

B7-H3 Contributes to the Development of Pathogenic Th2 Cells in a Murine Model of Asthma¹

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B7-H3 is a new member of the B7 family. The receptor for B7-H3 has not been identified, but it seems to be expressed on activated T cells. Initial studies have shown that B7-H3 provides a stimulatory signal to T cells. However, recent studies suggest a negative regulatory role for B7-H3 in T cell responses. Thus, the immunological function of B7-H3 is controversial and unclear. In this study, we investigated the effects of neutralizing anti-B7-H3 mAb in a mouse model of allergic asthma to determine whether B7-H3 contributes to the development of pathogenic Th2 cells and pulmonary inflammation. Administration of anti-B7-H3 mAb significantly reduced airway hyperreactivity with a concomitant decrease in eosinophils in the lung as compared with control IgG-treated mice. Treatment with anti-B7-H3 mAb also resulted in decreased production of Th2 cytokines (IL-4, IL-5, and IL-13) in the draining lymph node cells. Although blockade of B7-H3 during the induction phase abrogated the development of asthmatic responses, B7-H3 blockade during the effector phase did not inhibit asthmatic responses. These results indicated an important role for B7-H3 in the development of pathogenic Th2 cells during the induction phase in a murine model of asthma. *The Journal of Immunology*, 2008, 181: 4062–4071.

Allergic asthma is characterized by chronic airway inflammation with massive infiltration of eosinophils, increased mucus production in the bronchioles, and airway hyperreactivity (AHR)⁴ to a variety of specific and nonspecific stimuli. Although a variety of cell types is involved in allergic inflammation, there is substantial evidence that infiltration of Th2 cells is common in the lungs of allergic asthmatic subjects, as are increased levels of Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13, particularly in the late phase (1, 2). The level of Th2 cytokines appears to correlate with severity of disease. These cytokines are responsible for the recruitment and activation of other cell types, including eosinophils, which have been associated with lung injury. They also mediate the production of mucus by the airway epithelium, contributing to the airway obstruction that is a major component of the pathology of asthma (3–5). Although the critical roles of these Th2 cytokines in the pathogenesis of allergic asthma have been established, the mechanisms for the overproduction of Th2 cytokines in asthmatic responses have not been fully understood.

Optimal activation of Ag-specific T cells requires engagement of the TCR with Ag/MHC and a costimulatory signal provided by APCs (6, 7). It is well known that CD28-B7 (CD80 and CD86) interaction plays a major role in providing a costimulatory signal for T cells (8, 9). Blocking mAb to CD86 inhibits the development of specific IgE, pulmonary eosinophilia, and AHR in mice (10). CTLA-4 is a second receptor for CD80 and CD86, and CTLA-4-Ig, a soluble fusion protein, has also been effective in blocking AHR (11, 12). Blockade of these costimulatory pathways markedly reduced allergen-induced asthma in mice, suggesting a promising approach for the treatment of allergic disease in humans.

B7-H3 is a recently identified member of the B7 family (13, 14). Human B7-H3 protein is not expressed constitutively on monocytes, B cells, T cells, or NK cells, but it can be induced on these cell types by some stimulation (13, 15). Mouse B7-H3 mRNA expression is stimulated in dendritic cells (DCs) by IFN- γ , but suppressed by IL-4 (16). LPS stimulates B7-H3 protein expression on mouse DCs, B cells, and macrophages (16). The receptor for B7-H3 has not yet been identified, but it is thought to be expressed by activated T cells and is apparently distinct from other known T cell costimulatory receptors (13, 14). Some studies support roles for B7-H3 as a stimulator or an inhibitor of T cell responses. Initial studies showed that in conjunction with anti-CD3, B7-H3-Ig fusion protein costimulated human CD4⁺ and CD8⁺ T cell proliferation and IFN- γ production (13). A subsequent study also showed a costimulatory effect of B7-H3 on tumor immunity in a mouse model (17). However, recent studies have indicated that B7-H3 has an inhibitory function. Proliferation and cytokine production of purified human CD4⁺ T cells in response to anti-CD3 and anti-CD28 were inhibited by addition of B7-H3-transfected cells (18). Immobilized mouse B7-H3 inhibited mouse CD4⁺ and CD8⁺ T cell proliferation and IL-2 and IFN- γ production induced by anti-CD3 (16). Studies with B7-H3-deficient mice also support both stimulatory and inhibitory functions of B7-H3. B7-H3 has been proposed to promote T cell-mediated immune responses, based on the reduced rates of acute and chronic cardiac allograft rejection in B7-H3-deficient mice as compared with wild-type

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⁴ Abbreviations used in this paper: AHR, airway hyperreactivity; AB, Alcian blue; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived dendritic cell; BMMacs, bone marrow-derived macrophages; DC, dendritic cell; LN, lymph node; PAS, periodic acid-Schiff; Penh, enhanced pause; rmB7-H3, mouse rB7-H3.

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recipients (19). In contrast, under Th1-polarizing condition, B7-H3-deficient mice developed more severe airway inflammation than control wild-type mice (16). Thus, the immunological function of B7-H3 is controversial and still unclear. This study therefore examined the contribution of B7-H3 to the development of allergic asthma by administering a newly generated anti-B7-H3 mAb to a mouse model of asthma, particularly focusing on the role in induction and effector phases. Our present results suggest an important role for B7-H3 in the development of pathogenic Th2 cells during the induction phase, but not in the recruitment and/or activation of Th2 cells in the lung during the effector phase.

Materials and Methods

Animals and cells

Female BALB/c mice were purchased from CLEA Japan. All mice were 6–8 wk old at the start of experiments and kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee and complied with National Institutes of Health guidelines for animal care. Six-week-old female Sprague Dawley rats were purchased from Charles River Laboratories. Mice transgenic for the OVA_{323–339}-specific and I-A^d-restricted DO11.10 TCR- $\alpha\beta$ on a Rag-2^{-/-} BALB/c background were supplied by S. Koyasu (Keio University School of Medicine, Tokyo, Japan) (20). Stable L5178Y (murine T lymphoma) and P815 (murine mastocytoma) cells expressing B7-1, B7-2, B7-H1 (PD-L1), B7-DC (PD-L2), or B7RP-1 (B7-H2) were established previously (21–23). A cDNA fragment encoding the entire open reading frame of mouse B7-H3 was prepared by RT-PCR. The PCR product was cloned into pMKITneo vector and transfected into L5178Y and P815 by electroporation. Muirne B7-H4-transfected P815 cells were provided by M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan). These cells were cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μ M 2-ME.

Generation of anti-mouse B7-H3 mAbs

The anti-mouse B7-H3 mAbs were generated by immunizing Sprague Dawley rats with B7-H3-Ig, consisting of the extracellular domain (aa 1–242 of mouse B7-H3) (14) linked to the Fc portion of mouse IgG2a, emulsified in CFA (Difco). Three days after the final immunization, lymph node (LN) cells were fused with P3U1 myeloma cells. After hypoxanthine-aminopterin-thymidine selection, hybridomas (MJ8, rat IgG2b/ κ , and MJ18, rat IgG1/ κ) producing anti-B7-H3 mAb were selected by their reactivity to mouse B7-H3-transfected cells, but not to parental cells by flow cytometry, and then cloned by limiting dilution. The mAbs were purified from ascites by the caprylic acid and ammonium sulfate precipitation method, and purity was verified by SDS-PAGE analysis.

