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B7-DC Regulates Asthmatic Response by an IFN-γ-Dependent Mechanism[1]

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B7-H1 (PD-L1) and B7-DC (PD-L2) are the ligands for programmed death-1 (PD-1), which is a member of the CD28/CTLA-4 family and has been implicated in peripheral tolerance. We investigated the roles of B7-H1 and B7-DC in a murine OVA-induced allergic asthma model. B7-H1 was constitutively expressed on dendritic cells, macrophages, B cells, and T cells in the lungs of naive mice, and its expression could be dramatically increased after allergen challenge. In contrast, B7-DC expression was scarcely expressed on dendritic cells in naive mice, but was up-regulated after allergen challenge, although the up-regulation of B7-DC expression on macrophages was minimal. Treatment of mice with anti-B7-DC mAb at the time of allergen challenge, but not at the time of sensitization, significantly increased their airway hyper-reactivity and eosinophilia. Such treatment also resulted in the increased production of IL-5 and IL-13, and decreased IFN-γ production in the lungs and draining lymph node cells. These changes were diminished when mice were depleted of IFN-γ by anti-IFN-γ mAb pretreatment. Interestingly, treatment with anti-B7-H1 or anti-PD-1 mAb did not significantly affect the asthmatic response. These results suggest a unique role for B7-DC in the regulation of asthmatic response through an IFN-γ-dependent, but PD-1-independent, mechanism. ***The Journal of Immunology,*** **2004, 172: 2530–2541.**

Rapid advances in immunobiology, particularly the role of Th1/Th2 cells, have helped to improve our understanding of the inflammatory processes that mediate allergic asthma (1). Interactions between APCs and T cells appear to be the first steps in airway sensitization that ultimately lead to the generation of a Th2-type response. These T cell/APC interactions are characterized by the binding of an array of costimulatory molecules, as determined using various experimental models (2, 3). In murine asthma and rhinitis models, CD80/CD86 and CD28 were shown to be required for initial T cell activation (4–8). In contrast, the interaction between CD80/CD86 and CTLA-4 was reported to lead to the generation of an inhibitory signal that prevented excessive T cell activation (9, 10). Moreover, it has been suggested that inducible costimulator and its ligand (B7RP-1) may be associated with Th2 commitment and the induction of tolerance (11, 12). Accumulating evidence suggests that costimulatory molecules are critical elements for the promulgation of Th2-skewed allergic responses.

Programmed death-1 (PD-1)[3] is a transmembrane protein originally identified in a T cell line that was undergoing activation-induced cell death (13). Subsequent studies revealed that its expression was associated with cell activation rather than cell death (14, 15). PD-1 was expressed on activated T and B cells and on a subset of thymocytes (14). Structurally, PD-1 belongs to the CD28/CTLA-4 subfamily of the Ig superfamily and, as such, contains a single Ig V-like domain in its extracellular region (2, 16). PD-1-deficient C57BL/6 mice were shown to develop glomerulonephritis and lupus-like arthritis (17), whereas PD-1-deficient BALB/c mice were shown to develop autoantibody-mediated dilated cardiomyopathy (18). Additionally, PD-1-deficient mice that were crossed with H-2^d-specific TCR transgenic mice on an H-$2^{b/d}$ background exhibited growth retardation, splenomegaly, and lethal graft-vs-host disease (17). These studies support the idea that PD-1 plays a critical role in peripheral tolerance induction and autoimmune disease prevention.

Recently, two new members of the B7 family, i.e., B7-H1 (PD-L1) and B7-DC (PD-L2), were identified as ligands for PD-1 (19–22). The binding of PD-1 to B7-H1 or B7-DC reportedly inhibited TCR-mediated T cell proliferation and cytokine production, suggesting that the cross-linking of PD-1 by B7-H1 or B7-DC may lead to the down-regulation of T cell responses (20, 21). However, it was also shown that when resting T cells were stimulated with anti-CD3 mAb and B7-DC-Ig, they exhibited enhanced proliferation and IFN-γ production (22). Another group also reported enhanced proliferation and IFN-γ, GM-CSF, and IL-10 production by T cells that were stimulated with low doses of anti-CD3 mAb and B7-H1-Ig (19, 23). These conflicting results have made it difficult to decipher the biological significance of B7-H1 and B7-DC

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[3] Abbreviations used in this paper: PD-1, programmed death-1; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BALF, BAL fluid; DC, dendritic cell; DLN, draining lymph node.

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vis-à-vis PD-1. We theorized that a better understanding of the roles of these molecules might be obtained by using well-characterized disease models.

B7-H1 mRNA has been detected in various organs, including the heart, lung, thymus, spleen, kidney, and liver, and was shown to be up-regulated by IFN-γ in monocytes and dendritic cells (DCs) (19, 20). B7-DC mRNA has been detected in liver, lung, and spleen and was shown to be preferentially expressed in bone-marrow-derived and splenic DCs (21, 22). Recently, mAb specific for B7-H1 and B7-DC have become available (24, 25), which were used to show that B7-H1 was constitutively expressed on T cells, B cells, macrophages, and DCs and that it was up-regulated on T cells by CD3 ligation and on macrophages and DCs by IFN-γ, GM-CSF, or IL-4. In contrast, B7-DC expression was found to be inducible only on macrophages and DCs after their stimulation with IFN-γ, GM-CSF, or IL-4. The fact that cytokines can induce the expression of B7-H1 and B7-DC on APCs suggests that these ligands might play a role in regulating the activity of Th1/Th2 cells (26). This possibility prompted us to investigate the roles of these two ligands in a murine asthma models. Our study revealed distinct expression patterns of B7-H1 and B7-DC on a variety of immune cells in the lungs of these animals and provided a new scenario that B7-DC, but not B7-H1, might attenuate Th2-skewed allergic response by an IFN-γ-dependent, but PD-1-independent, mechanism.

Materials and Methods

Animals

Male BALB/c mice, 6–7 wk of age, were purchased from Charles River Japan (Kanagawa, Japan) and were housed in environmentally controlled specific pathogen-free conditions. All animals were maintained in-house for 1 wk before their use in this study. All procedures and protocols were approved by the animal research ethics committee at Kyushu University.

Sensitization and challenge

Mice were sensitized by i.p. injection of 10 μg of chicken OVA (Sigma-Aldrich, St. Louis, MO) and 0.3 mg of aluminum potassium sulfate (SERVA Electrophoresis, Heidelberg, Germany) on days 1 and 11, and then challenged with 5% OVA in saline mist for 20 min on days 19, 21, and 23. The OVA mist was generated using an ultrasonic nebulizer (NE-U17; Omron, Mie, Japan). Control mice were subjected to 0.9% saline sensitization and challenges, and were referred to as naive mice. Subgroups of animals received i.p. injections (250 μg/animal) of anti-mouse B7-H1 mAb (MIH6, rat IgG2a), anti-mouse B7-DC mAb (TY25, rat IgG2a), or control rat IgG (Sigma-Aldrich) 6 h before each sensitization and challenge. Other subgroups received i.p. injections (250 μg/animal) of anti-mouse PD-1 mAb (J43, hamster IgG) (14) or control hamster IgG (BD Biosciences, San Jose, CA) 6 h before each challenge. Two additional anti-mouse PD-1 mAbs, RMP1–14 (rat IgG2a) and RMP1–30 (rat IgG2b), were newly generated against mouse PD-1 transfectants. RMP1–14, but not RMP1–30, blocked the binding of both B7-H1-Ig and B7-DC-Ig to PD-1 transfectants just like J43 (27). These Abs or control rat IgG (Sigma-Aldrich) were administered to subgroups of mice i.p. (250 μg/animal) 6 h before each allergen challenge. In some experiments mice received i.p. injections of 500 μg/animal of anti-mouse IFN-γ mAb (R4-6A2, rat IgG1) or control rat IgG 8 h before their OVA challenges on days 19 and 21 to deplete their endogenous IFN-γ. The total dose of mAb was determined according to the previous study (28). All rat mAbs were purified from ascites by standard procedures using caprylic acid extraction (25). The anti-PD-1 mAb was purified from hybridoma supernatant using protein G columns. The purity of these mAbs was verified by SDS-PAGE analysis. Endotoxin levels were found to be $<$10 ng/ml in 1 mg/ml of the mAb solutions. Measurements of airway reactivity to inhaled acetylcholine, bronchoalveolar lavage, and collection of immune cells from the lungs and draining lymph nodes (DLN) were conducted 24 h after the last OVA challenge.

