Opposing Effects of Anti-Activation-Inducible Lymphocyte- Immunomodulatory Molecule/Inducible Costimulator Antibody on the Development of Acute Versus Chronic Graft-Versus-Host Disease

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Opposing Effects of Anti-Activation-Inducible Lymphocyte-Immunomodulatory Molecule/Inducible Costimulator Antibody on the Development of Acute Versus Chronic Graft-Versus-Host Disease

Shu-hei Ogawa,* Go Nagamatsu,* Masashi Watanabe,* Shiho Watanabe,* Tomohito Hayashi,* Shigeru Horita,‡ Kosaku Nitta,‡ Hiroshi Nihei,‡ Katsunari Tezuka,‡ and Ryo Abe‡∗

The functional role of inducible costimulator (ICOS)-mediated costimulation was examined in an in vivo model of alloantigen-driven Th1 or Th2 cytokine responses, the parent-into-F1 model of acute or chronic graft-vs-host disease (GVHD), respectively. When the Ab specific for mouse ICOS was injected into chronic GVHD-induced mice, activation of B cells, production of autoantibody, and development of glomerulonephritis were strongly suppressed. In contrast, the same treatment enhanced donor T cell chimerism and host B cell depletion in acute GVHD induced host mice. Blocking of B7-CD28 interaction by injection of anti-B7-1 and anti-B7-2 Abs inhibited both acute and chronic GVHD. These observations clearly indicate that the costimulatory signal mediated by CD28 caused the initial allore cognition resulting in the clonal expansion of alloreactive T cells, whereas the costimulatory signal mediated by ICOS played a critical role in the functional differentiation and manifestation of alloreactive T cells. Furthermore, treatment with anti-ICOS Ab selectively suppresses Th2-dominant autoimmune disease. The Journal of Immunology, 2001, 167: 5741–5748.

The balance of cytokines produced by Th1 cells and Th2 cells is a key factor influencing the character of immune response. These two Th subsets develop from the same Th precursor and differentiate via a complex developmental process. A number of factors have been shown to be involved in this process: cytokines (1–4) and chemokines (5), the dose of Ag (6, 7), APC (8, 9), and costimulation (10–14). Although accumulated data indicate that all influence the initiation of Th differentiation, the mechanism underlying polarization of Th subsets remains unclear. Among these factors, much attention has been placed to the role of CD28-mediated costimulatory signals in polarization of Th subsets. Some groups have suggested that Th2 cytokine production in vivo is less dependent on B7-CD28 signaling than Th1 cytokine production (14, 15). In contrast, other studies indicate a critical role of CD28-mediated costimulation for the development of Th2 cells and IL-4 production (16–19). We have previously shown that both Th1 and Th2 dominant immune responses could be abrogated by blocking of B7-CD28 interaction and were impaired in CD28-deficient mice (16, 21–25). Because CD28 ligation with specific Ab or natural ligands, B7-1 and B7-2, strongly enhances the production of Th1 cytokines, IL-2, IFN-γ, and Th2 cytokines, IL-4, IL-5, IL-6, and IL-10, how CD28 signaling plays a role in the polarization of Th subsets remains uncertain.

Recently, Hutloff et al. (26) identified a third member of the CD28 family, inducible costimulator (ICOS). They showed that triggering of ICOS significantly costimulates the proliferation of T cells. Subsequently, ligand for ICOS was cloned and designated B7 homologous protein (B7 h) (27, 28), B7-related protein-1 (29, 30), GL50 (31), or ligand of ICOS (32). Ligation of ICOS with Ab or its ligand has been shown to strongly enhance the production of the cytokines IL-4, IFN-γ, and IL-10 (29, 33). Interestingly, ICOS-mediated costimulation failed to affect the induction of IL-2 secretion. In vivo as well as in vitro studies provided evidence suggesting that ICOS is a costimulatory receptor in effector T cells rather than naive T cells (33, 34). The blocking of the ICOS signal with ICOS-Ig fusion protein has been shown to attenuate predominantly Th2 responses (33). More recently, ICOS knockout mice were generated and found to have smaller and fewer germinal centers and exhibit profound deficits in Ig isotype class switching to IgG1 and IgE. This defect is explained by the decrease in production of the Th2 cytokines IL-4 and IL-13 (35–37). These results suggested that ICOS is an important regulatory molecule for Th cell-dependent immune responses, particularly Th2-mediated responses.

