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Control Tolerance or Induction of Mucosal Inflammation in Chronic Experimental Colitis

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B7 Interactions with CD28 and CTLA-4 Control Tolerance or Induction of Mucosal Inflammation in Chronic Experimental Colitis


CD28-B7 interaction plays a critical costimulatory role in inducing T cell activation, while CTLA-4-B7 interaction provides a negative signal that is essential in immune homeostasis. Transfer of CD45RBhigh CD4+ T cells from syngeneic mice induces transmural colon inflammation in SCID recipients. This adoptive transfer model was used to investigate the contribution of B7-CD28/CTLA-4 interactions to the control of intestinal inflammation. CD45RBhigh CD4+ cells from CD28−/− mice failed to induce mucosal inflammation in SCID recipients. Administration of anti-B7.1 (but not anti-B7.2) after transfer of wild-type CD45RBhigh CD4+ cells also prevented wasting disease with colitis, abrogated leukocyte infiltration, and reduced production of proinflammatory cytokines IL-2 and IFN-γ by lamina propria CD4+ cells. In contrast, anti-CTLA-4 treatment led to deterioration of disease, to more severe inflammation, and to enhanced production of proinflammatory cytokines. Of note, CD25+ CD4+ cells from CD28−/− mice similar to those from the wild-type mice were efficient to prevent intestinal mucosal inflammation induced by the wild-type CD45RBhigh cells. The inhibitory functions of these regulatory T cells were effectively blocked by anti-CTLA-4. These data show that the B7-CD28 costimulatory pathway is required for induction of effector T cells and for intestinal mucosal inflammation, while the regulatory T cells function in a CD28-independent way. CTLA-4 signaling plays a key role in maintaining mucosal lymphocyte tolerance, most likely by activating the regulatory T cells. The Journal of Immunology, 2001, 167: 1830–1838.

C rohn’s disease and ulcerative colitis are two major forms of inflammatory bowel disease (IBD).5 Genetic predisposition, a defective mucosal barrier, and an abnormal host immune response to luminal Ags with a defect in immune homeostasis may all participate in the etiopathology. Dysregulation of mucosal T cells probably plays a key role in the pathogenesis, which results in secretion of proinflammatory mediators, accumulation of activated inflammatory cells, and tissue damage (1, 2). Studies in different animal models of experimental colitis have also definitely confirmed abnormal immune responses to potential immunogenic stimuli (e.g., luminal Ags) (1, 2). However, the stimuli responsible for the initiation and maintenance of signal-mediated lymphocyte activation in the intestinal mucosa in human IBD remain elusive.

Naïve T cell activation requires two distinct signals from the APC. The first signal is triggered by the engagement of the Ag-specific TCR-CD3 complex, and the second is a costimulatory signal. T cell recognition of Ag in the absence of costimulation may lead to anergy. CD28 is the most widely studied costimulatory molecule and is constitutively expressed on the surface of T cells. The natural ligands for CD28 have been identified as B7 molecules (B7.1 or CD80 and B7.2 or CD86) and have been demonstrated to be present or to be inducible on the surface of APC (3). CD28-B7 interaction results in an enhanced T cell proliferation, cytokine production, and resistance to apoptosis (3). Blockade of CD28-B7 interaction in vitro effectively prevents effector T cell responses (3). CD28−/− T cells exhibit defects in cell survival, clonal expansion, and differentiation into effector cells in response to Ag stimulation (4, 5).

Several lines of evidence have also demonstrated that B7-CD28 interactions play an important role in some immune diseases. Overexpression of B7 molecules has been found in the inflamed areas from patients with multiple sclerosis (6), rheumatoid arthritis (7), and psoriasis (8). We and others have reported increased expression of B7.1 and B7.2 molecules in the inflamed mucosa of IBD patients (9, 10). Administration of anti-B7.1 prevents the development of experimental autoimmune encephalomyelitis (EAE) in mice (11–13). Anti-B7.2 suppresses the onset of diabetes in nonobese diabetic mice (14) and prevents the development of lung mucosal allergic inflammation (15, 16). In vivo administration of B7 blocking agents reduces the severity of autoimmune diseases (14, 17, 18) and prolongs long-term graft survival (19, 20). In addition, CD28−/− mice are resistant to collagen-induced arthritis (21) and EAE induction (22) and show a delayed rejection of cardiac allografts (23). A second receptor for B7, the CTLA-4 molecule, is also important in immune regulation. CTLA-4 (CD152), a homologue of CD28, is up-regulated on the surface of activated T cells (24). It competes with CD28 to bind B7 and functions as a...
counterregulatory receptor that attenuates T cell responses by down-regulating T cell activation (25-27), by facilitating Ag-specific apoptosis (28), and by suppressing secretion of both Th1 and Th2 cytokines (26, 27, 29). Blockade of CTLA-4 signaling with neutralizing Ab was found to promote expansion of Ag-specific T cells (25), to enhance T cell IL-2 and IFN-γ secretion (29), and to augment antitumor immunity (30). Consistent with these findings, CTLA-4–/– mice develop a autoimmune lymphoproliferative disorder accompanied by extensive lymphocytic infiltration into multiple organs, resulting in tissue destruction and early lethality (31, 32). It has further been shown that administration of blocking anti-CTLA-4 exacerbates EAE (33, 34), autoimmune diabetes (35), and allograft rejection (23).