Flow cytometric analysis

Cells ($0.5\text{--}1 \times 10^6$) were first preincubated with unlabeled anti-CD16/32 mAb to avoid nonspecific binding of Abs to Fc γ R, and then incubated with biotinylated mAbs. After washing with PBS twice, the cells were incubated with PE-labeled streptavidin. Anti-CD86 (GL1) and anti-B7-H4 (clone 9) mAbs, rat IgG isotype control, and PE-labeled streptavidin were purchased from eBioscience. The hybridomas producing mAb against B7-1 (1G10) were purchased from American Type Culture Collection. Anti-B7RP-1 (HK5.3), anti-B7-H1 (MIH6), and anti-B7-DC (TY25) mAbs were prepared, as described previously (22, 23). After washing with PBS twice, the stained cells (live gated on the basis of forward and side scatter profiles and propidium iodide exclusion) were analyzed on a FACSCalibur (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences).

In vitro T cell proliferation assay

CD4⁺ T cells were purified from the spleen of DO11.10/Rag-2^{-/-} mice or BALB/c mice by passage through nylon wool column (Wako Biochemicals) and by using autoMACS columns with CD4⁺ T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Purified CD4⁺ T cells (1×10^5 /well) from DO11.10/Rag-2^{-/-} mice were cultured with bone marrow-derived DCs (BMDCs; 2×10^4 /well) and 2 μ M OVA_{323–339} peptide in the presence or absence of anti-B7-H3 mAbs (10 μ g/ml). Purified CD4⁺ T cells (1×10^5 /well) from BALB/c mice were stimulated with immobilized anti-CD3 (2C11) mAb (0.5 μ g/ml) and mouse rB7-H3 (rmB7-H3) (2 μ g/ml; R&D Systems) in the presence or

absence of anti-CD28 (PV-1) mAb (0.5 μ g/ml) and anti-B7-H3 mAbs (10 μ g/ml). Anti-CD28 (PV-1) mAb was provided by R. Abe (Tokyo University of Science, Chiba, Japan) and C. June (University of Pennsylvania, Philadelphia, PA). To assess proliferative responses, the cultures were pulsed with [³H]thymidine (0.5 μ Ci/well; GE Healthcare) for the last 6 h of a 48-h culture and harvested on a Micro 96 Harvester (Molecular Devices). Incorporated radioactivity was measured on a microplate beta counter (Micro β Plus; Wallac).

Induction of allergic airway inflammation and mAb treatment

Groups of 10 mice were sensitized by i.p. injection of 10 μ g of OVA (Sigma-Aldrich) with 2 mg of alum (Pierce) (OVA/alum) on days 0 and 14. On days 22, 24, 26, and 28, the mice inhaled aerosolized 30 ml of 1% OVA in PBS (OVA/PBS). The aerosol was generated by nebulizer (NE-U07; Omron). Some groups of mice were i.p. injected with 300 μ g of anti-B7-H3 mAb (MJ18) or control rat IgG (Sigma-Aldrich) on days 0, 2, 4, 7, 11, 14, 16, 18, 21, 24, and 27. Negative control animals were sensitized by i.p. injection of PBS with alum (PBS/alum) on days 0 and 14, and inhaled PBS in a similar manner. Two days after the last OVA inhalation, AHR was measured and bronchoalveolar lavage fluid (BALF) was collected, as described below. To examine the effect of anti-B7-H3 mAb in the induction phase, mice were injected with 300 μ g of anti-B7-H3 mAb or control rat IgG on days 0, 2, 4, 7, 11, 14, 16, and 18. To examine the effect in the effector phase, mice were injected with the same dose of anti-B7-H3 mAb or control rat IgG on days 21, 24, and 27.

Measurement of AHR

AHR was measured by methacholine (WAKO)-induced airflow obstruction. Mice were placed into whole-body plethysmographs (Buxco Research Systems) interfaced with computers using differential pressure transducers. Airway resistance is expressed as: enhanced pause (Penh) = ((Te/0.3Tr) – 1) $\times$ (2Pef/3Pif), where Te = expiratory time (s), Tr = relaxation time (s), Pef = peak expiratory flow (ml), and Pif = peak inspiratory flow (ml/s). Increasing doses of methacholine were administered by nebulization (for 2 min), and Penh were calculated over the subsequent 5 min.

Characterization of BALF

After AHR measurement, the trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged with 0.5 ml of PBS containing 10% FCS four times (2 ml total of BALF). The total number of cells was determined by Turk dye exclusion. Differential cell count was performed by staining of cytopspins with Diff-Quik (Sysmex International Reagents).

Histological analysis of lung sections

Lungs were removed from mice that were not subjected to the bronchial lavage procedure. Lungs were inflated and fixed by intratracheal instillation of 20% buffered formalin (pH 7.4) at a constant pressure of 25 cm H₂O for 24 h. After routine processing of successive dehydration, the lungs were embedded in paraffin and sectioned in a sagittal plain. Sections of 5 μ m thickness were stained with H&E or periodic acid-Schiff (PAS) and Alcian blue (AB) as a measure of mucus production. To quantify the infiltration of eosinophils in the lung tissue, cell counting was performed in a blinded manner based on a 5-point scoring system, as follows: 0, no eosinophil; 1, few eosinophils; 2, a ring of eosinophils one cell layer deep; 3, a ring of eosinophils two to four cells deep; 4, a ring of eosinophils more than four cells deep. Scoring was performed in at least 15 different fields for each lung section. Mean scores were obtained from six animals. Airway mucus levels were determined by counting PAS- or AB-positive cells as a percentage of total epithelial cells in a blinded manner, according to methods described (24).

Stimulation of bronchial LN cells in vitro

After AHR measurement, bronchial LN cells were isolated and pooled, and cultured in 96-well flat-bottom microculture plates at a density of 6×10^5 cells/well in the presence or absence of the indicated doses of OVA. To assess proliferative responses, the cultures were pulsed with [³H]TdR (0.5 μ Ci/well) for the last 6 h of a 72-h culture and harvested on a Micro 96 Harvester. Incorporated radioactivity was measured on a microplate beta counter. To determine the production of cytokines, cell-free supernatants were collected at 72 h and assayed for IL-4, IL-5, and IL-10 by ELISA using OptEIA kits (BD Biosciences), IL-13 using Quantikine Immunoassay (R&D Systems), and IFN- γ using Mouse IFN- γ ELISA Ready-SET-Go! kit (eBioscience), according to the manufacturer's instructions.

