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B7.2 Has Opposing Roles During the Activation Versus Effector Stages of Experimental Autoimmune Thyroiditis1

Karin E. Peterson,* Gordon C. Sharp, †‡ Haiwen Tang,** and Helen Braley-Mullen2*§

APCs provide costimulatory and down-regulatory signals to Ag-activated T cells through interactions between B7.1 and B7.2 on APCs with either CD28 or CTL Ag-4 expressed on T cells. Recipients of mouse thyroglobulin (MTg)-primed spleen cells activated in the presence of anti-B7.2 had decreased experimental autoimmune thyroiditis (EAT) severity compared with recipients of cells cultured with control rat Ig or anti-B7.1. Blocking B7.2 during in vivo priming also suppressed the ability of MTg-primed spleen cells to transfer EAT, implicating a role for B7.2 for priming and in vitro activation of EAT effector cells. In contrast, administration of anti-B7.2 or anti-B7.2 Fab to recipients of MTg-activated spleen cells increased the severity of EAT compared with recipients receiving control Ig. Thyroids from anti-B7.2-treated recipients had increased expression of IL-4 mRNA compared with thyroids from rat Ig-treated controls. Both B7.1 and B7.2 molecules were expressed in the thyroids of mice with EAT, although B7.2 was more prevalent than B7.1. Administration of both anti-B7.1 and anti-B7.2 to recipient mice suppressed the development of EAT, while anti-B7.1 treatment alone had no effect on EAT severity. The suppression of EAT was not observed when anti-B7.1 and anti-B7.2 treatment was delayed until 7 days after cell transfer, suggesting a requirement for B7 in the initiation of EAT in recipient mice. These results suggest that costimulation is required during the effector phase of EAT and that B7.2 may have opposing roles in the activation versus effector stages of autoreactive T cells. The Journal of Immunology, 1999, 162: 1859–1867.

Experimental autoimmune thyroiditis (EAT) is a chronic inflammatory autoimmune disease induced in genetically susceptible strains of mice by immunization with mouse thyroglobulin (MTg) and adjuvant (1) or by the adoptive transfer of MTg-sensitized donor spleen cells, activated in vitro with MTg, into recipient mice (2). MTg-sensitized cells, activated in vitro with MTg, induce a mild, chronic form of EAT in recipient mice characterized by a lymphocytic infiltrate of primarily CD4+ and CD8+ T cells and some macrophages and plasma cells (3). Activation of donor cells with MTg and IL-12 and/or anti-IL-2 receptor mAb induces a more severe and histologically distinct form of EAT with granulomatous histopathology (4, 5). CD4+ T cells are the primary effector cells for both lymphocytic and granulomatous EAT. MTg-specific CD4+ T cell lines and clones can transfer EAT to recipient mice (6), while depletion of CD4+ T cells from recipient mice can prevent or reverse the development of lymphocytic (7) and granulomatous EAT (4, 5).

Activation of CD4+ T cells requires two signals, an Ag-specific signal through the TCR and a secondary costimulatory signal between the T cell and APC. TCR ligation in the absence of a costimulatory signal can result in anergy or cell death (8). The major costimulatory molecules expressed by APC are B7.1 and B7.2, which bind to CD28 expressed on T cells (9). A requirement for T cell costimulation for the development of autoimmune has been observed in several animal models. Treatment with CTL Ag-4 (CTLA-4-Ig, which blocks both B7.1 and B7.2, suppressed the development of spontaneous autoimmunity in NZB/NZW F1 (10) and nonobese diabetic (NOD) mice (11), as well as experimentally induced autoimmune encephalomyelitis (EAE) (12) and collagen-induced arthritis (13). The specificity for B7.1 or B7.2 as the costimulatory ligand in the development of autoimmunity is not as clear. Anti-B7.1 inhibited the development of EAE, while anti-B7.2 exacerbated disease (14–16). In contrast, anti-B7.2 suppressed and anti-B7.1 accelerated the development of diabetes in NOD mice (11), while neither anti-B7.1 nor anti-B7.2 alone suppressed or accelerated the development of collagen-induced arthritis (17) or autoimmunity in NZB/NZW mice (18).

The ability to induce severe EAT with granulomatous histopathology by the adoptive transfer of MTg-primed spleen cells activated in vitro with MTg and IL-12, makes this an interesting model of autoimmunity for examining the roles of B7.1 and/or B7.2 in the activation and effector stages of autoreactive T cells. Surprisingly, a differential effect of blocking B7.2 was observed dependent upon the stage of EAT induction. Blocking B7.2 either during in vivo priming of donor mice or during in vitro activation of EAT effector cells suppressed the ability of MTg-primed spleen cells to transfer granulomatous EAT. However, treatment of recipient mice with anti-B7.2 increased EAT severity and granulomatous histopathology, suggesting opposing roles for B7.2 in the induction of EAT. Blocking both B7.1 and B7.2 during in vivo priming, in vitro activation, or development of thyroiditis in recipient mice suppressed EAT severity, suggesting that costimulation through B7.1 or B7.2 is not only necessary for the activation of primed effector cells, but is also required during the development of thyroiditis in recipient mice.
Materials and Methods

Mice

Female CBA/J mice 6–8 wk of age, obtained through Clarence Reeder at the National Institutes of Health, were used for all experiments.

