CD69 Controls the Pathogenesis of Allergic Airway Inflammation[1]

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Airway inflammation and airway hyperresponsiveness are central issues in the pathogenesis of asthma. CD69 is a membrane molecule transiently expressed on activated lymphocytes, and its selective expression in inflammatory infiltrates suggests that it plays a role in the pathogenesis of inflammatory diseases. In CD69-deficient mice, OVA-induced eosinophilic airway inflammation, mucus hyperproduction, and airway hyperresponsiveness were attenuated. Cell transfer of Ag-primed wild-type but not CD69-deficient CD4 T cells restored the induction of allergic inflammation in CD69-deficient mice, indicating a critical role of CD69 expressed on CD4 T cells. Th2 responses induced by CD69-deficient CD4 T cells in the lung were attenuated, and the migration of CD4 T cells into the asthmatic lung was severely compromised. The expression of VCAM-1 was also substantially altered, suggesting the involvement of VCAM-1 in the CD69-dependent migration of Th2 cells into the asthmatic lung. Interestingly, the administration of anti-CD69 Ab inhibited the induction of the OVA-induced airway inflammation and hyperresponsiveness. This inhibitory effect induced by the CD69 mAb was observed even after the airway challenge with OVA. These results indicate that CD69 plays a crucial role in the pathogenesis of allergen-induced eosinophilic airway inflammation and hyperresponsiveness and that CD69 could be a possible therapeutic target for asthmatic patients. *The Journal of Immunology,* **2009, 183: 8203–8215.**

Asthma is a chronic inflammatory disease of the lower airways that causes airway hyperresponsiveness (AHR)[4] to a wide variety of specific and nonspecific stimuli (1, 2). In most cases, the extent of AHR correlates with the level of airway inflammation. Hallmarks of asthma include airway inflammation predominated by eosinophils, mucus hyperproduction, and Th2 cytokines (IL-4, IL-5, and IL-13) (3–7). A suggestion for a Th2 paradigm for allergic diseases, wherein increased activation of Th2 cells that produce Th2 cytokines results in IgE production and the recruitment and activation of eosinophils, comes from observations of animal models previously studied. This notion has been supported by clinical studies in which the release of Th2-like cytokines from the lymphocytes of asthmatic patients was demonstrated (8, 9).

CD69 is a type II membrane protein expressed as a homodimer composed of heavily glycosylated subunits (10). CD69 is known as an early activation marker Ag of lymphocytes (11, 12). Freshly prepared thymocytes undergoing selection events express CD69, and regulatory roles for CD69 expression in T cell development in the thymus have been suggested (13, 14). The regulatory roles of CD69 in a collagen-induced arthritis model (15) and an anti-collagen Ab-induced arthritis model (16) were reported and multiple target processes were suggested; however, the role of CD69 in other inflammatory models, such as in the allergic airway inflammation, has not been clarified. More recently, a new function of CD69 in the lymphocyte trafficking has been proposed (17).

We herein investigated the role of CD69 using a mouse model of allergic asthma and found that CD69 plays a critical role in the induction of both Ag-induced eosinophilic airway inflammation and AHR. Furthermore, administration of anti-CD69 Ab resulted in a dramatic reduction in the extent of airway inflammation and AHR, suggesting that the CD69 mAb could be used for the treatment of asthmatic patients.

Materials and Methods

Mice

CD69-deficient (CD69KO) mice (16) were backcrossed with BALB/c 10 times. BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. GFP-transgenic (Tg) mice with a C57BL/6 background expressed an enhanced GFP in all tissue under the control of the β-actin promoter (18). All mice including OVA-specific αβ-TCR-transgenic (DO11.10 Tg) mice (19) were maintained under specific pathogen-free conditions. All animal care was conducted in accordance with the guidelines of Chiba University.

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[4] Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchioalveolar lavage; Cdyn, dynamic compliance; PAS, periodic acid-Schiff; RL, lung resistance; $S1P_1$, sphingosine 1 phosphate receptor 1; Tg, transgenic; KO, knockout; WT, wild type; HPRT, hypoxanthine phosphoribosyltransferase; Penh, enhanced pause.

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Immunofluorescent staining and flow cytometric analysis

In general, one million cells were incubated on ice for 30 min with the appropriate staining reagents according to a standard method (20). Intracellular staining of IL-4 and IFN-γ was performed as described previously (21). FITC-conjugated anti-IFN-γ Ab (XMG1.2), PE-conjugated anti-IL-4 Ab (11B11), PE-conjugated anti-KJ1-26 Ab, and allophycocyanin-conjugated anti-CD4 Ab (RM4-5; all from BD Biosciences) were used for detection.

OVA sensitization and OVA inhalation

CD69-deficient and wild-type (WT) BALB/c or C57BL/6 mice were immunized i.p. with 250 μg of OVA (chicken egg albumin from Sigma-Aldrich) in 4 mg of aluminum hydroxide gel (alum) on days 0 and 7. Where indicated, we used 50 μg of OVA in 2 mg of alum. Mice inhaled aerosolized 1% OVA in saline for 30 min using a supersonic nebulizer (model NE-U07; Omron) on days 14 and 16 to assess the degree of eosinophilic inflammation and AHR. In some experiments, mice were immunized on days 0 and 7 and received intranasal OVA (100 μg) challenges on days 14 and 16.

Collection and analysis of bronchioalveolar lavage (BAL) fluid

One day after the last OVA inhalation (on day 17), BAL was performed as previously described (22). All of the BAL fluid was collected and cells in 150-μl aliquots were counted. One hundred thousand viable BAL cells were cytocentrifuged onto slides by a Cytospin4 (Shandon) and stained with May-Grünwald Giemsa solution (Merck). Five hundred leukocytes were counted on each slide. Cell types were identified using morphological criteria. The percentages of each cell type were calculated.

Lung histology and immunohistochemistry

Mice were sacrificed by CO_2 asphyxiation 24 h after the last OVA inhalation on day 17 and the lungs were infused with 10% (v/v) formalin in PBS for fixation. The samples were sectioned and stained with Luna and periodic acid-Schiff (PAS) stain for the examination of pathological changes under a light microscope at ×100 or ×200 as described previously (22). The number of infiltrated mononuclear cells in the perivascular and peribronchiolar regions was determined by direct counting of three different fields per slide. The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, <0.5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75% as described elsewhere (23).