We have cloned the gene of a novel adhesion molecule from rat thymoma that belongs to the CD28 gene family, designated activation-inducible lymphocyte-immunomodulatory molecule (AILIM) (38). The predicted amino acid sequence demonstrated that AILIM is a rat homologue of human ICOS. Using a cross-hybridization technique, we cloned mouse AILIM and have generated mAbs (38, 57). Consistent with the reports concerning ICOS, cross-linking of

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AILIM/ICOS with Ab strongly enhanced the production of the cytokines IL-4, IFN-γ, and IL-10, but not IL-2 (29,33). To examine the role of AILIM/ICOS during in vivo development of Th1 and Th2 response, we tested the effect of anti-AILIM/ICOS mAb in the parent-into-F1 model of graft-vs-host disease (GVHD). The advantage of this murine system is that, depending on the parental strains injected, the same host develops either an anti-host-cell-mediated, Th1 cytokine-driven disease (acute GVHD) or an autoantibody-mediated, systemic lupus erythematosus-like Th2 cytokine-driven disease (chronic GVHD) in response to the same Ag (39,40). Our results indicate that administration of anti-AILIM/ICOS mAb selectively attenuates Th2-driven chronic GVHD, but Th1-driven acute GVHD is accelerated by the same treatment. Because blockade of CD28 signal with CTLA4-MAb or anti-B7 Ab completely abrogated both acute and chronic GVHD, our findings clearly indicated that CD28 and ICOS play a distinct role in T dependent immune responses. Furthermore, the manipulation of ICOS-mediated costimulation can be a therapeutic strategy for Th2-mediated immune disease.

Materials and Methods

Mice

Female C57BL/6, BALB/c, and (BALB/c × C57BL/6)F1 (CB6F1) mice were obtained from Sankyo Labo Service (Hamamatsu, Japan). The experiments described herein were conducted according to the principles set forth in Ref. 41.

Generation of anti-AILIM mAb

The anti-mouse AILIM/ICOS mAb (B10.5) was generated at JT Pharmaceutical Frontier Research Laboratories (57). Briefly, the purified membrane fractions of mouse AILIM/ICOS-expressing CHO-K1 cells (42) (2–3 × 10⁸) were injected four to five times at weekly intervals into 5- to 6-week-old female Wistar rats (Shizuoka Laboratory Animal Center). Freund’s complete adjuvant (ICN/Cappel, Aurora, OH) was used only for the first immunization. Popliteal lymph node cells were fused with a mouse myeloma cell lines, PAI, using polyethylene glycol 4000 (Boehringer Mannheim, Mannheim, Germany). Hybridomas were screened with their ability to bind to mouse AILIM/ICOS expressed on CHO-K1 or HB-ALL.

Reagents

Rat IgG2a anti-(4-hydroxy-3-nitrophenyl)acetyl mAb (20G2) was generated in our laboratory and was used as rat IgG2a control mAb (control-Ig). Rat anti-mouse B7-1 (RM80) and B7-2 (GL-1) Abs were generously provided by Dr. K. Okumura (Juntendo University School of Medicine, Tokyo, Japan), and Dr. R. J. Hodes (Experimental Immunology Branch, National Cancer Institute, and National Institute on Aging, National Institute of Health, Bethesda, MD), respectively (43,44). Human CTLA4-Ig fusion protein was prepared as described (45). A genetic fusion encoding AILIM-Ig construct was generated as described previously for the CTLA4-Ig construct. Briefly, a DNA sequence containing the signal sequence and extracellular domain of AILIM was PCR amplified and cloned into a vector containing human IgG1 constant region (AILIM-Ig). Several isolates were transfected into mouse plasmacytoma P3U1, and supernatants were tested for the presence of human IgG1 by ELISA. The specific binding of AILIM-Ig to B7 h was determined by indirect immunofluorescence and FACs analysis using B7 h + PT18 cells (data not shown). PE-conjugated anti-CD4 (GK1.5), anti-CD8α (53-67), anti-CD45R/B220 (RA3-2C2), and anti-mouse IgM (R6-60.2) were purchased from BD Pharmingen (San Diego, CA). The B cell hybridomas, anti-I-Ak (Y-3P), anti-I-H-D/Kk (28-8-6), anti-I-H-D/Kk (SF1-1.1), and anti-FeαR (2.4G2) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The mAbs were purified from culture supernatant and fluorescein-labeled according to standard techniques. Rat anti-mouse IgG (6HD5 and HMK12) were generously provided by Dr. K. Okumura (Juntendo University School of Medicine, Tokyo, Japan) (46).