To study the role of B7, CD28, and CTLA-4 costimulatory molecules in the control of intestinal inflammation, we used an adoptive transfer model of colitis in mice. Transfer of CD45RBhighCD4+ (naive) T cells from BALB/c mice to syngeneic SCID mice leads to the development of an IBD-like syndrome by 6–10 wk after T cell reconstitution, characterized by diarrhea, weight loss, transmural inflammation in the proximal colon, and a Th1 immune response by lamina propria CD4+ T cells (36, 37). The clinical, histopathological, and immunological features resemble those observed in human Crohn’s disease. Of particular importance, cotransfer of the reciprocal CD45RB–/–CD4+ T cells or CD25−/−CD4+ T cells together with pathogenic CD45RBlow cells prevents the development of colitis (36, 38), indicating that the CD45RB–/–CD25– subsets function as regulatory T cells to control mucosal inflammatory responses. Moreover, in vivo administration of anti-CTLA-4 has been recently reported to abrogate inhibition of colitis by CD25−CD4+ cells, suggesting that CTLA-4 signaling is involved in the immune-suppressive function of these regulatory cells (38). Targeted therapy directed against T cell costimulatory molecules such as CD40 ligand has also shed some light on the control of intestinal mucosal inflammation (36, 39). In this study, SCID mice were reconstituted with syngeneic CD45RBhighCD4+ cells from the wild-type (WT) or CD28–/–/ mice, and they were treated with either anti-B7.1 or anti-B7.2 or anti-CTLA-4 mAb to investigate the in vivo relevance of B7-CD28/CTLA-4 interaction to the control of intestinal inflammation. In addition, we also explored the potential role of CD28 and CTLA-4 costimulatory signals in the regulatory functions of CD45RB–/– and CD25−CD4+ T cells.

Materials and Methods

Mice

Female WT BALB/c mice were obtained from Harlan (Zeist, The Netherlands). Female BALB/c SCID mice were bred under standard pathogen-free conditions and maintained in a specific animal facility of the University Hospital Gasthuisberg, Catholic University of Leuven (Leuven, Belgium). Female BALB/c CD28–/– mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were all housed in a specific pathogen-free facility in microisolator cages with filtered air and free access to food and water. All mice were 6–8 wk of age when experiments were initiated.

Antibodies

Anti-mouse CD3ε (500A2, hamster IgG [H(2)], biotinylated anti-mouse CD4 (H129.19, rat IgG2a), biotinylated anti-mouse B7.1 (16–10A1, hamster IgG), FITC-conjugated anti-mouse CD45RB (16A, rat IgG2a), PE-conjugated anti-mouse CD4 (GK1.5, rat IgG2b), PerCP-conjugated anti-mouse CD4 (RM4-5, rat IgG2a), FITC-conjugated anti-mouse CD25 (7D4, rat IgM), and PE-conjugated anti-mouse CTLA-4 (UC10-4F10-11, Hlg) were purchased from BD PharMingen (San Diego, CA). Anti-mouse CD54 (KAT-1, rat IgG2a) was obtained from BioSource International (Nivelles, Belgium). Biotinylated anti-mouse F4/80 (CL:A3-1, rat IgG2b) was purchased from Serotec (Oxford, U.K.). Hamster anti-mouse B7.1 (16–10A1, IgG) (40), rat anti-mouse B7.2 (GL1, IgG2a) (41), and hamster anti-mouse CTLA-4 mAb (UC10-4F10-11) (25) were purified from culture supernatants of the hybridomas, which were obtained from the American Type Culture Collection (Manassas, VA). Hlg and rat IgG2a as controls were purchased from BioTrend (Cologne, Germany).

Purification of T cell subsets

CD4+ T cells and CD45RBhigh and CD45RB–/– CD4+ T cells were isolated from BALB/c spleen using a method as previously reported (36). Briefly, splenic CD4+ T cells were isolated by positive selection using mouse CD4 Dynabeads (L3T4) and mouse CD4 DETACHABead (Dynal, Oslo, Norway). These procedures resulted in ≥98% CD4+ T cells as assessed on a FACS sorter (Becton Dickinson). For isolation of CD4+ CD25+ T cells, purified splenic CD4+ T cells were stained with either anti-CD4-PE and anti-CD25-FITC or anti-CD25-FITC alone. CD4+ CD25+ subpopulation (∼10% of CD4+ T cells) were then sorted on a FACS Vantage. All populations were ≥99.0% pure on reanalysis.

