

ORIGINAL PAPER

4-1BB stimulation inhibits allergen-specific immunoglobulin E production and airway hyper-reactivity but partially suppresses bronchial eosinophilic inflammation in a mouse asthma model

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Summary

Background 4-1BB, a member of the tumour necrosis factor receptor superfamily, functions as a co-stimulatory molecule. Recently, stimulation of the 4-1BB pathway was shown to suppress antigen-specific CD4⁺ T cell and subsequent T cell-dependent humoral immune responses.

Objective We examined the effect of agonistic anti-4-1BB monoclonal antibody (mAb) treatment on allergic asthma, in which allergen-specific type 2 helper T cells (Th2) have been shown to play an important role.

Methods BALB/c mice were systemically sensitized with intraperitoneal injections of ovalbumin (OVA) and alum on days 0 and 14, and then challenged with inhaled OVA on days 28, 29 and 30. In test groups, the agonistic anti-4-1BB mAb was administered at the time of initial systemic sensitization with OVA. On day 31, mice were challenged with inhaled methacholine, and enhanced pause was measured as an index of airway hyper-responsiveness (AHR). Levels of OVA-specific IgE in serum, and levels of various cytokines in broncho-alveolar lavage (BAL) fluids were measured. The severity of airway inflammation was determined by differential cell counts in BAL fluids and histopathologic lung analysis. To evaluate local immunity, we cultured lymphocytes from draining perihilar lymph nodes and evaluated the proliferative response to OVA and the levels of IL-5 in the culture supernatant. In addition, the functional mechanism of 4-1BB stimulation was evaluated in splenocytes obtained at day 7 after systemic OVA sensitization.

Results We found that treatment with the anti-4-1BB mAb significantly decreased AHR and the production of allergen-specific IgE. Bronchial inflammation, however, had only partially improved and the levels of IL-4 and IL-5 in BAL fluids showed only a small degree of reduction compared with the control Ig-treated mice. Thoracic lymphocytes from anti-4-1BB-treated mice showed significant suppression of OVA-induced proliferation and IL-5 production. In anti-4-1BB-treated mice, splenocytes exhibited poor proliferation and marked apoptosis 7 days after systemic OVA challenge.

Conclusion These results suggest that stimulation of the 4-1BB pathway effectively suppresses some features of allergic asthma, including allergen-specific IgE production and AHR, through deletion of allergen-specific Th2 cells. However, we found that bronchial allergic inflammation was not strictly mediated by suppression of the Th2 immune response in this murine model of asthma. Despite these somewhat contradictory effects, intervention in the 4-1BB pathway might provide a potential novel immunotherapeutic approach for treatment of allergic asthma.

Keywords 4-1BB, activation-induced cell death, asthma, CD4⁺ T cells, tolerance

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Introduction

Allergic asthma is a chronic respiratory disease characterized by airway hyper-reactivity (AHR), eosinophilic bronchial inflammation and the presence of allergen-specific IgE. A hallmark of this asthma pathogenesis is a type 2 helper T cell (Th2)-dominant immune response. Despite our recent progress in understanding the pathophysiology of asthma, current therapies still rely on non-specific suppression of immune responses by corticosteroids, which may generate undesirable side-effects following long-term use. Thus, selective inhibition of pathogenic immune responses would represent a major advancement in the treatment of asthma.

Specific immune responses are based on T cell recognition of the antigen combined with a major histocompatibility complex displayed on antigen-presenting cells (APCs) (signal 1). In addition, full activation of antigen-specific T cells requires the interaction of co-stimulatory molecules on T cells and their respective ligands on APCs (signal 2). In allergic asthma, increased expression of a variety of co-stimulatory molecules has been observed in activated immune cells, indicating their involvement in establishment and maintenance of the disease state. In fact, blocking co-stimulatory pathways such as those involving CD28 [1–3], ICOS [4–7] and OX-40 [8–10] appears to prevent the amplification of allergic inflammation. These results indicate that modulation of a specific co-stimulatory pathway may be a novel immunotherapeutic strategy for asthma.

4-1BB (CD137) is a member of the TNF receptor superfamily. This receptor is expressed on activated T cells, whereas expression of its ligand (4-1BBL) is restricted to activated APCs such as dendritic cells, B cells and macrophages. It appears that 4-1BB is an especially potent co-stimulatory molecule for CD8⁺ T cells [11–13]; stimulation of 4-1BB markedly increased expansion and survival of CD8⁺ T cells, and 4-1BB plays a crucial role in the generation of effector and memory cytotoxic T cells [14, 15]. However, less is known about 4-1BB regulation of CD4⁺ T cell function.