Cytokine and chemokine expression in asthmatic lung tissue, BAL fluid, and lung CD4 T cells assessed by quantitative RT-PCR

Total RNA was isolated from the lung (three mice in each group) using the TRIzol reagent (Sigma-Aldrich). Reverse transcription was conducted with Superscript II RT (Invitrogen). The total BAL fluid was collected 1 day after the last OVA inhalation. CD4 T cells were purified by cell sorting, and the levels of expression of cytokines and chemokines were analyzed. Whole lung mononuclear cells were stimulated with OVA for 24 h, and CD4 T cells were subsequently collected by cell sorting. Quantitative RT-PCR was performed as described previously (24, 25). The primers for TaqMan probes for the detection of IL-4, IL-5, IL-13, IFN-γ, eotaxin 2, Gob-5 and Muc-5ac, and hypoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems. The expression was normalized by the HPRT signal.

Measurement of AHR

AHR was assessed 1 day after the last OVA inhalation by methacholine-induced airflow obstruction of conscious mice placed in a whole-body plethysmograph (model PLY3211; Buxco Electronics) as previously described (22). The respiratory parameters were obtained by exposing mice to 0.9% saline mist, followed by incremental doses of aerosolized methacholine. Airflow obstruction was monitored and analyzed by system XA software (model SFT3410; Buxco Electronics). The results are expressed as the average percentage of baseline enhanced pause (Penh) values after 0.9% saline exposure. Airway function was further assessed by measuring the changes in lung resistance (RL) and dynamic compliance (Cdyn) in response to increasing doses of inhaled methacholine (26–28). The maximum values of RL and Cdyn were taken and expressed as the percentage change from baseline after saline aerosol exposure.

Adoptive transfer of CD4 T cells

Splenic CD4 T cells of OVA-sensitized WT or CD69-deficient mice were purified using a $CD4^+$ T cell isolation kit (Miltenyi Biotec) and AutoMACS sorter (Miltenyi Biotec), yielding a purity of >98%. These cells were administered i.v. through the tail vein to OVA-sensitized CD69-deficient mice (1×10^7 cells/mouse) 1 day before the first airway challenge with aerosolized OVA (day 13). After cell transfer, the mice were exposed to allergen challenges via the airway on days 14 and 16 and assays were conducted on day 17.

Adoptive transfer of CD69-overexpressed Th2 cells

Naive CD4 T cells from DO11.10 Tg WT or CD69-deficient mice were stimulated with immobilized anti-TCR plus anti-CD28 mAb under Th2 conditions. On day 2, the mouse *CD69* (m*CD69*) gene was introduced by a retrovirus vector containing the m*CD69* gene (29). On day 5, the cultured cells were harvested and one or three million cells ($KJ1^+$ Th2 cells) were injected i.v. into normal BALB/c mice.

In vitro Th2 cell differentiation cultures

DO11.10 Tg $CD44^{low}CD4^+$ T cells (1.5×10^4) purified by cell sorting were stimulated with antigenic OVA peptide (Loh 15) and irradiated (3500 rad) T cell-depleted BALB/c APCs. The cultured cells were restimulated with immobilized anti-TCR-β mAb for 48 h, and the concentrations of cytokines in the supernatant were assessed by ELISA (21).

Visualization of OVA-primed CD4 T cells infiltrated into the lung after OVA inhalation

WT and CD69-deficient mice were immunized i.p. with 250 μg of OVA-alum on days 0 and 7. Splenic CD4 T cells were purified using a CD4 T cell isolation kit and a MACS sorter. The CD4 T cells were stained with either CFDA-SE (4 μM) or SNARF-1 (5 μM; Invitrogen) at 37°C for 15 min. Where indicated, GFP Tg mice (C57BL/6 background) were immunized with OVA and splenic CD4 T cells were purified using a CD4 T cell isolation kit and a MACS sorter. These CD4 T cells were administrated i.v. through the tail vain to normal C57BL/6 mice 24 h before OVA inhalation. One or 2 days after the last OVA inhalation, transferred T cells were monitored using a Leica M205FA fluorescence microscope equipped with a Keyence VB-7010 charge-coupled device (CCD) color camera system (30).

Assessment of the expression of VCAM-1 in the asthmatic lung

The lungs were fixed with in 4% paraformaldehyde (WAKO) after extensive washing. The tissues were embedded in Tissue-Tek OCT compound (Sakura Fine Technical). For microscopic analysis, an Alexa Fluor SFX kit (Invitrogen) was used according to the manufacturer's instructions. In brief, 10-μm cryostat sections were washed with PBS. Sections were blocked with Image-iT Fx Signal Enhancer for 30 min at room temperature and stained with rabbit anti-VCAM-1 Ab (H-276; Santa Cruz Biotechnology) in PBS containing 2% FCS for 15 h at 4°C. After washing with PBS, sections were treated with Alexa Fluor 555 goat anti-rabbit Ab for 3 h at room temperature. The specimen was analyzed using fluorescence microscopy (BZ-9000; Keyence) (31).

Anti-CD69 Ab treatment

BALB/c mice were immunized i.p. with 250 μg of OVA-alum on days 0 and 7. Twenty-four hours before the first airway challenge by OVA inhalation (on day 13), mice were injected i.p. with anti-CD69 mAb (H1.2F3, 250 or 500 μg/mouse). The mice were exposed to allergen challenges on days 14 and 16 and assays were conducted on day 17. Where indicated, anti-CD69 mAb was injected just after the second OVA challenge and BAL fluid was examined on days 17, 19, and 21. In one experiment (shown in Fig. 7), recipient mice transferred with GFP Tg CD4 T cells were challenged by OVA inhalation on day 15 and anti-CD69 mAb were injected 1 h before inhalation or 1day after inhalation, and the assay was conducted on day 17.

Statistical analysis

The Student *t* test was used.

