment with anti-CD80+86 mAbs. In histopathology, the anti-CD40L mAb treatment showed an apparent, but still incomplete, improvement of tissue destruction in the spleen. In contrast, irreversible destruction and atrophy were caused by the anti-CD80+86 mAb treatment (not shown). Taken together, our data suggested that the in vivo clinical manifestations of GVHD in the CD28(−/−)BMS mice were substantially improved by treatment with anti-CD40L mAb. In contrast, these GVHD manifestations were exacerbated by anti-CD80+86 mAb treatment.

**Table I. Hematologic reconstitution of peripheral blood at 28 days post-BMT**

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (×10^3/μl)</th>
<th>ALC (×10^3/μl)</th>
<th>ANC (×10^3/μl)</th>
<th>RBC (×10^6/mm³)</th>
<th>PLT (×10^9/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>6.2 ± 0.8</td>
<td>4.3 ± 0.7</td>
<td>1.7 ± 0.4</td>
<td>10.2 ± 0.7</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>CD28(+/-)BMS</td>
<td>2.8 ± 0.6*</td>
<td>0.9 ± 0.3*</td>
<td>1.7 ± 0.5</td>
<td>9.5 ± 0.7</td>
<td>4.5 ± 1.3*</td>
</tr>
<tr>
<td>CD28(−/−)BMS</td>
<td>2.9 ± 0.9*</td>
<td>1.6 ± 0.7*</td>
<td>1.2 ± 0.7</td>
<td>10.8 ± 0.4</td>
<td>5.1 ± 1.0*</td>
</tr>
</tbody>
</table>

*Peripheral blood cells were analyzed at day 28 post-BMT. Values for WBC (white blood cells), ANC (absolute neutrophil count), ALC (absolute lymphocytes count), and RBC are absolute cell numbers. Data represent the mean ± SD from 8 to 10 mice in each group. Statistically different from *BM and **CD28 (+/-)BMS (p < 0.05).
both CTLA-4 and CD40L, while the anti-CD80+86 mAb treatment clearly enhanced the expression of CTLA4, CD40L, and CD25 on T cells. The induction of these activation Ags was observed on the residual H-2Kd+ host-derived T cells, as well as on the H-2Kd+ donor-derived T cells (not shown). In the in vitro assay for T cell responses, the reduced host-alloantigen-specific T cell proliferative responses in the CD28(−/−)BMS mice was further diminished by the anti-CD40L mAb treatment (Fig. 6A). Interestingly, anti-CD80+86 mAb treatment clearly augmented alloantigen-specific T cell proliferative responses. In contrast with proliferative responses, no obvious difference in the generation of alloantigen-specific CTL was observed between nontreated, anti-CD40L mAb-treated, and anti-CD80+86 mAb-treated mice (Fig. 6B).

### Table II. Phenotype and alloreponses and T cell activation Ags in splenocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Total splenocytes (×10⁶ cells)</th>
<th>H-2Kd+ (%)</th>
<th>CD4 T cells (%)</th>
<th>CD8 T cells (%)</th>
<th>B cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>64.6 ± 11.5</td>
<td>86.7 ± 0.7</td>
<td>13.4 ± 1.9</td>
<td>10.5 ± 2.6</td>
<td>56.3 ± 15.1</td>
</tr>
<tr>
<td>CD28(+/+ BMS)</td>
<td>0.8 ± 1.4*</td>
<td>97.1 ± 0.4*</td>
<td>9.8 ± 3.0</td>
<td>17.7 ± 1.5*</td>
<td>4.9 ± 3.3*</td>
</tr>
<tr>
<td>CD28(−/−)BMS</td>
<td>1.7 ± 1.0*</td>
<td>95.1 ± 2.0*</td>
<td>6.9 ± 2.1*</td>
<td>12.8 ± 4.8</td>
<td>26.0 ± 2.3*</td>
</tr>
</tbody>
</table>

* All data represent the mean ± SD from three or four pooled samples in each group. Statistically different from ^BM and ^CD28(+/+ BMS (p < 0.05).