Ages and immunization

MTg was prepared from pooled mouse thyroids, as previously described (2). LPS (Escherichia coli 0111:B4) was purchased from Sigma (St. Louis, MO). Donor mice were injected twice with 150 µg MTg and 15 µg LPS (i.v.) at 10-day intervals (2).

Abs and injections

The anti-B7.2-producing hybridoma, GL1, was purchased from American Type Culture Collection (ATCC; Manassas, VA) (HB-253) (19). The anti-B7.1-producing hybridoma, 16-10A1 (ATCC HB-301) was provided by Dr. Hans Reiser (20), and the anti-CTLA-4-producing hybridoma (4F10) was provided by Dr. Jeffery Bluette (21). Anti-B7.1 and anti-B7.2 were purified from culture supernatant using protein G columns. Anti-B7.2 Fab fragments were generated using the papain-Fab fragment kit (Pierce, Rockford, IL). For blocking B7.1 or B7.2 during in vitro activation, anti-B7.1, anti-B7.2, or a combination of anti-B7.1 and anti-B7.2 was added to culture to a final concentration of 10 µg/ml of each Ab. Rat (Sigma) or hamster Ig (Jackson Immunoresearch, West Grove, PA) (10 µg/ml) were used as controls. For blocking B7.1 and/or B7.2 during in vivo priming, 100 µg of anti-B7.1, anti-B7.2, or rat and hamster Ig was injected i.p. to donor mice on days −1, 0, and +1 relative to each immunization with MTg and LPS. For treatment of recipient mice, 100 µg of anti-B7.1, anti-B7.2, or both Abs was injected i.p. every 3 days starting on the day of spleen cell transfer, unless otherwise indicated. Control mice received 100 µg of rat and/or hamster Ig according to the same schedule.

Adoptive transfer of EAT

Seven days after the secondary immunization with MTg and LPS, donor spleen cells were suspended at 10⁷ cells/ml in RPMI 1640 containing 25 mM HEPES buffer, 5% FBS (Sigma; Lot 504-0269), and 1% of the following: glutamine, vitamins, nonessential amino acids, sodium pyruvate, and 0.005 M 2-ME. Cells were cultured in 60-mm petri dishes (Corning 25100; Corning Glass, Corning, NY) at 4 ml/plate with 25 µg/ml MTg, anti-IL-2R mAb (M7/20) (22) (5% final concentration of culture supernatant), and 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN) (5). After 72 h at 37°C, cells were harvested and washed, and 3 × 10⁶ cells were injected i.v. into irradiated (600 rad) recipient mice (4).

Proliferation assay

For some experiments, proliferative responses of sensitized donor spleen cells were assessed, as previously described (23). Con A (0.5 µg/well), MTg (5 µg/well), or media were added to triplicate wells containing 5 × 10⁶ spleen cells. After 72 h, 0.5 µCi [3H]TdR was added to each well, and cells were harvested 16–20 h later. Results are expressed as the mean cpm of triplicate wells minus the mean cpm of cells cultured with medium alone (∆cpm).

ELISA assays

MTg-specific serum IgG Abs were determined by ELISA using MTg-coated ELISA plates. Serial dilutions of serum from individual mice were tested in duplicate, as previously described (24). Alkaline phosphatase-conjugated Abs specific for IgG1, IgG2A, and IgG2B (Zymed, San Francisco, CA) were used to assess the contributions of various IgG subclasses to the total IgG autoantibody response. Secondary Abs were titrated and used at an optimal concentration to provide maximal readings on MTg-coated ELISA plates and OD₅₀₅ < 0.05 on OVA-coated ELISA plates.

Evaluation of EAT

Thyroids were removed from recipient mice 19 to 21 days after cell transfer, the time of peak disease severity in this model (4). EAT severity (the extent of destruction of thyroid follicles) was scored on a scale of 1–5 (5), with 5 being destruction of thyroid follicles, with few or no remaining follicles. Thyroid lesions were also evaluated qualitatively (5). The inflammatory infiltrate in conventional (lymphocytic) EAT consists primarily of lymphocytes and some plasma cells, with relatively few neutrophils. Thyroids designated “G” in tables had granulomatous lesions, characterized by proliferation and enlargement of thyroid follicular cells with lymphocytes, histiocytes, multinucleated giant cells, and small to moderate numbers of neutrophils. The more severe (4–5+) granulomatous lesions had intense inflammation dominated by neutrophils, which also included microabscess formation, necrosis, fibrin deposition, multinucleated giant cells, mononuclear histiocytes, and focal fibrosis, which predominated over the follicular cell proliferative changes and mononuclear infiltrates characteristic of the milder granulomatous lesions. The granulomatous inflammation in thyroids with 4–5+ severity characteristically extended beyond the thyroid to involve the adjacent connective tissue and muscle.

Statistical analysis

All P values were determined using a two-tailed Student’s t test, in which each experimental group was compared individually with the isotype control group. Values of P ≤ 0.05 were designated by * and values of P ≤ 0.01 were designated by **.

