The Journal of Immunology

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J Immunol 2003; 171:4156-4163; ;
doi: 10.4049/jimmunol.171.8.4156
http://www.jimmunol.org/content/171/8/4156

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Blockade of B7-H1 Suppresses the Development of Chronic Intestinal Inflammation[1]

Takanori Kanai,[2]* Teruji Totsuka,* Koji Uraushihara,* Shin Makita,* Tetsuya Nakamura,* Kazutaka Koganei,‡ Tsuneo Fukushima,‡ Hisaya Akiba,§ Hideo Yagita,§ Ko Okumura,§ Utako Machida,† Hideyuki Iwai,† Miyuki Azuma,† Lieping Chen,¶ and Mamoru Watanabe*

A newly identified costimulatory molecule, programmed death-1 (PD-1), provides a negative signal that is essential for immune homeostasis. However, it has been suggested that its ligands, B7-H1 (PD-L1) and B7-dendritic cells (B7-DC; PD-L2), could also costimulate T cell proliferation and cytokine secretion. Here we demonstrate the involvement of PD-1/B7-H1 and B7-DC interaction in the development of colitis. We first examined the expression profiles of PD-1 and its ligands in both human inflammatory bowel disease and a murine chronic colitis model induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells to SCID mice. Second, we assessed the therapeutic potential of neutralizing anti-B7-H1 and/or B7-DC mAbs using this colitis model. We found significantly increased expression of PD-1 on T cells and of B7-H1 on T, B, and macrophage/DCs in inflamed colon from both inflammatory bowel disease patients and colitic mice. Unexpectedly, the administration of anti-B7-H1, but not anti-B7-DC, mAb after transfer of $CD4^+CD45RB^{high}$ T cells suppressed wasting disease with colitis, abrogated leukocyte infiltration, and reduced the production of IFN-γ, IL-2, and TNF-α, but not IL-4 or IL-10, by lamina propria $CD4^+$ T cells. These data suggest that the interaction of PD-1/B7-H1, but not PD-1/B7-DC, might be involved in intestinal mucosal inflammation and also show a possible role of interaction between B7-H1 and an as yet unidentified receptor for B7-H1 in inducing T cell activation. ***The Journal of Immunology,*** **2003, 171: 4156–4163.**

Crohn's disease (CD)[3] and ulcerative colitis (UC) are the two major forms of chronic inflammatory bowel disease (IBD). Although their etiologies remain unknown, increasing evidence suggests that dysregulation of mucosal T cells may play a key role in the pathogenesis of IBD, which results in secretion of proinflammatory mediators, accumulation of inflammatory cells, and tissue damage (1–6). Studies in animal models of experimental colitis have also definitely confirmed abnormal immune responses to potential immunogenic stimuli (e.g., luminal Ags) (7–10). However, the molecular mechanism responsible for the initiation and maintenance of lymphocyte activation in the intestinal mucosa remains elusive.

It is widely accepted that efficient T cell activation requires not only the TCR-mediated Ag-specific signal, but also a costimulatory signal provided by APCs (9, 11, 12). The B7 family of molecules provides signals that are critical for both stimulating and inhibiting T cell activation (13, 14). Engagement of CD28 by CD80 (B7-1) and CD86 (B7-2) stimulates and sustains T cell responses (15, 16), whereas engagement of CTLA-4 by the same ligands inhibits T cell responses (17). The studies using CTLA-4-deficient mice and anti-CTLA-4 mAb in vivo and in vitro demonstrated a critical negative regulatory role for CTLA-4 and prompted further studies on its role in regulating autoimmunity and peripheral tolerance (18–21). Thus, it is believed that the balance between stimulatory and inhibitory signaling is important for maintaining immune tolerance, and that breakdown of self-tolerance is involved in the pathogenesis of autoimmune diseases, including IBD.

Programmed death-1 (PD-1) is a homologue of CD28 and CTLA-4 that belongs to the Ig superfamily (22, 23). PD-1 has two tyrosines in its cytoplasmic tail, which form an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif (ITSM) (24, 25). Unlike CTLA-4, PD-1 is induced on T cells, B cells, and monocytes after activation (26). Like CTLA-4-deficient mice, PD-1-deficient mice develop autoimmune diseases, such as lupus-like glomerulonephritis and dilated cardiomyopathy (24, 27), but these mice display markedly different phenotypes compared with CTLA-4-deficient mice, suggesting differential regulatory roles for PD-1 and CTLA-4. Recently, B7-H1 (PD-L1) and B7-dendritic cells (B7-DC; PD-L2), which belong to the B7 family, were cloned (28, 29) and identified as potential ligands for PD-1 by several groups (30, 31). The PD-1 ligands show tissue distribution profiles distinct from those of the other B7 family members. The expression of B7-H1 is up-regulated upon activation of APCs, including DCs, monocytes and B cells (28, 30, 32). In addition, B7-H1 expression has been detected not only in lymphoid organs, but also in nonlymphoid organs, such as heart, lung, placenta, kidney, and liver (28, 30), while the expression of B7-DC is much more restricted, mainly found in lymphoid tissues and on DCs (29, 31, 32). B7-H1 expression is also found in the

*Department of Gastroenterology and Hepatology, †Molecular Immunology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan; ‡Department of Surgery, Yokohama City Hospital, Yokohama, Japan; §Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan; and ¶Department of Immunology, Mayo Graduate and Medical Schools, Mayo Clinic, Rochester, MN 55905

Received for publication March 10, 2003. Accepted for publication August 14, 2003.

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[1] This work was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture, and Science; the Japanese Ministry of Health and Welfare; the Japan Health Sciences Foundation; and the Ouchi Atsuko Medical Foundation.

[2] Address correspondence and reprint requests to Dr. Takanori Kanai, Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: taka.gast@tmd.ac.jp

[3] Abbreviations used in this paper: CD, Crohn's disease; DC, dendritic cell; IBD, inflammatory bowel disease; ITSM, immunoreceptor tyrosine-based switch motif; LP, lamina propria; LPMC, lamina propria mononuclear cells; NL, normal controls; PD-1, programmed death-1; PI, propidium iodide; UC, ulcerative colitis.

0022-1767/03/$02.00

majority of human cancers and leads to increased apoptosis of activated T cells (33).