Collection of lung cells and lymph node cells

Enzymatic digestion of the lungs was performed as previously described (29). Briefly, mice were exanguinated under a lethal dose of pentobarbital, and the lungs were carefully removed, cut, and minced. Two milliliters of complete Dulbecco's PBS containing 0.4 mg/ml collagenase type 1A, 330 U/ml hyaluronidase, 50 U/ml DNase, 10% FBS (all additives from Sigma-Aldrich), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Grand island, NY) were added to the minced lungs, and the suspension was incubated for 60 min at 37°C. The samples were then filtered through a 52-μm pore size nylon mesh. The single-cell suspensions were washed once with completed Dulbecco's PBS, and the erythrocytes were removed by lysis with NH_4Cl-Tris buffer. Cells were suspended in complete RPMI 1640 medium (Sigma-Aldrich) and were adjusted to a density of 2×10^6 cells/ml. DLN cells were collected from paratracheal and mediastinal lymph nodes and were dissociated into a single-cell suspension in complete RPMI 1640.

Flow cytometric analysis

Cells (10^6) were incubated with mAbs against CD3 (145-2C11, hamster IgG), CD4 (RM4-5, rat IgG2a), CD8α (53-6.7, rat IgG2a), CD11c (HL3, hamster IgG1), CD19 (ID3, rat IgG2a), CD86 (GL1, rat IgG2a), PD-1 (J43, hamster IgG), and F4/80 (rat IgG2b; Caltag Laboratories, An Der Grub, Austria). All fluorochrome-labeled mAbs and isotype control IgGs were purchased from BD PharMingen (San Diego, CA) unless otherwise noted. Biotinylated anti-B7-H1 (MIH6, rat IgG2a) and anti-B7-DC mAbs (TY25, rat IgG2a) were used in combination with allophycocyanin-labeled streptavidin. In some experiments, PE-labeled anti-B7-H1 mAb was used in combination with biotinylated anti-B7-DC mAb plus allophycocyanin-labeled streptavidin. Cells were first incubated with unlabeled anti-CD16/32 mAb for 20 min to prevent nonspecific binding via FcγR and were then incubated with FITC-, PE-, and PerCP-labeled mAbs and biotinylated mAb for 30 min. After washing with PBS containing 1% FBS, 0.02% NaN_3, and 0.02% EDTA, referred to as PBS^+, the cells were incubated with allophycocyanin-labeled streptavidin for 20 min. After washing with PBS^+, the cells were fixed with 4% paraformaldehyde (medium A; Caltag Laboratories) for 20 min, washed again with PBS^+, and stored at 4°C until analyzed.

Intracellular cytokine staining was performed as previously reported (29). DLN cells (5×10^6 cells/ml in complete RPMI 1640) were stimulated with OVA (0.4 mg/ml) for 24 h or with PMA (10 ng/ml; Sigma-Aldrich) and 2 μg/ml of ionomycin (Sigma-Aldrich) for 5 h; monensin (0.7 μl/well; GolgiStop; BD PharMingen) was added for the final 4 h of stimulation. The cells were then washed, and cell surface staining was performed using FITC-labeled anti-CD4, PerCP-labeled anti-CD8α, and allophycocyanin-labeled anti-CD3 mAbs, as described above. After fixation with 10% paraformaldehyde (medium A), cells were permeabilized with 0.1% saponin-containing solution (medium B; Caltag) for 10 min, after which they were stained with PE-labeled anti-IFN-γ mAb (XMG1.2, rat IgG1) or isotype control IgG1 for 30 min. After several washes, the cells were suspended in PBS containing 1% paraformaldehyde and analyzed.

It is known that a small number of DCs are double positive for CD11c and F4/80 (30, 31). This might make it difficult to for us distinguish DCs from lung macrophages. Thus, the assessment of costimulatory molecules on APCs in the lung samples was focused on two populations. One was composed of small $CD8\alpha^-CD11c^+$cells (determined by forward scatter) and was regarded as being a myeloid DC-dominant population; our preliminary experiments confirmed that one-third of these cells were also positive for F4/80. The other was composed of large $F4/80^+$ cells, 90% of which were negative for CD11c. This population was regarded as a macrophage-dominant population. Examination of $CD8\alpha^+CD11c^+$ cells, which were thought to be lymphoid DCs, was beyond the scope of our present study because they were extremely scarce in our lung (0.1–0.2% of total digested lung cells) and DLN (0.2–0.3% of total DLN cells) samples.

Measurement of airway hyper-reactivity (AHR)

Measurement of AHR was performed according to our previously described protocol (32). Briefly, animals were anesthetized with a mixture of ketamine and sodium pentobarbital i.p., and their tracheas were cannulated via tracheotomy. The animals were ventilated mechanically (model 687; Harvard Apparatus, South Natick, MA), with a tidal volume of 0.3 ml and at a frequency of 120 breaths/min. The airway opening pressure was measured with a differential pressure transducer (model TP-603T; Nihon Kohden, Tokyo, Japan) and was recorded continuously with a pen recorder (Nihon Kohden RJG-4124). Stepwise increases in acetylcholine (diluted in 0.9% saline; Sigma-Aldrich) concentrations (0.6–10 mg/ml/120 breaths) were administered with an ultrasonic nebulizer (Omron NE-U07). The data were expressed as the provocative concentration 200 (PC_{200}), i.e., the concentration at which airway pressure was 200% of its baseline value, which was calculated by log-linear interpolation for individual animals. Values of PC_{200} were expressed as log ($PC_{200} \times 100$).

Bronchoalveolar lavage (BAL) and cytokine measurements

Mice were exanguinated with a lethal dose of pentobarbital, and their lungs were gently lavaged with 1 ml of 0.9% saline via a tracheal cannula. Total and differential BAL cell counts were performed as previously described (32). Samples were centrifuged at 2000 rpm for 10 min, and the supernatants were stored at −80°C. Mouse IL-4, IL-5, IL-10, IL-13, and IFN-γ were quantified using ELISA kits (BioSource International, Camarillo, CA) according to the manufacturer's protocols.

Data analysis

Values were expressed as the mean ± SEM. Differences among groups were analyzed using unpaired *t* tests or an ANOVA together with a post hoc Bonferroni analysis. Nonparametric data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney *U* test. A value of $p < 0.05$ was considered significant.

Results

Differential expression of B7-H1 and B7-DC on lung leukocytes and DLN cells

The total cell numbers of DCs ($CD8\alpha^-CD11c^+$ cells) in the DLN and DCs, macrophages ($F4/80^+$ cells), and T cells ($CD3^+$ cells) in the lung were significantly elevated in allergen-challenged mice (Fig. 1*A*, *upper panels*). Allergen challenge also significantly increased the percentages of B7-$H1^+$ cells within the DC, macrophage, and B cell ($CD19^+$) populations in the lungs (Fig. 1, *A* and *B*). In contrast, the percentage of B7-$H1^+$ cells in the DC population in the DLNs was significantly reduced in allergen-challenged animals, although the absolute number of B7-$H1^+$ cells was elevated (data not shown). In contrast to the abundant expression of B7-H1, B7-DC expression in the lungs of naive mice was minimal. Significant B7-DC expression was only observed on DCs in the DLNs. Allergen challenge significantly elevated the expression of B7-DC on lung and DLN DCs and macrophages, but this elevated expression was limited, especially on lung macrophages. B7-DC expression was not evident on B or T cells.