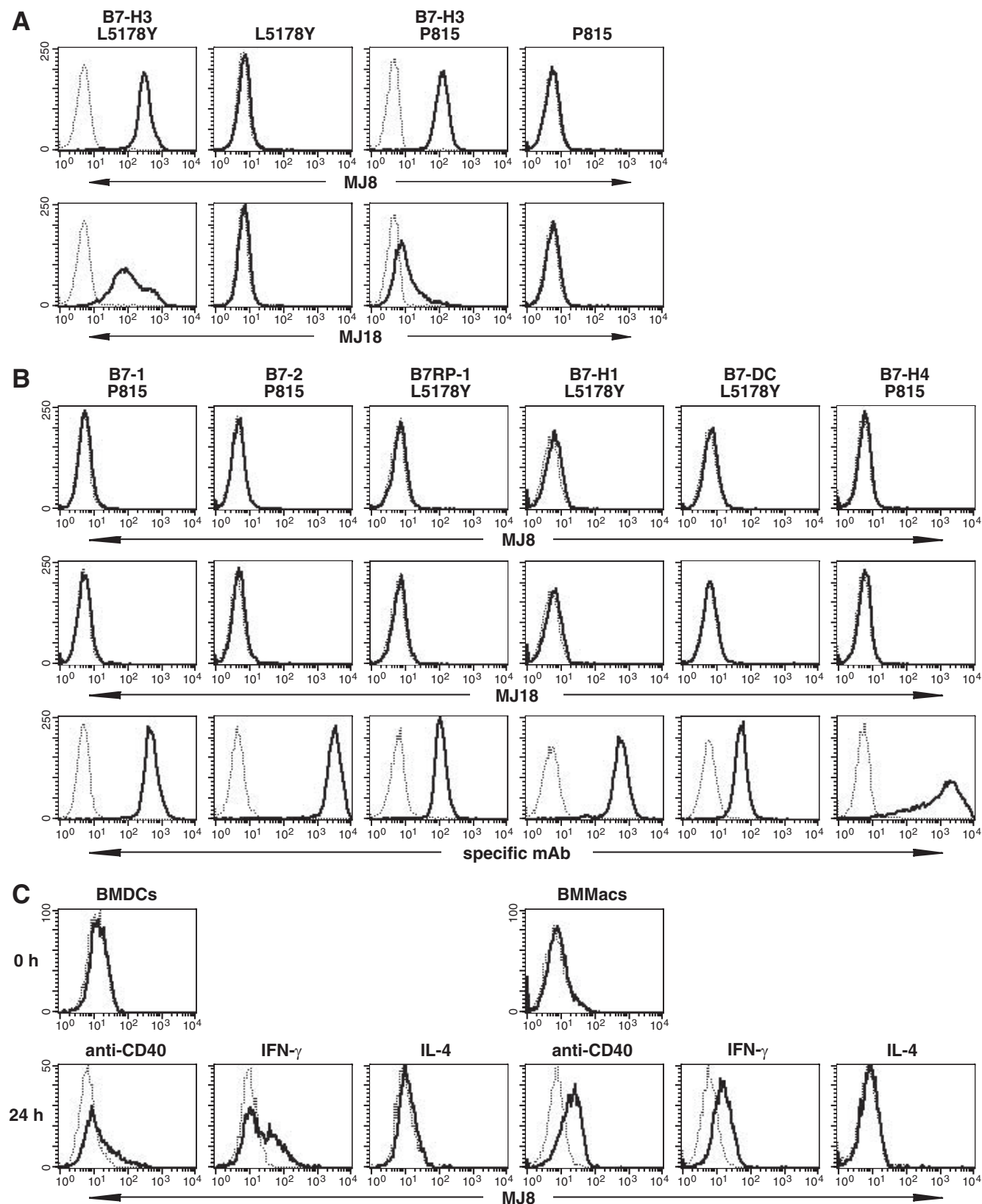
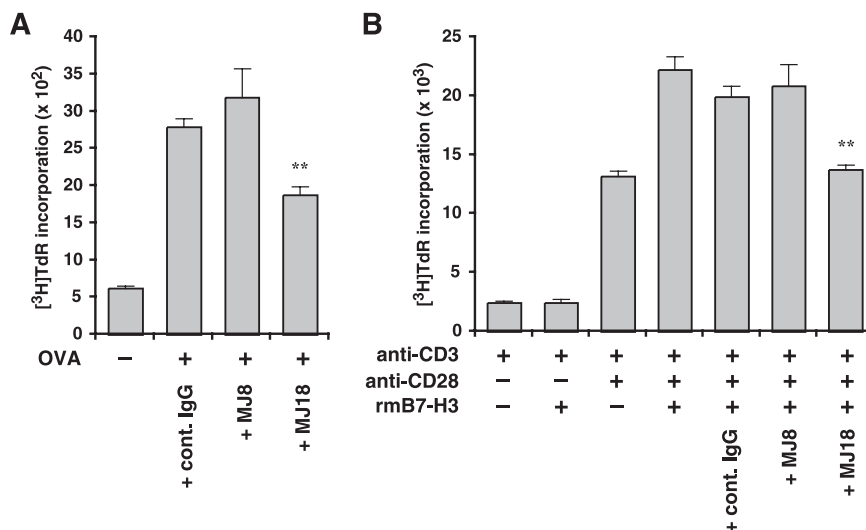


FIGURE 1. Characterization of anti-B7-H3 mAbs MJ8 and MJ18. *A*, Reactivity of anti-B7-H3 mAbs to mouse B7-H3 transfectants. L5178Y- and P815-derived B7-H3 transfectants and parental cells were stained with biotinylated MJ8, MJ18, or control rat IgG, followed by PE-labeled streptavidin. *B*, Anti-B7-H3 mAbs do not react to other B7 family molecules. L5178Y- or P815-derived transfectants (B7-1/P815, B7-2/P815, B7RP-1/L5178Y, B7-H1/L5178Y, B7-DC/L5178Y, and B7-H4/P815) were stained with biotinylated MJ8, MJ18, specific mAbs against each B7 family molecule, or control rat IgG, followed by PE-labeled streptavidin. *C*, Expression of B7-H3 on BMDCs and BMMacs. BMDCs and BMMacs were prepared by culturing bone marrow cells in the presence of GM-CSF or M-CSF, respectively. Cells were stimulated with anti-CD40 mAb, IFN- γ , or IL-4 for 24 h and stained with biotinylated MJ8 or control rat IgG, followed by PE-labeled streptavidin. Thick lines indicate the staining with the respective mAb, and the dotted lines indicate background staining with control IgG.

FIGURE 2. Effect of anti-B7-H3 mAbs on T cell responses. **A**, Purified CD4⁺ T cells from DO11.10/Rag-2^{-/-} mice were cultured with BMDCs and 2 μ M OVA_{323–339} peptide in the presence of anti-B7-H3 mAbs (MJ8 or MJ18) or control IgG for 48 h. Proliferative response was assessed by pulsing the cultures with 0.5 μ Ci of [³H]TdR for the last 6 h. Data are expressed as the mean $\pm$ SEM of triplicate wells. **B**, Purified CD4⁺ T cells from BALB/c mice were stimulated with immobilized anti-CD3 mAb and rmB7-H3 in the presence or absence of anti-CD28 mAb and anti-B7-H3 mAbs. Proliferative response was assessed at 48 h, as described above. **, $p < 0.01$ as compared with control IgG.