While B7-H1 and B7-DC are implicated in negative regulation of T cell responses via PD-1, several studies indicated that these molecules could costimulate T cell growth and cytokine production. When resting T cells were stimulated with suboptimal concentrations of anti-CD3 mAb, immobilized B7-H1-Ig moderately enhanced proliferation, strongly up-regulated IL-10 production, and modestly up-regulated IFN-γ and GM-CSF production in both human and mouse systems (28, 34). Similarly, when resting $CD4^+$ T cells were stimulated with immobilized anti-CD3 mAb and B7-DC-Ig, proliferation and IFN-γ production were strongly enhanced (29). Interestingly, B7-H1 and B7-DC could also serve as receptors on T cells (35) and DCs (36), respectively, and regulate their growth, activation, and death.

In this study we examined the expression profiles of PD-1 and PD-1 ligands in human IBD and a murine model of chronic colitis that was induced by adoptive transfer of $CD45RB^{high}$ $CD4^+$ T cells to SCID mice (37) and evaluated the roles of PD-1 ligands in the pathogenesis of these diseases.

Materials and Methods

Patients and samples

Tissue samples were obtained from the inflamed and uninflamed areas of intestinal mucosa of 17 patients with UC (14 surgical specimens and three biopsy specimens), and 16 patients with CD (12 surgical specimens and four biopsy specimens). The primary site of CD involvement was ileal in six, ileocolonic in six, and colonic in four patients. For the control, tissue samples were obtained from macroscopically and microscopically unaffected areas of 17 surgical colonic and ileal specimens from colon cancer patients. Mucosal biopsies were 2–3 mm in size. Histopathologic examination revealed no malignancy or inflammation. The mucosa was prepared immediately after stripping away the underlying submucosa by blunt dissection. All experiments were approved by the Tokyo Medical and Dental University Hospital committee on human subjects (Tokyo, Japan). Informed consent was obtained from all patients before the study. In the CD group, disease activity was defined using the Crohn's Disease Activity Index and endoscopic and histopathologic data. In the UC group, disease activity was defined by clinical criteria supplemented by endoscopic and histopathologic data.

Antibodies

Anti-murine B7-H1 (MIH6, rat IgG2a) (32) and anti-murine B7-DC (TY25, rat IgG2a) (32) mAbs, and anti-human B7-H1 (5H1 mouse IgG1) mAb (28) were obtained as described previously. MIH6 and TY25 specifically blocked the binding of mouse B7-H1-Ig and mouse B7-DC-Ig to mouse PD-1 transfectants, respectively (38), and acted antagonistically in vivo (38, 39). Anti-murine PD-1 (RMP1–14, rat IgG2a) was generated by immunizing Sprague Dawley rats with mouse PD-1-transfected BHK cells and fusing immune splenocytes with P3U1 myeloma cells and was screened for binding to mouse PD-1 transfectants. Anti-human PD-1 (MIH4, mouse IgG1) and anti-human B7-H1 (MIH1, mouse IgG1) mAbs were generated by immunizing C3H mice with human PD-1- or B7-H1-transfected L cells and fused immune splenocytes with P3U1 myeloma cells, and were selected by specific binding to each transfectant. All mAbs were purified from ascites or culture supernatants by standard procedures, using caprylic acid extraction and ammonium sulfate precipitation or protein A column, and the purity was verified by SDS-PAGE analysis. For in vivo use, the endotoxin level <1 endotoxin unit/mg of protein was confirmed. To verify that anti-mouse B7-H1 mAb treatment did not induce the depletion of B7-H1-expressing $CD4^+$ T cells, colitic SCID mice at 6 wk after $CD4^+CD45RB^{high}$ transfer were injected i.p. with either anti-B7-H1 mAb or control IgG at a dose of 1 mg/body weight. Seventy-two hours after the injection, $CD4^+$ T cell numbers in spleen and colon were determined. After isolating splenocytes, dead or apoptotic cells were detected by annexin V-FITC/propidium iodide (PI) staining (MBL, Nagoya, Japan).

For flow cytometry and immunohistochemistry, biotinylation of these mAbs was performed by the standard method. FITC- or PE-conjugated or biotinylated anti-human CD3 (UCHT1, mouse IgG1), anti-human CD14 mAb (M5E2, mouse IgG2a), anti-human CD19 mAb (HIB19, mouse IgG1), anti-mouse CD4 (RM4-5, rat IgG2a), anti-mouse F4/80 (rat IgG2b), anti-mouse CD11c (HL3, hamster IgG), anti-mouse CD45RB (16A, rat IgG1), and isotype-matched control IgGs were purchased from BD PharMingen (San Diego, CA).

Immunohistochemistry

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. For immunohistochemistry, colonic samples were embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at -80°C. Cryostat sections (6 μm) were fixed in cold acetone, and detection of human and mouse PD-1, B7-H1, and B7-DC was performed by the avidin-biotin complex method. Briefly, after blocking, the sections were incubated with primary unlabeled mAbs, followed by biotin-conjugated, goat anti-mouse IgG (1/200; Vector Laboratories, Burlingame, CA) or biotinylated mAbs. The deposition of the biotin on tissue sections was detected by streptavidin-biotinylated HRP complex (Vectastain ABC kit; Vector Laboratories) and diaminobenzidine. Then the sections were counterstained with hematoxylin.

Preparation of mononuclear cells from the intestinal mucosa

In human studies, lamina propria mononuclear cells (LPMCs) were isolated from surgically resected intestinal specimens using enzymatic techniques as previously described (7). Briefly, the dissected mucosa was incubated in Ca^{2+} Mg^{2+}-free HBSS containing 2.5% FBS and 1 mM DTT (Sigma-Aldrich, St. Louis, MO) to remove mucus. The mucosa was then incubated in medium containing 0.75 mM EDTA (Sigma-Aldrich) for 60 min at 37°C. During this treatment, intraepithelial lymphocytes and epithelial cells were released from the tissue, and the remaining tissues containing LPMCs were collected and incubated in medium containing 0.02% collagenase (Worthington Biochemical Corp., Freehold, NJ). The fraction was pelleted twice through a 40% isotonic Percoll solution, and the cells were centrifuged over a Ficoll-Hypaque density gradient (40 and 60%). The purity of the resulting LPMCs was analyzed by flow cytometry. In murine studies, LPMCs were isolated from the colon as described previously (40). Briefly, the entire intestine was opened longitudinally, washed with PBS, and cut into small pieces. The pieces were incubated with Ca^{2+} Mg^{2+}-free HBSS containing 1 mM DTT for 30 min to remove mucus and then serially incubated twice in medium containing 0.75 mM EDTA for 60 min each time. The supernatants from these incubations were collected, pooled, and treated with 1 mg/ml collagenase and 0.01% DNase (Worthington Biochemical Corp.) in medium for 2 h. The cells were pelleted twice through a 40% isotonic Percoll solution and then further purified by Ficoll-Hypaque density gradient centrifugation (40/75%) at the interface.