Establishment of experimental colitis and Ab treatment

SCID mice were reconstituted by i.p. injection of syngeneic CD45RBhigh CD4+ T cells either alone or cotransferred with syngeneic CD45RB–/– CD4+ or CD25−/−CD4+ cells (4 × 107/mouse of each cell population). Colitic SCID mice, reconstituted with syngeneic CD45RBhigh CD4+ T cells from spleen of normal mice, were treated i.p. with either anti-B7.1 or anti-B7.2 or a combination of both Ab and anti-CTLA-4 at the dose of 250 μg/mouse twice a week starting at the beginning of T cell transfer up to 8 wk. Mock-treated controls received similar injections of Hlg and rat IgG2a on the same schedule. All mice were monitored weekly for weight, soft stool or diarrhea, and rectal prolapse. All mice were sacrificed 8 wk after T cell transfer or when they exhibited a loss of >20% of original body weight. Colonic tissues were obtained for histologic and cytologic examination.

Histologic examination and immunohistochemistry

Intestinal tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5-μm) were cut and stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. Microscopic sections were graded according to the number and severity of lesions. The mean degree of inflammation in the colon was assessed using a scoring system as described previously (36).

Colonic samples for immunohistochemistry were embedded in OCT compound and snap-frozen in liquid nitrogen. The frozen sections were stored at −80°C until processed. Five-micrometer cryostat sections of colonic tissue were cut and stained for the presence of B7.1, B7.2, CD4, and F4/80 using an avidin-biotin-peroxidase complex technique as previously described (36). Briefly, serial sections were incubated for 30 min with 5 μg/ml rat anti-mouse B7.2. The rabbit anti-rat IgG (Dako, Glostrup, Denmark; 1:400) was chosen as second Ab. After three washes with PBS, the avidin-biotin-peroxidase complex (Dako) was added, and sections were incubated for 30 min. Staining for B7.1, CD4, or F4/80 Ag was performed using either biotinylated anti-B7.1 or anti-CD4 or anti-F4/80 as primary Ab at a concentration of 5 μg/ml, followed by incubation with the avidin-biotin-peroxidase complex. The color reaction was developed with 0.05% 3-amino-9-ethylcarbazole (Janssen, Beerse, Belgium). The slides were counterstained with hematoxylin. Negative controls were obtained by incubating sections with irrelevant isotype-matched Hlg or rat IgG2a, or by omitting the primary Ab.

Cell preparation and cytokine analysis

Colonic lamina propria CD4+ T cells were isolated using a method as described previously (36). For induction of cytokine production by lamina propria CD4+ T cells, 96-well culture plates (Nunc, Roskilde, Denmark) were precoated with anti-CD3ε (final concentration, 5 μg/ml) in 100 μl PBS at 37°C for 4 h and washed with PBS three times to remove unbound Ab. CD4+ T cells (5 × 106/ml) were incubated in the presence of coated anti-CD3ε and mitomycin C-treated (50 μg/ml for 30 min at 37°C) mouse mastocytoma P815 cells (5 × 106/ml) transfected with mouse CD80 at 37°C in 5% CO2 humidified air. Samples were performed in triplicate or quadruplicate in a total volume of 200 μl/well. After 48 h of culture, supernatants were harvested and assayed for IFN-γ, IL-2, and IL-4 by ELISA as described previously (36).
**Results**

**CD28\(^{-/-}\)/CD45RB\(^{high}\)/CD4\(^{+}\) T cells fail to induce intestinal mucosal inflammation in SCID recipients**

To evaluate the role of the CD28-B7 costimulatory pathway in the induction of intestinal mucosal inflammation, CD45RB\(^{high}\)/CD4\(^{+}\) T cells from the WT and CD28\(^{-/-}\) mice were isolated and reconstituted into SCID recipients (4 × 10\(^{7}\)/mouse). After an 8-wk period of observation, SCID mice reconstituted with CD28\(^{-/-}\)/CD45RB\(^{high}\)/CD4\(^{+}\) T cells appeared healthy and none of them developed wasting disease with colitis, as evidenced by gradual increase of body weight (Fig. 1A) and absence of diarrhea and prolapse. Histological analysis of colonic sections revealed normal mucosa of colon with absence of inflammatory cell infiltrates (Fig. 1B), being indistinguishable from that in naive SCID mice. In contrast, WT CD45RB\(^{high}\) cell-reconstituted mice developed severe colitis. Histologic analysis showed transmural inflammation with large numbers of leukocyte infiltrates in colon (Fig. 1C), similar to our earlier report (36). Lower numbers of CD4\(^{+}\) T cells were recovered from the colon of CD28\(^{-/-}\)/CD45RB\(^{high}\) cell-reconstituted mice (0.24 ± 0.03 × 10\(^{6}\)/colom, n = 12) as compared with those from WT cell-reconstituted mice (2.11 ± 0.51 × 10\(^{6}\)/colom, n = 8) (p < 0.001). Immunohistochemical analysis also demonstrated few CD4\(^{+}\) T cells and macrophages in the lamina propria in CD28\(^{-/-}\)/CD45RB\(^{high}\) cell-reconstituted mice (data not show). In additional experiments, SCID mice were also reconstituted with either CD28\(^{-/-}\)/CD45RB\(^{low}\)/CD4\(^{+}\) T cells (n = 5) or cotransferred with both CD28\(^{-/-}\)/CD45RB\(^{high}\) and CD28\(^{-/-}\)/CD45RB\(^{low}\) cells (n = 5). None of these cell subsets was able to induce mucosal inflammation after an 8-wk observation. The data show that activation of CD45RB\(^{high}\)/CD4\(^{+}\) T cells and induction of mucosal inflammation are dependent on the CD28-B7 costimulatory pathway.