PCR analysis

Total RNA was isolated from 6 × 10⁶ in vitro activated spleen cells or from individual thyroid lobes using Trizol (Life Technologies, Gaithersburg, MD). Total RNA was converted to cDNA by Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer Cetus, Branchburg, NJ) and oligo(dT)₁₁–₁₅ primers. To determine the relative amounts of target cDNA, 1/5 and 1/25 dilutions of each cDNA sample were analyzed. Cytokine message levels were measured using specific primers for IL-2, IL-4, IL-10, IL-12, and IFN-γ, as previously described (24, 25). B7 mRNA was measured using the forward primer AGTGTGTCATCAAAGTCCG and reverse primer CTAAGGGAAGACGGTCT (26). B7 mRNA was detected using the forward primer ATGGACCCAGATGACCACAT and reverse primer TTTCTCAAGCTCTCATTGC (26). HPRT was used as a housekeeping gene to verify that similar amounts of RNA were amplified. Samples were amplified at 28 cycles for IFN (and HPRT), 34 cycles for IL-12 and IL-10, and 42 cycles for IL-2 and IL-4. PCR products were separated by gel electrophoresis, stained with ethidium bromide, and analyzed on a digital imager. Cytokine message level was determined as a ratio of cytokine message versus HPRT message for each sample.

Cytokine ELISA

Supernatants were collected from 72-h spleen cell cultures. IL-2, IL-4, IL-10, and IFN-γ levels were measured using sandwich ELISA. JESS-2A5 (PharMingen, San Diego, CA) and biotinylated SXC-1 (PharMingen) were used to measure IL-10. IL-12 and IFN-γ were measured using mouse monoclonal Ab (ATCC HB-188) and rat anti-human Ab (PharMingen) were used to measure IL-4, while biotinylated XMG1.2 and R4-6A2 (ATCC HB170) were used to measure IFN-γ. IL-2 (Endogen, Cambridge, MA) ELISA kits were used to determine IL-2 levels.

Immunohistochemistry

Immunohistochemistry was performed as previously described (27). Thyroid lobes were removed from recipient mice 19 days after cell transfer, snap frozen in OCT (VWR Scientific, Torrance, CA) with liquid nitrogen, and stored at −70°C before cutting. Cryostat sections (6 µm) were cut, fixed in acetone for 10 min, and stored at −70°C. Sections were blocked with 1% BSA in PBS before incubation with primary Ab. Unconjugated 16-10A1 and GL1 were used to detect B7.1 and B7.2, respectively, while rat Ig (Sigma) and hamster Ig (Jackson Immunoresearch) were used to control for nonspecific binding. Biotinylated goat anti-hamster or goat anti-rat IgG (Caltag, Burlingame, CA) were used to detect anti-B7-1 and anti-B7-2, respectively. Sections were then incubated with 0.3% H₂O₂, followed by avidin-biotin-horseradish peroxidase (Vectorstain Elite ABC; Vector, Burlingame, CA) and VIP peroxidase substrate (Vector). To determine whether the anti-B7-1 and anti-B7-2 given to recipients bound to B7.1 and B7.2 molecules in the thyroid, frozen thyroid sections from recipient mice given anti-B7-1 and/or anti-B7-2 were incubated with anti-hamster Ig or anti-rat Ig to detect anti-B7-1 and anti-B7-2 Ab, respectively. Slides were then incubated with 0.3% H₂O₂, avidin-biotin-horseradish peroxidase, and VIP substrate to detect positive cells.

Results

Suppression of granulomatous EAT by blocking B7.2 during in vivo priming and in vitro activation

To determine the concentration of Ab necessary to block B7.1 or B7.2 in vitro, 5–50 µg/ml of anti-B7-1 or anti-B7-2 was added to MTg-pulsed spleen cells. A total of 10 µg/ml of each Ab resulted...
in maximal suppression of MTg-specific proliferation (data not shown). Anti-B7.1 suppressed MTg-specific proliferation approximately twofold, while anti-B7.2 suppressed proliferation to near background levels (Table I). To determine the costimulation requirements during in vitro activation of MTg-specific effector cells, anti-B7.1 and/or anti-B7.2 were added to MTg-primed spleen cell cultures at a final concentration of 10 μg/ml. MTg-primed spleen cells cultured with anti-B7.2 or anti-B7.2 Fab transferred less severe EAT than spleen cells cultured with rat Ig (Table I, Expts. 1 and 2), indicating a requirement for B7.2 costimulation during in vitro activation of EAT effector cells. EAT induced by spleen cells cultured in vitro with anti-B7.1 was generally similar to that induced by cells cultured with rat Ig (Table I, Expt. 1). To determine whether B7.2 was also necessary for in vivo priming of effector cells, 100 μg of anti-B7.2 was given i.p. to donor mice on days −1, 0, and +1 relative to each immunization with MTg and LPS. Recipients receiving cells from anti-B7.2-treated donor mice had decreased EAT severity compared with recipients given rat Ig, while IL-2 production by spleen cells cultured with anti-B7.1 was not consistently decreased (Fig. 2). IFN (and IL-10) levels were not decreased in supernatants of cells cultured with anti-B7.1, anti-B7.2, or both anti-B7.1 and anti-B7.2. IFN levels were also not decreased by anti-B7.1 and/or anti-B7.2 when cells were cultured in the absence of IL-12. IL-4 was undetectable in any of the culture supernatants at 24, 48, or 72 h. Spleen cells cultured with anti-B7.2 had decreased IL-4 mRNA, as determined by RT-PCR, compared with spleen cells cultured with rat Ig (data not shown).