Flow cytometry

Flow cytometric, two-color analysis was performed as previously described (7, 41). For staining of freshly isolated LPMCs and PBMCs from humans or splenocytes from mice, $0.5–1 \times 10^6$ cells were incubated with biotinylated anti-human or mouse PD-1, anti-human or mouse B7-H1, or anti-mouse B7-DC mAb, followed by PE-conjugated streptavidin (BD PharMingen) and FITC-conjugated anti-human CD3, anti-human CD14, or anti-human CD19, anti-mouse CD4 mAb, anti-mouse F4/80 mAb, or anti-mouse CD11c mAb, or with fluorochrome-conjugated control IgG. After staining, flow cytometry and data analysis were performed using FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

Induction of colitis and treatments

Induction of colitis by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice was performed essentially as described previously (41). Briefly, $CD4^+$ T cells were purified from the spleen of healthy BALB/c mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (96–97% pure as estimated by FACS) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5; BD PharMingen) and FITC-conjugated anti-CD45RB (16A; BD PharMingen) mAbs and sorted into $CD45RB^{high}$ (brightest staining, 30%) and $CD45RB^{low}$ (dullest staining, 30%) fractions on a FACSVantage (BD Biosciences). Each BALB/c SCID mouse was injected i.p. with 200 μl of PBS containing 5×10^5 $CD4^+CD45RB^{high}$ T cells. These mice were administered i.p. 500 μg of control IgG, 250 μg of anti-B7-H1 mAb (MIH6) plus 250 μg of control IgG, 250 μg of anti-B7-DC mAb (TY25) plus 250 μg of control IgG, or 250 μg of anti-B7-H1 mAb plus 250 μg of anti-B7-DC mAb three times per week starting from the same day of T cell transfer over a period of 7 wk. Rat IgG (Sigma-Aldrich) was used as the control IgG.

Clinical and histological scoring

Mice were weighed and monitored for appearance and signs of soft stool and diarrhea weekly. Clinical score was assessed 7 wk after T cell transfer

as the sum of three parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, gross bloody stool) (39). For histological scoring, the area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (41).

Cytokine ELISA

$CD4^+$ T cells were purified from LPMCs using the anti-CD4 (L3T4) MACS magnetic separation system and then were cultured in 200 μl of 10% FCS/RPMI 1640 medium supplemented with 1 μg/ml soluble anti-CD28 mAb (37.51; BD PharMingen) in 96-well plates (Costar, Cambridge, MA) that were precoated with 10 μg/ml anti-CD3ϵ Ab (145-2C11; BD PharMingen) in PBS overnight at 4°C. Culture supernatants were collected after 48 h and assayed for cytokine contents by specific ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

The results were expressed as the mean ± SEM. Groups of data were compared by Mann-Whitney *U* test. A value of $p < 0.05$ was considered statistically significant.

Results

Expression of PD-1 and B7-H1 in human IBD

To explore whether interaction between PD-1 and B7-H1 was involved in the development of IBD, we first examined the expression of PD-1 and B7-H1 in human IBD by immunohistochemistry. As shown in Fig. 1, $PD\text{-}1^+$ and $B7\text{-}H1^+$ mononuclear cells in the lamina propria (LP) were markedly increased in inflamed mucosa from patients with UC or CD compared with normal controls (NL). To further determine the surface cell phenotypes of infiltrated mononuclear cells expressing PD-1 and B7-H1, we performed two-color flow cytometric analysis of LPMCs and PBMCs. $CD3^+PD\text{-}1^+$ LPMCs were significantly increased in both UC and CD compared with NL (NL, 3.1 ± 0.5%; UC, 35.2 ± 5.0%; CD, 25.1 ± 3.8%; NL vs UC, $p = 0.0037$; NL vs CD, $p = 0.0129$; Fig. 2, *A* and *B*). In addition, the increased $CD3^+PD\text{-}1^+$ LPMCs in UC and CD were mainly $CD4^+$, and partly $CD8^+$, T cells (data not shown). Furthermore, we found that $CD19^+PD\text{-}1^+$ LP B cells were also significantly increased in both UC and CD compared with NL (data not shown). Interestingly, we found that $CD3^+PD\text{-}1^+$ PBMCs were also significantly increased in UC, but not CD, compared with NL (NL, 5.2 ± 0.1%; UC, 24.5 ± 5.0%; CD, 6.4 ± 1.0%; NL vs UC, $p = 0.0116$; NL vs CD, $p = 0.9484$; Fig. 2, *C* and *D*). Similarly to PD-1 expression, $CD3^+B7\text{-}H1^+$ LPMCs were increased in UC and CD compared with NL (NL, 4.8 ± 5.0%; UC, 35.1 ± 21.2%; CD, 28.2 ± 12.5%; NL vs UC, $p = 0.031$; NL vs CD, $p = 0.048$; Fig. 3, *A* and *B*). In addition, $CD19^+B7\text{-}H1^+$ LP B cells were increased in UC, but not CD, compared with NL (NL, 10.7 ± 9.1%; UC, 39.9 ± 27.9%; CD, 17.3 ± 13.0%; NL vs UC, $p = 0.0033$; NL vs CD, $p = 0.5887$), and macrophage-gated $B7\text{-}H1^+$ cells were also increased in both UC and CD (NL, 5.2 ± 6.3%; UC, 21.1 ± 13.2%; CD, 28.9 ± 15.6%; NL vs UC, $p = 0.048$; NL vs CD, $p = 0.021$; Fig. 3, *A* and *B*). $CD3^+B7\text{-}H1^+$ and $CD19^+B7\text{-}H1^+$ PBMCs were also increased in UC, but not CD, compared with NL ($CD3^+B7\text{-}H1^+$ in NL, 5.8 ± 5.2%; UC, 24.8 ± 10.2%; CD, 11.1 ± 8.9%; NL vs UC, $p = 0.014$; NL vs CD, $p = 0.594$; $CD19^+$ $B7\text{-}H1^+$ in NL, 21.1 ± 10.9%; UC, 31.1 ± 11.1%; CD, 20.8 ± 12.1%; NL vs UC, $p = 0.049$; NL vs CD, $p = 0.970$; Fig. 3, *C* and *D*). Unlike LPMCs, $CD14^+$ monocytes from all three groups constitutively expressed B7-H1, and no significant difference was observed (NL, 74.0 ± 21.1%; UC, 63.2 ± 19.8%; CD, 85.9 ± 21.4%; Fig. 3, *C* and *D*). Additionally, further flow cytometric analysis revealed that $CD3^+B7\text{-}H1^+$ T cells in PBMCs were mainly $CD4^+$ and in part $CD8^+$ (data not shown). The increased expression of both PD-1 and B7-H1 on LPMCs from IBD patients suggested that the PD-1/B7-H1 interaction could participate in the pathogenesis or regulation of IBD.