**Overexpression of B7.1 and B7.2 in the inflamed colon of colitic SCID mice**

In situ expression of B7.1 and B7.2 in the inflamed colon was studied by immunohistochemistry. Colonic samples were obtained from colitic SCID mice 8 wk after reconstitution with syngeneic WT CD45RB\(^{high}\)/CD4\(^{+}\) or CD45RB\(^{low}\)/CD4\(^{+}\) T cells (36). Fig. 2 shows that the number of B7.1- and B7.2-positive cells was markedly increased in the inflamed mucosa. The majority of positive cells were located in the lamina propria and submucosa, but some in the tunica muscularis and subserosa. The positive cells were mostly diffusely distributed, but also formed aggregates, suggesting a granulomatous type of inflammation. Intestinal epithelial cells were negative for B7. In contrast, only weak expression of B7.1 and B7.2 was observed in the colonic tissue from SCID mice reconstituted with CD45RB\(^{low}\)/CD4\(^{+}\) T cells (Fig. 2, C and D). These results show that B7 molecules are overexpressed in inflamed mucosa of colitic SCID mice and support the concept that B7 may participate in the immunopathology of colitis.

**Anti-B7.1 but not anti-B7.2 prevents experimental colitis in reconstituted SCID mice**

After confirming increased expression of B7.1 and B7.2 in the inflamed mucosa of colitic SCID mice, we asked whether blockade of the B7 costimulatory signaling could interfere with the development of intestinal mucosal inflammation. SCID mice reconstituted with syngeneic WT CD45RB\(^{high}\)/CD4\(^{+}\) T cells were treated with i.p. injection of either anti-B7.1, anti-B7.2 mAb, a combination of both Abs, or control Hlg or rat IgG2a at the dosage of 250 μg/mouse twice weekly starting at the beginning of T cell transfer and continuing up to 8 wk. Hlg- or rat IgG2a-treated mice developed wasting disease with severe colitis and loss of body weight (Fig. 3A), soft stool or diarrhea with increased mucus, and anorectal prolapse. These mice had an enlarged colon with a significantly thickened wall. Transmural inflammation with increased leukocyte infiltrates was commonly seen in the ascending and transverse colon. Epithelial lesions included loss of goblet cells, crypt abscesses, and extensive ulceration (Fig. 4A). Moreover, architectural changes such as crypt elongation, villous transformation of the surface, and crypt branching were also observed. Anti-B7.2-treated mice still developed colitis with comparable severity, time course, and histological scores to control IgG-treated recipients (Figs. 3A and 4B). In contrast, anti-B7.1-treated mice appeared healthy with gradual increase of body weight and absence of diarrhea (Fig. 3A). Histological features of colon were almost normal (Fig. 4C), and histological scores were significantly decreased compared with those in Hlg-treated mice (Fig. 3B). In
addition, a mixture of mAb to B7.1 and B7.2 also prevented wasting disease with colitis, being indistinguishable from anti-B7.1 alone (Fig. 3A). Histological analysis of colonic tissue revealed normal mucosa, similar to findings in Fig. 4C (data not shown).

Consistent with clinical and histological findings, lamina propria CD4⁺ T cell recovery in the colon was significantly lower from anti-B7.1-treated mice (0.36 ± 0.11 × 10⁶) compared with that from either anti-B7.2-treated (1.92 ± 0.43 × 10⁶) or Hlg-treated (2.08 ± 0.31 × 10⁶) or rat IgG2a-treated mice (2.14 ± 0.45 × 10⁶) (p < 0.001). Splenomegaly was also reduced in anti-B7.1-treated mice, and only a few lymphocytes were observed in the white pulp (data not shown). Immunohistochemistry further proved that anti-B7.1 treatment significantly reduced infiltrates of leukocytes (CD4⁺ T cell, F4/80 macrophages) and expression of accessory molecules CD54, CD40, B7.1, and B7.2 in the colon (data not shown).

Colonic lamina propria CD4⁺ T cells were isolated from the colonic samples of colitic SCID mice treated with either anti-B7.1 or anti-B7.2 or controls. CD4⁺ T cells (5 × 10⁶/ml) were incubated in the presence of coated anti-CD3ε (5 μg/ml) and mouse mastocytoma P815 cells transfected with mouse CD80 (5 × 10⁵/ml) at 37°C in 5% CO₂ humidified air. Fig. 3, C and D show that IFN-γ and IL-2 were significantly reduced in the supernatants of CD4⁺ T cell cultures from anti-B7.1-treated mice compared with those from either anti-B7.2- or control IgG-treated recipients (p < 0.005). IL-4 was found to be at or below the detection limit of the assay. Thus, anti-B7.1 treatment down-regulates proinflammatory cytokine secretion without evidence for induction of a Th2 shift. Moreover, the data indicate that B7.1 is the dominant costimulatory molecule that potentiates lamina propria T cell activation and differentiation during the induction of colitis, similar to what has been reported in EAE (11–13).