Induction of GVHD and in vivo treatment with anti-AILIM mAb, anti-B7 mAbs, CTLA4-Ig, control-Ig, and AILIM-Ig

Single-cell suspensions of donor spleen cells (6 × 10⁷) from C57BL/6 and BALB/c mice in PBS were injected i.v. via the tail vein into unirradiated CB6F1, hosts to induce acute and chronic GVHD, respectively. Control mice consisted of un.injected age- and sex-matched F1 mice. Experimental mice received 200 µg control-Ig, anti-AILIM mAb, anti-B7 mAbs, CTLA4-Ig, and AILIM-Ig i.v. at the time of GVHD induction (day 0) and day 1 and 100 µg i.p. on days 2, 3, and 4 after cell transfer.

Flow cytometry

Spleen cell suspensions from donor cell-injected GVHD mice were prepared in FACS medium (PBS plus 0.1% BSA (Sigma, A-2153, St. Louis, MO) and 0.1% sodium azide). Cells (10⁵/tube) were incubated first with unlabeled anti-Fc (2.4G2) to block nonspecific binding and then stained with Abs specific for CD4, CD8, CD45R/B220, IgM, H-2Kk, and H-2D/Kk. We used a FACSCalibur and FACSVerse with CellQuest software (BD Biosciences, San Jose, CA) for two-color or four-color flow cytometric analysis, respectively.

Serum Ab levels

Serum IgG levels were assessed by ELISA as previously reported using Abs purchased from Southern Biotechnology Associates (Birmingham, AL). Briefly, ELISA plates (Nalg Nunc International, Roskilde, Denmark) were coated with goat anti-mouse IgG (H + L). Serum samples, diluted 1/500, 1/1000, and 1/2000 in PBS, were added in duplicate and incubated for 1 h at room temperature or overnight at 4°C. The plates were washed with ELISA buffer (PBS plus 0.05% Tween 20 (Wako, Osaka, Japan)) and incubated for 1 h at room temperature with HRP-conjugated goat Abs specific for IgG1 and IgG2a. After washing, ABTS (Sigma, St. Louis, MO) was added to detect HRP activity by OD₄₅₀. The plates were read with an automated ELISA reader (Bio-Rad Microplate Reader model 3550; Bio-Rad, Hercules, CA). Total IgE was assessed by ELISA as described above. ELISA plates were coated with rat anti-mouse IgE (H/D5) and developed with biotin-conjugated rat anti-mouse IgE (HMK12) and detected HRP-conjugated streptavidin (Sigma). For measuring autoantibodies specific for dsDNA, ELISA plates were precoated with 0.001% protamine sulfate in deionized H₂O, then coated with 5 µg/ml dsDNA (Sigma) in 0.015 M sodium citrate with 0.15 M NaCl, and then developed HRP-conjugated goat Abs specific for IgG. Ab concentrations were calculated by using the linear ranges of the dilution and standard curves generated with purified mouse IgG1 (1 mg/ml; Zymed, San Francisco, CA), mouse IgG2a (Zymed), mouse IgE (SPE-7; Seikagaku, Tokyo, Japan), and serum from (New Zealand black × New Zealand white)F1 mice for anti-dsDNA.

Measurement of cytokines

IL-2 and IL-4 were measured by ELISPOT as previously reported (47). Briefly, 96-well ELISA plates were coated with anti-IL-2 or IL-4 Abs. Then wells were washed and blocked with RPMI 1640 plus 5% FCS for 1 h. Freshly isolated splenocytes from donor cell-injected GVHD mice were cultured in a concentration of 1 × 10⁶, 2 × 10⁵, or 4 × 10⁴ cells/well for 5 h. Then plates were washed with PBS plus 0.05% Tween 20. Wells were incubated with biotinylated anti-IL-2 or IL-4 for 1 h at 37°C. After washing, streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at room temperature. Plates were washed again with PBS, 5-bromo-4-chloro-3-indolyl phosphate (Sigma) substrate was added overnight, and colored spots were counted using a stereomicroscope. The number of spots per 10⁵ CD3⁺ splenocytes is shown.