In the DLN cells of naive mice, a higher percentage of DCs was found to coexpress CD86 and B7-H1, but the overall percentages of $CD86^+$B7-$H1^+$ and $CD86^-$B7-$H1^+$ cells were reduced in the OVA-challenged mice (Fig. 1*B*). In contrast, $CD86^+$B7-DC^+ DCs in the DLNs were elevated in allergen-challenged mice. As shown in Fig. 1*C*, one-third of the B7-$H1^+$ DCs coexpressed B7-DC, whereas one-half of the B7-DC^+ DCs coexpressed B7-H1. In lung DCs, $CD86^+$ cells were increased after OVA challenge, but only a subpopulation of B7-$H1^+$ and B7-DC^+ DC were found to coexpress CD86 (Fig. 1*B*). An even lower percentage of DCs coexpressed both B7-H1 and B7-DC in the lung (Fig. 1*C*). As for $CD8\alpha^+$ DCs in DLNs, the expression patterns of B7-H1 and B7-DC were similar to those of $CD8\alpha^-$ DCs (data not shown).

B7-H1 was constitutively expressed on lung macrophages in naive mice, and this expression was further enhanced after OVA challenge. B7-DC expression on lung macrophages in naive mice was minimal, but was somewhat enhanced by allergen challenge. Interestingly, CD86 expression on lung macrophages was diminished after allergen challenge. In sum, our results showed that B7-H1 was abundantly expressed on DCs and macrophages, and that there was limited enhancement of B7-DC expression on lung DCs and macrophages in response to allergen challenge.

Expression of PD-1 on T cells in the lungs and on DLN cells

The total number of $CD4^+$ T cells was significantly increased in both the lungs and DLNs, whereas the number of $CD8^+$ T cells was increased only in the DLNs (Fig. 2). PD-1 expression on $CD4^+$ and $CD8^+$ T cells was significantly increased in the DLN cells of OVA-challenged mice. PD-1 was rarely expressed on $CD4^+$ and $CD8^+$ T cells in the lungs of naive mice. The expression of PD-1 on lung $CD4^+$ T cells was increased in OVA-challenged mice, but PD-1 expression on lung $CD8^+$ T cells was limited. Collectively, these data showed that allergen challenge induced a significant expansion and activation in both $CD4^+$ and $CD8^+$ T cells in the DLNs and the preferential infiltration of $CD4^+$ T cells expressing PD-1 in the lung.

Treatment with anti-B7-H1 or anti-B7-DC mAb during allergen sensitization did not affect asthmatic response

Flow cytometric analysis revealed that allergen challenge increased B7-H1 and B7-DC expression on $CD8\alpha^-$ DCs and macrophages, as well as the expression of PD-1 on T cells, in the lungs and DLNs. Airway reactivity to inhaled acetylcholine was significantly higher in OVA-challenged and control IgG-treated mice compared with naive mice. In assessing the effects of treatment with neutralizing anti-B7-H1 and anti-B7-DC mAbs during the sensitization phase on the development of allergen-induced asthmatic responses, we found that such treatment did not affect AHR (Fig. 3*A*). The numbers of lymphocytes, neutrophils, and eosinophils in the BAL fluid (BALF) of OVA-challenged and control IgG-treated mice were significantly increased compared with those of naive mice (Fig. 3*B*). Although treatment with anti-B7-H1 mAb did not affect this inflammatory cell profile, treatment with anti-B7-DC mAb resulted in an elevation in the number of neutrophils in the BALF.

Treatment with anti-B7-DC mAb, but not anti-B7-H1 mAb during allergen challenge accelerated the asthmatic response

Treatment with anti-B7-H1 mAb had no effect on AHR (Fig. 4*A*) or the BALF inflammatory cell profile (Fig. 4*B*). However, treatment with anti-B7-DC mAb during OVA challenge significantly augmented AHR and BALF eosinophilia compared with those in control IgG-treated mice. The effects of mAb treatment on the inflammatory responses were further evaluated by enumerating the T and B cells in the digested lungs. OVA challenge significantly increased the number of $CD4^+$, but not $CD8^+$, T cells; this increase was not affected by either anti-B7-H1 or anti-B7-DC mAb treatment. Interestingly, the number of $CD8^+$ T cells was significantly reduced in response to anti-B7-DC mAb treatment. The number of B cells was not affected by OVA challenge and/or each mAb treatment.

Treatment with anti-PD-1 mAb during the allergen challenge phase did not affect the asthmatic response

Consistent with the observations in Fig. 4, airway reactivity and the lymphocyte, neutrophil, and eosinophil counts in the BALF were significantly enhanced in the OVA-challenged mice. Surprisingly, these asthmatic responses were not affected by treatment with any of three anti-PD-1 mAbs, J43, RMP1–14, or RMP1–30 (Fig. 5).

Treatment with anti-B7-DC mAb enhanced IL-5 and IL-13 and reduced IFN-γ production

OVA challenge dramatically increased IL-4, IL-5, and IL-13; moderately increased IL-10; and slightly increased IFN-γ production in the BALF (Fig. 6*A*). IL-5 and IL-13 production in the BALF was further enhanced by the treatment with anti-B7-DC mAb, but not control IgG. The production of IL-4 and IL-10 production was not affected by anti-B7-DC mAb treatment. Interestingly, IFN-γ production in the BALF was substantially suppressed