**Anti-CTLA-4 exacerbates the development of colitis**

Colitic SCID mice were then injected i.p. with either anti-CTLA-4 or Hlg at the dose of 250 μg twice a week starting at the time of T cell transfer. Because our preliminary experiments had demonstrated that anti-CTLA-4-treated mice developed severe colitis after 6-wk administration, both anti-CTLA-4- and Hlg-treated mice were sacrificed after 6 wk. The clinical manifestations during treatment were monitored as described above. Hlg-treated mice at that time point had a slight decrease of body weight and soft stool (Fig. 5A). Diarrhea with increased mucus was observed in two of eight mice. Histological analysis of colonic sections revealed that Hlg-treated mice had already developed an active mild colitis with leukocyte infiltration in the lamina propria, occasionally in the submucosa and the muscular layers, and absence in the serosa (Fig. 6A). Leukocyte infiltration was characterized by focal aggregation at the apical areas of villi and the basal areas, or by diffuse infiltration in the lamina propria. Epithelial lesions included mucin depletion, crypt branching, crypt elongation, and transformation. Ulceration was not found in all samples. In contrast, anti-CTLA-4-treated mice started progressive weight loss already 2–3 wk after T cell transfer (Fig. 5A). By 6 wk they had severe wasting disease with colitis, diarrhea with increased mucus in the stool, marked

**FIGURE 2.** Overexpression of B7.1 and B7.2 in inflamed mucosa of colitic SCID mice. Colonic samples were obtained from SCID mice 8 wk after reconstitution with syngeneic WT CD45RBhighCD4⁺ (A and B) or CD45RBlowCD4⁺ T cells (C and D) and stained immunohistochemically with anti-B7.1 (A and C) and anti-B7.2 (B and D). Original magnification, ×100.
decrease of body weight ($p < 0.05$), and anorectal prolapse. Colonic sections revealed transmural inflammation with significantly increased leukocyte infiltration in any part of the colon from all mice. Epithelial lesions included mucin depletion, loss of goblet cells, crypt abscesses, and ulceration, occasionally with a mountain-peak appearance (Fig. 6B). The mucosal abnormalities were also seen in the ileocecum. Histological scores of colonic samples were significantly increased in anti-CTLA-4-treated mice ($6.5 \pm 2.4$) compared with HIg-treated mice ($3.1 \pm 1.5$) ($p < 0.01$). The CD4$^+$ T cell recovery from the inflamed colon was also markedly increased in these mice ($2.45 \pm 0.84 \times 10^6$) compared with that from the HIg-treated recipients ($1.02 \pm 0.36 \times 10^6$) ($p < 0.01$).

Additionally, splenomegaly with large numbers of lymphocyte infiltrates in the white pulp was commonly seen (data not shown). We also analyzed cytokine secretion by lamina propria CD4$^+$ T cells after in vitro stimulation. Lamina propria CD4$^+$ T cells ($5 \times 10^5$/ml) from both anti-CTLA-4- and HIg-treated mice 6 wk after T cell transfer were stimulated with coated anti-CD3e (5 $\mu$g/ml) and mouse mastocytoma P815 cells transfected with mouse B7.1 ($5 \times 10^5$/ml). After 48 h of culture, the supernatants were harvested and assayed for IFN-$\gamma$ and IL-2 by ELISA. $\ast$, $p < 0.005$ vs control Ig- and anti-B7.2-treated groups.

**FIGURE 3.** Administration of anti-B7.1 but not anti-B7.2 prevents mucosal inflammation in colitic SCID mice. A. Colitic SCID mice reconstituted with WT CD45RB$^{low}$CD4$^+$ T cells ($4 \times 10^5$/mouse) were treated i.p. with either anti-B7.1 or anti-B7.2 or a combination of both Abs or HIg or rat IgG2a at the dose of 250 $\mu$g twice a week starting at the time of T cell transfer over a 8-wk period. The change of weight over time is expressed as percent of the original weight. Data represent the mean $\pm$ SEM of each group from three independent experiments. B. Histological scores of colonic sections from all groups. C and D, Lamina propria CD4$^+$ T cells were isolated from the colon of colitic SCID mice treated with either anti-B7.1 or anti-B7.2 or control Igs. CD4$^+$ T cells ($5 \times 10^5$/ml) were incubated in the presence of coated anti-CD3e (5 $\mu$g/ml) and mouse mastocytoma P815 cells transfected with mouse B7.1 ($5 \times 10^5$/ml). After 48 h of culture, the supernatants were harvested and assayed for IFN-$\gamma$ and IL-2 by ELISA. $\ast$, $p < 0.005$ vs control Ig- and anti-B7.2-treated groups.