Results

Attenuated OVA-induced eosinophilic inflammation and AHR in CD69-deficient mice

The aim of this study was to evaluate the role of CD69 in the development of allergic airway inflammation and AHR. WT and

CD69-deficient mice were sensitized with OVA-alum on days 0 and 7 and subsequently received an OVA inhalation on days 14 and 16 (Fig. 1*A*). The expression of CD69 was induced in infiltrating leukocytes in WT animals after the OVA sensitization and challenge (data not shown). One day after the last inhalation, the BAL fluid was harvested and examined for infiltrating leukocytes. A summary of the infiltrated cell types is shown in Fig. 1*B*. The frequencies of eosinophils in the BAL fluid samples were lower in CD69-deficient mice in comparison to WT mice. The lung sections were subjected to Luna staining and the perivascular and peribronchiolar regions were assessed for eosinophilic infiltration (Fig. 1*C*). The number of infiltrated eosinophils was significantly reduced in the CD69-deficient mice in comparison to the WT after receiving the OVA airway challenge. Next, the levels of mRNA expression of Gob-5 and Muc-5ac, molecular makers for goblet cell hyperplasia and mucus production, were assessed in the lungs of CD69-deficient mice (Fig. 1, *D* and *E*). As expected, Gob-5 and Muc-5ac expression levels were reduced in the CD69-deficient mice in comparison to the WT. The AHR as measured by methacholine-induced airflow obstruction with a whole-body plethysmograph was not obviously observed in the OVA-sensitized and OVA-challenged CD69-deficient mice (CD69KO/OVA) in comparison to the WT mice (WT/OVA) (Fig. 1*F*).

To further examine the role of CD69 in the pathogenesis of allergic asthma, we used a different experimental asthma model wherein WT and CD69-deficient mice were immunized i.p. and challenged intranasally with OVA (24). The numbers of total infiltrated leukocytes and eosinophils in the BAL fluid were all significantly decreased (Fig. 1*G*), and the level of AHR was dramatically reduced (Fig. 1*H*) in the CD-69-deficient mice compared with WT. Similar results were obtained by the experiments using low-dose OVA-sensitization and OVA challenge (Fig. 1*I*). Our results indicate that the OVA-induced airway inflammation and AHR are therefore attenuated in CD69-deficient mice.

Attenuated Th2 responses induced by CD69-deficient CD4 T cells in the lung

We next examined the mRNA expression levels of IL-4, IL-5, IL-13, IFN-γ, eotaxin 2, IL-2, and TNF-α in CD4 T cells in the BAL fluid of OVA-sensitized and OVA-challenged CD69-deficient mice shown in Fig. 1. CD4 T cells in the BAL fluid were purified by cell sorting and RNA was subsequently prepared. Although the mRNA levels of IL-4, IFN-γ, IL-2, and TNF-α were not significantly changed, there was a substantial decrease in the expression of IL-5, IL-13, and eotaxin 2 in the CD69-deficient mice (Fig. 2*A*). Lung mononuclear cells from OVA-sensitized and OVA-challenged CD69-deficient mice were stimulated in vitro with OVA for 24 h, and the CD4 T cells were subsequently purified by cell sorting. A dramatic decrease in the mRNA expression of IL-4, IL-5, and IL-13 and a moderate decrease in eotaxin 2 were observed in the lung CD4 T cells from CD69-deficient mice after Ag stimulation in vitro (Fig. 2*B*). Spleen cells and BAL fluid mononuclear cells from OVA-sensitized and OVA-challenged CD69-deficient mice were stimulated in vitro with PMA and ionomycin for 4 h, and intracellular IFN-γ/IL-4 profiles of CD4 T cells were assessed. Although the number of IL-4-producing Th2 cells recovered from the spleen was not significantly changed, a substantial decrease in the number of Th2 cells in the BAL fluid was seen in the CD69-deficient mice (Fig. 2*C*). These results suggest that functional Th2 cell numbers in the lung were reduced in the inflamed lung of CD69-deficient mice.

We then examined the efficiency of Th2 cell differentiation using OVA-specific DO11.10 TCR Tg CD69-deficient CD4 T cells in vitro (Fig. 2*D*). Intracellular IFN-γ/IL-4 profiles revealed that the number of IL-4-producing Th2 cells was lower in the CD69-deficient groups at all Ag doses. The production of IL-4, IL-5, IL-13, and IFN-γ in the culture supernatant of the in vitro differentiated Th2 cells was also measured and moderate reduction of the production of Th2 cytokines was seen in the CD69-deficient DO11.10 Tg Th2 cells (Fig. 2*E*). Th2 cell survival in the in vitro culture was not significantly altered in the absence of CD69 expression (data not shown). Thus, the attenuated OVA-induced airway inflammation and AHR observed in CD69-deficient mice could be in part due to the weaker Th2 responses induced by CD69-deficient Th2 cells in the lung.

Requirement of CD69 on Ag-primed CD4 T cells in the induction of eosinophilic airway inflammation and AHR

To investigate the cellular basis underlying the requirement of CD69 in the pathogenesis of allergic asthma, we performed cell transfer experiments in which splenic CD4 T cells from OVA-primed WT and CD69-deficient mice were adoptively transferred into OVA-sensitized CD69-deficient mice (Fig. 3*A*). As shown in Fig. 3*B*, eosinophilic infiltration was restored by the transfer of OVA-primed WT CD4 T cells into the CD69-deficient mice. Luna staining revealed that the transfer of OVA-primed WT CD4 T cells (Fig. 3*C*) resulted in substantial restoration of the infiltration of eosinophils in the perivascular and peribronchiolar regions, whereas transfer of unprimed WT CD4 T cells or primed CD69-deficient CD4 T cells failed to restore the eosinophilic infiltration. Similarly, the development of AHR was observed upon transfer of OVA-primed WT CD4 T cells into CD69-deficient mice (Fig. 3*D*). The restoration of the AHR was confirmed by a direct measurement of RL and Cdyn in anesthetized, tracheostomized, intubated, and mechanically ventilated CD69-deficient mice (Fig. 3, *E* and *F*). Furthermore, CD69-deficient mice responded with mucus hyperproduction when they received OVA-primed WT $CD4^+$ T cells (Fig. 3, *G* and *H*). Allergic inflammation and AHR were induced, although to a relatively milder extent, when OVA-primed WT CD4 T cells were transferred into nonsensitized CD69-deficient mice, and the extent of disease was reduced in the CD69-deficient CD4 T cell groups (data not shown). These results indicate that CD69 molecules on Ag-primed CD4 T cells play an important role in the induction of eosinophilic airway inflammation and AHR.