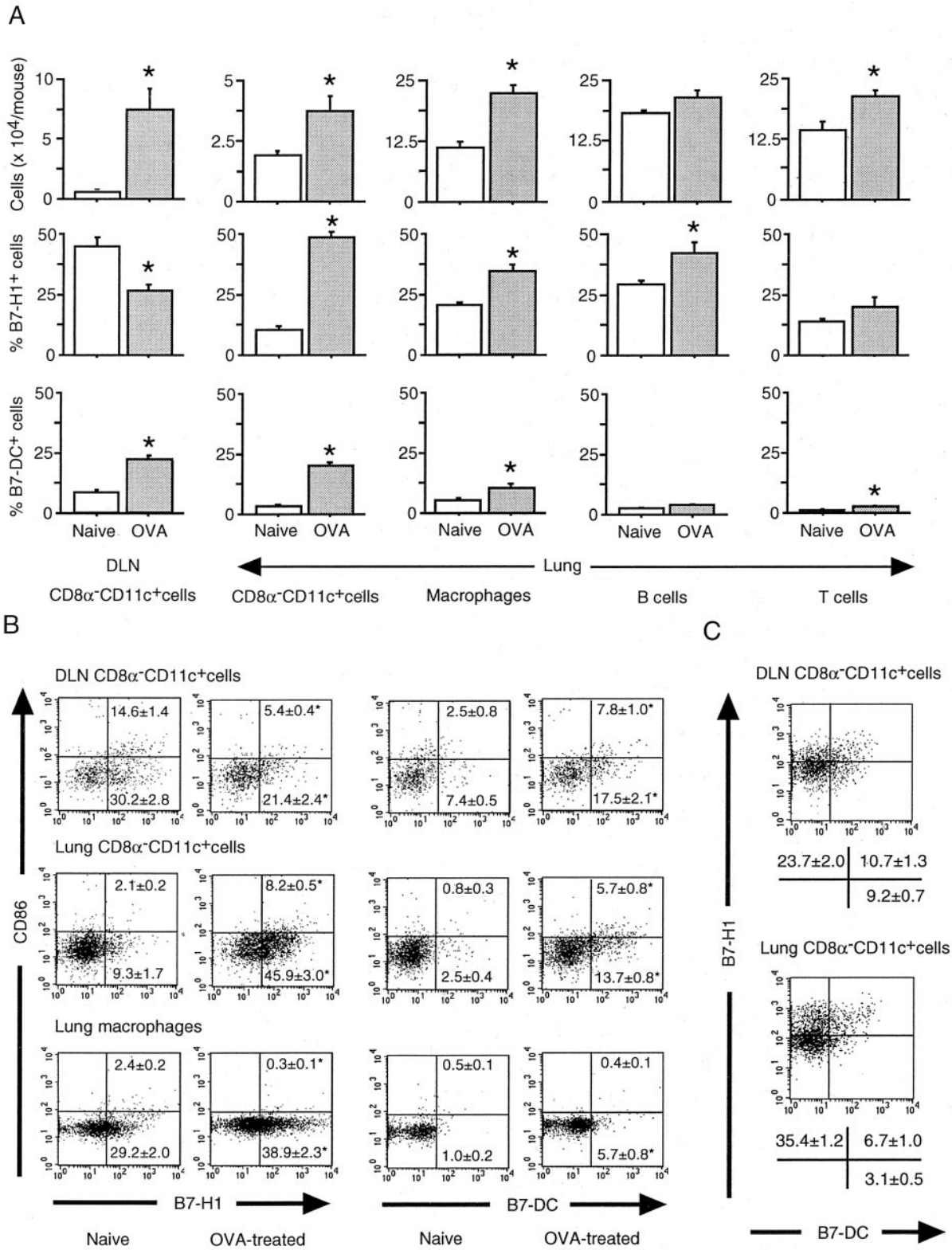

FIGURE 1. Expression of B7-H1 and B7-DC on immune cells in the lung and DLN. A single-cell suspension was prepared from enzymatically digested lungs and DLNs of naive mice or OVA-sensitized/challenged mice (two to four animals per sample). Mice were sensitized with i.p. OVA on days 1 and 11 and were challenged with OVA inhalation on days 19, 21, and 23. Samples were obtained 24 h after the last OVA challenge. Cells were stained with FITC-labeled anti-CD86 mAb; PE-labeled anti-CD11c, anti-F4/80, or anti-CD19 mAb; PerCP-labeled anti-CD3 or anti-CD8α mAb; and biotinylated anti-B7-H1 or anti-B7-DC mAb, followed by allophycocyanin-labeled streptavidin. *A*, The absolute numbers and percentages of B7-H1- or B7-DC-positive cells within $CD8\alpha^{-}CD11c^{+}$ (DCs), $F4/80^{+}$ (macrophages), $CD19^{+}$ (B cells), and $CD3^{+}$ (T cells) lymphocytes are shown, respectively. The columns indicate the mean ± SEM from five to seven mice. B, An electronic gate of flow cytometry was set on each immune cell type, and the expression of the indicated costimulatory molecules is shown as a dot plot. Each dot plot represents three to five independent experiments. The quadrant markers were set so that >99% of control Ig-stained cells were included in the lower left quadrant. The numbers indicate the mean ± SEM percentages of cells with B7-H1 or B7-DC single- or double-positive for CD86. *C*, The expression of B7-H1 and B7-DC was shown by dual staining with PE-labeled anti-B7-H1 mAb and biotinylated anti-B7-DC mAb in combination with allophycocyanin-labeled streptavidin (*C*). *, $p < 0.05$ compared with naive mice.

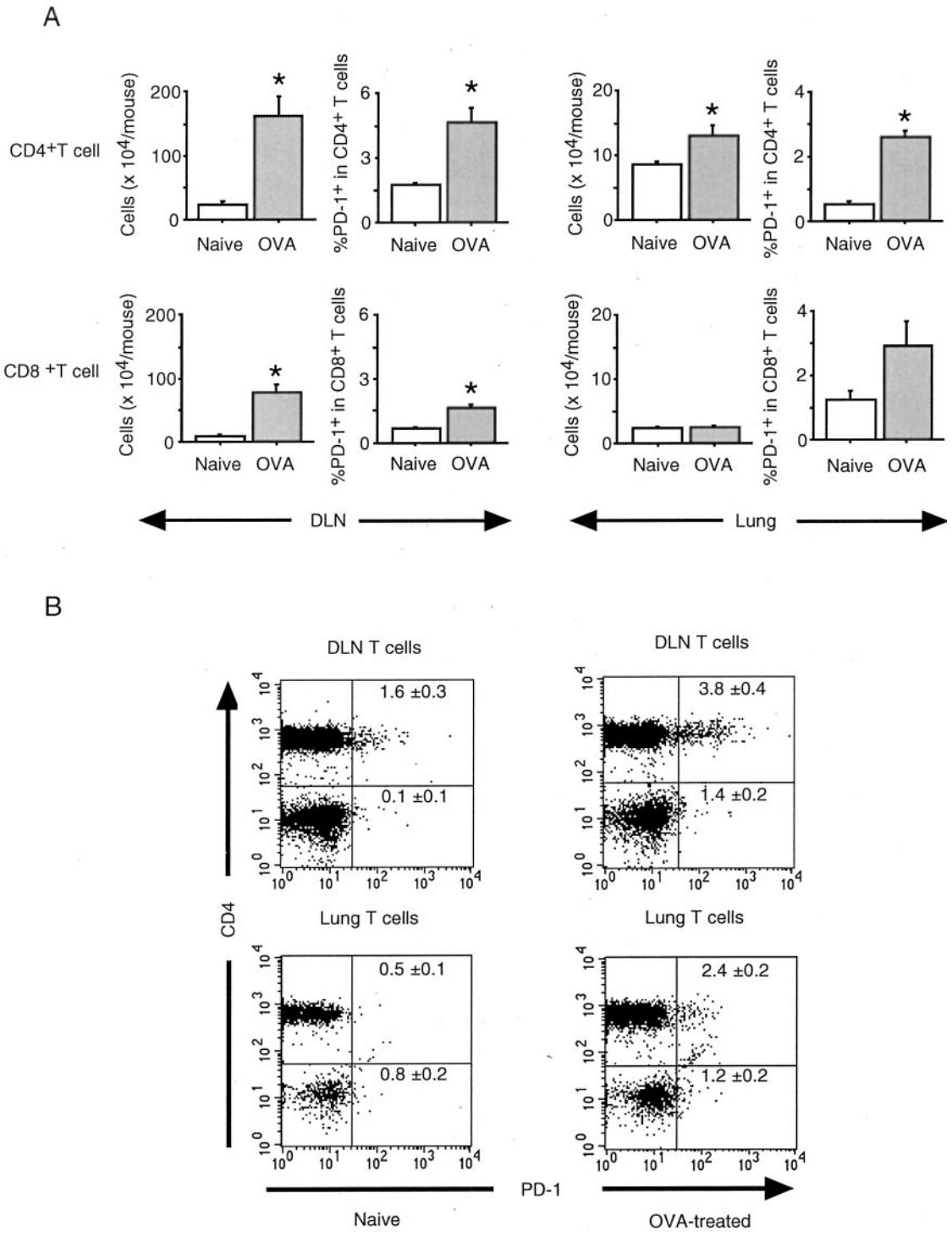

FIGURE 2. Expression of PD-1 on T cells in the lung and DLN. Single-cell suspension was prepared from enzymatically digested lungs and DLNs of naive mice or OVA-sensitized/challenged mice (two to four animals per sample). Mice were sensitized with i.p. OVA on days 1 and 11 and were challenged with OVA inhalation on days 19, 21, and 23. Samples were obtained 24 h after the last OVA challenge. Cells were stained with FITC-labeled anti-CD4 mAb, PE-labeled anti-PD-1 mAb, PerCP-labeled anti-CD8α mAb, and allophycocyanin-labeled anti-CD3 mAb. *A*, The absolute numbers CD4 T cells ($CD4^+CD8^-CD3^+$ cells) and CD8 T cells ($CD4^-CD8^+CD3^+$ cells) and the percentages of PD-1-positive cells in each subset. The columns indicate the mean ± SEM from four or five mice. *B*, An electronic gate of flow cytometry was set on $CD3^+$ cell, and then PD-1 expression is shown as dot plots with reference to CD4 expression. The numbers indicate the mean ± SEM percentages of PD-1-positive cells for $CD4^+$-$CD3^+$ cells or $CD4^-CD3^+$ cells. Each dot plot represents four to five independent experiments. ∗, $p < 0.05$ compared with naive mice.

by anti-B7-DC mAb treatment, which was further confirmed by the reduced intracellular staining of T cells for IFN-γ of DLN T cells that were stimulated with OVA in vitro. The expression of IFN-γ by both DLN $CD4^+$ and $CD8^+$ T cells from mice treated with anti-B7-DC mAb was significantly reduced compared with that seen in control IgG-treated mice (Fig. 6*B*). In contrast, the expression of IFN-γ by both $CD4^+$ and $CD8^+$ DLN T cells that were stimulated with PMA and ionomycin was not inhibited in the