IFN-γ was measured by ELISA. Spleen cell suspensions from donor cell-injected GVHD mice were cultured for 48 h at a concentration of 1 × 10⁶ cells/ml without exogenous stimulation in 24-well plates (Corning Costar, Corning, NY) and supernatants were placed on anti-IFN-γ-coated 96-well plates and incubated for 1 h at room temperature. After washing, wells were incubated with biotinylated anti-IFN-γ for 1 h at 37°C, and then streptavidin-alkaline phosphatase was added for 1 h at room temperature. Finally, ABTS was added to detect HRP activity. The plates were read with an automated ELISA reader (Bio-Rad).

Histology and immunohistochemistry

A part of kidney tissues were immediately frozen in liquid nitrogen. Frozen sections (4 µm) were dried and fixed in acetone for 10 min. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse Ig (γ-chain specific; Sigma) for 30 min at room temperature and were extensively washed with PBS. Specific staining was visualized by a fluorescence microscope. The stained sections were examined, and two or three glomeruli per mouse were photographed under a fluorescence microscope (AX80; Olympus, Tokyo, Japan). The intensity of immunofluorescence was graded as negative (point 0), trace (point 0.5), 1+ (point 1.0), 2+ (point 2.0), and 3+ (point 3.0). The assessment was performed by two observers who did not know the background data (see Table III).
Anti-host cytotoxicity by GVHD spleen cells was determined directly, with no in vitro restimulation, by assessing killing of an H-2d tumor line. P815, a murine DBA/2-derived mastocytoma line, was incubated with $^{51}$Cr (0.2 mCi) for 1 h in 50% FCS, washed twice, and diluted to $10^6$ cells/ml to serve as a target. Single-cell suspensions of spleen cells ($10^7$ cells/ml in complete medium) were serially diluted and incubated for 4 h at 37°C with target cells at four E/T ratios. Chromium release into the supernatant was measured by gamma counter. The percent cytotoxicity was calculated as 

$$\text{percent cytotoxicity} = \frac{\text{experimental } ^{51} \text{Cr release} - \text{spontaneous } ^{51} \text{Cr release}}{\text{maximal } ^{51} \text{Cr release} - \text{spontaneous } ^{51} \text{Cr release}} \times 100\%.$$ 

**T cell functional analysis**

To assess T cell function, proliferative response against Con A (Sigma) was measured by $[^3H]$thymidine incorporation. Freshly isolated splenocytes from donor cell-injected GVHD mice ($2 \times 10^7$ cells/well) were cultured with Con A for 48 h in 96-well plates (Falcon), with $[^3H]$thymidine (0.5 μCi) during the last 12 h. The $[^3H]$thymidine incorporation per CD3$^+$ cell was calculated as ($[^3H]$thymidine incorporation/well)/(2 x $10^5$ x proportion of CD3$^+$ cell in $2 \times 10^5$ cells). The proportion of CD3$^+$ was analyzed by flow cytometry.

**Statistical analysis**

All statistical analyses were performed using Student’s t test. $p < 0.05$ was considered statistically significant.

**Results**

**Injection of anti-AILIM mAb inhibits development of chronic GVHD**

To identify the role of AILIM/ICOS-mediated costimulatory signal in development of chronic GVHD and to compare it with that of the CD28-mediated signal, CB6F1 mice were treated with anti-mouse AILIM mAb or with CTLA4-Ig at the time of BALB/c spleen cell transfer. Serum was taken from chronic GVHD-induced CB6F1 mice that had been treated with anti-AILIM mAb, CTLA4-Ig, or control Ig. As shown in Fig. 1, the serum level of IgG1, IgG2a, and IgE were increased in control-Ig treated mice, indicating that polyclonal B cell activation accompanying hypergammaglobulinemia occurred in these mice. The CTLA4-Ig treatment abrogated elevation of all Ig subclasses tested. Similar to the CTLA4-Ig treatment, anti-AILIM mAb treatment strongly inhibited IgE increase in chronic GVHD. Injection of anti-AILIM mAB enhanced IgG1 production at the early stage; then IgG1 level rapidly declined to the level observed after CTLA4-Ig treatment. In contrast, IgG2a levels were not altered by anti-AILIM mAB treatment.