Recent studies have demonstrated that intervention in the 4-1BB co-stimulatory pathway has therapeutic implications. An agonistic anti-4-1BB monoclonal antibody (mAb) was shown to have marked therapeutic effects on the regression of established tumours [16, 17]. In addition, *in vivo* blocking of 4-1BB significantly decreased inflammation induced by viruses [18, 19]. Interestingly, it was reported that an agonistic anti-4-1BB mAb markedly inhibited the development of experimental autoimmune encephalomyelitis and spontaneous systemic lupus erythematosus [20–22]. The mechanisms underlying these findings might be related to deletion of autoreactive CD4⁺ T and B cells [20], suggesting that it may be valuable to examine the involvement of 4-1BB with CD4⁺ T cells in allergic asthma.

To the best of our knowledge, there have been few studies on this topic, although a previous report showed that an agonistic anti-4-1BB mAb could abrogate T cell-dependent antibody production and stimulate activation-induced cell death (AICD) in CD4⁺ T cells [20]. Based on these observations, we hypothesized that an agonistic anti-4-1BB mAb administered with an allergen might have the potential to prevent asthma through the induction of AICD of allergen-specific Th2 cells and subsequent inhibition of allergen-specific IgE production. Here, we tested whether stimulation of the 4-1BB pathway at initial allergen sensitization could induce suppression of the characteristic features of asthma, including AHR, bronchial eosinophilic inflammation and production of allergen-specific IgE in a mouse model of asthma.

Materials and methods

Experimental design

Female BALB/c mice were divided into three groups, and the numbers of mice using each experimental set were five to ten in each group. Two asthma groups were either intraperitoneally administered agonistic anti-4-1BB mAb (3H3) [11] or control rat IgG (Sigma Chemical Co., St Louis, MO, USA) at the same time as the initial systemic sensitization with ovalbumin (OVA). Negative control mice (sham) were treated with vehicle phosphate-buffered saline (PBS). An additional set of experiments was performed to obtain splenocytes 7 days after the first systemic sensitization with OVA. All animal experiments were approved by the Animal Research Ethical Review Board of our institute.

Sensitization and challenge by allergen

All mice were housed in a pathogen-free facility, and were used at 6 weeks of age. On day 0, mice were immunized by intraperitoneal injection with 20 µg chicken OVA (Grade V, Sigma) and 2 mg Al(OH)₃ (alum), followed by a booster injection with the same amount of OVA and alum on day 14. Agonistic anti-4-1BB mAb (200 µg per mouse) or rat Ig (control Ig, 200 µg per mouse) was intraperitoneally administered immediately after the first immunization. On days 28, 29 and 30, mice were challenged with inhaled antigen (5% OVA in PBS) for 30 min per day; for this, mice were placed in a plastic chamber (30 × 30 × 15 cm) and administered aerosolized OVA solution via an ultrasonic nebulizer. Sham-treated (sham) mice underwent the same schedule for sensitization and challenge, but received vehicle (PBS) rather than OVA.

Determination of airway responsiveness

Twenty-four hours after the final aerosol challenge, AHR was assessed in conscious and unrestrained mice by

whole-body plethysmography. Individual mice were placed in a plastic chamber and exposed to increasing concentrations of aerosolized methacholine solution (0, 2.5, 5 and 10 mg/mL; Sigma) for 3 min. The 'enhanced pause' (Penh) value was calculated automatically based on the mean pressure generated in the plethysmography chamber during inspiration and expiration. Every Penh, combined with the time of each phase, was recorded for 3 min with 10 s intervals after each aerosol exposure, and then averaged. Values are presented as percent changes in Penh at each methacholine concentration, as compared with baseline readings.

Enzyme-linked immunosorbent assay for immunoglobulins

The levels of serum OVA-specific IgE were measured using an ELISA. Briefly, 96-well plates were coated with 100 µL OVA in PBS (5 µg/mL) and incubated overnight at 4 °C. Plates were washed three times with 0.1% Tween 20 in PBS (PBS-T) and then blocked with 10% fetal bovine serum (FBS) in PBS for 1 h. Samples diluted in PBS (1 : 50) were added (100 µL) and incubated for 2 h at room temperature. After the samples were washed five times with PBS-T, rat anti-mouse IgE (PharMingen, San Diego, CA, USA) (1 : 2000) was added to each well and the plates were incubated at room temperature for 1 h. The plates were then washed three times with PBS-T, each well was treated with 100 µL horseradish-peroxidase (HRP)-conjugated goat anti-rat IgG (PharMingen) (1 : 1000), and the plates were incubated at room temperature for 1 h. Samples were then washed seven times with PBS-T, and colour was developed for 30 min using 100 µL 3,3',5,5'-tetramethylbenzidine substrate (PharMingen) followed by addition of 100 µL 2N H₂SO₄. The plates were read at 450 nm using a microplate reader. Values were converted into arbitrary units (AU) using the sera from immunized mice as a standard. The levels of serum OVA-specific IgG1 were determined as described above, except that OVA-specific IgG1 was detected using biotinylated rat anti-mouse IgG1 followed by HRP-conjugated streptavidin. The serum levels of total IgE were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Bronchoalveolar lavage and analysis

After AHR measurement, mice were anaesthetized and killed by cardiac puncture. Bronchoalveolar lavage (BAL) was obtained by instilling and withdrawing 2 mL saline via a tracheal cannula. BAL fluids were centrifuged at 400 g for 5 min, cell pellets were re-suspended in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) and the total number of BAL cells was determined. Slides for differential cell counts were prepared using cytopspin techniques and stained with Diff-Quik. For each sample,

two investigators blinded to the treatment group performed differential cell counts of 300 cells, and the values were averaged. The levels of IL-4, IL-5, IFN-γ, IL-12, IL-1β and TNF-α in BAL fluids were determined using an ELISA kit (R&D Systems).