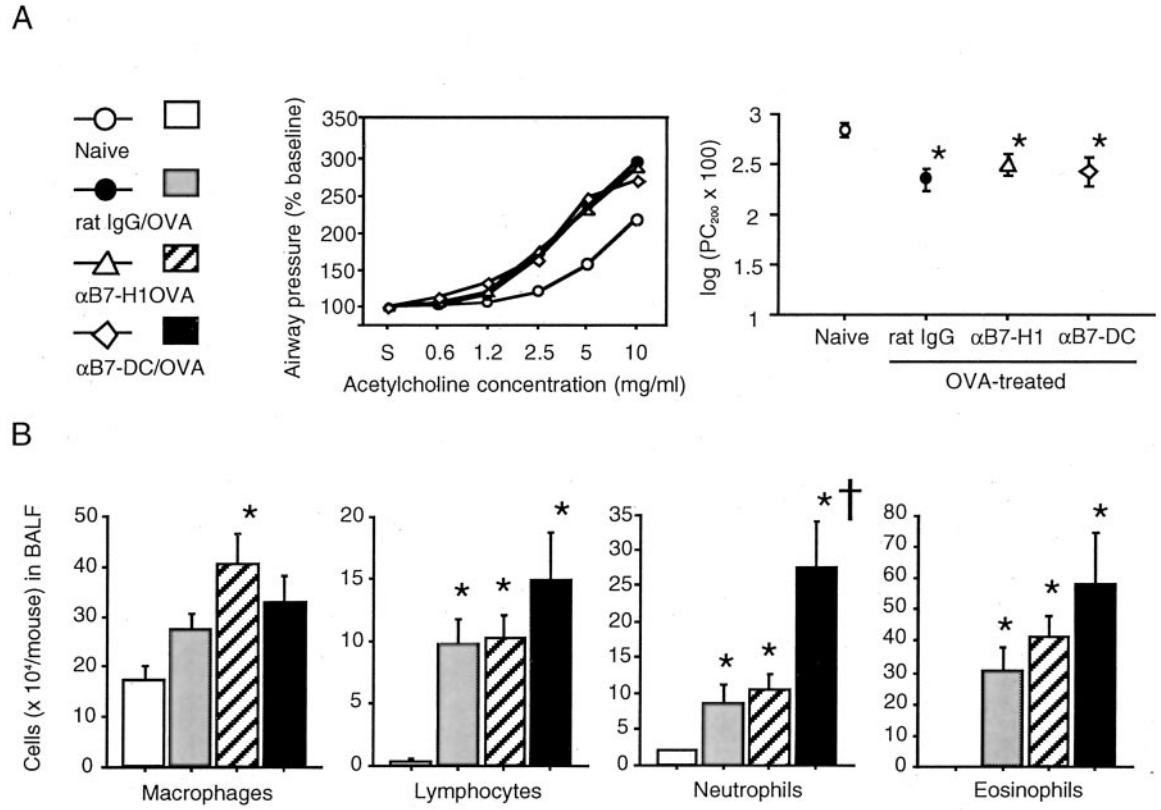

FIGURE 3. Effect of anti-B7-H1 or anti-B7-DC mAb treatment during allergen sensitization on the development of asthmatic responses. Mice were sensitized with i.p. OVA on days 1 and 11, and challenged with OVA inhalation for 20 min on days 19, 21, and 23. AHR and inflammation were assessed 24 h after the last OVA challenge. Subgroups of animals received i.p. injection with 250 μg/animal of anti-B7-H1 mAb, anti-B7-DC mAb, or control rat IgG 6 h before every sensitization. Control mice (naive mice) were subjected to sensitization and challenges with saline. Each group consisted of five to eight mice. Anesthetized mice were ventilated mechanically via tracheotomy, and airway-opening pressure was measured. *A*, Stepwise increases in acetylcholine dose were given with an ultrasonic nebulizer. The data are expressed as a dose-response curve. The provocative concentration 200 (PC_{200}), at which airway pressure was 200% of its baseline value, is expressed as log ($PC_{200} \times 100$). *B*, After the measurement of airway reactivity, bronchoalveolar lavage was performed, and BALF was processed for differential cell counts. Data are expressed as the mean ± SEM. ∗, $p < 0.05$ compared with naive mice; †, $p < 0.05$ compared with control rat IgG-treated mice.

anti-B7-DC mAb-treated mice. These results showed that anti-B7-DC mAb treatment suppressed the allergen-specific production of IFN-γ on T cells from allergen-challenged mice.

Depletion of IFN-γ nullified the effects of anti-B7-DC mAb treatment

Although neutralization of IFN-γ did not affect AHR, it did result in a significant increase in the number of eosinophils in the BALF (Fig. 7). This finding was consistent with previous reports that depletion of IFN-γ augmented eosinophilic inflammation without affecting AHR (28, 33). The augmented AHR and eosinophilia that occurred as a result of anti-B7-DC mAb treatment were not seen in IFN-γ-depleted mice. Similarly, the enhanced production of IL-5 and IL-13 in the BALF that was observed in anti-B7-DC mAb-treated mice (Fig. 6*A*) was reversed in the IFN-γ-depleted animals. These results indicated that the exaggerated effects of the anti-B7-DC mAb treatment on the asthmatic response were mediated by an IFN-γ-dependent mechanism.

Discussion

In this study we demonstrated that treatment with anti-B7-DC mAb at the time of allergen challenge, but not at the time of sensitization, augmented the asthmatic responses; this conclusion was based on the demonstration of an increase in lung eosinophilia and AHR in these animals. Such treatment also resulted in increased IL-5 and IL-13 and decreased IFN-γ production in the BALF. These effects of anti-B7-DC mAb treatment were diminished in animals that were depleted of IFN-γ. Interestingly, the treatment of animals with anti-PD-1 mAb did not affect the asthmatic responses in this model.

Despite the abundant expression of B7-H1 on DCs and macrophages in the inflamed lung and DLN cells after allergen challenge, surprisingly, treatment with anti-B7-H1 mAb did not affect lung eosinophilia and AHR in these animals. It is unlikely that our anti-B7-H1 mAb failed to effectively bind and block B7-H1, as treatment with this same Ab effectively enhanced immune responses that were mediated by PD-1/B7-H1 interactions in animals belonging to the autoimmune nonobese diabetic (NOD) (27) and hapten-induced contact hypersensitivity (34) models. Although PD-1 has been implicated in the negative regulation of T cell functions (16–18, 20, 21, 35), several reports (19, 36, 37) have shown that its ligand, i.e., B7-H1, plays a role in providing costimulation for T cell effector functions. It has been shown that B7-H1 on tumor cells promoted apoptosis in effector CTL and that this apoptotic effect was probably mediated by PD-1 or receptors other than PD-1 (36, 38). Treatment with B7-H1Ig was shown to prolong cardiac allograft survival, and this prolongation was accompanied by reduced expression of IFN-γ within the graft (39). Thus, it is possible that the regulatory and