Induction of DCs and macrophages from bone marrow cells and immunization

BMDCs were prepared by culturing bone marrow cells from BALB/c mice, according to the procedure developed by Lutz et al. (25). Briefly, bone marrow cells (2×10^6 /100 mm bacteriological petri dish) were cultured in 10 ml of RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin and streptomycin, and 50 μ M 2-ME, and supplemented with 20 ng/ml GM-CSF (eBioscience). On day 3, another 10 ml of RPMI 1640 medium containing 20 ng/ml GM-CSF was added, and on day 6 half of the culture supernatant was collected and centrifuged, and the cell pellet was resuspended in 10 ml of fresh RPMI 1640 medium containing 20 ng/ml GM-CSF and given back into the original plate. On day 8, CD11c⁺ BMDCs were isolated by incubation with anti-CD11c-coupled magnetic beads and positive selection over an autoMACS column, according to the manufacturer's instructions. The purity was checked by flow cytometric analysis (>98% CD11c⁺). For Ag pulsing, 100 μ g/ml OVA was added to the cultures, and then the cells were further cultured overnight. OVA-pulsed BMDCs were washed in PBS and immunized (3×10^5 cells) into the hind footpads of BALB/c mice ($n = 5$). Mice were i.p. administrated with 300 μ g of anti-B7-H3 mAb (MJ18) or control rat IgG on days 0, 2, and 5 after immunization, and popliteal LNs were harvested on day 8. In another experiment, BALB/c mice ($n = 5$) were immunized s.c. in the neck with OVA/alum and i.p. administrated with 300 μ g of anti-B7-H3 mAb or control rat IgG on days 0, 2, and 4 after immunization, and cervical LNs were harvested on day 7. LN cells were pooled from five mice in each group and restimulated with the indicated doses of OVA at 6×10^5 cells/0.2 ml in 96-well plates. The proliferative response and cytokine production were measured, as described above.

Bone marrow-derived macrophages (BMMacs) were also prepared by culturing bone marrow cells from BALB/c mice. Bone marrow cells (5×10^6 /100 mm bacteriological petri dish) were cultured in 10 ml of RPMI 1640 medium with 50 ng/ml M-CSF (Wako). On day 3, nonadherent cells were aspirated, and 10 ml of RPMI 1640 medium containing 50 ng/ml M-CSF was added, and on day 6 adherent cells on the bottom of the plate were collected by 0.1% EDTA/PBS (>98% CD11b⁺). Purified BMMacs and BMDCs were stimulated with anti-CD40 (FGK45) mAb (5 μ g/ml), IFN- γ (20 ng/ml), or IL-4 (20 ng/ml) at 37°C for 24 h.

Statistical analysis

Significant differences between two experimental groups were analyzed by unpaired Student's *t* test. The results are expressed as the mean $\pm$ SEM. Values of $p < 0.05$ were considered significant.

Results

Establishment of anti-mouse B7-H3 mAbs

We immunized Sprague Dawley rats with B7-H3-Ig chimera protein and screened the hybridomas producing mAb that reacted with B7-H3 transfectants, but not parental cells. Two mAbs, designated MJ8 and MJ18, were selected. As represented in Fig. 1A, MJ8 reacted strongly with B7-H3-transfected cells (B7-H3/L5178Y and

B7-H3/P815), but not with parental L5178Y and P815 cells. MJ18 bound to B7-H3-transfected cells weakly, but not to parental cells. Both MJ8 and MJ18 failed to bind to the other B7 family-transfected cells (B7-1/P815, B7-2/P815, B7RP-1/L5178Y, B7-H1/L5178Y, B7-DC/L5178Y, and B7-H4/P815) (Fig. 1B), indicating that MJ8 and MJ18 are specific for B7-H3. We also examined the expression of B7-H3 on BMDCs and BMMacs, which were prepared by culturing bone marrow cells with GM-CSF or M-CSF, respectively, by staining with MJ8. As shown in Fig. 1C, although B7-H3 expression was not found on unstimulated BMDCs and BMMacs, it was detectable on both BMDCs and BMMacs after stimulation with anti-CD40 mAb or IFN- γ for 24 h, but not with IL-4. Because B7-H3 expression was inducible on BMDCs, we further tested whether the anti-B7-H3 mAbs would affect the stimulation of Ag-specific CD4⁺ T cells by BMDCs. Purified CD4⁺ T cells from DO11.10/Rag-2^{-/-} mice were cultured with BMDCs and 2 μ M OVA_{323–339} peptide in the presence of anti-B7-H3 mAbs or control IgG. As shown in Fig. 2A, the addition of OVA_{323–339} peptide induced CD4⁺ T cell proliferation, and this proliferation was not affected by MJ8. In contrast, addition of MJ18 significantly inhibited the CD4⁺ T cell proliferation. Similar activities of MJ8 and MJ18 were observed when purified CD4⁺ T cells from BALB/c mice were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of rmB7-H3 (Fig. 2B). No difference in CD4⁺ T cell proliferation was observed when rmB7-H3 was added to the culture with anti-CD3 mAb alone. However, rmB7-H3 significantly enhanced CD4⁺ T cell proliferation stimulated by anti-CD3 and anti-CD28 mAbs. This enhancement was not affected by addition of control IgG or MJ8. In contrast, the addition of MJ18 mostly abrogated the enhancement by rmB7-H3. Collectively, our in vitro results indicate a substantial involvement of B7-H3 in the CD4⁺ T cell proliferative responses in vitro, which can be blocked by MJ18.

Anti-B7-H3 mAb treatment suppresses the development of asthmatic responses

To explore the contribution of B7-H3 to the pathogenesis of asthma, we administrated anti-B7-H3 mAb (MJ18) or control rat IgG from day 0 to 27 to a murine model of asthma. BALB/c mice were sensitized by injection of OVA with alum on days 0 and 14, and then challenged by inhalation of aerosolized OVA on days 22, 24, 26, and 28. On day 30, the control IgG-treated mice developed a typical asthmatic phenotype characterized by AHR to methacholine (Fig. 3A) and accumulation of eosinophils in BALF (Fig. 3B).