**Cytokine analysis of spleen cells cultured with anti-B7.1 or anti-B7.2**

Culture supernatants from spleen cells cultured with either anti-B7.2 alone or both anti-B7.1 and anti-B7.2 had decreased IL-2 compared with supernatants from cells cultured with rat Ig, while IL-2 production by spleen cells cultured with anti-B7.1 was not consistently decreased (Fig. 2). IFN (and IL-10) levels were not decreased in supernatants of cells cultured with anti-B7.1, anti-B7.2, or both anti-B7.1 and anti-B7.2. IFN levels were also not decreased by anti-B7.1 and/or anti-B7.2 when cells were cultured in the absence of IL-12. IL-4 was undetectable in any of the culture supernatants at 24, 48, or 72 h. Spleen cells cultured with anti-B7.2 had decreased IL-4 mRNA, as determined by RT-PCR, compared with spleen cells cultured with rat Ig (data not shown).

**B7.1 and B7.2 expression in thyroids of recipients with EAT**

B7 expression on thyroid infiltrating cells, but not on thyrocytes, was observed in patients with Hashimoto’s thyroiditis or Graves disease (28). The infiltrating cells in the target organs in other

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**Table I. Suppression of EAT by blocking B7.2 during in vivo priming or in vitro culture of MTg-primed spleen cells**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Ab to Donors</th>
<th>Ab in Culture</th>
<th>EAT Severity</th>
<th>G Lesions</th>
<th>Recipient Anti-MTG IgG</th>
<th>MTG-Specific Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rat Ig</td>
<td></td>
<td>0 0 2 1 0 4/5</td>
<td>0.500 ± 0.085</td>
<td>5.004 ± 0.869</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1</td>
<td>0 1 2 1 0 3/5</td>
<td>0.196 ± 0.050</td>
<td>2.350 ± 0.120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td>4 1 0 0 0 0**</td>
<td>0/5 0.078 ± 0.023</td>
<td>389 ± 271</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1 + anti-B7.2</td>
<td>4 0 0 0 0 0**</td>
<td>0/4 0.000 ± 0.000</td>
<td>179 ± 89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Rat Ig</td>
<td>0 0 5 1 0 6/6</td>
<td>0.643 ± 0.047</td>
<td>2.261 ± 0.258</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td>4 1 0 0 0 0**</td>
<td>0/5 0.274 ± 0.053</td>
<td>837 ± 650</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2 Fab</td>
<td>0 4 1 0 0 0* 0**</td>
<td>0/5 0.531 ± 0.058</td>
<td>157 ± 373</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Donor mice were immunized with MTg and LPS as described in Materials and Methods. Seven days after the second immunization, donor spleen cells were cultured with MTg, anti-IL-2R, and IL-12. After 72 h, 3.35 × 10⁷ spleen cells were injected i.v. into recipient mice.

A total of 100 μg of anti-B7.2 (GL1) or rat Ig was injected i.p. to donor mice on days −1, 0, and +1 relative to each immunization with MTg and LPS.

Anti-B7.1 (16-10A1), anti-B7.2 (GL1), anti-B7.2 Fab, or both anti-B7.1 and anti-B7.2 were added to spleen cell cultures as indicated at a final concentration of 10 μg/ml.

Number of recipient mice with various degrees of severity of EAT 21 days after transfer. *p ≤ 0.05 compared to rat Ig control; **p ≤ 0.01. Statistical analysis was done using the Student’s t test.

Number of mice per total in group with granulomatous thyroid lesions.

Mean OD₅₅₀ ± SE of 1:400 dilutions of serum from four to five individual mice per group obtained 21 days after cell transfer.

Average ΔCPM of MTG-specific proliferation ± SEM. A total of 10 μg/ml of anti-B7.1 or anti-B7.2 was added to culture as indicated.
human autoimmune diseases (29) and experimental models of autoimmunity (15, 30) also expressed high levels of B7.1 and/or B7.2. Immunohistochemistry analysis of thyroids with 3–5×10^6 cells (Fig. 3A) appeared to be higher than the number of B7.1^+ cells (Fig. 3A), although it is possible that the anti-B7.2 Ab has a higher affinity for its Ag and is therefore more readily detectable. To examine the kinetics of B7.1 and B7.2 expression, thyroids were removed from recipient mice at various times after cell transfer and analyzed for B7.1 and B7.2 mRNA expression by RT-PCR. Although B7.1 and B7.2 mRNA were undetectable in normal thyroids, both B7.1 and B7.2 mRNA were detected as early as 5 to 7 days after cell transfer (Fig. 4). The increased expression of B7.1 and B7.2 mRNA was maintained through peak severity of EAT at day 21. These results demonstrate that both B7.1 and B7.2 mRNA are expressed in autoimmune thyroid lesions and therefore may be involved in the development of EAT in recipient mice.