Lung histology

For histopathological assessment, the lungs were removed, fixed in 4% formalin overnight and embedded in paraffin. The tissues were sliced into 4 µm sections and stained with haematoxylin and eosin. The degree of lung inflammation was evaluated using the previously described methods [23]. Briefly, lung lesions, including alveolar septal infiltrates, perivascular infiltrates and peribronchial infiltrates, were subjectively graded as 1, 2, 3 or 4 based on a severity scale of minimal, mild, moderate or marked, respectively. This grading was performed by a pathologist blinded to the treatment groups. Individual lesion scores were summed from each mouse to generate an overall histopathology score.

Analysis of lymphocytes obtained from perihilar lymph nodes

Perihilar lymph nodes were removed and single-cell suspensions were prepared by gently pressing organs through a steel mesh with a rubber plunger. Samples from sham mice were pooled as necessary to obtain 5×10^5 cells per well. The cell suspensions were centrifuged at 400 g for 5 min at 4 °C, and the cells were re-suspended in PBS/3% bovine calf serum and washed twice. The samples were then suspended in 50 µL aliquots of wash buffer and incubated for 30 min at 4 °C with saturating concentrations of fluorochrome-labelled mAbs (fluorescein isothiocyanate (FITC)-conjugated anti-CD4-, anti-CD45 (B220)- or phycoerythrin (PE)-conjugated anti-CD8). Cell samples were then washed twice with 200 µL wash buffer, re-suspended in 50 µL wash buffer, fixed in 0.5% paraformaldehyde and stored in the dark at 4 °C. Samples were analysed using a flow cytometry instrument (Becton Dickinson, Franklin Lakes, NJ, USA), and populations were counted based on a discrete histogram population.

For evaluation of the proliferative response to OVA in each study group, another set of cells was aliquotted into 96-well plates with 2×10^6 cells per well in RPMI media containing 2 mM glutamine, 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. These samples were cultured in triplicate in the presence of various concentrations of OVA (0, 30, 100 and 300 µg/mL) in a final volume of 200 µL for 96 h in an incubator (37 °C, 5% CO₂+95% air). During the final 8 h of culture, 1 µCi [³H] thymidine was added to each well. At the end of the incubation, cells were harvested and radioactivity was counted using a β

counter. Cell culture supernatants were also harvested at 96 h, and IL-5 and IFN- γ levels were determined using an ELISA kit (R&D Systems).

Analysis of splenocytes

As a preliminary experiment, the expression of 4-1BB on CD4⁺ T lymphocytes was observed for the first week after the initial sensitization with OVA by using FACS analysis of splenocytes. The results revealed that 4-1BB-expressed CD4⁺ T cells were significantly increased up to more than 10% of total CD4⁺ T cells in the sensitized group, while less than 1% of CD4⁺ cells expressed 4-1BB in non-immunized group.

Spleens were also obtained from a set of experimental mice 7 days after the first sensitization. Single-cell suspensions were prepared, and cell proliferation assays were performed in both 4-1BB treated and untreated groups as described above. In addition, apoptotic CD4⁺ T cells were detected by flow cytometric analysis of splenocytes cultured for 24 h with OVA (100 μ g/mL) and stained with anti-CD4-FITC plus annexin-V-PE.

Statistical analysis

The data are expressed as mean $\pm$ SEM. For comparison between groups, Mann-Whitney and Kruskal-Wallis tests were used. Statistical significance was defined as $P < 0.05$.

Results

Effect of anti-4-1BB treatment on allergic asthma

We examined the effects of 3H3 treatment on AHR of asthma (which was significantly increased in the asthmatic group) and found that 3H3 treatment markedly decreased AHR compared with control Ig-treated mice (Fig. 1). Measurements of serum IgE revealed markedly less OVA-specific IgE in 3H3-treated mice compared with control Ig-treated mice (median values of 20 and 776 AU/mL, respectively; $P < 0.0001$) (Table 1). Similarly, OVA-specific IgE levels were undetectable in BAL fluid from 3H3-treated mice compared with the detectable levels in control Ig-treated mice (data not shown). Total IgE levels did not differ between control Ig- and 3H3-treated groups (Table 1), indicating that the effect of 3H3 was specific for the antigen. The inhibitory effect of 3H3 was also evident in IgG1 production; OVA-specific IgG1 production was abolished in 3H3-treated mice compared with control Ig-treated mice (median values of undetectable and 1526 pg/mL, respectively; $P < 0.0001$) (Table 1).