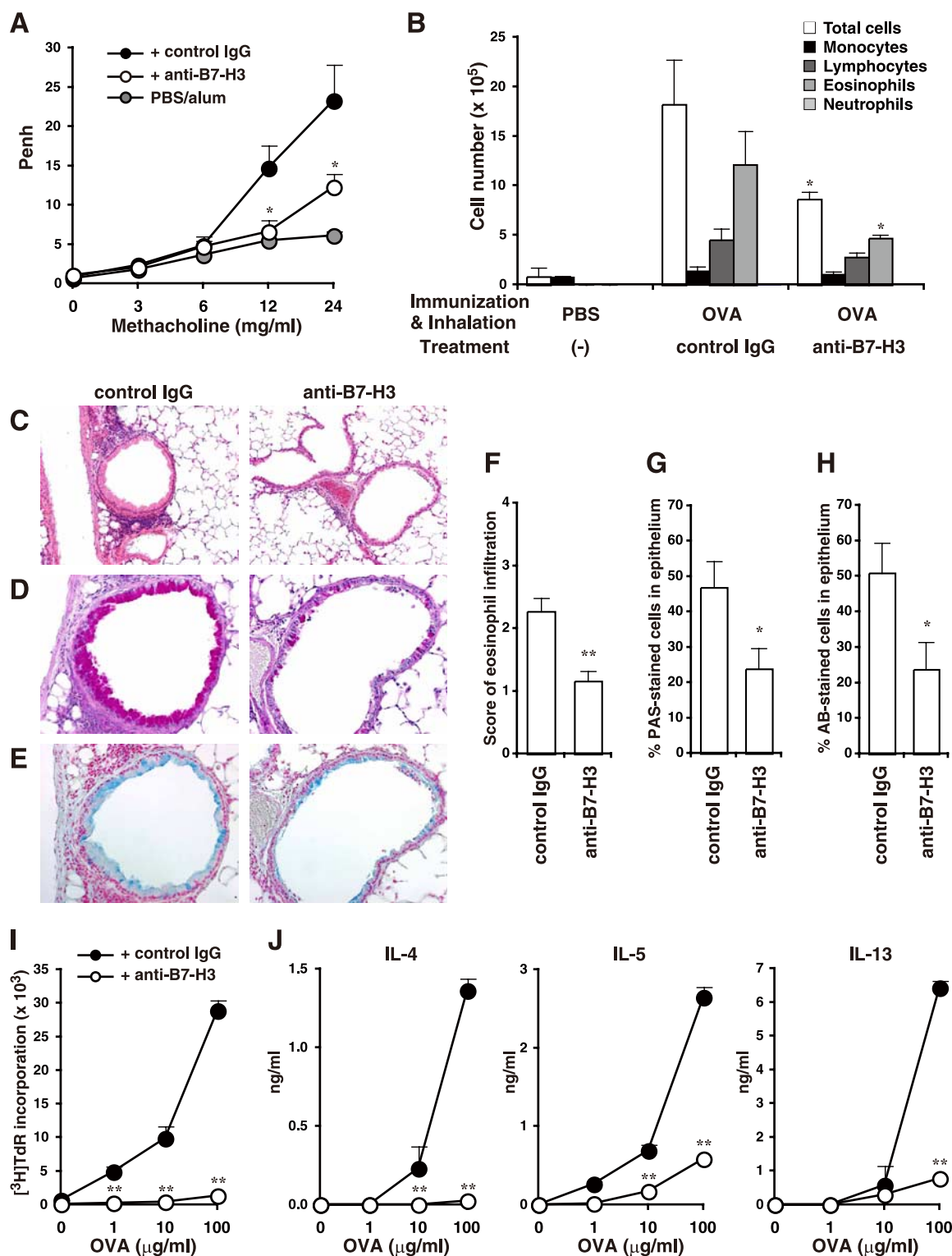


FIGURE 3. Anti-B7-H3 mAb inhibits the development of murine asthma. BALB/c mice were immunized with OVA/alum or PBS/alum on days 0 and 14, and challenged with OVA/PBS or PBS aerosol on days 22, 24, 26, and 28. Some groups of mice were i.p. injected with anti-B7-H3 mAb or control rat IgG from day 0 to 27. **A**, Two days after the last challenge, individual mice were assessed for AHR. Results are presented as the mean Penh $\pm$ SEM of 10 mice in each group after exposure to increasing concentrations of inhaled methacholine. **B**, BALF was collected from individual mice, and cellular composition of the airway infiltrates is shown. **C–H**, Lung tissue was stained with H&E ($\times 100$) (**C**), PAS ($\times 200$) (**D**), and AB ($\times 200$) (**E**). Data are representative of 10 mice in each group analyzed. The infiltration of eosinophils is defined as the average of the scores (**F**). Airway mucus levels are expressed as the percentage of PAS-positive (**G**) or AB-positive (**H**) cells to total epithelial cells. **I** and **J**, Bronchial LN cells were isolated and cultured with indicated concentrations of OVA. For estimating proliferation, 0.5 μ Ci of [^{3} H]TdR was added during the last 6 h of a 72-h culture (**I**). Cytokine production in the culture supernatants at 72 h was determined by ELISA (**J**). Results are expressed as the mean $\pm$ SEM of triplicate samples. *, $p < 0.05$ and **, $p < 0.01$ as compared with control IgG. Similar results were obtained in three independent experiments.