Effect of anti-B7.1 and/or anti-B7.2 on the development of EAT in recipient mice

To determine whether B7.1 and/or B7.2 costimulation was necessary for the development of thyroid lesions, 100 μg of anti-B7.1 or anti-B7.2, or both anti-B7.1 and anti-B7.2 was given to recipient mice every 3 days beginning on the day of cell transfer. Anti-B7.1 (hamster Ab) was detected using anti-hamster Ig (Fig. 3C), but not anti-rat Ig (Fig. 3F), but not anti-hamster Ig (Fig. 3E), in thyroid sections from anti-B7.2-treated recipient mice, while both anti-B7.1 and anti-B7.2 were detected in thyroid sections from recipients given both anti-B7.1 (Fig. 3G) and anti-B7.2 (Fig. 3H). This demonstrates that both anti-B7.1 and anti-B7.2 Abs can gain access to the thyroid after i.p. injection, indicating that the B7.1 and/or B7.2 molecules in the thyroid can be blocked by anti-B7.1 and/or anti-B7.2, respectively.

Recipient mice receiving both anti-B7.1 and anti-B7.2 had decreased EAT severity compared with recipients receiving control rat and hamster Ig (Table II; Fig. 5, A and D), indicating that T cell costimulation was necessary for the development of maximal EAT in recipient mice. Although treatment of recipient mice with anti-B7.1 suppressed the production of anti-MTg autoantibody, it had little effect on EAT severity or the development of granulomatous lesions (Table II, Fig. 5B). Surprisingly, recipients treated with anti-B7.2 consistently had more severe EAT than recipients receiving control rat and hamster Ig (Table II, Fig. 5C), suggesting a possible regulatory role for B7.2 during the effector stage of EAT. Treatment of recipients with anti-B7.2 Fab also resulted in increased EAT severity (Table II, Expt. 4), indicating that the increase in EAT severity was not due to cross-linking and stimulation of recipient cells by the anti-B7.2 Ab. Thyroids from anti-B7.2 or anti-B7.2 Fab-treated recipients had more fibrosis, follicular proliferation, and fibrin deposition, with increased neutrophils and histiocytes and fewer lymphocytes compared with thyroids from recipients receiving rat Ig (Fig. 5, A versus C). The lack of suppression of EAT severity by either anti-B7.1 or anti-B7.2 alone suggests that either costimulatory molecule can be utilized for EAT development. The suppression of autoantibody by anti-B7.1, or anti-B7.1 and anti-B7.2 was observed for both IgG1 and IgG2A (Table II).

Kinetics of anti-B7.1/anti-B7.2-induced suppression of EAT

The suppression of EAT by treatment of recipient mice with anti-B7.1 and anti-B7.2 could be due to blocking costimulation of effector cells in the periphery of recipient mice before infiltration into the thyroid or to suppression of effector cell costimulation in the thyroid, since both B7.1 and B7.2 are blocked in the thyroid (Fig. 3C–H). To determine when B7.1 and B7.2 signaling was needed during the development of thyroiditis, anti-B7.1 and anti-B7.2 or anti-B7.1 alone were administered to recipient mice beginning on day 0 until the removal of thyroids on day 19, as in the previous experiments, or beginning on day 7, when infiltrating cells are readily detectable in the thyroids of recipient mice, until day 19. Mice receiving anti-B7.1 and anti-B7.2 from day 0 to day 19 had decreased EAT severity compared with controls, while delaying treatment with anti-B7.1 and anti-B7.2 until day 7 (day 7 to 19) had no effect on EAT severity (Table III). These results suggest that the suppression of EAT by the combination of anti-B7.1 and anti-B7.2 is most likely due to blocking costimulation either before or during the initial infiltration of effector cells into the thyroid. The ability of anti-B7.2 to increase EAT severity was independent of when anti-B7.2 was given to recipients, since anti-B7.2 increased EAT severity when given from day 0 to day 19 or from day 7 to day 19 (Table III). This suggests that the enhancement of EAT by blocking B7.2 can occur in the thyroid, after the initial infiltration of cells into the thyroid. Recipients receiving anti-B7.2 from day 0 to day 7 also had increased EAT severity compared with rat Ig-treated controls (data not shown). However, the enhancement of EAT by anti-B7.2 treatment from day 0 to day 7 may be due to the presence of residual Ab, since the in vivo t_1/2 of anti-CD40L, an Ab that also blocks cell to cell interactions, is 12 days (31). Recipients receiving both anti-B7.1 and anti-B7.2
from day 0 to day 7 had decreased EAT compared with controls (data not shown), although this may also be due to the persistence of residual Ab after day 7.