It has been postulated that hypergammaglobulinemia is the result of continuous host B cell activation by donor T cells (48). In fact, in control-Ig-treated host mice, elevated expression of MHC class II Ag on B cells was observed from 8 to 60 days after induction of chronic GVHD (Fig. 2 and Table I). Blocking the CD28-mediated costimulatory signal by injection of CTLA4-Ig inhibited MHC class II elevation. Anti-AILIM mAb treatment was more effective in the prevention of B cell activation at the early stage of chronic GVHD (day 8) and then became similarly as effective as CTLA4-Ig treatment at 24 and 60 days after induction.

**Generation of autoantibody is one of the major characteristics of chronic GVHD.** As expected, we observed sharp elevation of anti-dsDNA Ab in control-Ig treated chronic GVHD mice. As shown in Fig. 1D, both CTLA4-Ig and anti-AILIM mAb abrogated the production of anti-dsDNA Ab.

An immune complex glomerulonephritis is often observed in chronic GVHD mice (49). Nineteen and 94 days after induction of chronic GVHD mice were treated with control-Ig, anti-AILIM mAb, or anti-B7-1 plus B7-2 mAb at the time of parental cell transfer as described in Materials and Methods. Eight, 24, and 60 days after donor cell transfer, splenocytes were stained with FITC-conjugated anti-I-A$^b$ mAb and PE-conjugated anti-B220 mAb. I-A$^b$ expression on B220$^+$ cells is shown. Similar results were obtained in four additional experiments.

**FIGURE 1.** Anti-AILIM mAb treatment prevents the hyperproduction of polyclonal IgG and autoantibody in chronic GVHD. Chronic GVHD mice received control-Ig (□), anti-AILIM mAb (○), or CTLA4-Ig (△) at the time of parent cell transfer as described in Materials and Methods. At 1, 2, 3, 6, 9, and 12 wk, all mice were bled from the tail vein (n = 6 per group) and total IgG1 (A), IgG2a (B), IgE (C), and IgG anti-dsDNA Ab (D) in serum was determined by ELISA. Bars represent group mean ± SE. Similar results were obtained in two additional experiments with four to five mice in each group. * $p < 0.05$; + $p < 0.01$ compared with the chronic GVHD control-Ig-treated group.

**Table 1.** Anti-AILIM mAb treatment prevents polyclonal B cell activation in chronic GVHD mice.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control-Ig</th>
<th>Anti-AILIM</th>
<th>Anti-B7-1 + Anti-B7-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>257.94 ± 10.24</td>
<td>219.05 ± 7.27$^*$</td>
<td>116.78 ± 5.12$^*$</td>
</tr>
<tr>
<td>24</td>
<td>241.88 ± 39.81</td>
<td>146.97 ± 8.84$^*$</td>
<td>156.24 ± 1.55</td>
</tr>
<tr>
<td>60</td>
<td>152.72 ± 20.14</td>
<td>100.26 ± 11.04$^*$</td>
<td>107.04 ± 9.88</td>
</tr>
</tbody>
</table>

$^*$ Chronic GVHD mice were treated with control-Ig, anti-AILIM mAb, or anti-B7-1 plus B7-2 mAb at the time of parental cell transfer as described in Materials and Methods. Eight, 24, and 60 days after donor cell transfer, splenocytes were stained with FITC-conjugated anti-I-A$^b$ mAb and PE-conjugated anti-B220 mAb. Numbers indicated percentages relative to the normal host mean fluorescence intensity of I-A$^b$ on B220$^+$ cells. Results represent mean values ± SD of three mice per group. Similar results were obtained in four additional experiments. * $p < 0.05$ compared with the chronic GVHD control group treated with control-Ig.
chronic GVHD, mice were sacrificed, and their kidneys were evaluated for the presence of immune complex deposit (Fig. 3 and Table II). In control-Ig-treated host mice, immune complex deposits were observed at 19 days, and the extent of deposit was elevated at 94 days. However, only low levels of immune complex deposits were detected in CTLA4-Ig-treated mice at 19 and 94 days. The kidney sections from anti-AILIM mAb-treated mice showed a small amount of immune complex deposits at 19 days. The extent of deposits was slightly elevated at 94 days but still much lower than that of control-Ig-treated mice.

**Anti-AILIM mAb treatment accelerates acute GVHD**

We have previously shown that blockade of CD28 by the injection of CTLA4-Ig blocks the development of acute GVHD (21). To evaluate the role of AILIM/ICOS in acute GVHD, we tested the effect of anti-AILIM mAb treatment to acute GVHD-induced mice and compared it with that of CTLA4-Ig and control-Ig treatment.