CD69-overexpressed Th2 cells restored the defect in the induction of eosinophilic inflammation of CD69-deficient Th2 cells

To further investigate whether the eosinophilic airway inflammation is dependent on the expression of CD69 on Th2 cells, OVA-specific TCR$\alpha\beta$ Tg (DO11.10 Tg) CD69-deficient CD4 T cells were cultured under Th2 conditions for 2 days and the developing Th2 cells were infected with a retrovirus vector containing the mouse *CD69* gene (Fig. 4*A*). The overexpression of CD69 was confirmed (Fig. 4*B*). The CD69-overexpressed Th2 cells (3×10^6) were transferred to normal BALB/c recipient mice and after 1 and 3 days, the recipient mice were challenged by OVA inhalation, and the BAL fluid was collected on day 10. As shown in Fig. 4*C*, eosinophilic infiltration was lower in mice receiving CD69-deficient DO11.10 Tg Th2 cells (CD69 KO mock infected) compared with those receiving WT Th2 cells (WT mock infected). Eosinophilic infiltration was restored in the mice that received CD69-overexpressed Th2 cells (CD69 mCD69 infected). These results indicate that overexpression of CD69 overcame the defect in the induction of allergic eosinophilic inflammation of CD69-deficient Th2 cells.

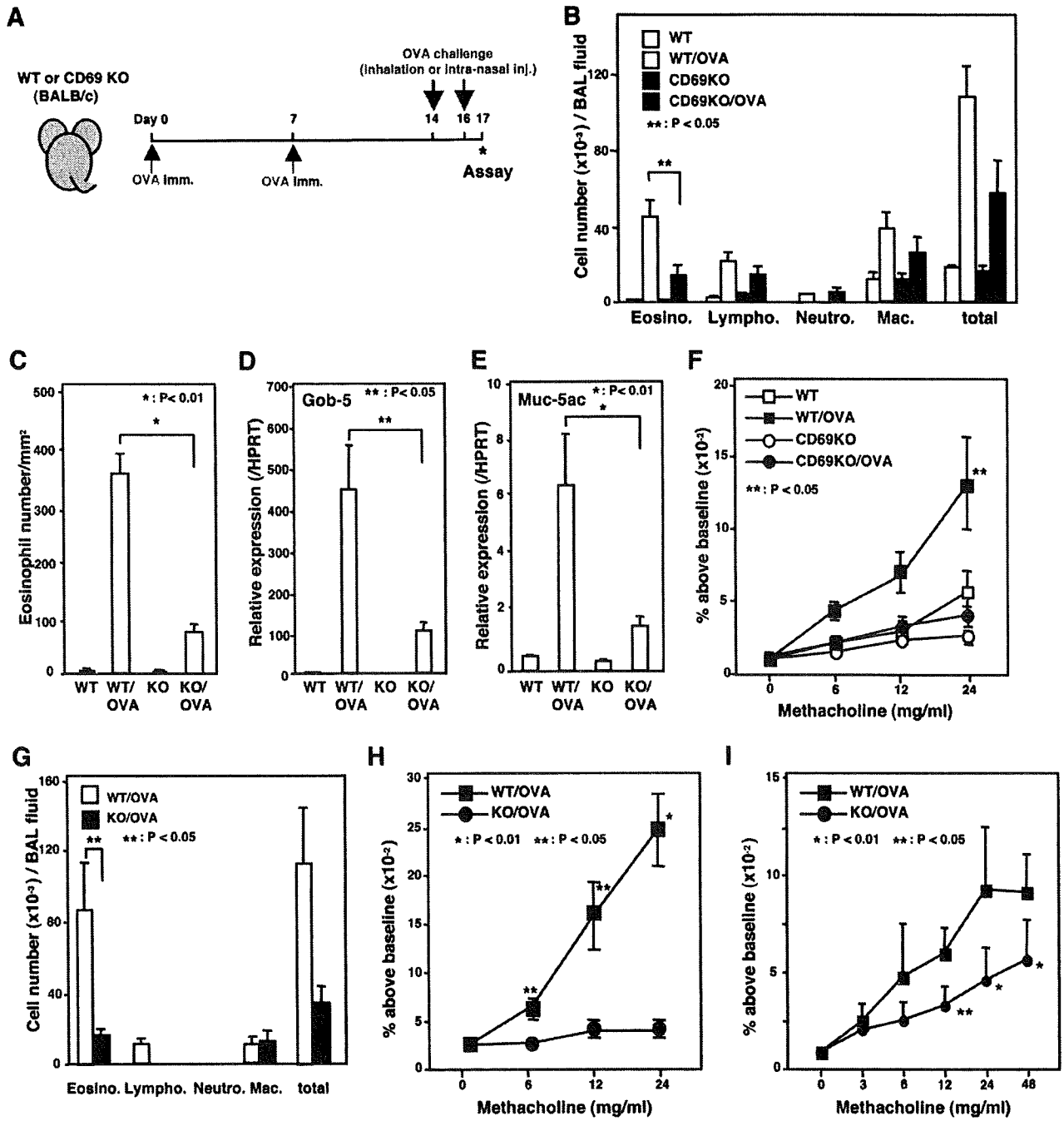


FIGURE 1. Inhibition of OVA-induced eosinophilic inflammation and AHR in CD69-deficient mice. *A*, Six-week-old WT or CD69-deficient (CD69 KO) mice were sensitized with OVA-alum and were subsequently challenged with or without OVA on days 14 and 16. One day after the last challenge, assays were performed. *B*, OVA-induced eosinophilic infiltration in the BAL fluid. Six- week-old WT and CD69-deficient mice were sensitized with OVA-alum and were subsequently challenged with (WT/OVA or CD69KO/OVA) or without (WT or CD69KO) OVA on days 14 and 16. One day after the last challenge, the mice were sacrificed