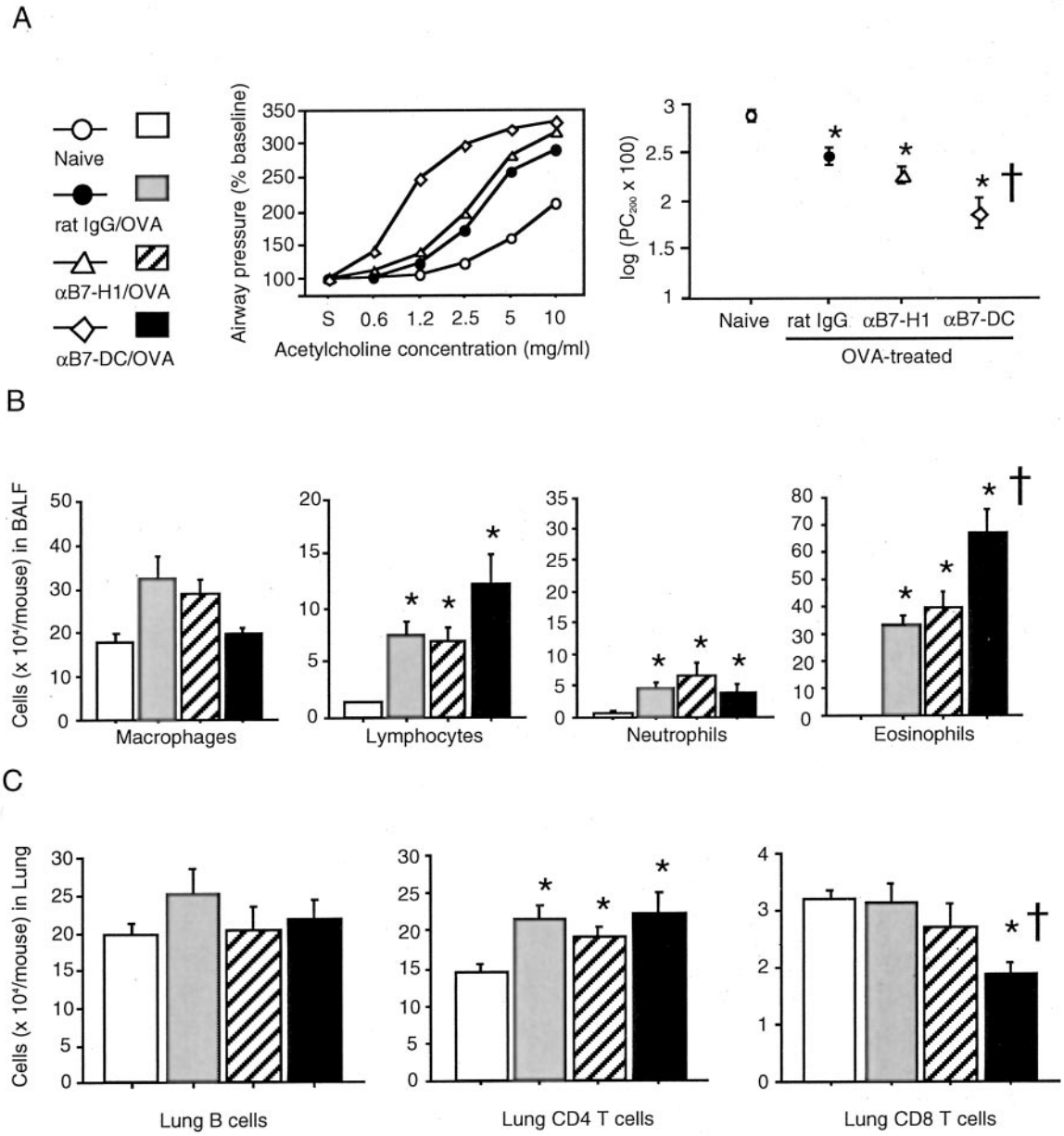

FIGURE 4. Effect of anti-B7-H1 or anti-B7-DC mAb treatment during allergen challenge on asthmatic responses. Mice were sensitized and challenged with OVA as described in Fig. 3. AHR (*A*) and inflammation (*B*) were assessed for animals treated with anti-B7-H1 mAb, anti-B7-DC mAb, or control rat IgG 6 h before every OVA challenge (days 19, 21, and 23). Control mice (naive mice) were subjected to sensitization and challenges with saline. Each group consisted of five to eight mice. *C*, Single-cell suspension was obtained by enzymatic digestion of the lungs (two or three animals per sample). Cells were stained with FITC-labeled anti-CD4 mAb, PerCP-labeled anti-CD8α mAb, and allophycocyanin-labeled anti-CD3 mAb and were analyzed by flow cytometry. Values are expressed as the mean ± SEM. The absolute numbers of B cells, CD4 T cells, and CD8 T cells were calculated by multiplying the total cell count. ∗, $p < 0.05$ compared with naive mice; †, $p < 0.05$ compared with control rat IgG-treated mice.

costimulatory functions that are mediated by PD-1 and unknown receptor(s) can offset the B7-H1-mediated effects in the experimental asthma model.

It should be noted that the augmentation of the asthmatic response by anti-B7-DC mAb was only observed when treatment was given during the allergen challenge, but not sensitization, period. This finding suggests that B7-DC preferentially mediates the effector phase of the asthmatic response, which includes the migration and activation of pathogenic Th2 cells into the lung, but not in the induction phase, which results in the development of pathogenic Th2 cells. It is noteworthy that anti-B7-DC mAb treatment altered the cytokine profiles of the BALF, with increases seen in the Th2 cytokines, IL-5 and IL-13, and a decrease in Th1 cytokine, IFN-γ. Furthermore, the number of $CD8^+$ T cells that was recruited into the lung was significantly reduced, whereas the number of recruited $CD4^+$ T cells was not affected. As $CD8^+$ T cells are the dominant producers of IFN-γ, the diminished recruitment of $CD8^+$ T cell into the lung may partly account for the reduction in IFN-γ seen in that organ. The inhibition of IFN-γ production by $CD4^+$ and $CD8^+$ T cells was only observed in the response to a specific allergen, i.e., OVA, and not in response to nominal TCR signals, suggesting that this inhibition was Ag specific. Anti-B7-DC mAb treatment during the effector phase might alter the balance between Th1/CTL and Th2 cells. In a murine model of allergic asthma, accumulating evidence suggests that Th2 cytokines, including IL-4, IL-5, and IL-13 mediate allergen-induced