Histological examination of the extent and anatomical distribution of allergic inflammation revealed intense inflammation in the lungs of control Ig-treated mice. The

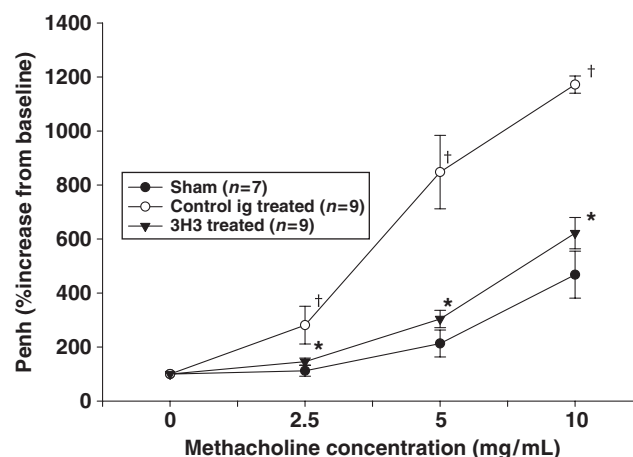


Fig. 1. Effect of agonistic anti-4-1BB mAb (3H3) on airway hyper-reactivity (AHR). AHR was significantly increased in the asthmatic group and 3H3 treatment markedly decreased AHR compared with control Ig-treated mice. Penh means enhanced pause, which represents the airway resistance determined by non-invasive body plethysmography and 'n' shows the number of mice used in the each study group. $^{\dagger}P < 0.01$, $^*P < 0.03$.

Table 1. Effect of anti-4-1BB mAb on immunoglobulin levels in serum

	Sham (n = 8)	Control Ig (n = 10)	3H3 (n = 10)
Total IgE (pg/mL)	443 $\pm$ 97	5,528 $\pm$ 283*	5,045 $\pm$ 295*
OVA-specific IgE (AU/mL)	0.7 $\pm$ 0.2	788 $\pm$ 105*	25 $\pm$ 29**†
OVA-specific IgG1 (pg/mL)	U.D.	1,576 $\pm$ 345**	UD

* $P < 0.05$ compared with sham group; $^{\dagger}P < 0.05$ compared with control Ig-treated group; ** $P < 0.0001$.

UD, undetectable.

infiltrates surrounding the bronchi and vessels comprised a mixture of eosinophils, neutrophils and mononuclear cells (Fig. 2b). Even though there were substantial degrees of peribronchial and perivascular infiltrates in 3H3-treated mice (Fig. 2c), the overall histopathological lesion score was lower in these mice than in control Ig-treated mice (summed lesion score: 5.63 ± 0.38 for the 3H3-treated group and 7.0 ± 0.38 for the control Ig-treated group, $P < 0.05$). An examination of inflammatory cells in the BAL fluid samples revealed that compared with the sham treatment, OVA sensitization and challenge induced a significant increase in total cell numbers (Fig. 3a), and in the proportions of eosinophils, lymphocytes and neutrophils, with a concomitant decrease in the proportion of alveolar macrophages (Fig. 3b). In addition, treatment with 3H3 partially reduced the total number of inflammatory cells. The proportions of lymphocytes, eosinophils and neutrophils in BAL fluid samples were generally lower in 3H3-treated mice, whereas the proportion of alveolar macrophages was increased compared with control Ig-treated mice (Fig. 3).