and the BAL fluid was collected. The absolute cell numbers of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. Four mice from each group were used in this experiment. **, $p < 0.05$. *C*, The lung tissue specimens were fixed and stained with Luna. The number of peribronchiolar eosinophilic infiltrates is shown with the SEM. *, $p < 0.01$. *D* and *E*, mRNA expression of Gob-5 (*D*) and Muc-5ac (*E*) in the lung tissue was determined by quantitative RT-PCR. The relative intensity (/HPRT signal, mean of three samples) is shown with SDs. *F*, One day after the last OVA challenge, AHR in response to increasing doses of methacholine was measured in a whole-body plethysmograph. The mean values of the percent above baseline are shown with the SEM for four mice. A total of five independent experiments was performed with similar results. **, $p < 0.05$ (between WT/OVA and CD69KO/OVA groups). *G*, OVA-sensitized CD69-deficient mice were challenged intranasally with 100 μg of OVA twice while under anesthesia on days 14 and 16. One day after the last challenge, the mice were sacrificed and the BAL fluid was collected. The absolute cell number of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. Four mice from each group were used in this experiment. **, $p < 0.05$. *H*, AHR was monitored by measuring Penh. Mean values of the percent above baseline are shown with SDs of four mice. *, $p < 0.01$ and **, $p < 0.05$. *I*, CD69-deficient mice were immunized i.p. with 50 μg in 2 mg of alum on day 0 and were subsequently challenged intranasally with 30 μg of OVA on days 7, 8, and 9. One day after the last challenge, AHR was monitored by measuring RL. Mean values of the percent above baseline are shown with SDs of four mice. *, $p < 0.01$ and **, $p < 0.05$.

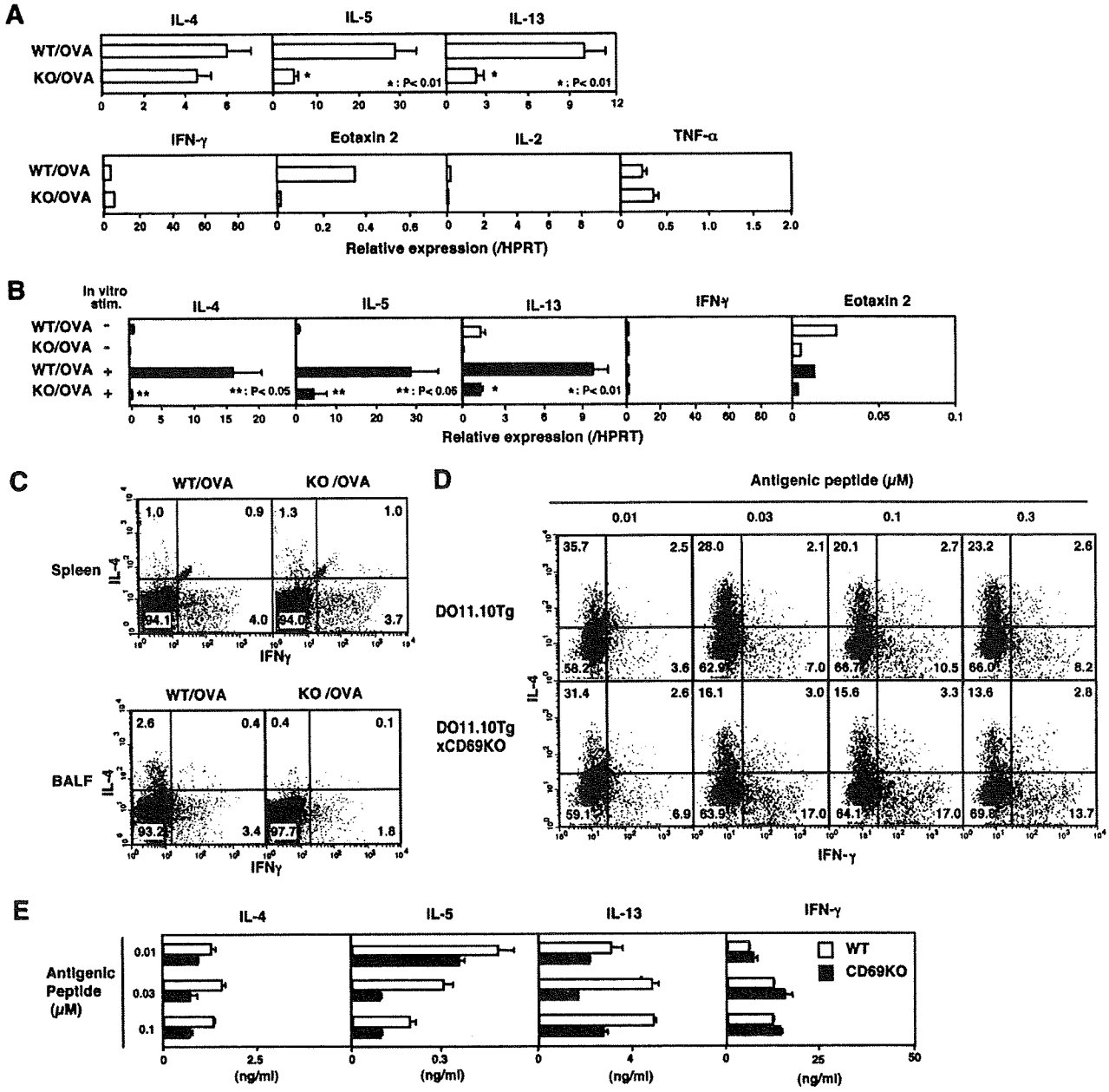
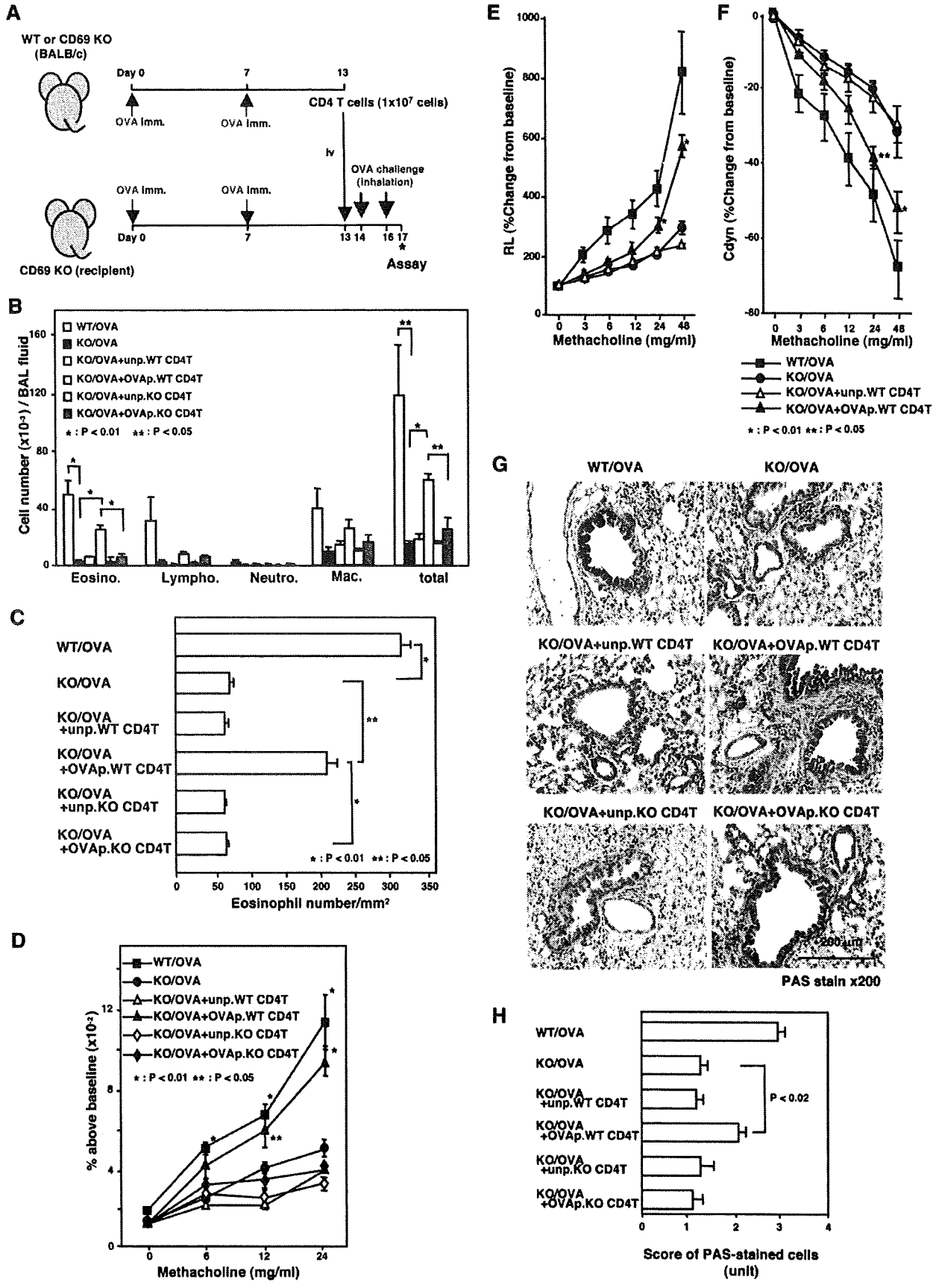