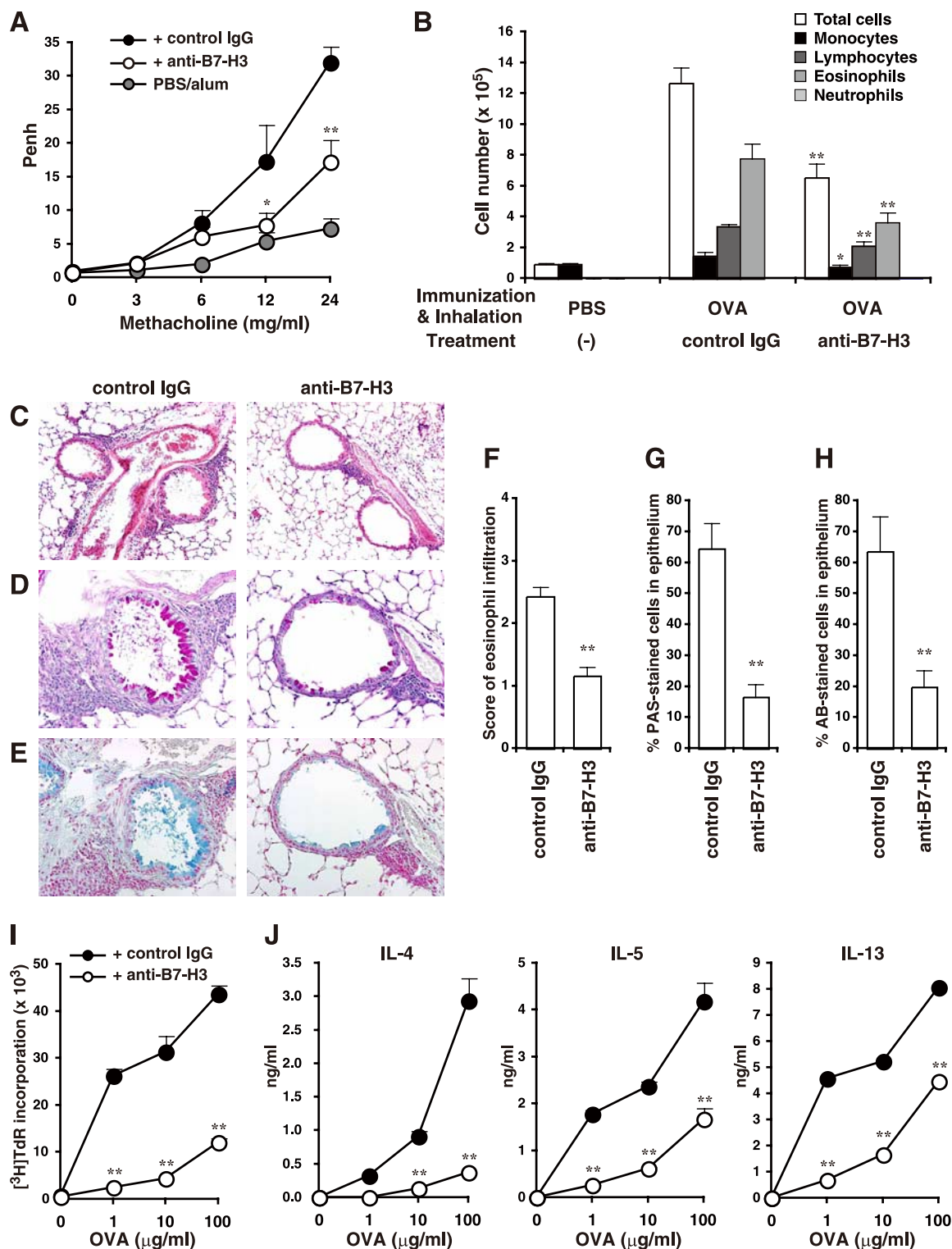


FIGURE 4. Effect of anti-B7-H3 mAb treatment at the induction phase of murine asthma. BALB/c mice were immunized and challenged, as described in Fig. 3. Some groups of mice were i.p. injected with anti-B7-H3 mAb or control rat IgG from day 0 to 18. **A**, Two days after the last challenge, individual mice were assessed for AHR. Results are presented as the mean Penh $\pm$ SEM of 10 mice in each group after exposure to increasing concentrations of inhaled methacholine. **B**, BALF was collected from individual mice, and cellular composition of the airway infiltrates is shown. **C–H**, Lung tissue was stained with H&E ($\times 100$) (**C**), PAS ($\times 200$) (**D**), and AB ($\times 200$) (**E**). Data are representative of 10 mice in each group analyzed. The infiltration of eosinophils is defined as the average of the scores (**F**). Airway mucus levels are expressed as the percentage of PAS-positive (**G**) or AB-positive (**H**) cells to total epithelial cells. **I** and **J**, Bronchial LN cells were isolated and cultured with indicated concentrations of OVA. For estimating proliferation, 0.5 μ Ci of [^{3} H]TdR was added during the last 6 h of a 72-h culture (**I**). Cytokine production in the culture supernatants at 72 h was determined by ELISA (**J**). Results are expressed as the mean $\pm$ SEM of triplicate samples. *, $p < 0.05$ and **, $p < 0.01$, as compared with control IgG. Similar results were obtained in three independent experiments.

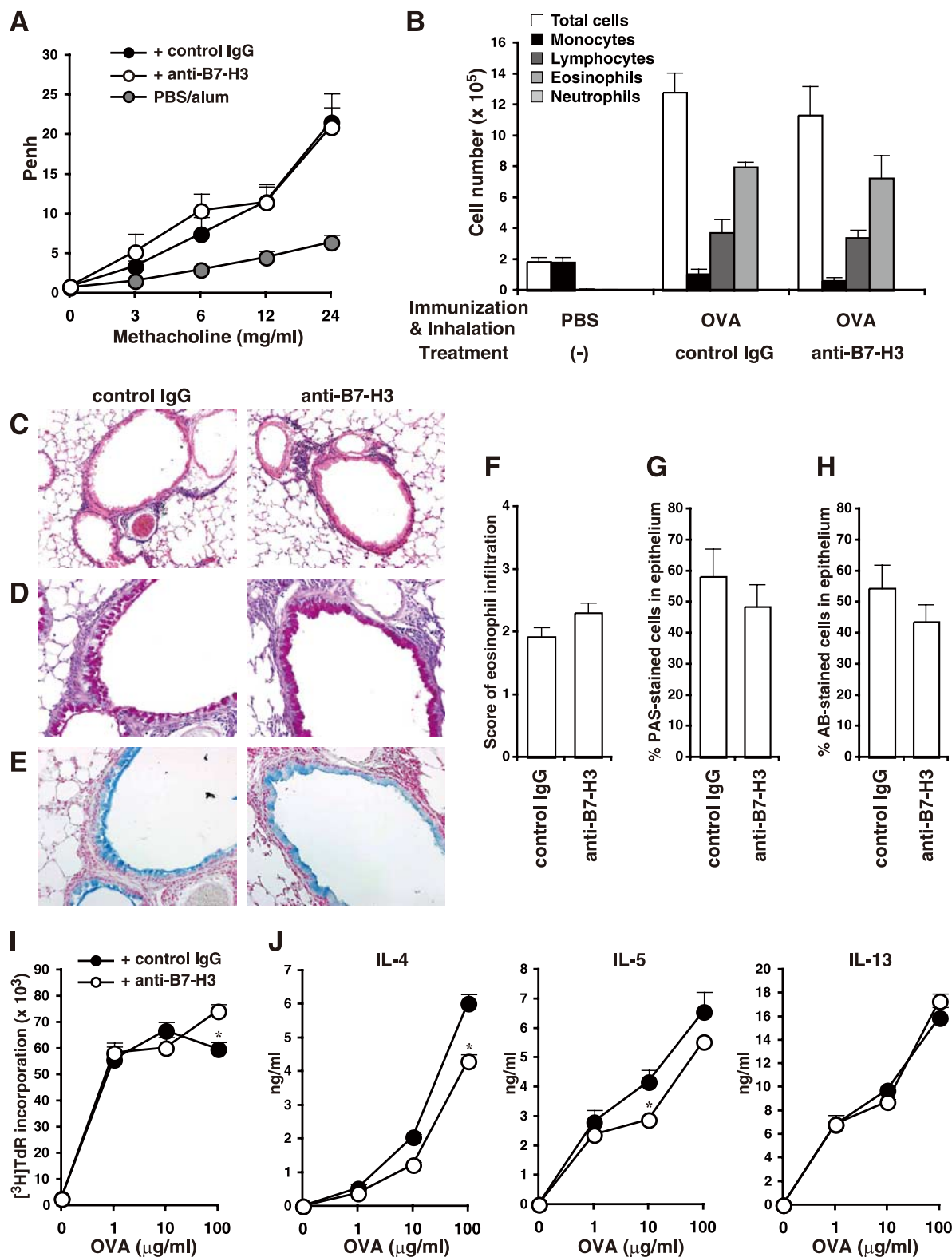


FIGURE 5. Effect of anti-B7-H3 mAb treatment at the effector phase of murine asthma. BALB/c mice were immunized and challenged, as described in Fig. 3. Some groups of mice were i.p. injected with anti-B7-H3 mAb or control rat IgG from day 21 to 27. **A**, Two days after the last challenge, individual mice were assessed for AHR. Results are presented as the mean Penh $\pm$ SEM of 10 mice in each group after exposure to increasing concentrations of inhaled methacholine. **B**, BALF was collected from individual mice, and cellular composition of the airway infiltrates is shown. **C–H**, Lung tissue was stained with H&E ($\times 100$) (**C**), PAS ($\times 200$) (**D**), and AB ($\times 200$) (**E**). Data are representative of 10 mice in each group analyzed. The infiltration of eosinophils is defined as the average of the scores (**F**). Airway mucus levels are expressed as the percentage of PAS-positive (**G**) or AB-positive (**H**) cells to total epithelial cells. **I** and **J**, Bronchial LN cells were isolated and cultured with indicated concentrations of OVA. For estimating proliferation, 0.5 μ Ci of [^{3} H]TdR was added during the last 6 h of a 72-h culture (**I**). Cytokine production in the culture supernatants at 72 h was determined by ELISA (**J**). Results are expressed as the mean $\pm$ SEM of triplicate samples. *, $p < 0.05$ as compared with control IgG. Similar results were obtained in three independent experiments.