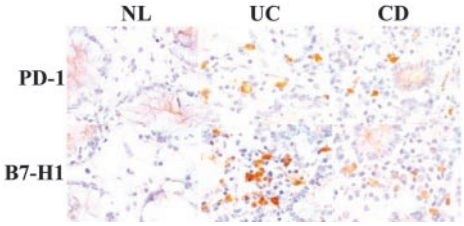

FIGURE 1. Expression of PD-1 and B7-H1 in gut samples from NL and UC or CD patients. Cryostat sections of gut samples from NL and UC or CD patients were stained with anti-human PD-1 (MIH4) and anti-human B7-H1 (5H1) mAbs. Representatives of four separate samples in each group are shown. Original magnification, ×200.

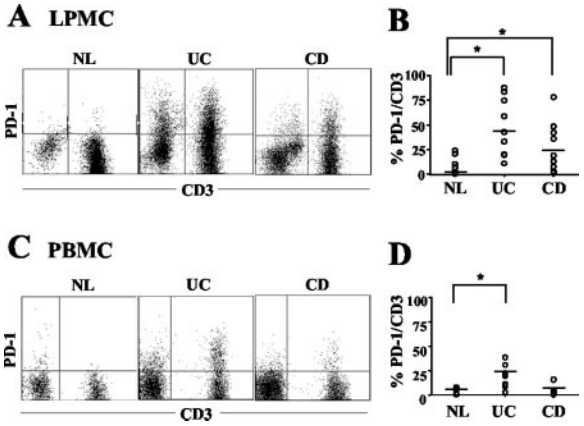

FIGURE 2. Expression of PD-1 on freshly isolated LPMCs and PBMCs from NL and UC or CD patients. LPMCs (*A*) and PBMCs (*C*) were stained with either FITC-conjugated anti-CD3 and biotinylated anti-PD-1 mAbs, followed by PE-conjugated streptavidin, or fluorochrome-conjugated control Ig (not shown). Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as a dot plot (4-decade log scale), and quadrant markers were positioned to include >98% of control Ig-stained cells in the *lower left*. In *A* and *C*, representative FACS profiles are presented. In *B* and *D*, individual values in LPMCs and PBMCs from eight or nine NL and 10 each of patients with UC or CD are shown, respectively. ∗, $p < 0.05$.

Overexpression of PD-1 and PD-1 ligands (B7-H1 and B7-DC) in murine chronic colitis

We next examined the expression profiles of PD-1, B7-H1, and B7-DC in a murine IBD model induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells to SCID mice by immunohistochemistry. Colonic samples were obtained from colitic SCID mice 7 wk after T cell transfer and from normal BALB/c mice as a control. Fig. 4 shows that $PD\text{-}1^+$, $B7\text{-}H1^+$, and $B7\text{-}DC^+$ cells were markedly increased in the inflamed mucosa from colitic mice compared with normal mice. The majority of $PD\text{-}1^+$ cells were located in the LP and submucosa, but some were found in the tunica muscularis and subserosa. Of note, $B7\text{-}H1^+$ cells were diffusely distributed like $CD4^+$ T cells, suggesting that B7-H1 was at least in part expressed on $CD4^+$ LP T cells. In contrast, the number of $B7\text{-}DC^+$ cells was less than that of $B7\text{-}H1^+$ cells and appeared to overlap with the number of $CD11c^+$ DCs.

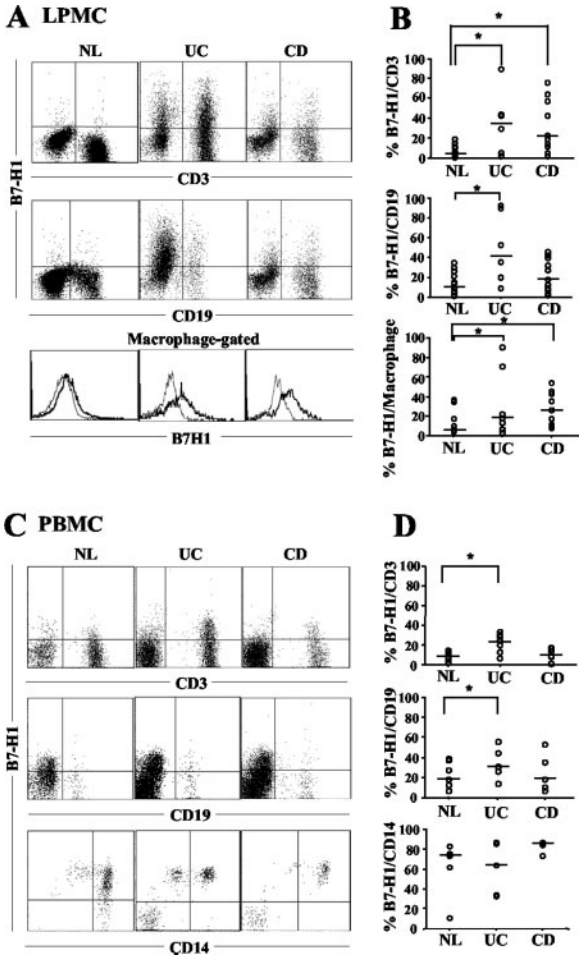

FIGURE 3. Expression of B7-H1 on freshly isolated LPMCs and PBMCs from NL and UC or CD patients. LPMCs (*A*) and PBMCs (*C*) were stained with either FITC-conjugated anti-CD3, anti-CD19, or anti-CD14 mAb and biotinylated anti-B7-H1 mAb, followed by PE-conjugated streptavidin or fluorochrome-conjugated control Ig (not shown). Samples were analyzed by flow cytometry as described in Fig. 2. *A*, Representative data showing the increased expression of B7-H1 on $CD3^+$, $CD19^+$, and macrophage/DC-gated LPMCs. *B*, Expression of B7-H1 on $CD3^+$, $CD19^+$, and macrophage/DC-gated LPMCs from NL (12 cases), UC (12 cases), and CD (12 cases). $*$, $p < 0.05$. *C*, Representative data showing the expression of B7-H1 on $CD3^+$, $CD19^+$, and $CD14^+$ PBMCs. *D*, Expression of B7-H1 on $CD3^+$, $CD19^+$, and $CD14^+$ PBMCs from NL (eight cases), UC (seven cases), and CD (seven cases). $*$, $p < 0.05$.