**CD25$^+$CD4$^+$ regulatory T cells function in a CD28-independent way**

CD45RB$^{low}$CD4$^+$ T cells do not induce colitis but are able to prevent colitis development if cotransferred with WT CD45RB$^{high}$ cells into SCID mice (36). More recently, it has been reported that CD25$^+$CD4$^+$ cells among the CD45RB$^{low}$CD4$^+$ T cells function as the regulatory T cells to inhibit intestinal inflammation (38, 42) and other experimental autoimmunity models (43), and that regulatory T cell activity depends on IL-10 secretion (44). Therefore, we addressed the question whether these regulatory T cells also require CD28 costimulation. This was studied by cotransfer of CD45RB$^{low}$ or CD25$^+$CD4$^+$ T cells from CD28$^{-/-}$ mice together with disease-inducing WT CD45RB$^{high}$CD4$^+$ T cells. As shown in Fig. 7, A and B, cotransfer of WT CD45RB$^{low}$ or CD25$^+$CD4$^+$ T cells effectively inhibited wasting disease with colitis over an 8-wk...
period of observation. No detectable pathological changes were observed in the bowel wall, consistent with earlier reports (36, 38). Of particular note, cotransfer of CD45RBlow or CD25

3

T cells (5 x 10^5/ml) were isolated from both groups and cultured under the same conditions as shown in Fig. 3. After 48 h of culture, the supernatants were harvested and assayed for IFN-γ and IL-2 by ELISA. *, p < 0.05; **, p < 0.01 vs controls.

diarrhea. The colon appeared normal when animals were sacrificed. Histological analysis of colonic sections illustrated only few leukocyte infiltrates in the lamina propria. Transmural inflammation and epithelial hyperplasia were effectively inhibited. Histological scores were significantly decreased compared with those in WT CD45RB^high cell-reconstituted mice (p < 0.01), but not different from those in SCID mice cotransferred with WT CD45RB^low or CD25^-CD4^+ cells (Fig. 7C). Colonic lamina propria CD4^+ T cell recovery from mice cotransferred with either CD45RB^low or CD25^-CD4^+ cells was also decreased compared with that from recipients reconstituted with WT CD45RB^high cells alone (p < 0.01, Fig. 7D). Moreover, lamina propria CD4^+ T cells, when stimulated with Con A (1 μg/ml), also secreted lower levels of IL-2 and IFN-γ compared with those from WT CD45RB^high-CD4^+ cell-reconstituted mice (data not shown). These findings indicate that the regulatory functions of CD45RB^low-CD4^+ and CD25^-CD4^+ T cells are CD28 independent.

Function of CD25^-CD4^+ regulatory T cells is eliminated by anti-CTLA-4

CTLA-4 was reported to be constitutively expressed on a subset of regulatory CD25^-CD4^+ T cells (38, 42). We also analyzed expression of CTLA-4 on freshly isolated CD25^-CD4^+ T cells and found that CD25^-CD4^+ T cells from CD28^-/- mice expressed comparable levels of CTLA-4 as the WT subsets (data not shown). To further illustrate the role of B7-CTLA-4 interaction in the effector functions of these regulatory T cells, WT CD45RB^high-CD4^+ cell-reconstituted SCID mice were cotransferred with CD25^-CD4^+ cells from either WT or CD28^-/- mice, and they were treated with either anti-CTLA-4 or Hlg. As indicated in Fig. 8, after 8 wk of administration, Hlg-treated mice appeared healthy as evidenced by gradual increase of body weight and absence of diarrhea. No detectable pathological changes were seen in the colon. In contrast, all of anti-CTLA-4-treated mice developed wasting disease with colitis. Histological analysis of the colon revealed the same features as shown in Fig. 4A. Therefore, the data, together with previous reports (38, 42), substantiate the critical role of B7-CTLA-4 interaction for the functioning of the regulatory T cells that control inflammatory responses in the gut.
Discussion

Experiments presented here were designed to investigate the B7-CD28/CTLA-4 interaction in the induction of experimental colitis in SCID mice and to explore its role in the activity of regulatory T cells. Administration of anti-B7.1 but not anti-B7.2 starting at the beginning of T cell reconstitution effectively prevented mucosal inflammation, as evidenced by decreased inflammatory cell numbers and down-regulated proinflammatory cytokine production (i.e., IFN-γ and IL-2) by lamina propria CD4+ T cells. In contrast, anti-CTLA-4 treatment deteriorated mucosal immune responses with enhanced inflammatory damage in the intestine and up-regulation of local proinflammatory cytokine secretion. Of note, CD45RBhighCD4+ T cells from CD28−/− mice failed to induce intestinal mucosal inflammation after transfer into SCID recipients. CD45RBlow and CD25+CD4+ T cells from CD28−/− mice, similar to the WT subsets, still prevented severe colitis induced by reconstitution with WT CD45RBhigh cells of SCID mice. Therefore, our findings provide evidence that the CD28-B7 costimulatory pathway plays an important role in promoting pathogenic CD4+ T cell activation and differentiation and that CTLA-4-B7 interaction delivers a negative signal to prevent or down-regulate inflammatory responses in the intestine. Moreover, these data also implicate that CD28-B7 interaction is not required for the effector functions of regulatory T cells.