The major pathology of acute GVHD is the expansion of host-reactive donor T cells and depletion of host immune competent cells. In our experiments, control-Ig-treated GVHD mice showed significant expansion of donor CD4^+ and CD8^+ T cells and reduction of host B cells in proportion as well as in absolute number (Table III). Consistent with the previous reports, donor expansion and host B cell depletion were abrogated by the administration of CTLA4-Ig (21). In contrast, anti-AILIM mAb injection enhanced the proportion of donor T cells from control-Ig-treated mice, CD4^+ T cells (from 9.66% to 14.07%), and CD8^+ T cells (from 9.64% to 25.11%). The proportion of B cells was also further decreased in anti-AILIM mAb-treated mice (from 30.08% to 22.05%). At day 12, control-Ig treated GVHD spleens contained an average of 16.6 × 10^6 of both CD4 and CD8 donor T cells, whereas <1 × 10^6 donor T cells were present in CTLA4-Ig treated mice. In contrast, anti-AILIM mAb treatment strongly accelerated expansion of donor CD8^+ T cells (from 16.68 × 10^6 to 28.07 × 10^6) and depletion of host B cells (from 5.21 × 10^7 to 2.44 × 10^7).

**FIGURE 3.** Anti-AILIM mAb treatment inhibits renal IgG deposition in chronic GVHD mice. Nineteen and 94 days after induction of chronic GVHD, mice were sacrificed, and their kidneys were evaluated for the presence of immune complex deposit as described in Materials and Methods. A and D, Control-Ig treatment (n = 4); B and E, anti-AILIM mAb treatment (n = 4); C and F, anti-B7-1 plus anti-B7-2 mAb treatment (n = 3); G, normal CB6F1. Similar results were obtained in two additional experiments.

**Table II. Scoring for the glomerular deposition of mouse IgG in chronic GVHD mice**

<table>
<thead>
<tr>
<th>Days</th>
<th>Control-Ig</th>
<th>Anti-AILIM</th>
<th>Anti-B7-1 + Anti-B7-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>2.75 ± 0.25</td>
<td>2.25 ± 0.25*</td>
<td>1.67 ± 0.24**</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 3)</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>2.75 ± 0.25</td>
<td>1.75 ± 0.25**</td>
<td>1.17 ± 0.24**</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td>(n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

* Chronic GVHD mice were treated with control-Ig, anti-AILIM mAb, or anti-B7-1 plus B7-2 mAb at the time of parental cell transfer as described in Materials and Methods. Two or three frozen glomeruli sections per mouse were stained with FITC-conjugated anti-mouse IgG and photographed under a fluorescence microscope. The intensity of immunofluorescence was graded as negative (point 0), trace (point 0.5), 1+ (point 1.0), 2+ (point 2.0), and 3+ (point 3.0).

**Anti-AILIM mAb treatment altered the cytokine expression in acute and chronic GVHD**

It has been established that the various pathology of acute and chronic GVHD was caused by hyperproduction of Th1 and Th2 cytokines, respectively. The strong and unique effect of anti-AILIM mAb treatment in acute (Fig. 6, A–C) and chronic (Fig. 6, D–F) GVHD therefore led us to examine the production of cytokines at 12 days after cell transfer by ELISA or ELISPOT. In anti-B7-1 plus anti-B7-2 mAb-treated mice, secretion of IL-2, IL-4, and IFN-γ remained at basal level, i.e., equal to the level that normal CB6F1 mice produced. IL-4 secretion was reduced in anti-AILIM mAb-treated mice, whereas this treatment did not show any effect on IL-2 and IFN-γ secretion.

**Evaluation of the in vivo effect of anti-AILIM mAb injection**

The data obtained and described above indicate that injection of anti-AILIM mAb inhibits Th2-driven chronic GVHD and partially accelerates Th1-driven acute GVHD. To determine the in vivo effect of anti-AILIM mAb, the expression of AILIM on splenic T cells of chronic GVHD-induced mice, which were treated with anti-AILIM mAb or control Ab, was evaluated. As shown Fig. 7,
T cells from control Ab-treated mice expressed AILIM on both CD4+ and CD8+ T cells. In contrast, anti-AILIM mAb treatment almost completely abrogated the expression of AILIM on spleen cells (Fig. 7, C and D) as well as lymph node cells (data not shown). Two possible explanations can be considered: 1) Ab injection depleted AILIM expressing T cells; 2) AILIM molecules were down-modulated. To delineate these two possibilities, spleen cells from Ab-treated mice were placed in an in vitro culture for 24 h and were tested for the expression of AILIM, with positive results (Fig. 7, G and H). These results indicated that anti-AILIM mAb injection caused down-modulation of surface expression of AILIM molecules and suggested that inhibitory effects of anti-AILIM mAb treatment may be the result of the blockade of AILIM/ICOS-B7 h interaction.