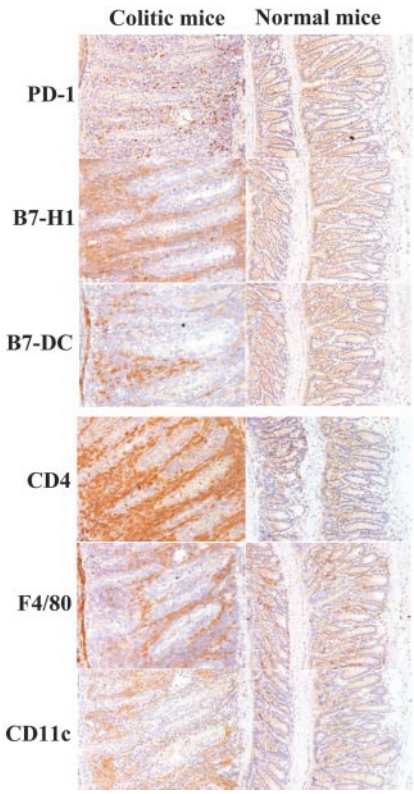

FIGURE 4. Expression of PD-1, B7-H1, and B7-DC in murine intestinal mucosa. Cryosections of the colon from colitic mice at 6 wk after T cell transfer (*left*) and normal BALB/c mice (*right*) were stained with anti-PD-1, anti-B7-H1, anti-B7-DC, anti-CD4, anti-F4/80, and anti-CD11c mAbs. Representatives of three samples in each group are shown. Original magnification, ×200.

To clarify the expression of PD-1, B7-H1, and B7-DC in LPMC and splenocytes subpopulations, we next conducted two-color flow cytometric analysis (Fig. 5). In this colitis model, $CD8^+$ T cells and B cells are absent because SCID mice are reconstituted with only $CD4^+$ donor T cells. Therefore, we focused on $CD4^+$, $CD11b^+$, and $CD11c^+$ cells in this analysis. $CD4^+$ LP T cells from colitic mice were markedly increased, and PD-1 expression on these cells was significantly increased compared with that in normal mice. As expected, most $CD4^+$ LP T cells from colitic mice strongly expressed B7-H1, while $CD4^+$ LP T cells from normal mice did not. In sharp contrast, $CD4^+$ LP T cells from colitic or normal mice did not express B7-DC. $CD11b^+$ and/or $CD11c^+$ macrophages/DCs were also markedly increased in colitic mice (Fig. 5*A*). Increased expression of B7-H1 was found on most $CD11b^+$ and $CD11c^+$ cells from colitic mice. Expression of B7-DC was found on ~12% of $CD11b^+$ cells and ~44% of $CD11c^+$ cells from colitic mice. Similarly, albeit to a lesser extent, the expression of PD-1 and B7-H1 on splenic $CD4^+$ T cells and the expression of B7-H1 on $CD11b^+$ and/or $CD11c^+$ splenic macrophages/DCs from colitic mice were significantly increased compared with those from normal BALB/c mice. Interestingly, splenic $CD11b^+$ and/or $CD11c^+$ DCs did not express B7-DC, suggesting that these DCs were less activated compared with the colitic LP DCs.

These results indicated PD-1 expression on a part of infiltrating $CD4^+$ T cells, B7-H1 on both infiltrating T cells and $CD11b^+$ or $CD11c^+$ potential APCs, and B7-DC on a part of $CD11b^+$ or $CD11c^+$ APCs in the LP of colitic mice, suggesting a possible contribution of PD-1/B7-H1 and B7-DC interaction to the development or regulation of colitis in this model.

Anti-B7-H1, but not anti-B7-DC, mAb prevents experimental colitis

After confirming the increased expression of B7-H1 and B7-DC in the inflamed mucosa of colitic mice, we asked whether blockade of these molecules could affect the development of colitis. To do so, SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells were treated with i.p. injection of anti-B7-H1 (MIH6) mAb, anti-B7-DC (TY25) mAb, a combination of both mAbs, or control rat IgG at the dosage of 250 μg/mouse three times per week starting on the day of T cell transfer and continuing up to 7 wk. The control IgG-treated mice developed severe colitis, which was clinically manifested by loss of body weight (Fig. 6*A*) and soft stool or diarrhea with increased mucus (Fig. 6*B*). These mice had an enlarged colon with a significantly thickened wall (not shown).

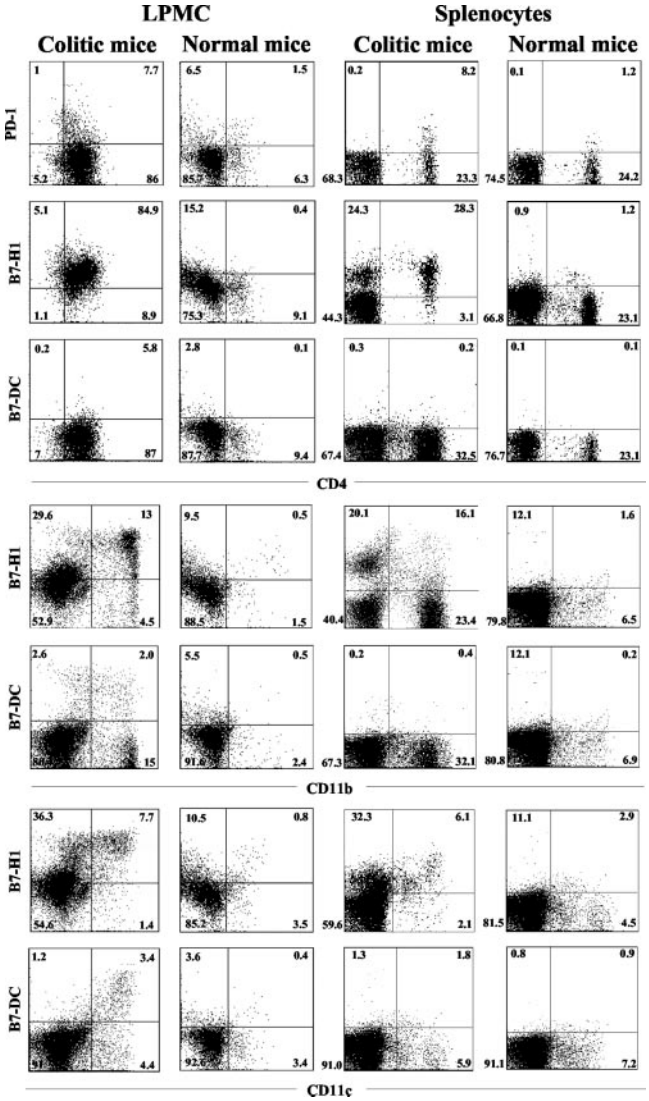

FIGURE 5. Expression of PD-1, B7-H1, and B7-DC on LPMCs and splenocytes. Freshly isolated LPMCs and splenocytes from colitic mice at 7 wk after T cell transfer and normal BALB/c mice were stained with FITC-labeled anti-CD4, anti-CD11b, or anti-CD11c mAb and biotin-labeled anti-PD-1, anti-B7-H1, or anti-B7-DC mAb, followed by PE-labeled streptavidin. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as a dot plot (4-decade log scale), and quadrant markers were positioned to include >98% of control Ig-stained cells in the *lower left*. Percentages in each quadrant are indicated. Representatives of three mice in each group are shown.