FIGURE 2. Decreased Th2 responses induced by CD69-deficient CD4 T cells. *A*, mRNA expression of IL-4, IL-5, IL-13, IFN-γ, eotaxin2, IL-2, and TNF-α in CD4 T cells in the BAL fluid. CD4 T cells in the BAL fluid were obtained from OVA-sensitized and OVA-challenged CD69-deficient mice 1 day after the last OVA inhalation, and the levels of mRNA expression of the indicated cytokines and a chemokine were determined by quantitative RT-PCR. The mean values of four samples are shown with SDs. *B*, mRNA expression of IL-4, IL-5, IL-13, IFN-γ, and eotaxin 2 expression in lung CD4 T cells. Whole lung mononuclear cells obtained from OVA-sensitized and OVA-challenged CD69-deficient mice were stimulated in vitro with antigenic OVA for 24 h. The $CD4^+$ T cells were collected by cell sorting, and the levels of mRNA expression of the indicated cytokines and a chemokine were determined by quantitative RT-PCR. The mean values of four samples are shown with SDs. *C*, Intracellular IFN-γ/IL-4 profiles of CD4 T cells from the spleen and BAL fluid mononuclear cells. Spleen cells and BAL fluid mononuclear cells obtained from OVA-sensitized and OVA-challenged CD69-deficient mice were stimulated in vitro with PMA and ionomycin for 4 h, and intracellular IFN-γ/IL-4 profiles of CD4 T cells were examined. The percentages from each quadrant are also shown. *D*, OVA-specific TCR Tg (DO11.10 TCR Tg) naive CD4 T cells were stimulated with increasing doses of antigenic peptides (0.01–0.3 μM) and irradiated APCs. Five days later, the cells were restimulated with immobilized anti-TCR mAb for 6 h, and intracellular IFN-γ/IL-4 profiles were examined. The percentages from each quadrant are also shown. Three independent experiments were performed with similar results. *E*, Cytokine production was assessed using in vitro-differentiated Th2 cells as in *C*. Mean values are shown with SDs ($n = 5$).

Impaired migration of CD69-deficient T cells into the asthmatic lung

To evaluate the ability of CD69-deficient CD4 T cells to migrate into the inflamed lung tissue, we used color-coded lymphocytes and an imaging model to visualize the migration of OVA-specific Th2 cells into the lung after allergen inhalation. WT and CD69-deficient mice were immunized with OVA on days 0 and 7, and the spleen CD4 T cells were stained with either CFDA-SE or SNARF-1. CFDA-SE-labeled WT CD4 T cells (2×10^7) and SNARF-1-labeled CD69KO T cells (2×10^7)