To evaluate this hypothesis, we generated AILIM-Ig, a fusion protein that blocks the AILIM/ICOS-B7 h (27–32) interaction, and compared its in vivo effect with anti-AILIM mAb on the development of chronic GVHD. As shown in Fig. 8, AILIM-Ig treatment effectively reduced the serum level of polyclonal IgG1 and IgE, although less effectively than anti-AILIM mAb treatment. Neither anti-AILIM mAb nor AILIM-Ig had any effect on production of IgG2a. These results are consistent with the idea that the inhibitory effect of anti-AILIM mAb treatment is the result of the blockade of AILIM/ICOS-B7 h interaction.

**Table III. Effect of anti-AILIM mAb treatment on splenic lymphocyte populations and donor/host chimerism in GVHR at day 12**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Donor CD4 Cells (×10^6)</th>
<th>No. of Donor CD8 Cells (×10^6)</th>
<th>No. of Host B Cells (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-Ig</td>
<td>16.61 ± 1.28 (9.66)</td>
<td>16.68 ± 3.82 (6.64)</td>
<td>52.1 ± 5.3 (30.8)</td>
</tr>
<tr>
<td>Anti-AILIM</td>
<td>16.73 ± 1.88 (14.07)</td>
<td>28.07 ± 3.54 (25.11)</td>
<td>24.4 ± 7.0 (22.05)</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>0.94 ± 0.09 (0.66)</td>
<td>0.84 ± 0.07 (0.59)</td>
<td>73.0 ± 17.1 (55.82)</td>
</tr>
<tr>
<td>CB6F1</td>
<td></td>
<td></td>
<td>80.0 ± 10.6 (57.15)</td>
</tr>
</tbody>
</table>

* Number of donor T or B lymphocytes in the spleen (mean ± SD).

Discussion

In this study, we investigated the functional role of AILIM/ICOS in the T cell response to alloantigens in vivo by using models of parent into F1 GVHD. We tested the effect of anti-AILIM mAb treatment at the time of parental cell transfer in acute or chronic GVHD. In chronic GVHD, donor CD4+ T cells are stimulated by MHC class II Ag on host B cells and in turn stimulate these B cells to become activated and produce polyclonal Ig as well as autoantibodies. We found that the anti-AILIM mAb treatment inhibited the up-regulation of the MHC class II Ag expression on B cells (Fig. 2), elevation of polyclonal IgG1, IgE, but not IgG2a in serum (Fig. 1, A–C). This treatment also effectively suppressed the induction of autoimmune responses in this model, production of anti-DNA Ab (Fig. 1D) and development of glomerulonephritis (Fig. 3). In contrast to the strong effect of the anti-AILIM mAb on chronic GVHD, this treatment did not suppress the development of acute GVHD. In fact, the major characteristics of acute GVHD, expansion of donor T cells and host B cell depletion, were more apparent in mice treated with anti-AILIM mAb than those in mice treated with control rat Ab, indicating that anti-AILIM mAb accelerates the development of acute GVHD. The mechanisms underlying this opposing effect of anti-AILIM mAb injection on chronic vs acute GVHD are partially explained by the
supernatants were assayed for IFN-γ after 48 h without exogenous stimulation, and the resultant supernatant from acute (C) or chronic (F) GVHD day 12 mice (n = 4/group) was determined by ELIS-POT in which cells were cultured for 5 h without re-stimulation. Splenocytes from acute (C) or chronic (F) GVHD day 12 mice (n = 4/group) were cultured for 48 h without exogenous stimulation, and the resultant supernatants were assayed for IFN-γ by ELISA. Bars represent group means ± SE. Similar results were obtained in three additional experiments. * p < 0.05 compared with the chronic GVHD control-Ig treated group; n.d., not detectable. CBF1, CB6F1.