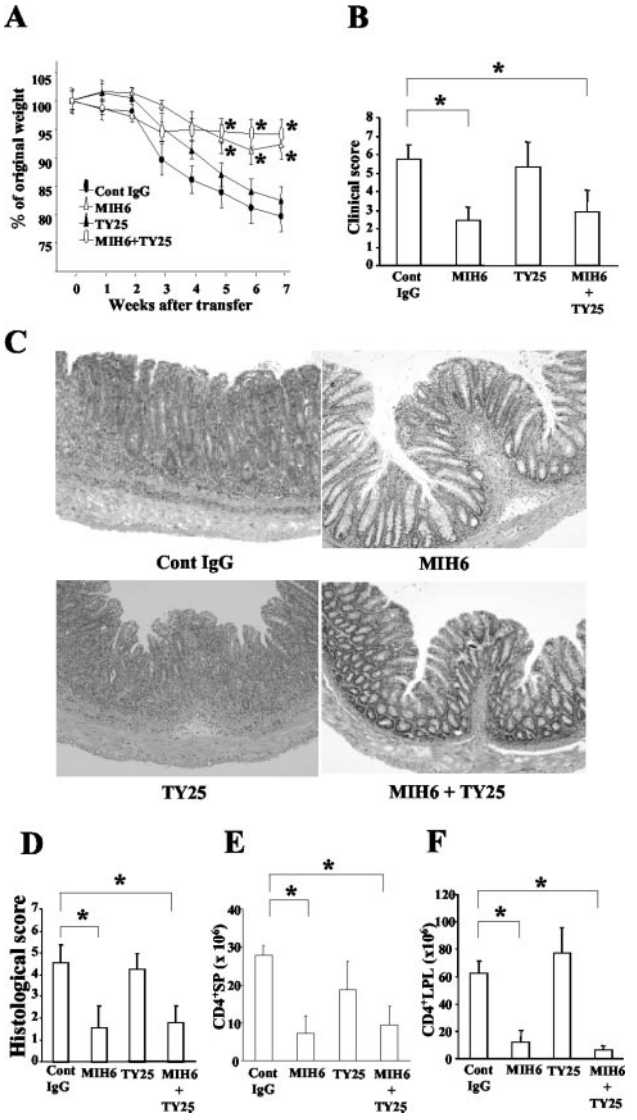

FIGURE 6. Administration of anti-B7-H1, but not anti-B7-DC, mAb prevents the development of colitis. SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells (4×10^5/mouse) were treated i.p. with control IgG, anti-B7-H1 (MIH6), and/or anti-B7-DC (TY25) mAb at a dose of 250 μg three times per week over 7 wk starting at the time of T cell transfer. *A*, Change in weight over time is expressed as percentage of the original weight. Data represent the mean ± SEM of 15 mice in each group from three independent experiments. ∗, $p < 0.05$ vs control IgG-treated group. *B*, Clinical scores at 7 wk after T cell transfer. Data are indicated as the mean ± SEM of 15 mice in each group. ∗, $p < 0.05$. *C*, Histological examination of the colon at 7 wk after T cell transfer. Original magnification, ×100. *D*, Histological scoring at 7 wk after T cell transfer. Data are indicated as the mean ± SEM of seven mice in each group. ∗, $p < 0.05$. Splenocytes (SP; *E*) and LPMCs (*F*) were isolated from the colon at 7 wk after T cell transfer, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 15 mice in each group. ∗, $p < 0.05$.

Transmural inflammation with increased leukocyte infiltrates was commonly seen in the ascending and transverse colon (Fig. 6, *C* and *D*). Epithelial lesions included loss of goblet cells and crypt abscesses. Moreover, architectural changes, such as crypt elongation, villous transformation of the surface, and crypt branching, were also observed. The anti-B7-DC mAb-treated mice developed colitis with severity, time course, and histological scores comparable to those of the control IgG-treated recipients (Fig. 6, *A–D*). In contrast, the mice treated with anti-B7-H1 mAb appeared healthy and were free of diarrhea. The combined treatment with mAbs to B7-H1 and B7-DC did not result in further improvement compared with anti-B7-H1 mAb alone (Fig. 6, *A* and *B*). Histological features and histological scores of the colon from the mice treated by anti-B7-H1 mAb were significantly improved compared with those treated by anti-B7-DC mAb or control IgG. Again, there was no additive effect by the combination of anti-B7-H1 and anti-B7-DC mAbs (Fig. 6, *C* and *D*).

Consistent with the clinical and histological findings, $CD4^+$ T cell recovery from the spleen and LP from the mice treated with anti-B7-H1 mAb alone or in combination with anti-B7-DC mAb was significantly reduced compared with that from the anti-B7-DC mAb- or control IgG-treated mice (spleen ($\times 10^6$): control IgG,