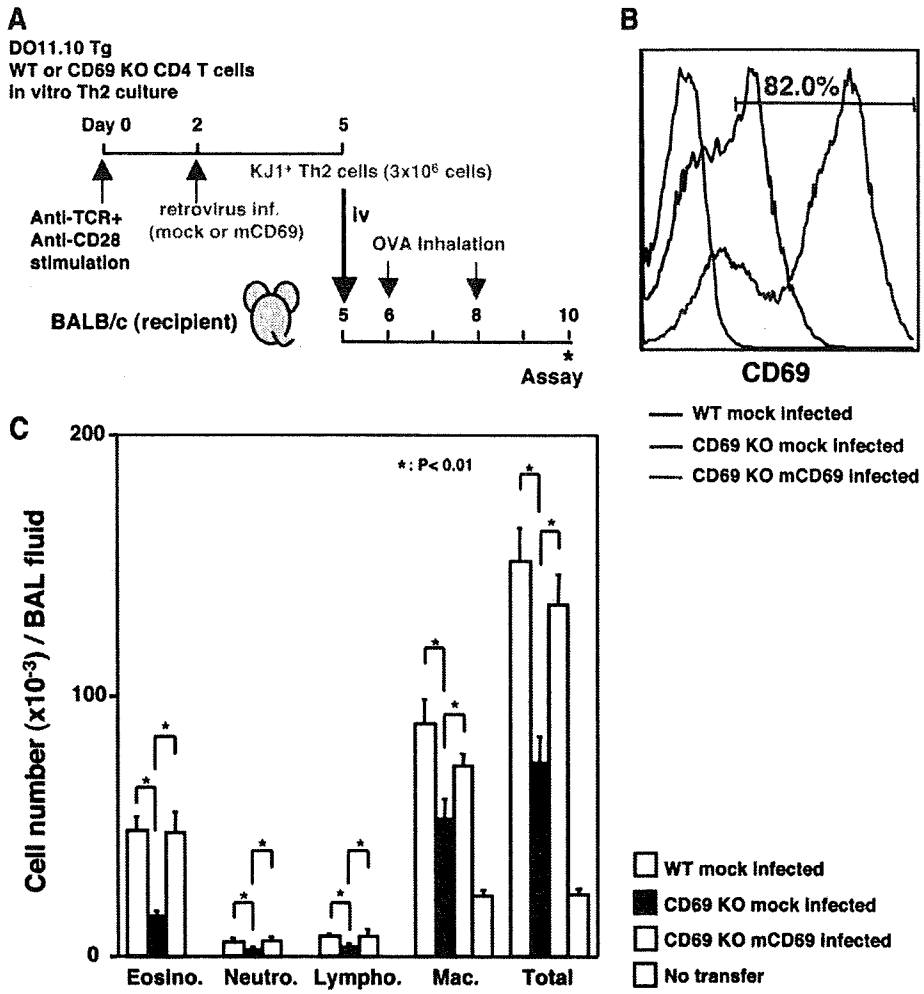


FIGURE 4. Induction of airway inflammation in CD69-deficient mice following adoptive transfer of CD69-overexpressing T cells. *A*, A schematic overview of the study protocol for the induction of asthma. Naive CD4 T cells from DO11.10 Tg WT or CD69-deficient (CD69KO) mice were stimulated with immobilized anti-TCR plus anti-CD28 mAb under Th2 conditions. On day 2, the mouse CD69 (mCD69) gene was introduced by a retrovirus vector containing the mCD69 gene. On day 5, the cultured cells were harvested and one or three million cells ($KJ1^+$ Th2 cells) were injected i.v. into normal BALB/c mice. The recipient mice were exposed to airway challenge with aerosolized OVA on days 6 and 8. BAL fluid was collected on day 10. *B*, A representative CD69 expression profile of the transferred $CD4^+$ Th2 cells (WT mock infected, CD69 KO mock infected, and CD69 KO mCD69 infected). The proportion of CD69-overexpressing cells in the CD69 KO mCD69-infected CD4 T cells was also indicated. *C*, Two days after the last OVA inhalation, the mice were sacrificed and the BAL fluid was collected. Cells in the BAL fluid were analyzed as in Fig. 1*A*. The absolute cell numbers of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. Five mice from each group were used in this experiment. *, $p < 0.01$. Three independent experiments with titration of transferred cells were performed and similar results were obtained.

were mixed and i.v. transferred into normal syngeneic C57BL/6 mice. One day later, the recipient mice were challenged by OVA inhalation (Fig. 5*A*). The mice were sacrificed and the excised lungs were examined using a Leica M205FA fluorescence microscope equipped with a Keyence VB-7010 CCD color camera system as described in *Materials and Methods*. This system allows us

FIGURE 3. OVA-induced eosinophilic inflammation and AHR were restored by adoptive transfer of OVA-primed WT but not CD69-deficient CD4 T cells. *A*, Splenic CD4 T cells from OVA-sensitized WT or CD69-deficient mice were administered i.v. into OVA-sensitized CD69-deficient mice (1×10^7 cells/mouse) 1 day before the first airway OVA challenge (day 13). The recipient mice were exposed to OVA challenges by inhalation on days 14 and 16. All assays were performed on day 17. *B*, We analyzed the cells in the BAL fluid from WT (WT/OVA), CD69-deficient (KO/OVA) mice without cell transfer, and CD69-deficient mice that received unprimed WT CD4 T cells (unp. WT), OVA-primed WT CD4 T cells (OVAp.WT), unprimed CD69-deficient CD4 T cells (unp.KO), or OVA-primed CD69-deficient CD4 T cells (OVAp.KO). The absolute cell numbers of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. Four mice from each group were used in this experiment. *, $p < 0.01$ and **, $p < 0.05$. *C*, The lung specimens were fixed and stained with Luna. The results of the peribronchiolar eosinophilic infiltrates are shown with the SEM. *, $p < 0.01$ and **, $p < 0.05$. *D*, OVA-induced AHR was induced as in Fig. 1. OVA-sensitized CD69-deficient mice received i.v. 1×10^7 CD4 T cells from unprimed (unp.) or OVA-primed (OVAp.) WT or CD69-deficient (KO) mice 1 day before the first airway challenge with aerosolized OVA. The level of AHR was monitored by measuring Penh. Mean values of the percent above baseline are shown with the SEM ($n = 4$). *, $p < 0.01$ and **, $p < 0.05$ (between WT/OVA, ■ and CD69KO/OVA, ●, and between KO/OVA + OVAp.WT CD4T, ▲, and KO/OVA + OVAp.KO CD4T; ♦). *E* and *F*, The AHR was also assessed by measuring RL (*E*) and Cdyn (*F*). Mean values of the percent changes above baseline are shown with the SEM ($n = 5$). *, $p < 0.01$ and **, $p < 0.05$ (between KO/OVA + OVAp.WT CD4T, ▲, and KO/OVA, ●). *G*, The lung specimens were fixed and stained with PAS. A representative staining pattern in each group is shown. Original magnification, ×200. *H*, The numerical scores of PAS-stained cells.