Colitis in this model is dependent on the transfer of CD45RBhighCD4+ cells, which home after activation to the gut wall where further activation leads to proinflammatory cytokine production. CD28−/−CD45RBhighCD4+ T cells failed to cause intestinal inflammation when reconstituted into SCID recipients. These findings indicate that the induction of effector response of this subset of T cells and their homing to the lamina propria is CD28 dependent. Previous work has shown that CD28−/− T cells have decreased proliferative responses to Ags and costimulatory stimulation and produce low levels of IL-2 (4, 5). CD28−/− mice have a deficient T cell activation, as evidenced by the findings that they exhibit delayed cardiac allograft rejection after transplantation (23) and are resistant to collagen-induced arthritis with low IFN-γ secretion by lymph node cells in response to collagen stimulation (21). Requirement of CD28 for disease induction might depend on interaction with either B7.1 or B7.2 or both. In the present study, B7.1 and B7.2 were both highly expressed in the inflamed mucosa of colitic SCID mice. We suggest that this increase in expression of B7.1 and B7.2 is induced either directly by luminal bacterial products or by activated lamina propria T cells via CD40-CD40 ligand interaction (36, 39). Anti-B7.1 but not anti-B7.2 effectively prevented mucosal inflammation and significantly down-regulated proinflammatory cytokine secretion (e.g., IL-2, IFN-γ) by lamina propria CD4+ T cells and leukocytic infiltrates in the intestine. Thus, B7.1 appears to be predominantly involved in the Th1-mediated mucosal inflammation in this adoptive transfer model of colitis, which is consistent with some previous reports suggesting a predominant involvement of B7.1 in Th1-mediated pathology. B7.1 is generally not detected on resting APC, whereas low levels of B7.2 are constitutively expressed on macrophages and dendritic cells in naïve mice. Upon cell activation, both B7.1 and B7.2 are up-regulated, although expression of B7.1 often follows that of B7.2 (3). The slower rate of dissociation of B7.1 binding to CD28, compared with B7.2, may enable it to provide more potent costimulatory functions (45). Moreover, the differences in B7.1 and B7.2 binding determinants of CD28 also raise the possibility that these molecules play a distinct role in T cell effector response (46). Some previous work has suggested that B7.1 is preferentially involved in the Th1 immune response, while B7.2 regulates the development of Th2 cells (11–13, 15, 16). However, not all data support such a conclusion. Anti-B7.2 but not anti-B7.1 effectively prevents the onset of diabetes but not insulin in nonobese diabetic mice (14). Moreover, mice lacking either B7.1 or B7.2 are still susceptible to EAE induction (22). These conflicting data may be attributed to several factors, such as a strain dependency in the susceptibility of animals and in the development of immune responses. Moreover, we cannot rule out the possibility that differences in the affinity or in the catabolism of both Abs could be responsible for the different outcome of in vivo treatment. The same anti-B7.2 mAb, which was ineffective in the present model, has been previously shown to suppress the onset of diabetes and experimental allergic asthma (14–16). Therefore, the respective roles of B7.1 and B7.2 in the induction of mucosal immune responses still need to be further investigated, and in vivo studies using recipients deficient for B7 single molecules seem warranted.

Lack of B7-CD28 interaction prevents disease induction in this model. The potential sequence of events by which CD28-B7 co-stimulation is involved in induction of mucosal immunopathology is likely at an early stage of the disease. Once CD45RBhighCD4+ cells are transferred into SCID mice, interaction in gut-draining lymph nodes with APC (i.e., dendritic cells) involving CD28-B7 interactions leads to initial triggering with low levels of IL-2 secretion (3), up-regulation of IL-12Rβ2 expression (47), and polarization toward Th1 differentiation. CD4+ T cells, especially after CD28 cross-linking, also express high levels of CD40 ligand, which interacts with CD40 expressed on APC to promote IL-12 secretion (36) and B7 expression. Furthermore, IL-12 may act synergistically with B7.1 to abrogate mucosal T cell tolerance and to induce intestinal inflammation (48). According to recent report (49), this priming occurs in regional Ag-draining lymph nodes and will be followed by homing of the Ag-primed cells to the lamina propria of the gut. After priming and homing to the gut, B7 molecules might still play a role in further enhancing and/or prolonging T cell activity. Increased IL-12Rβ2 expression on lamina propria T cells after B7 signal triggering has been observed in inflamed mucosa of Crohn’s disease (50). We and others have also reported increased expression of B7.1 and B7.2 molecules in the inflamed mucosa of IBD patients (9, 10). Further elucidation of the role of these molecules in the persistence of colitis is still unclear and would require delayed treatment after disease onset.