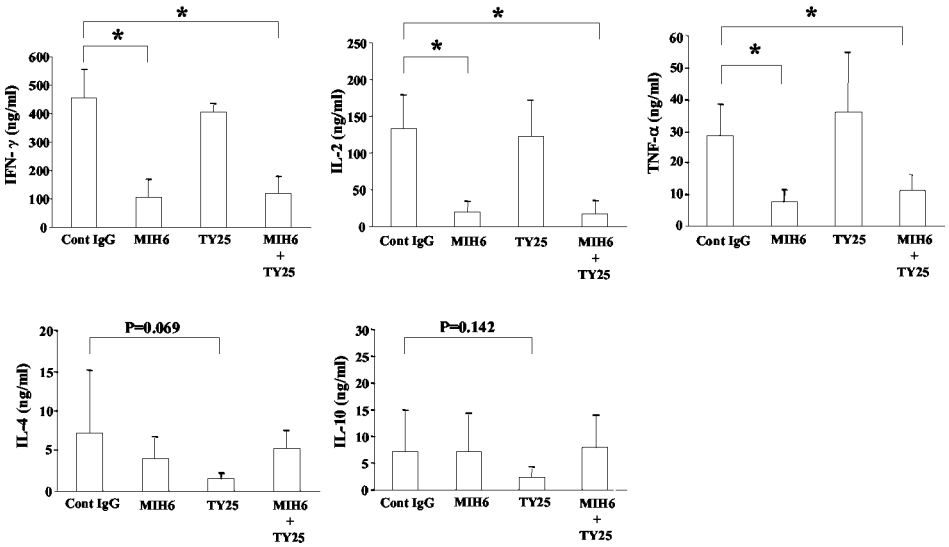

FIGURE 7. Effect of anti-B7-H1 and/or anti-B7-DC mAb treatment on cytokine production by $CD4^+$ LPMCs. LP $CD4^+$ T cells were isolated from control IgG, anti-B7-H1 (MIH6), and/or anti-B7-DC (TY25)-treated mice at 7 wk after T cell transfer and stimulated with anti-CD3ϵ and anti-CD28 mAbs for 48 h. IFN-γ, IL-2, TNF-α, IL-4, and IL-10 concentrations in culture supernatants were measured by ELISA. Data are represented as the mean ± SEM of seven mice in each group. *, $p < 0.05$.

27.8 ± 10.7; anti-B7-H1, 7.0 ± 4.6; anti-B7-DC, 18.6 ± 11.6; anti-B7-H1/B7-DC, 9.3 ± 5.0; LP ($\times 10^6$): control IgG, 52.4 ± 26.8; anti-B7-H1, 12.0 ± 7.5; anti-B7-DC, 77.4 ± 21.0; anti-B7-H1/B7-DC, 6.0 ± 2.4; Fig. 6, *E* and *F*). In addition, immunohistochemical analysis demonstrated that anti-B7-H1 or anti-B7-H1/B7-DC treatment significantly reduced infiltration of $CD4^+$ T cells, $F4/80^+$ macrophages, and $CD11c^+$ DCs (data not shown).

To determine the effect of anti-B7-H1 and/or anti-B7-DC mAb treatment on Th1/Th2 development, we measured IFN-γ, IL-2, TNF-α, IL-4, and IL-10 production by anti-CD3/CD28 mAb-stimulated $CD4^+$ LP T cells. As shown in Fig. 7, the production of Th1 cytokines (IFN-γ, IL-2, and TNF-α) was significantly reduced in $CD4^+$ LP T cells from mice treated with anti-B7-H1 mAb alone or anti-B7-H1/B7-DC mAbs compared with that from the anti-B7-DC mAb- or control IgG-treated mice ($p < 0.05$). In contrast, the production of Th2 cytokines (IL-4 and IL-10) was not significantly affected by these treatments, although treatment with anti-B7-DC mAb alone tended to suppress IL-4 and IL-10 production.

Finally, to exclude the possibility that anti-B7-H1 mAb treatment might deplete the activated $CD4^+$ T cells expressing B7-H1, we treated mice with established colitis at 6 wk after the transfer of $CD4^+$ $CD45RB^{high}$ cells with a single dose of anti-B7-H1 mAb or control IgG (1 mg/body weight) and determined the numbers of $CD4^+$ T cells in the spleen and colon 72 h later. As shown in Fig. 8*A*, there were no differences between the mice treated with anti-B7-H1 mAb and those treated with control IgG, indicating that anti-B7-H1 mAb did not induce the depletion of B7-H1-expressing T cells in vivo. We also determined the number of apoptotic cells in splenocytes by PI/annexin V-FITC staining (Fig. 8, *B* and *C*). There were no significant differences in the numbers of dead (PI^+ annexin V^+) or apoptotic (PI^- annexin V^+) cells between the anti-B7-H1 mAb- and control IgG-treated mice. These results indicated that anti-B7-H1 mAb did not induce apoptotic cell death in activated T cells expressing B7-H1 in vivo.

Discussion

The present study was designed to investigate whether PD-1 and its ligands (B7-H1 and B7-DC) are involved in the pathogenesis of human IBD and murine experimental colitis. Both PD-1 and PD-1 ligands were significantly up-regulated on LPMCs in inflamed mucosa from IBD patients and experimental colitic mice. Especially B7-H1 was broadly found on T cells, B cells, macrophages/monocytes, and DCs in individuals with colitis. Importantly, blockade of B7-H1 by neutralizing mAb ameliorated the development of experimental colitis. These findings suggested that B7-H1 plays a critical role in the development of colitis.

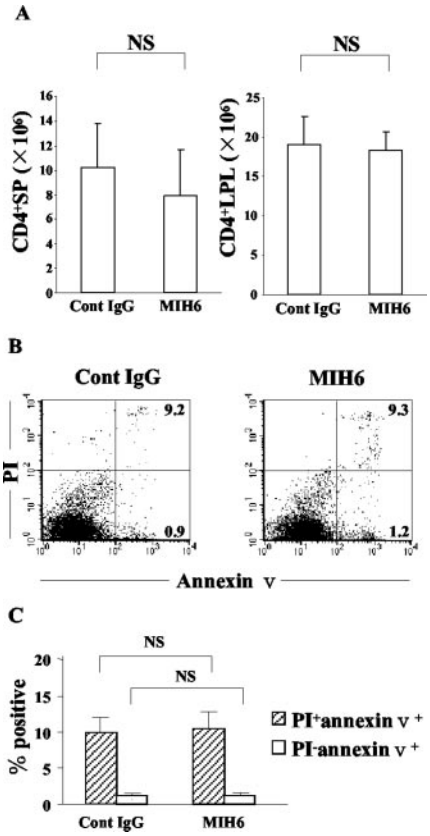

FIGURE 8. Anti-B7-H1 mAb treatment does not induce depletion or apoptosis of $CD4^+$ LPMCs and splenocytes. Mice with established colitis at 6 wk after the transfer of $CD4^+$ $CD45RB^{high}$ cells were injected i.p. with either anti-B7-H1 mAb (MIH6) or control rat IgG at a dose of 1 mg/body. *A*, Seventy-two hours after the injection, $CD4^+$ T cell numbers in the spleen and colon were determined. Data are represented as the mean ± SEM of four mice in each group. *B*, After isolating splenocytes, the number of apoptotic cells was determined by the annexin V-FITC/PI. Data represent one experiment from a mouse treated with anti-B7-H1 mAb or control IgG. *C*, Data are represented as the mean ± SEM percentage of PI^+ annexin V^+ (dead) cells and the percentage of PI^- annexin V^+ (apoptotic) cells from four mice in each group.