**Cytokine analysis in thyroids from recipient mice**

The suppression or enhancement of inflammation by blocking costimulation has been associated with alterations in cytokine production (14, 32, 33). To determine whether the effects of treatment of recipients with anti-B7.2 or the combination of anti-B7.1 and anti-B7.2 were associated with changes in cytokine production in the target organ, thyroids from recipient mice were examined for mRNA expression by RT-PCR. Although intercytokine comparison is not valid using RT-PCR due to variations in primer annealing and variable amplification, the ratio of a particular cytokine between samples can be semiquantitatively compared to provide some indication of relative mRNA levels (24, 25). Thyroids were removed on day 19 after cell transfer, and one thyroid lobe was used for histology, while the other lobe was used for isolation of RNA (24). Thyroids from recipients given anti-B7.1 and anti-B7.2 had decreased IL-2, IL-4, IL-13, and IFN-γ mRNA expression compared with mRNA from thyroids of recipients treated with rat Ig and hamster Ig (Fig. 6). IL-2, IL-4, IL-10, IL-12, IL-13, and IFN-γ mRNA were not expressed in thyroids from naive mice, indicating that the cytokine mRNA expression was from the infiltrating cells. A statistically significant increase in IL-4 mRNA expression was observed in thyroids from recipients given anti-B7.1 and anti-B7.2 or anti-B7.2 Fab fragments (Fig. 6). IL-13 (Fig. 6) and IL-10 (data not shown) mRNA expression was increased in some, but not all, experiments. No consistent changes in mRNA expression were observed for IL-2, IFN (Fig. 6), or IL-12 (data not shown).
The requirement for costimulation in the development of autoimmune diseases has been demonstrated in several experimental models using CTLA-4-Ig or a combination of anti-B7.1 and anti-B7.2 to block the B7.1 and B7.2 costimulatory molecules (10–13, 34, 35). However, treatment with anti-B7.1 or anti-B7.2 alone resulted in either exacerbation or amelioration of disease severity, depending upon the model of autoimmunity (11, 14). In the current study, blocking B7.2 had differential effects on EAT severity, depending on the stage of EAT development. Blocking B7.2 during vitro activation of effector cells or during in vivo priming of donor mice (Table I) suppressed the ability of MTg-primed spleen cells to transfer EAT, while blocking B7.2 in recipient mice resulted in more severe granulomatous EAT (Table II).

Ag (MTg) presentation during donor priming and in vitro activation of MTg-primed effector cells is required for the adoptive transfer of EAT (2, 36, 37). The requirement for B7.2 and not B7.1 during in vitro activation may be explained by the differences in B7.1 and B7.2 expression on splenic APCs, since B7.2 is prevalent on splenocytes, while only a low level of B7.1 is expressed (38). When B7.2 is blocked, the levels of B7.1 may be too low to allow effective T cell costimulation. However, optimal concentrations of either B7.1- or B7.2-deficient APCs stimulated OVA-transgenic T cells to proliferate and produce cytokines, suggesting that B7.1 can provide costimulatory signals in vitro when B7.2 is absent (39). Blocking both B7.1 and B7.2 during in vitro activation of effector cells suppressed the transfer of EAE to recipient mice (16). However, neither Ab alone suppressed EAE or proliferative responses to myelin basic protein, although recipients of cells cultured with anti-B7.2 had less severe relapses compared with controls (16). The differences between the ability of anti-B7.2 to suppress adoptive transfer of EAT versus EAE may be the source of cells activated in vitro (spleen versus lymph node), the level of B7.1 expression on the APCs, or differences in costimulation requirements in the two model systems. In both models, suppression of disease correlated with a decrease in IL-2 production during Ag activation (Fig. 2) (16). Studies are in progress to determine whether blocking B7.2 during in vitro activation induces anergy to MTg.

The expression of costimulatory molecules in inflammatory autoimmune lesions has been demonstrated in autoimmune thyroiditis (28), rheumatoid arthritis (40), and multiple sclerosis (29), as well as in the murine models of EAE (15) and diabetes (30). Expression of B7.1 and B7.2 in thyroids of mice with EAT, but not normal thyroids, was observed by immunohistochemistry (Fig. 3).

**Discussion**

The requirement for costimulation in the development of autoimmune lesions has been demonstrated in several experimental

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**Table II. Effects of blocking B7.1 and/or B7.2 during the effector stage of EAT**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Ab to Recipients</th>
<th>EAT Severity</th>
<th>G Lesions</th>
<th>Recipient Ab</th>
<th>Recipient Anti-MTg IgG1</th>
<th>Recipient Anti-MTg IgG2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rat + hamster Ig</td>
<td>0 0 0 0 1 3 1</td>
<td>5/5</td>
<td>0.647 ± 0.087</td>
<td>0.807 ± 0.014</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1</td>
<td></td>
<td></td>
<td>0.421 ± 0.047</td>
<td>0.528 ± 0.059</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td></td>
<td></td>
<td>0.742 ± 0.069</td>
<td>0.818 ± 0.031</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1 + anti-B7.2</td>
<td>0 1 3 1</td>
<td>4/5</td>
<td>0.228 ± 0.015</td>
<td>0.307 ± 0.044</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Rat + hamster Ig</td>
<td>0 0 0 0 1 3 1</td>
<td>5/5</td>
<td>0.575 ± 0.040</td>
<td>0.725 ± 0.066</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1</td>
<td></td>
<td></td>
<td>0.323 ± 0.091</td>
<td>0.399 ± 0.076</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td></td>
<td></td>
<td>0.614 ± 0.040</td>
<td>0.799 ± 0.076</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1 + anti-B7.2</td>
<td>1 3 0 0 2 3</td>
<td>0/4</td>
<td>0.130 ± 0.054</td>
<td>0.189 ± 0.092</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Rat + hamster Ig</td>
<td>0 0 0 1 2 2 0</td>
<td>4/5</td>
<td>1.006 ± 0.086</td>
<td>1.185 ± 0.100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td></td>
<td></td>
<td>0.765 ± 0.135</td>
<td>0.888 ± 0.136</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.1 + anti-B7.2</td>
<td>0 0 0 1 2 3</td>
<td>5/5</td>
<td>0.706 ± 0.057</td>
<td>0.747 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Rat + hamster Ig</td>
<td>0 0 0 0 1 4 1</td>
<td>5/5</td>
<td>0.839 ± 0.013</td>
<td>0.749 ± 0.011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-B7.2</td>
<td></td>
<td></td>
<td>0.989 ± 0.034</td>
<td>0.752 ± 0.026</td>
<td></td>
</tr>
</tbody>
</table>