In addition to CD28 and its interaction with B7, we also studied the involvement of the CTLA-4 molecule. Blockade of the CTLA-4 signal with neutralizing mAb precipitated the development of intestinal mucosal inflammation induced by CD45RBhighCD4+ T cells. This was characterized by an accelerated onset of wasting disease, an increased severity of colitis, and an up-regulated secretion of Th1

FIGURE 8. Anti-CTLA-4 blocks the effector functions of CD25−CD4+ cells. WT CD45RBhighCD4+ cell-reconstituted SCID mice were cotransferred with CD25−CD4+ cells from either WT or CD28−/− (KO) mice and treated with either anti-CTLA-4 or Hlg at the dosage of 250 μg twice a week starting at the beginning of T cell transfer up to week 8. The change of weight over time is expressed as percent of the original weight. Data represent the mean of each group from one experiment.
proinflammatory cytokines. These results are consistent with earlier reports, showing that anti-CTLA-4 deteriorates clinical disease and augments T cell immune responses in other experimental diseases including EAE (33, 34), autoimmune diabetes (35), and allograft rejection (23). Blockade of CTLA-4 signaling with mAb promotes T cell expansion and enhances both Th1- and Th2-type effector responses (25, 29). After T cell transfer and development of colitis, CTLA-4 expression on lamina propria T cells was clearly increased (data not shown), providing a target for the effect of anti-CTLA-4. Because anti-CTLA-4 treatment resulted in the deterioration of colitis, the CTLA-4 expressed on these pathogenic cells can to some degree regulate functional responsiveness of effector T cells in vivo. The most likely mechanism by which anti-CTLA-4 deteriorates mucosal inflammation is that it directly blocks negative signals for effector T cells. Whether these effects occur at the level of priming in lymph nodes or after migration to the lamina propria is still unclear. Our findings are different from a recent report (38) showing that administration of anti-CTLA-4 to mice reconstituted with WT CD45RB<sup>hi</sup> cells alone does not affect the development of colitis. The discrepancy could be attributed to the dosage and schedule of Ab administration as well as the genetic background of animals used.

CD25<sup>+</sup> CD4<sup>+</sup> T cells have been identified as important regulatory T cells, also in experimental colitis, and they represent a subset of CD45RB<sup>lo</sup>CD4<sup>+</sup> T cells. Whether these cells act at the initiation of T cell priming or rather suppress effector T cells after induction of intestinal inflammation by WT CD45RB<sup>hi</sup> cells. CD28 and/or CTLA-4 for their suppressive activity. CD45RB<sup>lo</sup> T cells that can be attributed to the dosage and schedule of Ab administration as well as the genetic background of animals used.

Interaction of CTLA-4 on these regulatory T cells with B7 on APC may provide a sufficient costimulatory signal for their expansion and their activity (53), leading to the down-regulation of intestinal inflammation. However, whether and how CTLA-4-B7 interaction affects the effector functions of these regulatory T cells is in fact not yet clearly understood. Recent work reported that CTLA-4 is constitutively expressed at high levels on CD25<sup>+</sup> CD4<sup>+</sup> T cells and that CTLA-4 might be involved in the activity of these cells (38, 42). We also observed increased expression of CTLA-4 on both freshly isolated CD45RB<sup>lo</sup> and CD25<sup>+</sup> CD4<sup>+</sup> T cells. Interestingly, blockade of CTLA-4 signaling and of TGFB-reversed the suppressive effects of CD25<sup>+</sup> CD4<sup>+</sup> T cells and regulatory T cells from IL-10<sup>−/−</sup> mice were able to prevent colitis (44). CTLA-4 cross-linking leads to CD4<sup>+</sup> T cell TGFB production (54), suggesting that TGFB may mediate the inhibitory effects of CTLA-4 signaling. TGFB is a pleiotropic cytokine with generally antiinflammatory and immunosuppressive properties. Taken together, these data suggest that TGFB and IL-10 secretion by regulatory T cells after CTLA-4 ligation plays a role in their effector functions. In our work, anti-CTLA-4 treatment effectively reversed the activity of regulatory CD25<sup>+</sup> CD4<sup>+</sup> or CD45RB<sup>lo</sup>CD4<sup>+</sup> T cells. The most obvious explanation is that anti-CTLA-4 abrogates a B7-dependent costimulatory signal for activation for these regulatory T cells. However, it is also possible that in vivo administration of anti-CTLA-4 directly eliminates regulatory T cells or interferes with their activity in another way, because triggering of CTLA-4 has been previously shown to induce T cell apoptosis (28).

In conclusion, our findings presented here indicate that CD28-B7.1 costimulatory pathway preferentially participates in regulating the Th1 response and induction of mucosal immune responses in colitic SCID model. In contrast, CTLA-4 triggering seems to play an essential role in maintenance of tolerance against luminal Ags. These data may shed some light on the pathogenic mechanisms involved in human IBD, where the role of regulatory T cells should be further investigated. Also the exact role of B7.1 and B7.2 in the pathogenesis of IBD needs to be further evaluated, eventually by targeting with mAb. Such experiments might increase the insight into the immunoregulatory mechanisms in the intestine and might provide a new therapeutic approach in human IBD.

References

induction and effector phases of experimental autoimmune encephalomyelitis.