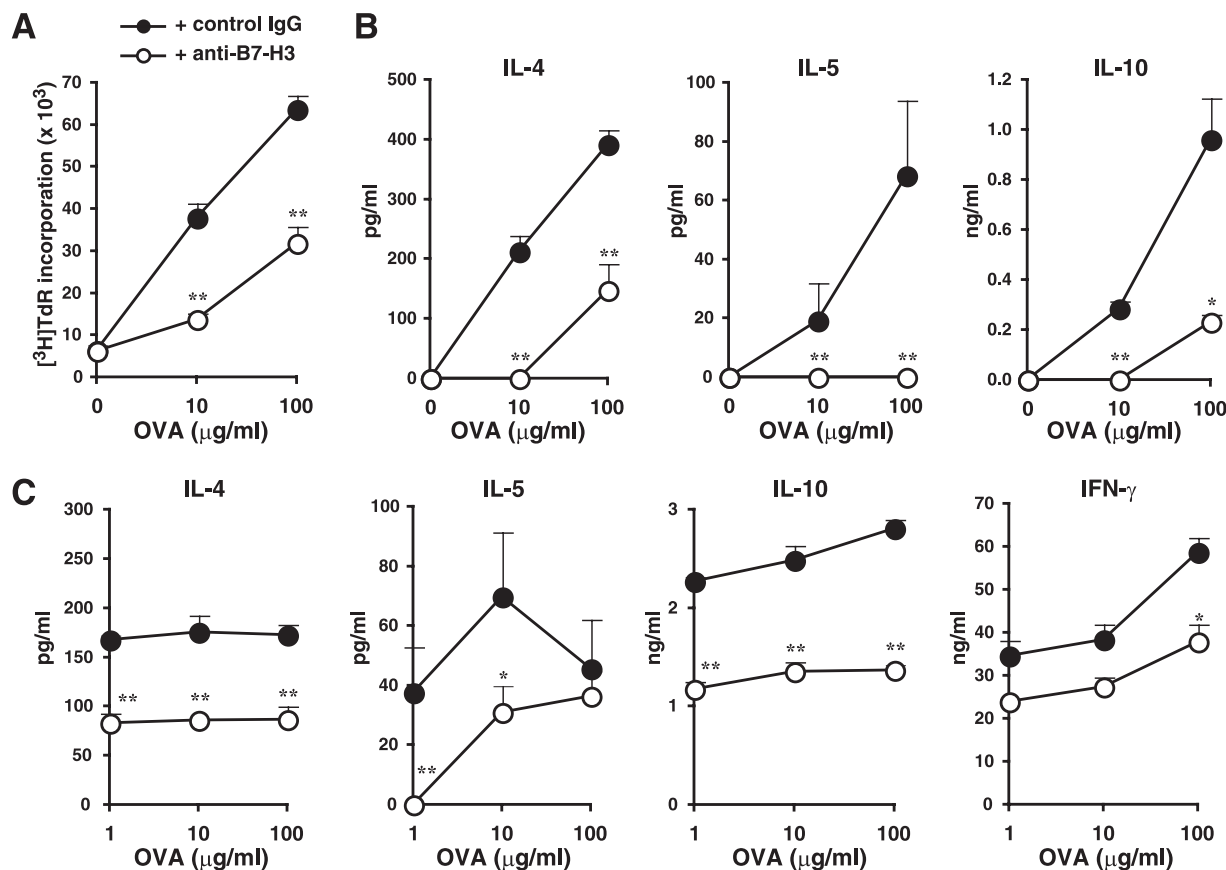


FIGURE 6. Anti-B7-H3 mAb inhibits priming of CD4⁺ T cells by OVA/alum- and OVA-pulsed BMDCs. *A*, BALB/c mice were immunized in the neck with OVA/alum on day 0 and i.p. injected with anti-B7-H3 mAb or control rat IgG on days 0, 2, and 4. LN cells were isolated on day 7 and cultured with indicated concentrations of OVA. For estimating proliferation, 0.5 μCi of [³H]TdR was added during the last 6 h of a 72-h culture. *B*, Cytokine production in the culture supernatants at 72 h was determined by ELISA. *C*, BALB/c mice were immunized with OVA-pulsed BMDCs in footpads on day 0 and i.p. injected with anti-B7-H3 mAb or control rat IgG on days 0, 2, and 5. LN cells were isolated on day 8 and cultured with indicated concentrations of OVA. Cytokine production in the culture supernatants at 72 h was determined by ELISA. Results are expressed as the mean ± SEM of triplicate samples. *, $p < 0.05$ and **, $p < 0.01$ as compared with control IgG. Similar results were obtained in two independent experiments.

Histological examination showed massive infiltration of eosinophils around the bronchioles (Fig. 3, *C* and *F*), and mucus overproduction in the bronchioles (Fig. 3, *D*, *E*, *G*, and *H*). In contrast, development of AHR (Fig. 3*A*), accumulation of eosinophils (Fig. 3, *B*, *C*, and *F*), and overproduction of mucus (Fig. 3, *D*, *E*, *G*, and *H*) were greatly reduced in the anti-B7-H3-treated mice.

The amelioration of asthma by anti-B7-H3 mAb might result from modulation of OVA-specific CD4⁺ T cell responses. To address this possibility, bronchial LN cells were prepared on day 30, and proliferative response and Th2 cytokine production (IL-4, IL-5, and IL-13) against various doses of OVA were assessed. As represented in Fig. 3*I*, OVA-specific proliferative response was almost abrogated in LN cells from the anti-B7-H3-treated mice as compared with those from the control IgG-treated mice. The production of IL-4, IL-5, and IL-13 was also significantly reduced in the anti-B7-H3-treated mice as compared with the control IgG-treated mice (Fig. 3*J*). These results indicate that B7-H3 plays a critical role in the development of allergic asthma and Th2 responses in this model.

Effect of anti-B7-H3 mAb treatment during induction or effector phase of allergic asthma

Although the above results indicated a critical contribution of B7-H3 to the development of allergic asthma, it was not clear whether B7-H3 played a role during the induction phase of pathogenic Th2 cell development or during the effector phase of pathogenic Th2 cell migration and activation in the lung. We next ex-

amined whether B7-H3 played a role during the induction phase or the effector phase of allergic asthma. OVA-immunized BALB/c mice were treated with anti-B7-H3 mAb or control IgG at the time of OVA sensitization from day 0 to 18 for the induction phase or from day 21 to 27 before the first OVA inhalation for the effector phase. Administration of anti-B7-H3 mAb at the induction phase significantly inhibited development of AHR (Fig. 4*A*), accumulation of eosinophils in BALF (Fig. 4*B*), and eosinophilia and mucus overproduction in the lung (Fig. 4, *C–H*). In addition, anti-B7-H3-treated mice showed significantly reduced OVA-specific proliferative response and production of Th2 cytokines (IL-4, IL-5, and IL-13) in LN cells as compared with control IgG-treated mice (Fig. 4, *I* and *J*). In contrast, anti-B7-H3 mAb treatment at the effector phase did not inhibit development of AHR (Fig. 5*A*), accumulation of eosinophils, and mucus overproduction in the lung (Fig. 5, *B–H*), or OVA-specific proliferative response and Th2 cytokine production in LN cells (Fig. 5, *I* and *J*). These results indicate a critical contribution of B7-H3 to the induction phase of allergic asthma, but B7-H3 does not play a major role in the effector phase, including the migration and activation of pathogenic Th2 cells in the lung.