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Effects of treatment with either anti-CD40L or a mixture of anti-CD80+86 mAbs on body weight in the CD28(−/−)BMS. Mean body weight was compared between the BM mice (closed circles) and the CD28(−/−)BMS mice treated with control reagents (closed triangles), anti-CD40L mAb (closed squares), or anti-CD80+86 mAbs (open squares). Error bars represent the SEM. Each group consists of 8 to 10 mice. The data shown are representative of three experiments. *, Statistically different from the CD28(−/−)BMS mice (p < 0.05). †, A mouse died.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Hematologic reconstitution of peripheral blood. Hemopoietic reconstitution was monitored in BM mice (closed circles) and CD28(−/−)BMS mice treated with control reagents (closed triangles), anti-CD40L mAb (closed squares), or anti-CD80+86 mAbs (open squares). The absolute cell counts were determined using an automated cell counter or by manual counting of a blood smear. Values are the mean ± SD from 5 to 10 mice. The count on day 0 represents the average values of 8-wk-old normal CBF1 mice. RBC, red blood cells; ALC, absolute lymphocyte count.
Discussion

Our data demonstrated that an acute GVHD can be induced by recipient T cells lacking CD28, although the GVHD is not lethal. The manifestation of GVHD was especially obvious in the hematopoietic and lymphoid tissues. These pathologies were similar to those seen in GVHD mice treated with CTLA4Ig (8, 9) or a combination of anti-CD80+86 mAbs (16). Speiser et al. (26) have also observed the development of lethal GVHD induced by T cells from CD28−/− mice. However, in their experimental design without BMT, it was not possible to estimate the subsequent BMT-related immune responses after the early allogeneic reaction. In our GVHD model induced by CD28−/− donor T cells, we observed neither weight loss nor obvious histologic manifestations of GVHD in the liver or the intestine, and this might result in a complete survival. In contrast, lymphoid tissues in peripheral blood and the spleen were apparently damaged, and vigorous T cell activation against host alloantigen was evident in the CD28−/− BMS mice (Table I). Our results demonstrated the disparity between the lethality and the lymphocyte abnormalities in the spleen and blood, suggesting that abnormalities in the lymphoid organs were mediated more by the CD28-independent mechanism. Wallace et al. (9) also suggested that the lethality of acute GVHD was more dependent upon CD28 costimulation than the other GVHD-associated abnormalities.

In this study, anti-CD40L mAb treatment in the CD28−/− BMS mice efficiently ameliorated the delay of hematologic reconstitution and reduced the expression of activation Ags (such as CD40L, CTLA4, and CD25) and allogeneic T cell proliferative responses. These results suggest that the CD40L→CD40 pathway may be a potent CD28-independent activation pathway. Prior reports (27, 28) demonstrated that treatment with anti-CD40L mAb inhibited the tissue-destructive properties of allogeneic CD4+ T cells and allogeneic CTL responses when splenocytes were injected into sublethally or nonirradiated recipients. These findings are compatible with an active involvement of the CD40L→CD40 pathway in the pathogenesis of acute GVHD. In a highly immunogenic organ allograft model, the simultaneous blockade of the CD28-B7 and CD40-CD40L pathways was required for long term acceptance of grafts (29). Furthermore, Tang et al. (30) reported that successful tolerance induction in Th2-mediated contact hypersensitivity required blockade of CD40L and CTLA4Ig treatment. We previously suggested that the delay of hematologic reconstitution by treatment with anti-CD80+86

FIGURE 5. Effects of anti-CD40L or anti-CD80+86 mAb treatment on CD40L, CTLA4, and CD25 expression in splenic T cells. Splenocytes at 28 days post-BMT were stained with FITC-anti-H-2Kd mAb, cyochrome-anti-CD3 mAb, and either PE-anti-CD40L, PE-anti-CTLA4, or biotinylated anti-CD25 mAb, or with appropriate fluorochrome-conjugated control Igs (not shown). For staining with biotinylated mAb, PE-streptavidin was used for visualization. Samples were analyzed by flow cytometry. An electronic gate was set on CD3+ lymphocytes; differential expression for CD40L, CTLA4, and CD25 is presented in histogram form. Markers were placed so as not to include >1% of control Ig-stained cells. Data are representative of two individuals in each group of mice.