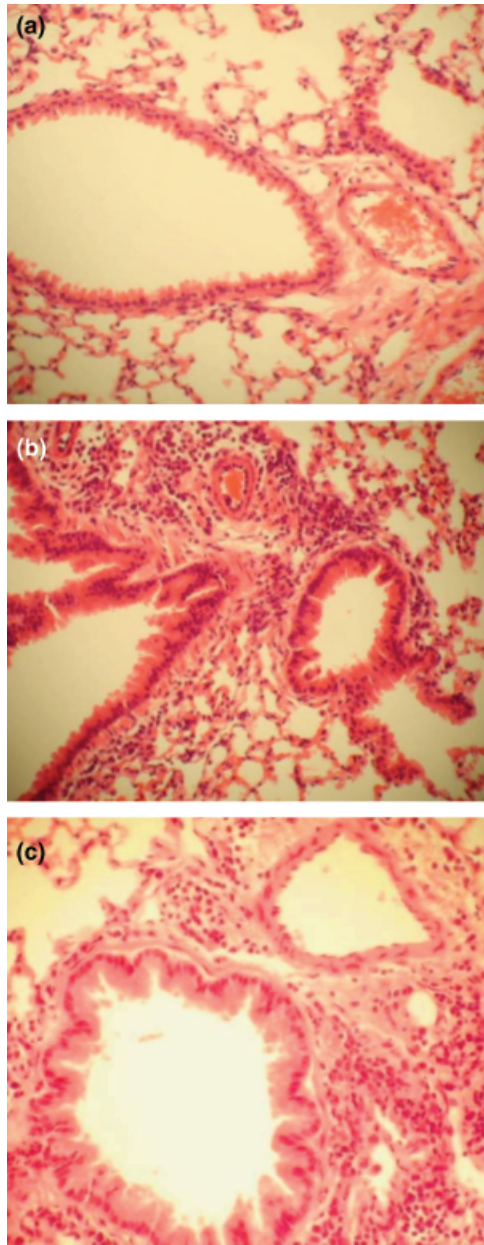


Fig. 2. Lung histology in each study group. Histological examination of the extent and anatomical distribution of allergic inflammation reveals intense inflammation in the lungs of control Ig-treated mice (b). Although the degree of inflammation is partially improved in the 3H3 treated mice, there are substantial degrees of peribronchial and perivascular infiltrates in 3H3-treated mice (c). a, Lung section from sham mice; b, lung section from control Ig-treated mice; c, lung section from 3H3-treated mice visualized by haematoxylin and eosin staining and light microscopy ($\times 200$).

Measurements of the levels of various cytokines in the BAL fluid samples revealed that the levels of IL-5 were significantly lower in 3H3-treated mice compared with control Ig-treated mice. No significant differences were observed in IL-12 and IFN- γ levels, whereas the levels of

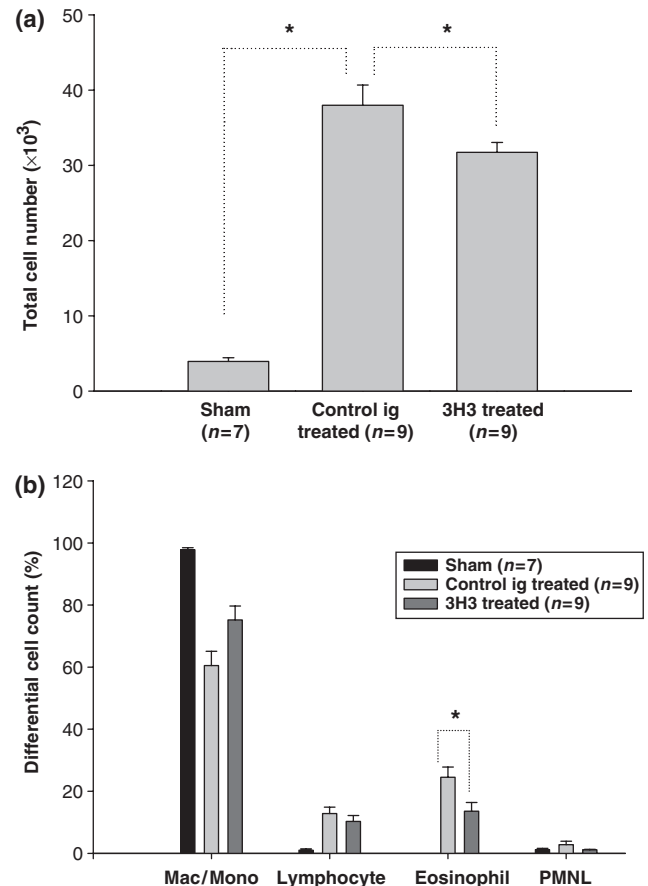


Fig. 3. Effect of anti-4-1BB mAb on cell counts in bronchoalveolar lavage (BAL) fluids. The inflammatory cells in the BAL fluid samples reveal that ovalbumin sensitization and challenge induced a significant increase in total cell numbers. Treatment with 3H3 partially reduced the total number of inflammatory cells (a). The proportions of lymphocytes, eosinophils and neutrophils in BAL fluid samples were generally lower in 3H3-treated mice (b). 'n' shows the number of mice used in the each study group. a, Total cell numbers in BAL fluids; b analysis of differential cell counts in BAL fluids; * $P < 0.05$ Mac/Mono, macrophage and monocytes; PMNL, polymorphonuclear leucocytes.

IL-1 β and TNF- α were significantly increased in both control Ig- and 3H3-treated groups compared with the sham group (Table 2).