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*See footnote a in Table I.

*See footnote b in Table I.

*See footnote c in Table I.

*See footnote d in Table I.

*See footnote e in Table I.
and by RT-PCR (Fig. 4), suggesting that B7.1 and B7.2 are expressed on the infiltrating cells and not on thyrocytes. The expression of B7 by infiltrating cells was also observed in human autoimmune thyroiditis, while thyrocytes did not express B7 (28). In multiple sclerosis, both B7.1 and B7.2 were observed in the infiltrating cells in the central nervous system, with a predominance of B7.1 (29). The level of B7.1 and B7.2 expression in a particular target organ and the time of expression of B7.1 and B7.2 may determine which of these molecules is most critical for development of a given autoimmune disease (9). The predominant expression of B7.1 in the central nervous system during EAE correlated with the suppressive effects of anti-B7.1, which blocked clinical relapses of EAE, suppressed disease severity, and prevented epitope spreading (15). In diabetes, the predominant expression of B7.2 in the pancreas correlated with the suppression of diabetes by anti-B7.2, but not anti-B7.1 (41). Both B7.1 and B7.2 were expressed in thyroids from recipient mice with EAT, with B7.2+ cells predominating over B7.1+ cells (Fig. 3, A and B). However, this did not correlate with the ability of anti-B7.1 or anti-B7.2 to suppress EAT severity in recipient mice, since anti-B7.2 treatment of recipients increased EAT severity, while anti-B7.1 alone had no effect (Table II). The enhancement of EAT severity by blocking B7.2 in recipients may be due to T cells receiving costimulation only through B7.1 or by blocking interactions between B7.2 and CTLA-4, thereby preventing down-regulation of activated T cells. Blocking CTLA-4 after the activation of EAE effector cells (42) or after the onset of EAE (43) increased EAE severity, while blocking CTLA-4 in BDC2.5/NOD TCR-transgenic mice increased the severity of spontaneous diabetes (44). The ability of anti-B7.2 to enhance EAT severity when treatment with anti-B7.2 was delayed until 7 days after cell transfer (Table III) suggests that anti-B7.2 may be inhibiting B7.2-CTLA-4 interactions instead of B7.2-CD28 interactions. This is consistent with preliminary results indicating that recipient mice given anti-CTLA-4 have increased EAT severity and granulomatous histopathology (Table IV) similar to that observed for recipients given anti-B7.2 (Table II). However, if the enhancement of EAT is due to blocking CTLA-4 signaling, it is unclear why recipients given both anti-B7.1 and anti-

![Image](A)

![Image](B)

![Image](C)

![Image](D)

**FIGURE 5.** Effect of anti-B7.1, anti-B7.2, or anti-B7.1 and anti-B7.2 on granulomatous EAT. Hematoxylin and eosin (H&E) stained thyroid sections (×100) from recipient mice treated with rat and hamster Ig (A), anti-B7.1 (B), anti-B7.2 (C), or anti-B7.1 and anti-B7.2 (D), as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Ab to Recipients</th>
<th>Days Given</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>4+</th>
<th>5+</th>
<th>G Lesions</th>
<th>Recipient Anti-MTg IgG1</th>
<th>Recipient Anti-MTg IgG2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat/hamster Ig</td>
<td>0–19</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3/4</td>
<td>0.650 ± 0.041</td>
<td>0.543 ± 0.037</td>
</tr>
<tr>
<td>Anti-B7.1/B7.2</td>
<td>0–19</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0**</td>
<td>0.063 ± 0.014</td>
<td>0.125 ± 0.110</td>
</tr>
<tr>
<td>Anti-B7.1/B7.2</td>
<td>7–19</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>5/5</td>
<td>0.795 ± 0.107</td>
<td>0.658 ± 0.097</td>
</tr>
<tr>
<td>Anti-B7.2</td>
<td>0–19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>4/4</td>
<td>0.564 ± 0.080</td>
<td>0.500 ± 0.116</td>
</tr>
<tr>
<td>Anti-B7.2</td>
<td>7–19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4/4</td>
<td>1.131 ± 0.036</td>
<td>0.566 ± 0.097</td>
</tr>
</tbody>
</table>

* See footnote a in Table I.