Pattern of cytokine secretion of spleen cells in these mice (Fig. 6). In both chronic and acute GVHD, anti-AILIM mAb strongly inhibited the expansion of IL-4-secreting cells, whereas secretions of IFN-γ as well as IL-2 were not affected by this treatment. It has been well established that IL-4 plays a pivotal role for the Ig class switching to IgG1 and IgE, whereas IgG2a class switching is controlled by IFN-γ. It is therefore most likely that reduction of IL-4 secretion by anti-AILIM mAb treatment resulted in the suppression of chronic GVHD. Consistent with our hypothesis, anti-IL-4 treatment is reported to be effective for the prevention of autoantibody production and lupus-like glomerulonephritis (50).

It was found that although acute GVHD is Th1 driven, not only IL-2 and IFN-γ but also IL-4 secretion is increased (Fig. 6). Treatment with anti-B7 Abs abrogated secretion of all cytokines, whereas anti-AILIM mAb treatment selectively inhibited IL-4 secretion and had no effect on IFN-γ secretion (Fig. 6). It has been proposed that immune deficiency in GVHD is dependent on IFN-γ production by donor cells (51, 52). Also, donor CD8+ T cells are a significant population of anti-host effectors, which deplete host B cells (52). The inability of anti-AILIM mAb treatment on the alteration of IFN-γ secretion contributes to the failure of this treatment in the suppression of acute GVHD. Furthermore, it is reported that IL-4 protects activation-induced cell death of B cells (53); thus, inhibition of IL-4 secretion may be the mechanism of acceleration of host B cell depletion in anti-AILIM mAb-treated mice.

The role of ICOS in the polarization of Th1 and Th2 subsets is a matter of controversy. Coyle et al. (34) found that ICOS was selectively expressed on Th2 clones but not Th1, and ICOS-Ig inhibited lung mucosal inflammation induced by Th2 but not Th1 effector populations. In contrast, Gonzalo et al. (54) showed that ICOS-Ig treatment in vivo significantly inhibited IFN-γ production but had no effect on IL-4 production after in vivo injection of SEB. Kopf et al. (55) reported that blocking of ICOS-mediated costimulation with ICOS-Ig reduced both Th1 and Th2 cytokine production. Recently, ICOS knockout mice have been generated (35–37). It was found that they have smaller and fewer germinal centers and exhibit profound deficits in Ig isotype class switching to IgG1 and IgE. This defect is explained, at least partly, by the decrease in production of Th2 cytokines IL-4 and IL-13. These results are consistent with
the profound inhibitory effect of anti-AILIM/ICOS mAb to polyclonal IgG1 and IgE production in chronic GVHD described above. Furthermore, Dong et al. (35) reported that ICOS knockout mice showed greatly enhanced susceptibility to Th1-driven experimental autoimmune encephalomyelitis, which resembles the partial enhancement of acute GVHD by anti-AILIM mAb treatment observed here.

Our results indicate that the blockade of CD28/B7 interaction by CTLA4-Ig or anti-B7-1 plus anti-B7-2 mAb treatment abrogated both acute and chronic GVHD, whereas blockade of ICOS-B7 h interaction by anti-AILIM mAb treatment selectively inhibited development of chronic GVHD. These results suggest that CD28-mediated T cell costimulation is critically required for the initiation of alloreactivity of T cells, including expansion, presumably with autocrine IL-2, cytokine production, and up-regulation of ICOS and other cell surface receptors. In contrast, ICOS-B7 h interaction may be dispensable for primary T cell response to allografts but play an important role for the differentiation of immune responses.

Via et al. have previously reported that early administration of CTLA4-Ig, at the time of GVHD induction, prevented the development of both acute and chronic GVHD, whereas delayed CTLA4-Ig administration, after the establishment of Th1 and Th2 effector responses (day 7), was unable to alter acute GVHD but did reverse chronic GVHD (56). They interpreted these results such that CD28-B7 interaction is equally required for both Th1 and Th2 responses; however, once effector mechanisms become established, only Th2-driven responses require further costimulation for the continued expansion of alloreactive CD4+ T cells. The effect of anti-AILIM mAb treatment resembles the function of delayed CTLA4-Ig administration. Because a CD28-costimulatory signal is necessary for the full expression of ICOS (data not shown), the blocking effect of delayed administration of CTLA4-Ig in their study resembles the partial enhancement of acute GVHD by anti-AILIM mAb treatment resembling the function of delayed CTLA4-Ig in their study.

B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2.

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