Response of allergen-specific CD4⁺ T cells to 3H3 treatment at the time of systemic sensitization

To evaluate the possible mechanisms underlying the effects of 3H3 on asthma, we first evaluated the regional immune response through examination of lymphocytes obtained from perihilar lymph nodes. The total number of perihilar lymphocytes was significantly lower in 3H3-treated mice than in control Ig-treated mice (Fig. 4a). This was because of decreases in all lymphocyte subsets, including B cells, CD4⁺ T cells and CD8⁺ T cells, as evidenced by the observation that their proportions did

Table 2. Levels of various cytokines in bronchoalveolar lavage fluids

	Sham (n = 5)	Control Ig (n = 5)	3H3 (n = 5)
IL-4 (pg/mL)	22.1 ± 5.0	30.9 ± 5.3	24.7 ± 3.4
IL-5 (pg/mL)	33.0 ± 5.9	53.8 ± 5.1*	41.2 ± 4.9†
IFN- γ (pg/mL)	49.1 ± 4.8	40.4 ± 3.9	38.6 ± 5.3
IL-12 (pg/mL)	61.8 ± 5.5	52.1 ± 4.6	45.1 ± 3.9
IL-1 β (pg/mL)	12.5 ± 3.6	28.7 ± 4.2*	20.5 ± 4.8*
TNF- α (pg/mL)	21.5 ± 6.2	52.5 ± 5.8*	42.5 ± 9.2*

* $P < 0.05$ compared with sham group; † $P < 0.05$ compared with control Ig-treated group.

not differ between control Ig- and 3H3-treated mice (Fig. 4b). Proliferation assays showed that perihilar lymphocytes from 3H3-treated mice responded poorly to antigen stimulation (Fig. 4c) and secreted significantly lower amounts of IL-5 than did control Ig-treated thoracic lymphocytes (Table 3). IFN- γ levels were below the detection levels in all study groups.

To further clarify the underlying mechanism of 3H3-induced palliation of the allergen-specific T cell response, we then examined OVA-induced apoptosis and proliferation in splenocytes isolated from 3H3- and control Ig-treated mice. Three independent evaluations of apoptosis yielded similar results, showing that a greater proportion of CD4⁺ T cells underwent apoptosis in 3H3-treated mice as compared with the control Ig-treated mice (Fig. 5a). Proliferation of splenocytes exposed to OVA was considerably suppressed in 3H3-treated mice, while a dose-dependent proliferation occurred in control Ig-treated mice (Fig. 5b).

Discussion

We demonstrated that a single administration of agonistic anti-4-1BB mAb at the time of sensitization could nearly abolish allergen-specific IgE production and attenuate AHR in a mouse model of asthma, although only a partial improvement of eosinophilic airway inflammation was observed. These results suggest that agonistic anti-4-1BB mAbs may have a suppressive effect on the development of some features of allergic asthma, and that modulation of a co-stimulatory pathway may be potentially useful as an immunotherapeutic agent for asthmatics.

It appears that the observed effects of the anti-4-1BB mAb are mechanistically associated with the deletion of allergen-specific CD4⁺ T cells. Recently, it was shown that 4-1BB stimulation causes AICD of antigen-specific CD4⁺ T cells [20] or allo-stimulated CD4⁺ T cells (manuscript in submission) *in vivo*. Here, we demonstrated that anti-4-1BB mAb administration at the time of initial systemic sensitization induced significantly enhanced apoptosis of OVA-specific CD4⁺ T cells following OVA exposure. Once allergen-specific CD4⁺ T cells are deleted by 4-1BB