Anti-B7-H3 mAb treatment inhibits priming Th2 response by OVA/alum

To further evaluate whether B7-H3 plays a critical role in priming of CD4⁺ T cells, BALB/c mice were immunized s.c. in the neck with OVA/alum and treated with anti-B7-H3 mAb or control IgG

on days 0, 2, and 4. Cervical LN cells were harvested 7 days after immunization and restimulated *in vitro* with OVA, and proliferative response and Th2 cytokine production (IL-4, IL-5, and IL-10) were assessed. As represented in Fig. 6A, OVA-specific proliferative response was significantly reduced in LN cells from the anti-B7-H3-treated mice as compared with those from the control IgG-treated mice. Production of Th2 cytokines was also significantly reduced in the anti-B7-H3-treated mice as compared with the control IgG-treated mice (Fig. 6B). We also examined the effect of anti-B7-H3 mAb treatment on the induction of regulatory T cells, which were determined by flow cytometric analysis of CD4⁺CD25⁺ Foxp3⁺ cells in LN cells. The anti-B7-H3-treated mice exhibited no significant difference in the frequency of CD4⁺CD25⁺ Foxp3⁺ cell population within total CD4⁺ T cells ($1.64 \pm 0.12\%$) as compared with the control IgG-treated mice ($1.70 \pm 0.26\%$).

Anti-B7-H3 mAb treatment inhibits priming of CD4⁺ T cell responses by OVA-pulsed BMDCs

We further tested the effect of anti-B7-H3 mAb treatment on DC-induced CD4⁺ T cell responses. BMDCs were prepared by culturing bone marrow cells from BALB/c mice with GM-CSF for 8 days, and then pulsed overnight with OVA. A total of 3×10^5 BMDCs was subsequently injected into the hind footpads of syngeneic mice, which were treated with anti-B7-H3 mAb or control rat IgG on days 0, 2, and 5. Popliteal LN cells were harvested on day 8 and restimulated with OVA *in vitro*. Consistent with the previously published results (26), LN cells from the control IgG-treated mice produced IL-4, IL-5, IL-10, and IFN- γ when restimulated with OVA *in vitro* (Fig. 6C). In contrast, LN cells from the B7-H3-treated mice produced significantly reduced levels of IL-4, IL-5, IL-10, and IFN- γ (Fig. 6C). An addition of anti-B7-H3 or control IgG in the cultures had no effect (data not shown). Taken together, our present results suggest that B7-H3 plays a critical role in the induction phase of asthmatic response, particularly the priming of Th2 response by DCs.

Discussion

To explore the contribution of B7-H3 to the development of allergic asthma, we first generated anti-mouse B7-H3 mAbs (MJ8 and MJ18), which bound to B7-H3 cDNA transfectants, but not to those expressing the other B7 family molecules (B7-1, B7-2, B7RP-1, B7-H1, B7-DC, and B7-H4). Moreover, MJ18, but not MJ8, significantly inhibited CD4⁺ T cell proliferation stimulated by BMDCs or anti-CD3 and anti-CD28 mAbs with rmB7-H3. These results indicate that MJ18 used in this study is specific for mouse B7-H3 and can interrupt the interaction between B7-H3 and its putative receptor. The anti-B7-H3 mAb-treated mice showed a substantial reduction of the OVA-induced asthmatic responses. Moreover, the administration of anti-B7-H3 mAb during the induction phase effectively ameliorated the asthmatic responses, although it was not effective during the effector phase. *In vitro* restimulation of draining LN cells showed that the anti-B7-H3 mAb treatment significantly reduced OVA-specific proliferative response and production of Th2 cytokines (IL-4, IL-5, and IL-13). These results suggest that B7-H3 plays its major role in the development of pathogenic Th2 cells during the induction phase, but not in the recruitment or activation of Th2 cells in the lung during the effector phase. In support of this notion, we also found that the anti-B7-H3 mAb treatment inhibited the priming of OVA-reactive Th2 cells induced by OVA/alum immunization or OVA-pulsed BMDC immunization. Collectively, these results suggest that the most critical role of B7-H3 in the asthmatic responses is to facilitate the priming of pathogenic Th2 cells, and that B7-H3 delivers

a positive signal to CD4⁺ T cells leading to enhanced proliferation and cytokine production.

Several recent studies, however, indicated that B7-H3 might have inhibitory functions in certain circumstances. Under Th1-, but not Th2-polarizing conditions, B7-H3-deficient mice developed more severe airway inflammation than wild-type mice (16). B7-H3-deficient mice also developed experimental autoimmune encephalomyelitis 2 days earlier than wild-type mice, but disease severity and incidence were comparable to wild-type mice (16). These results suggested that B7-H3 could be involved in the suppression of Th1-mediated immune responses. Thus, the present suppression of asthmatic response by anti-B7-H3 mAb might be due to an augmentation of Th1 responses. However, this possibility seems unlikely because the anti-B7-H3 mAb treatment inhibited not only Th2-type cytokines, but also Th1-type IFN- γ production in LN cells from the mice primed by OVA-pulsed BMDCs. Moreover, IFN- γ was not detectable in OVA-restimulated LN cells from the anti-B7-H3-treated mice in Figs. 3 and 4 (data not shown). Therefore, the anti-B7-H3 mAb treatment inhibited Th2 development, but this did not result in a dramatic shift to Th1 responses. These results also suggest that the anti-B7-H3 mAb treatment potentially has an inhibitory effect on Th1-mediated immune responses. Further studies are required to address this possibility using Th1-polarized disease models.

The immunization of BALB/c mice with OVA/alum preferentially induces a Th2 response and high levels of serum anti-OVA IgG1 and IgE Abs. The blockade of B7-H3 abrogated the development of asthmatic responses and Th2 responses in LN cells. However, it should be noted that the anti-B7-H3 mAb treatment did not affect the serum levels of anti-OVA IgG1 and IgE even when mice were treated with anti-B7-H3 during both induction and effector phases (data not shown). These results indicate that B7-H3 does not contribute to the Ab production in the OVA-induced asthma model. We previously indicated that the blockade of B7RP-1, which is another member of the B7 family, by neutralizing anti-B7RP-1 mAb, abolished the development of CXCR5⁺ CD4⁺ T cells and germinal center B cells in the spleen in response to Ag immunization (27). The chemokine receptor CXCR5 confers responsiveness to B lymphocyte chemokine (CXCL13), which is produced by follicular stroma cells in the spleen, LN, and Peyer's patches (28, 29). CXCR5 is constitutively expressed by circulating B cells and is required for their migration into B cell follicles in the secondary lymphoid organs (30, 31). A subset of CD4⁺ T cells also expresses CXCR5, which mediates their migration to the B cell follicles, where they provide cognate help to B cells (32–35). Thus, CXCR5⁺ CD4⁺ T cells are referred to as follicular B helper T cells and differ from Th1 and Th2 cells (35, 36). The differential role of B7-H3 in Th2 response and Ab production may be explained by differential contributions of Th2 cells and CXCR5⁺ CD4⁺ T cells. Further studies are needed to address this possibility.

In conclusion, a potent inhibition of the inflammatory response in mouse model of asthma was observed with anti-B7-H3 mAb administration. B7-H3 appears to play a critical role in the development of pathogenic Th2 cells, and thus represents a novel target for intervention of allergic asthma.

Disclosures

The authors have no financial conflict of interest.

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