FIGURE 6. Anti-host T cell proliferative responses and cytotoxicity after treatment with either anti-CD40L or anti-CD80+86 mAbs. Purified pooled splenic T cells from two to six spleens from each group at 28 days post-BMT were cocultured with irradiated BALB/c (H-2d) splenocytes at the responder:stimulator ratio of 1:2 in the absence (A) or the presence of rIL-2 (40 U/ml) (B). A. After 5 days, cultures were pulsed with 1 μCi/well of [3H]thymidine for 18 h, and incorporated radioactivity was measured. *, Statistically different (p < 0.05). B. After a 6-day culture with rIL-2, cells were harvested, and cytotoxicity against A20 (H-2d) target cells was measured. Each bar or plot represents the mean ± SEM from three or four pooled samples. Data are representative of two experiments.
mAbs may come from the incomplete inhibition of Th2 cell activation (16). The fact of excellent recovery in hemopoiesis, especially in lymphogenesis, by the anti-CD40L treatment in the CD28(-/-)BMS mice strongly supports our previous proposal. CD40 signaling via CD40L in the APC induces not only B7 but also the expression of other costimulatory molecules that may result in augmentation of the APC function. CD44H and ICAM-1 are possible candidates for alternative costimulators induced by CD40L (21, 22). The CD28 and CD40 pathways are crucial independent regulators of T cell mediated immune responses.

It should be noted that treatment with the anti-CD40 mAb significantly increased peripheral blood lymphocytes in the GVHD mice. At present, we are unable to understand the reason why the blockade of CD40-CD40L enhances the number of peripheral lymphocytes. The same schedule of administration of anti-CD40L mAb in syngeneic BMT did not show a significant increase of peripheral lymphocytes (J.O., unpublished observations), and therefore, this manifestation might be associated with allo- neic T cell transfer. It has been shown that CD40L can induce both Fas expression and susceptibility to Fas-mediated apoptosis (31). But CD40 ligation also counteracts apoptosis of B cells and dendritic cells by up-regulating Bcl-xL or Bcl-2 (32, 33). Thus, CD40 can act as either a negative or positive regulator in APC. If the former situation is dominant in our GVHD model, the blockade of CD40 signaling may inhibit apoptosis of B cells and other APC and consequently may enhance T and B lymphocyte expansion. Further studies will be required to clarify this issue.

Treatment with anti-CD80+86 mAbs in the CD28(-/-)BMS mice revealed that CTLA4 acted protectively in the development of acute GVHD, since the blockade of CTLA4 signal by anti-CD80+86 mAbs clearly exacerbated the manifestations of acute GVHD. Interestingly, blockade of the CTLA-4 signal in the CD28(-/-)BMS mice strongly enhanced the expression of CD40L and CTLA4 on donor T cells, indicating that the CD28-mediated activation pathway was not essential for induction of CD40L and CTLA-4. These observations are consistent with previous findings (34, 35). The enhancement of CD40L expression in the anti-CD80+86 mAb-treated CD28(-/-)BMS mice suggested that the development of GVHD may be mediated mainly by the CD28-dependent activation pathway in these mice. Our results also suggested that the use of anti-CD80 and/or CD86 mAbs, or possibly CTLA4-Ig, could interfere with negative signals through CTLA4, resulting in an incomplete blockade of T cell activation. Therefore, the selective manipulation of the CD28 signal alone may be a better approach to preventing acute GVHD. Otherwise, the combination of CD28 and CD40 antagonists might be desirable for attenuating the disadvantage of blockade of CTLA4 regulatory signals.

In conclusion, our results suggested that CD28-B7 and CD40L-CD40 may be two crucial pathways in the pathogenesis of acute GVHD. In addition, the regulatory mechanism through CTLA4-B7 may protect the development of GVHD.

Acknowledgments

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References