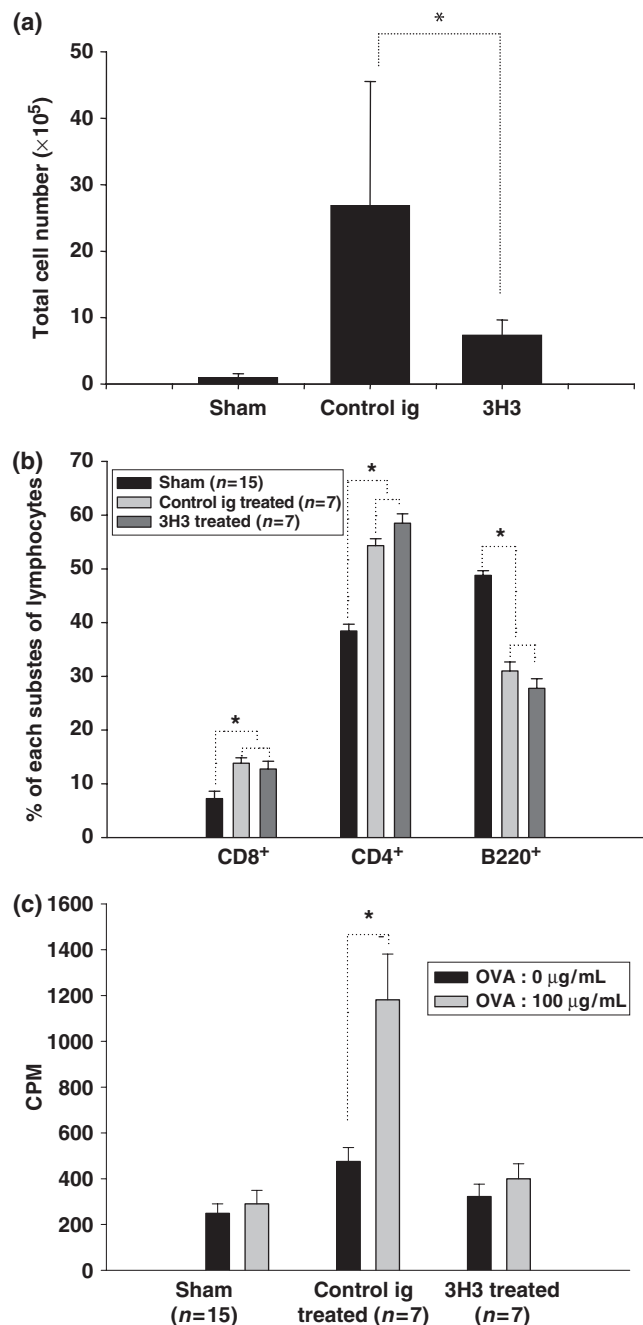


Fig. 4. Effect of anti-4-1BB mAb on lymphocytes from draining perihilar lymph nodes. The total number of perihilar lymphocytes is significantly lower in 3H3-treated mice than in control Ig-treated mice (a). Their proportions of subsets of lymphocytes do not differ between control Ig- and 3H3-treated mice (b). Cellular proliferation shows that perihilar lymphocytes from 3H3-treated mice respond poorly to antigen stimulation (c). 'n' shows the number of mice used in the each study group. a, The total number of lymphocytes; b proportions of lymphocyte subsets; c, proliferation of cultured thoracic lymphocytes; * $P < 0.01$.

stimulation through AICD, it is possible that CD4⁺ T cells may not differentiate into effector cells. Thus, re-exposure to antigen may fail to induce a CD4⁺ T cell immune response [24]. In addition, as anti-4-1BB-mediated

Table 3. IL-5 levels in culture supernatants of perihilar lymphocytes

Ovalbumin concentration (mg/mL)	0	30	100	300
Sham (<i>n</i> = 15), pg/mL	< 0.3	< 0.3	< 0.3	< 0.3
Control Ig (<i>n</i> = 4), pg/mL	0.4 ± 0.4	92.2 ± 7.8	203.7 ± 95.9	336.6 ± 121.5
3H3 (<i>n</i> = 4), pg/mL	0.4 ± 0.4	< 0.3*	24.5 ± 14.8*	41.0 ± 29.3*

**P* < 0.001 compared with control Ig-treated group.

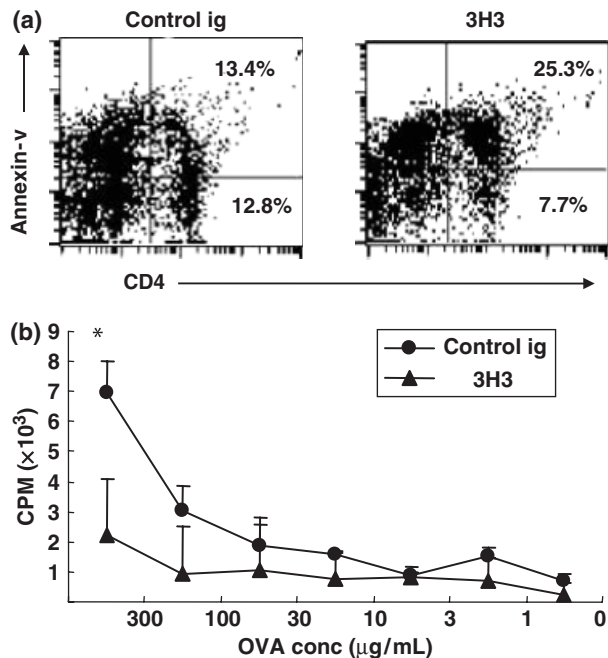


Fig. 5. Analysis of splenocytes obtained from mice treated with anti-4-1BB mAb simultaneous to the initial systemic sensitization. The splenocytes were obtained 7 days after initial sensitization. The evaluation of apoptosis shows that a greater proportion of CD4⁺ T cells underwent apoptosis in 3H3-treated mice as compared with control Ig-treated mice (a). Proliferation of splenocytes exposed to ovalbumin is considerably suppressed in 3H3-treated mice (b). a, A representative image of apoptosis in primed CD4⁺ T cells; b, lymphocyte proliferation after stimulation for 48 h with various concentrations of ovalbumin (*n* = 3 in each group, **P* < 0.05).

abrogation of antibody production could occur via induction of CD4⁺ T cell tolerance [11], it is possible that deletion of allergen-specific CD4⁺ T cells is a prerequisite for induction of CD4⁺ T cell tolerance. Thus, our results suggest that anti-4-1BB mAb-mediated inhibition of OVA-specific Ig production may be achieved through deletion of the T cell subset and subsequent induction of allergen-specific CD4⁺ T cell tolerance.

In this study, we consistently found that lymphocytes obtained from draining lymph nodes did not expand following repeated allergen exposure, and the production of allergen-specific IgE and IgG1 was almost completely suppressed. Furthermore, the effect of anti-4-1BB stimulation should be considered specific for the OVA-mediated

immune response, as total IgE and IgG levels showed no difference between the control Ig- and anti-4-1BB mAb-treated groups.