* Recipients were given 100 µg of anti-B7.2, 100 µg of anti-B7.1, and 100 µg of anti-B7.2, or 100 µg of rat Ig and 100 µg of hamster Ig. Abs were given every 3 days starting at the indicated day after cell transfer and ending at day 19 after cell transfer.

* See footnote d in Table I.
Leishmania donovani increased in all experiments, and IFN was not affected (Fig. 6). In longer required, did not have enhanced EAT severity (Table III). B7.2, beginning on day 7 when costimulation was apparently no longer required, did not have enhanced EAT severity (Table III). All results are from cDNA at a 1/25 dilution. *p ≤ 0.05 relative to the control Ig, as determined by Student’s t test.

B7.2, beginning on day 7 when costimulation was apparently no longer required, did not have enhanced EAT severity (Table III).

The increase in IL-4 mRNA expression in thyroids from mice given anti-B7.2 is surprising since other Th2 cytokines were not increased in all experiments, and IFN was not affected (Fig. 6). In Leishmania donovani-infected mice, treatment with anti-B7.2 increased the number of IL-4- and IFN-producing cells (45). Blocking CTLA-4 during a response to staphylococcal enterotoxin B increased IL-4, but not IFN, production by CD4+ T cells (46), suggesting that signaling through CTLA-4 may suppress Th2 differentiation. Although IL-4-deficient mice can develop granulomatous EAT (24), administration of anti-IL-4 to CBA/J recipient mice reduced granulomatous histopathology, but not EAT severity (Braley-Mullen, unpublished results), suggesting that IL-4 may be involved in promoting granulomatous histopathology in the thyroid.

The suppression of EAT by blocking B7.1 and B7.2 in recipient mice indicates that costimulation is required during the effector stage of EAT. Administration of anti-B7.1 Fab after the onset of clinical signs suppressed relapses in EAE, suggesting that costimulation was required during the effector stage of autoimmunity (15). A requirement for costimulation after adoptive transfer was suggested when spleen cells from diabetic NOD mice rapidly induced diabetes in transgenic NOD.scid mice expressing B7.1 on pancreatic β cells, but not in nontransgenic NOD.scid mice (47). The suppression of EAT by blocking both B7.1 and B7.2 in recipient mice is apparently due to a requirement for costimulation of MTg-primed effector cells during early development of thyroid lesions, since blocking B7.1 and B7.2 starting on the day of transfer suppressed EAT, while delaying treatment until day 7 had no effect (Table III). Treatment of recipients with both anti-B7.1 and anti-B7.2 also suppressed the expression of IL-2, IL-4, and IFN-γ mRNA in the thyroid (Fig. 6). The decreased levels of cytokine mRNA are likely to be due to the overall decrease in inflammatory cells in the thyroid, since the level of CD3 mRNA was also decreased in thyroids from anti-B7.1- and anti-B7.2-treated recipients (data not shown).

The lack of complete suppression of EAT by both anti-B7.1 and anti-B7.2 (Table II) may be due to use of an insufficient amount of Ab, or to a role for other costimulatory molecules/pathways in the development of EAT. It is also possible that activation of some EAT effector cells may be costimulation independent (48). In murine lupus and autoimmune oophoritis, complete long-term suppression of disease was only observed with the inhibition of both the CD28-B7 and CD40-CD40L pathways (35, 36). However, in our studies, treatment of recipients with a combination of anti-B7.1, anti-B7.2, and anti-CD40L did not further suppress EAT (data not shown).

In conclusion, these results demonstrate that B7.2 has opposing roles in the regulation of EAT. Blocking B7.2 costimulation before effector cells are fully active (during in vivo priming or in vitro activation) suppresses the ability of MTg-primed spleen cells to induce EAT, and also inhibits proliferation and IL-2 production in response to MTg, suggesting that costimulation through B7.2 is necessary for activation of EAT effector cells (Table I). However, during the development of thyroid lesions in recipient mice, inhibition of B7.2 increased EAT severity (Table II), suggesting that

Table IV. Effects of blocking CTLA-4 during the effector stage of EAT  

<table>
<thead>
<tr>
<th>Ab to Rat and hamster Ig</th>
<th>G</th>
<th>Recipient Anti-MTg IgG1</th>
<th>Recipient Anti-MTg IgG2A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EAT severity</td>
<td>Lesions</td>
<td>Anti-MTg IgG1</td>
</tr>
<tr>
<td></td>
<td>0+ 1+ 2+ 3+ 4+ 5+</td>
<td>5/5</td>
<td>0.633 ± 0.075</td>
</tr>
<tr>
<td></td>
<td>Anti-CTL-A4</td>
<td>0 0 0 5 0 0</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* See footnote c in Table I. ** See footnote d in Table I.
B7.2 may be important for the down-regulation of inflammatory responses in the thyroid. Inhibition of both anti-B7.1 and anti-B7.2 in recipient mice resulted in decreased EAT severity, suggesting that costimulation is necessary during the priming, in vitro activation, and effector stages of MTh-primed EAT effector cells. Studies are underway to determine the role of B7.1 and/or B7.2 in the resolution of granulomatous EAT lesions, as well as defining the role of CTLA-4 in the development and resolution of EAT lesions.

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References