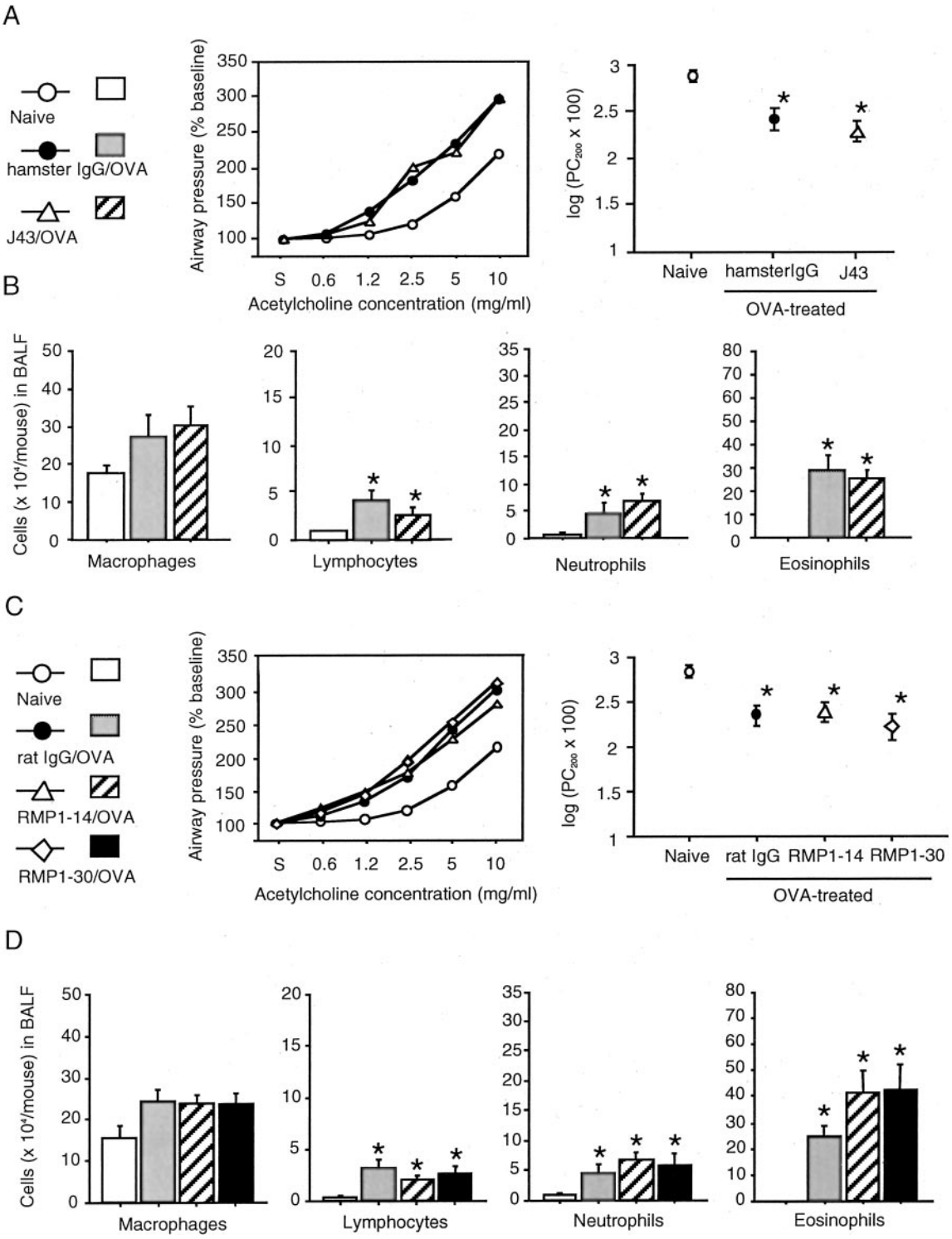

FIGURE 5. Effect of anti-PD-1 mAb treatment during allergen challenge on asthmatic responses. Mice were sensitized and challenged with OVA as described in Figs. 3 and 4. AHR (*A* and *C*) and inflammation (*B* and *D*) were assessed for animals treated with anti-PD-1 mAbs (J43, RMP1–14, or RMP1–30) or control IgG (hamster IgG for J43 or rat IgG for RMP1–14/RMP1–30) 6 h before every OVA challenge (days 19, 21, and 23). Control mice (naive mice) were subjected to the sensitization and challenges with saline. Each group consisted of nine mice. Values are expressed as the mean ± SEM. *, $p < 0.05$ compared with naive mice.

airway eosinophilia and AHR, whereas IFN-γ and IL-12 down-regulate these responses (40). Allergen-induced airway eosinophilia was shown to be augmented by treatment with anti-IFN-γ mAb during allergen challenge (28) and in IFN-γ-deficient mice (33). Therefore it is possible that blockade of B7-DC might initially suppress IFN-γ production, resulting in enhanced Th2 cell activation and eosinophil recruitment into the lung. In support of this idea, we found that the increased severity of the asthmatic response that was seen in anti-B7-DC mAb-treated mice diminished after the neutralization of endogenous IFN-γ. These data suggest an initial and critical role of IFN-γ in the B7-DC-mediated regulation of the asthmatic response.

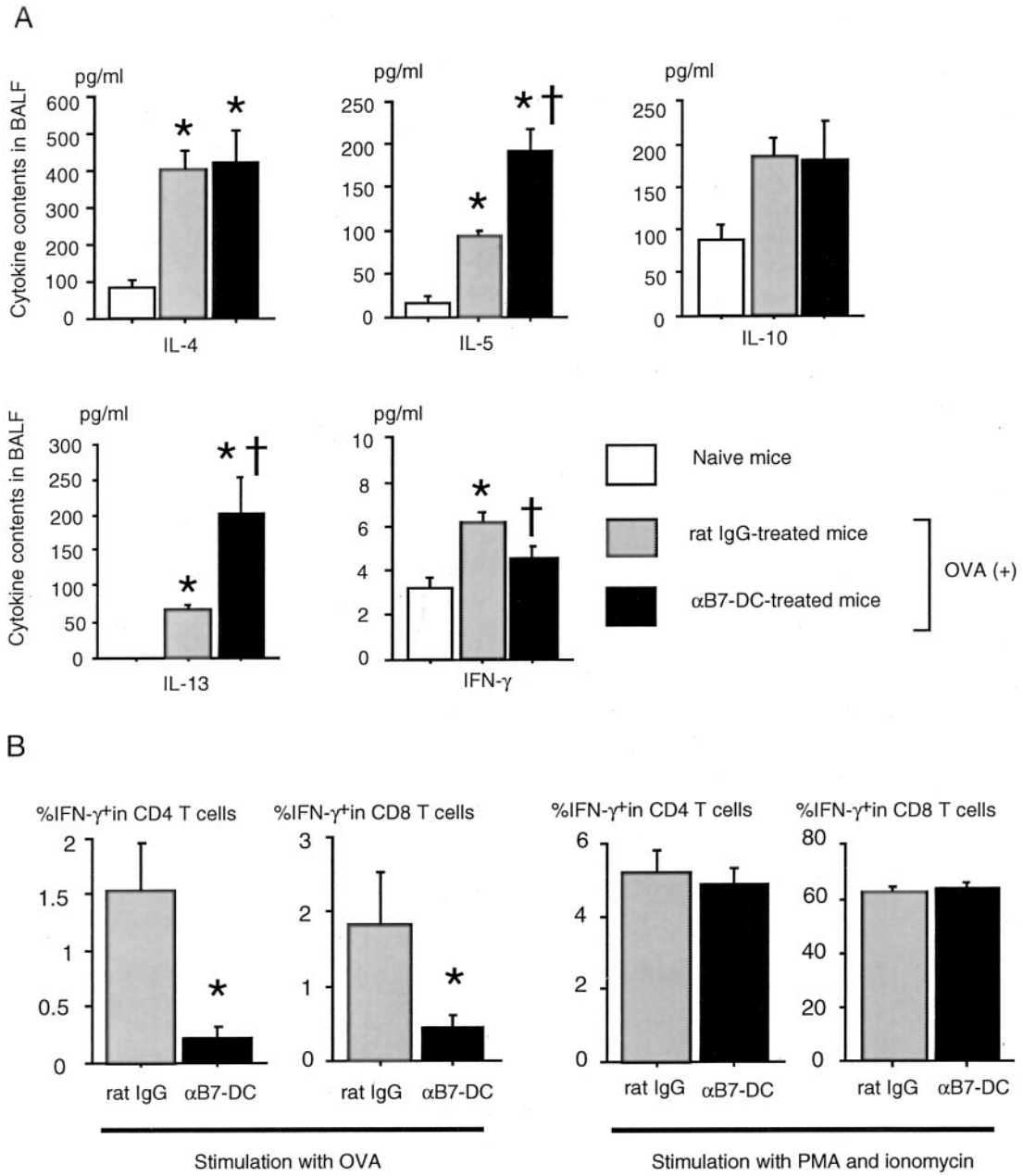

FIGURE 6. Effect of anti-B7-DC mAb treatment during allergen challenge on cytokine profile in BALF. *A*, Concentrations of IL-4, IL-5, IL-10, IL-13, and IFN-γ in BALF were measured by ELISA for naive and OVA-challenged mice. The OVA-challenged mice received i.p. injection of 250 μg/animal of either anti-B7-DC mAb or control rat IgG 6 h before every OVA challenge (days 19, 21, and 23). Control mice (naive mice) were subjected to sensitization and challenges with saline. Values are expressed as the mean ± SEM. Each group consisted of five to eight mice. *, $p < 0.05$ compared naive mice; †, $p < 0.05$ compared with control rat IgG-treated mice. *B*, IFN-γ-producing CD4 T cells in DLN were assessed by intracellular cytokine staining. Single-cell suspensions of DLN were stimulated with OVA (0.4 mg/ml) for 24 h or with PMA (10 ng/ml) plus ionomycin (2 μg/ml) for 5 h. Monensin was added during the last 4 h of stimulation. Cells were stained with FITC-labeled anti-CD4 mAb, PerCP-labeled anti-CD8α mAb, and allophycocyanin-labeled anti-CD3 mAb, fixed and permeabilized with 0.1% saponin, then stained with PE-labeled anti-IFN-γ mAb or isotype-matched IgG1. The columns indicate the mean ± SEM of the percentage of IFN-γ-positive cells from four mice. *, $p < 0.05$ compared with naive mice; †, $p < 0.05$ compared with control rat IgG-treated mice.

In contrast to the abundant expression of B7-H1, B7-DC expression in the inflamed lung was limited, especially on lung macrophages. Consistent with previous reports (6, 41–43), we observed reduced expression of CD86 and B7-DC on lung macrophages compared with other peripheral tissues. Similar findings were reported in the CNS of mice with experimental autoimmune encephalomyelitis (44). Interestingly, treatment with anti-B7-DC mAb, but not anti-B7-H1 mAb similarly augmented disease in the experimental autoimmune encephalomyelitis model.