By flow cytometric analysis, we first determined the expression of PD-1, B7-H1, and B7-DC on LP T cells and APCs, including B cells, macrophages, and DCs. We found that the expression of PD-1 was significantly increased on $CD3^+$ LP T cells from both UC and CD patients, and that the expression of B7-H1 was increased on $CD3^+$ LP T cells and macrophages from both UC and CD patients. In UC patients, B7-H1 expression was also increased on $CD19^+$ LP B cells. Interestingly, the increased expression of PD-1 and B7-H1 on $CD3^+$ T cells and of B7-H1 on $CD19^+$ B cells was also found in PBMCs from UC patients, suggesting that PD-1 and B7-H1 expression in PBMCs may become a useful clinical marker for the diagnosis of UC. In colitic mice, we found that the numbers of PD-1^+, B7-$H1^+$, and B7-DC^+ LP cells were markedly increased. Consistent with the human results, B7-H1 was expressed on various cells, including $CD4^+$ T cells, $CD11b^+$, and/or $CD11c^+$ macrophages/DCs. The expression of B7-DC, however, was restricted to macrophages/DCs. The differential expression patterns of B7-H1 and B7-DC thus suggest different roles for these molecules in the pathogenesis of IBD.

The most important finding from this study is that the administration of anti-B7-H1 mAb, but not anti-B7-DC mAb, prevented the development of experimental colitis in association with the decreased expansion of pathogenic T cells and the down-regulated Th1 cytokine production (i.e., IFN-γ, IL-2, and TNF-α) by LP $CD4^+$ T cells. This effect is unlikely to be caused by Ab-mediated depletion of B7-$H1^+$ pathogenic T cells, because administration of anti-B7-H1 mAb to mice with established colitis did not deplete $CD4^+$ T cells in the spleen and colon or induce apoptosis of splenic $CD4^+$ T cells 72 h after mAb injection (Fig. 8). Since it has recently been reported that B7-H1 is constitutively expressed on vascular endothelium in peripheral tissues, including heart, liver, lung, and kidney (42), it is possible that the anti-B7-H1 mAb treatment might affect the migration of PD-1^+ T cells into the inflamed mucosa. However, since we could not detect the expression of B7-H1 on vascular endothelium in normal or inflamed colon (Fig. 4), it seems unlikely that anti-B7-H1 mAb treatment affected the accumulation of PD-1^+ T cells in the colon. Thus, our results suggest that endogenous B7-H1 might be required for the expansion and differentiation of adoptively transferred $CD4^+CD45B^{high}$ T cells in vivo. Consistent with this idea, we previously demonstrated that immobilized B7-H1-Ig could costimulate proliferation and cytokine production by human peripheral blood T cells (28). In addition, cell-associated B7-H1 costimulated the proliferation of murine $CD4^+$ T cells in vitro (34). Furthermore, the administration of B7-H1-Ig enhanced $CD4^+$ T cell responses to keyhole limpet hemocyanin and increased Th cell-dependent synthesis of 2,4,6-trinitrophenyl hapten-specific IgG2a in vivo (34). It is thus possible that B7-H1 may be involved in promoting $CD4^+CD45B^{high}$ T cell activation in our model.

PD-1-deficient mice spontaneously develop systemic autoimmune diseases (24, 27). In addition, some experiments have shown that the ligation of PD-1 by B7-H1 inhibited proliferation and cytokine production by activated T cells (30). These results support a role for PD-1 as a negative regulator of T cell responses. However, the cytoplasmic domain of PD-1 contains an ITSM in addition to an immunoreceptor tyrosine-based inhibition motif (24, 25). The ITSM has been implicated in the costimulatory function of CD150 on T cells (44). Thus, the PD-1 molecule on $CD4^+CD45RB^{high}$ naive T cells may transmit a positive costimulatory signal via its ITSM. The actual contribution of PD-1 to the development of experimental colitis would be determined by adoptive transfer of $CD4^+CD45RB^{high}$ T cells from PD-1-deficient mice.

Alternatively, B7-H1 may costimulate naive T cells via an as yet unidentified receptor other than PD-1. This possibility is supported by our recent findings that B7-H1 could costimulate proliferation and cytokine secretion of PD-$1^{-/-}$ T cells to the same level as PD-$1^{+/+}$ T cells (44). More importantly, B7-H1 mutants that lost binding capacity to PD-1 were still capable of costimulating proliferation and cytokine secretion of T cells from both normal and PD-1-deficient mice (44). Thus, it is tempting to speculate that this alternative receptor may be expressed on $CD4^+CD45RB^{high}$ naive T cells, while PD-1 appears only after activation. Since we here assessed the effect of anti-B7-H1 mAb in the preventive protocol, this putative costimulatory receptor for B7-H1 might be dominant for the onset of colitis. This possibility will be verified by identification of the second B7-H1 receptor.

Another possibility is that the anti-B7-H1 mAb treatment might disturb costimulatory signaling via B7-H1. We recently reported that ligation of B7-H1 on T cells could transmit a costimulatory signal into T cells (35). The anti-B7-H1 mAb treatment might inhibit the ligation of B7-H1 by PD-1 or the alternative receptor and thus suppress the activation of pathogenic T cells. Further characterization of the effect of anti-B7-H1 mAb on T cell activation in vitro and in vivo is required to address this possibility.

A relevant question is why anti-B7-DC mAb did not affect the development of colitis, albeit B7-DC was up-regulated. One explanation may be the lower and limited expression of B7-DC in the inflamed mucosa. Alternatively, B7-DC may not interact with the costimulatory B7-H1 receptor, although a recent study has shown that B7-DC could also costimulate naive $CD4^+$ T cells from PD-1-deficient mice (45). The identity of the second B7-DC receptor with the second B7-H1 receptor remains to be determined. It may also be worth noting that treatment with anti-B7-DC mAb alone tended to suppress IL-4 and IL-10 production by LP $CD4^+$ T cells. The preferential contribution of B7-DC to Th2 development would be more appropriately addressed in Th2-mediated disease models such as allergic asthma.

In summary, our present results indicated a critical contribution of B7-H1 to the development of experimental colitis in mice. Since B7-H1 expression was also up-regulated in the mucosa of UC and CD patients, B7-H1 may be a novel target for clinical intervention in human IBD.

Acknowledgments

We thank Hiroshi Nishikawa for his invaluable technical assistance, and Drs. Motomi Yamazaki and Mieko Sakae for helpful discussion.

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