In terms of the role of anti-4-1BB antibody on Th1/Th2 imbalance, the levels of IL-4 and IL-5 in BAL fluids and the levels of IL-5 secreted from OVA-stimulated lymphocytes obtained from perihilar regional lymph nodes appeared to decrease after administration of the anti-4-1BB antibody, while there were no apparent differences in the levels of IFN- γ . Although further detailed studies will be required to elucidate fully the effect of the anti-4-1BB antibody on the Th1/Th2 balance, these results suggest that treatment of agonistic anti-4-1BB antibody could at least partially suppress Th2 cells in our asthma model, but did not seem to have a significant effect on Th1 cells.

With regard to AHR, the anti-4-1BB mAb treatment dramatically suppressed AHR even though pulmonary eosinophilic inflammation was only partially blocked. It has been demonstrated that AHR can be induced without pulmonary eosinophilia or allergen-specific IgE [23], and activated CD4⁺ T cells have been critically implicated in AHR development [25, 26], although the precise mechanism underlying this is not yet known. Taken together, these observations suggest that in this study, anti-4-1BB mAb treatment caused attenuation of AHR as well as abrogation of allergen-specific IgE production through induction of CD4⁺ T cell tolerance, even though substantial bronchial eosinophilic inflammation persisted. However, further studies should be undertaken to clarify the exact mechanism(s) by which 4-1BB stimulation affects AHR.

At present, it is not clear why anti-4-1BB mAbs differentially inhibit asthma features despite the fact that they effectively induce pathogenic allergen-specific CD4⁺ T cell deletion in our murine model of asthma. This discrepancy might be associated with the observation that 4-1BB stimulation caused allergen-specific Th2 cells to undergo AICD, leading to suppression of AHR and IgE production but not complete repression of eosinophilic inflammation. However, it is also possible that the eosinophilic infiltrates in our study were not entirely dependent on the Th2 immune response, but instead may also have been induced through other mechanisms. Recent evidence has indicated that not all characteristic features of allergic asthma are necessarily correlated with each other. For example, AHR and eosinophilic pulmonary

inflammation were found to occur in the absence of IgE in asthmatic mice [27–30] and AHR could be independently induced without eosinophilic inflammation or allergen-specific IgE [23]. It is possible that in this study, chemical mediators from various inflammatory cells other than OVA-specific CD4⁺ T cells might have contributed to the observed pulmonary inflammation. Although the allergen-specific CD4⁺ Th2 immune response is the key mechanism for pulmonary eosinophilia in allergic asthma, other immune reactions such as innate immunity [31] are capable of inducing tissue eosinophilia in the absence of a Th2 response. The present study showed that the BAL fluid levels of IL-4 and IL-5, the important cytokines for eosinophilic inflammation of the asthmatic airway, were only partially reduced following 3H3 treatment. Partial reduction of airway inflammation observed in this study may be associated with the secretion of important cytokines from other inflammatory cells. In addition, the BAL fluid levels of IL-1 β and TNF- α , which represent activation of innate immune cells, were also increased in the asthmatic groups regardless of the anti-4-1BB mAb treatment.

In general, experimental protocols involving allergen sensitization and challenge may provide direct insights into the immune response during allergic asthma. Strong systemic allergen sensitization with an adjuvant followed by airway inhalation challenge results in considerable accumulation of eosinophils in the airway, in a manner that may operate independent of allergen-specific IgE [29]. In contrast, a model of exclusively active airway sensitization in the absence of adjuvant leads to a modest increase in AHR and allergen-specific IgE, and limited accumulation of eosinophils in the lung [32]; in this mouse model, all features of asthma showed a close association [33]. The latter protocol, which mimics a more natural mode of sensitization to airborne allergens, seems to imply that antigen-specific IgE plays an important role in asthmatic bronchial inflammation, despite our observation that airway inflammation persists in the absence of OVA-specific IgE in the present study. However, the exact reasons behind this apparent discrepancy remain to be determined.

In terms of the therapeutic potential of anti-4-1BB antibody as a biologic agent, further investigations using other dosing regimens in which the agonistic anti-4-1BB antibody is administered to systemically sensitized mice just prior to or just after OVA inhalation challenge are clearly needed as asthma patients already have allergen-specific Th2 cells present. In addition, although the results of this study did not show the dramatic anti-inflammatory effect of anti-4-1BB antibody on allergic inflammation, this does not necessarily negate the possible therapeutic potential of the anti-4-1BB antibody, as the common animal models of asthma may not exactly represent the human condition.

In conclusion, our data suggest that intervention in the 4-1BB co-stimulatory pathway can selectively suppress allergen-specific CD4⁺ T cells. Importantly, these observations might offer a novel immunotherapeutic approach for controlling allergic asthma at the level of the pathogenic immune response.

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