We cannot completely rule out the possibility that the anti-B7-DC mAb (TY25) might have acted as an activator of B7-DC and that it activated Th2 cells. Recent reports suggested that the ligation of B7-DC by the cross-linking of IgM Abs may activate DCs and promote T cell effector functions (45, 46). However, both intact TY25 and Fab of TY25 were found to block the B7-DC-mediated cellular responses in a similar manner in vitro (H. Yagita, unpublished observations). In addition, the in vivo treatment of NOD mice (27) and mice with contact hypersensitivity (34) with

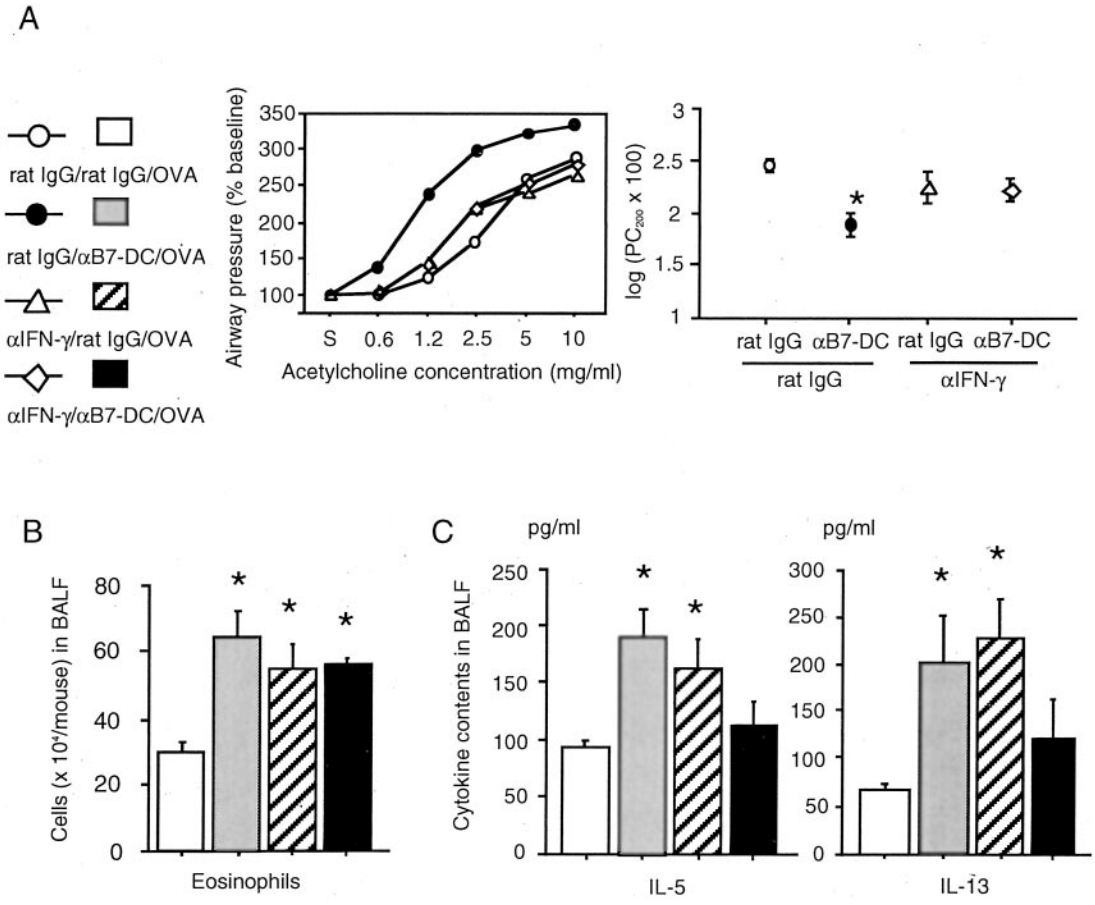

FIGURE 7. Effect of anti-B7-DC mAb in IFN-γ-depleted mice. OVA-sensitized mice received i.p. injection of 500 μg/animal of anti-IFN-γ mAb or control rat IgG 8 h before the first and second OVA challenges (days 19 and 21) for depleting endogenous IFN-γ. The mice subsequently received i.p. injection of 250 μg/animal of either anti-B7-DC mAb or control rat IgG 6 h before every OVA challenge (days 19, 21, and 23). These mice were used for assessment of AHR (*A*), eosinophil count in BALF (*B*), and IL-5 and IL-13 ELISA (*C*) in BALF. Data are represented as the mean ± SEM of five to seven mice in each group. *, $p < 0.05$ compared with naive mice.

TY25 failed to affect their pathological outcomes. These results support the idea that TY25 treatment can block the interaction of B7-DC with its receptor.

B7-DC is known to provide a negative signal, via an immunoreceptor tyrosine-based inhibitory motif in the PD-1 cytoplasmic region (21). A recent study has shown that blockade of B7-DC on DCs augmented IFN-γ production by alloreactive T cells (47). Thus, the augmentation of asthmatic response by anti-B7-DC mAb might be due to blocking of a negative signal provided by B7-DC on IL-5 and IL-13 production. However, this speculation does not explain the reciprocal decrease in IFN-γ in BALF by anti-B7-DC mAb treatment, whereas mouse B7-DC transfectants inhibited IFN-γ production by OVA peptide-stimulated $CD4^+$ T cells from DO11.10 transgenic mice in vitro (21). In addition, the diminished augmentation of IL-5 and IL-13 production by anti-B7-DC mAb in mice pretreated with anti-IFN-γ mAb renders it difficult to fully explain the whole phenomena in the context of inhibitory function of B7-DC.

Given the original concept that PD-1 ligation delivers an inhibitory signal, a remaining question is how B7-DC can stimulate IFN-γ production. Intriguingly, the treatment with anti-PD-1 mAbs did not affect airway eosinophilia and AHR. Previous studies using the same anti-mouse PD-1 mAb (J43), effectively blocked PD-1-mediated regulation of experimental models for autoimmune encephalomyelitis, autoimmune diabetes, graft-vs-host response, and contact hypersensitivity (27, 34, 44, 48). We included newly established anti-PD-1 mAb, RMP1–14, which blocks the binding of both B7-H1-Ig and B7-DC-Ig to PD-1 in vitro. We have recently found that RMP1–14 treatment exacerbated experimental autoimmune encephalomyelitis (H. Yagita, unpublished observation), as did J43 (44). Tseng et al. (22) showed that naive T cells that were costimulated with B7-DC-Ig in the presence of anti-CD3 mAb enhanced their production of IFN-γ. but not that of IL-4 and IL-10. A recent report demonstrated that a B7-DC mutant with impaired PD-1 binding capacity was still able to provide costimulation for the production of IFN-γ by T cells (49). Furthermore, it was shown that DCs from B7-DC-deficient mice diminished their ability to costimulate proliferation and IFN-γ production by $CD4^+$ T cells and that this costimulation was independent of PD-1 (50). These reports provide strong evidence of the existence of a second receptor, in addition to PD-1, which is capable of delivering a costimulatory signal. In our model, B7-DC might function through such a novel receptor independently of PD-1.

In the IFN-γ-depleted mice, the treatment with anti-B7-DC mAb failed to enhance IL-5 and IL-13 production. If it is the case that B7-DC could deliver costimulatory signals to Th2 cells for production of IL-5 and IL-13 as well as to Th1 cells (and CTL) for IFN-γ production, and the ability for costimulation would be more potent for Th1 cells, the regulation of asthmatic responses by

IFN-γ could overcome the potential enhancement of IL-5 and IL-13 production. To clarify this issue, further studies are now underway using B7-DC$^{-/-}$ and IFN-γ$^{-/-}$ mice.

Two unique properties of B7-DC have been suggested by the above findings. First, as suppression of Th2 responses is specific to B7-DC, but not to B7-H1, the disparate effects of B7-H1 and B7-DC blockade on different immune responses and disease models raise the possibility that B7-H1 and B7-DC (or PD-1 and an unknown receptor) may preferentially regulate Th1 and Th2 responses (26). Second, the B7-DC blockade is observed in the effector phase of the immune response. These findings suggest that the regulation of B7-DC activity may have therapeutic implications for the control of asthma in humans. Further investigation of the molecular and cell biology of B7-H1 and B7-DC will undoubtedly lead to a broadening of our understanding of the mechanisms of immune diseases, including asthma.

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