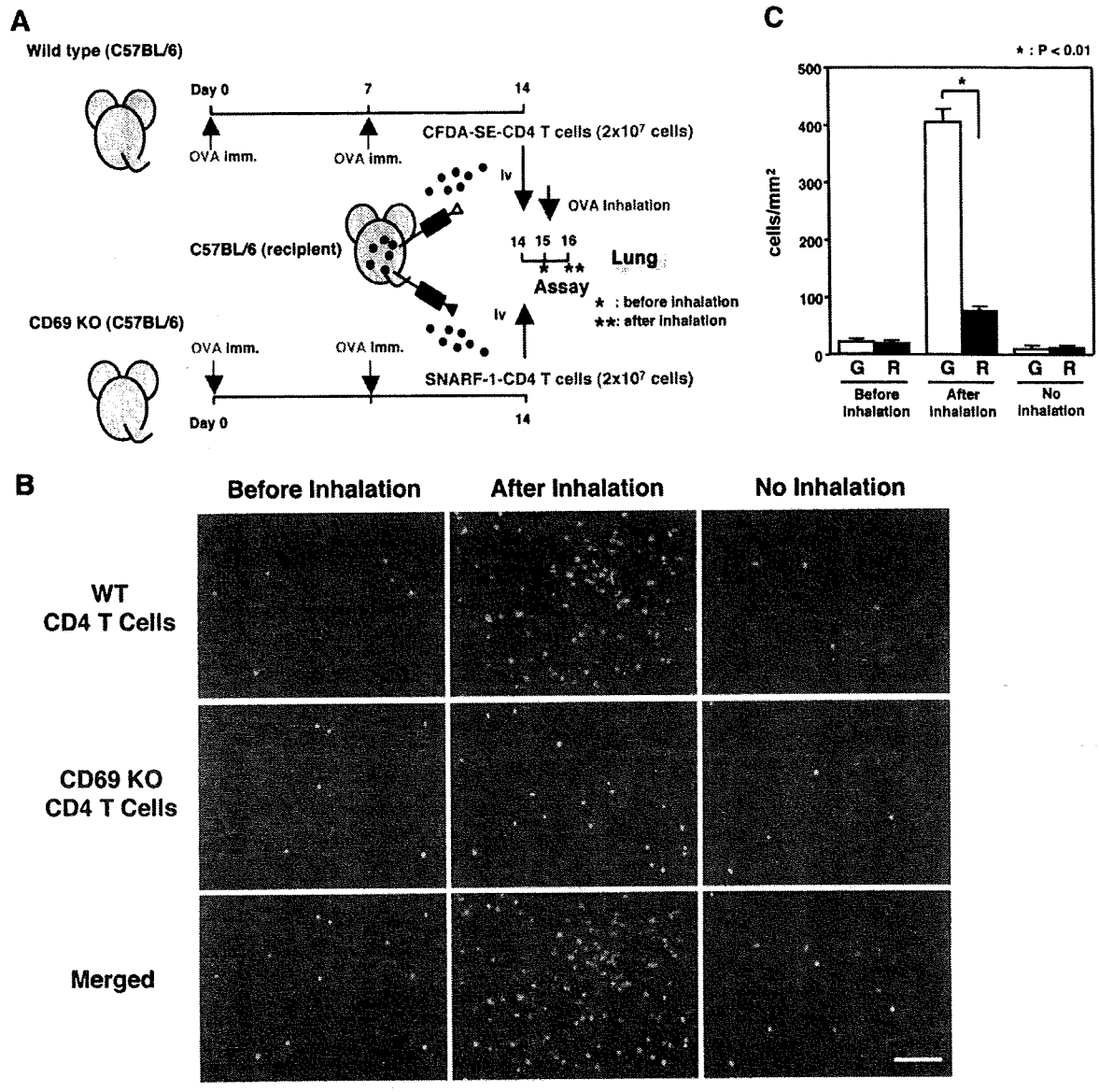


FIGURE 5. Color-coded fluorescence imaging of $CD4^+$ T cell infiltration into the lung during OVA-induced allergic asthma. *A*, A schematic overview of the study protocol for the induction of asthma. WT and CD69-deficient (CD69KO) mice were immunized with OVA on days 0 and 7. Splenic $CD4^+$ T cells were purified and labeled with either CFDA-SE (4 μM) or SNARF-1 (5 μM) at 37°C for 15 min. Twenty million CD4 T cells were injected i.v. into normal C57BL/6 mice on day 14. The recipient mice were exposed to airway challenge with aerosolized OVA on day 15. *B*, CFDA-SE- or SNARF-1-labeled CD4 T cells in the excised lungs were monitored before OVA inhalation on day 15 and 24 h after OVA inhalation on day 16 using the M205FA fluorescence microscope equipped with the VB-7010 CCD color camera system. Bar, 100 μm. *C*, Summary of the accumulation of fluorescent cells. Data are from 15 fields from three mice with the SD. □, Green fluorescent cells; ■, red fluorescent cells. *, $p < 0.01$ by Student's *t* test.

to monitor the green and red fluorescent T cells at the near surface area of the lung. As can be seen in Fig. 5, *B* and *C*, CFDA-SE-labeled green fluorescent cells from WT mice accumulated dramatically 1 day after inhalation. In contrast, the accumulation of the SNARF-1-labeled red fluorescent cells from CD69-deficient mice was marginal (Fig. 5*C*). Similar results were obtained when we used the opposite color-coded pattern for WT and CD69-deficient mice (data not shown). These results indicate that the migration of CD69-deficient CD4 T cells into the inflamed lung was impaired in comparison to that of WT CD4 T cells.

Reduced VCAM-1 induction and Th2 cell migration in the asthmatic lung of mice received CD69-deficient Th2 cells

VCAM-1 expression is induced in the asthmatic lung, and the VCAM-1/VLA-4 interaction is critical for the migration of T cells and the induction of Th2-dependent airway inflammation (32, 33). To test the involvement of VCAM-1 in the CD69-dependent airway inflammation, an immunofluorescent staining analysis with anti-VCAM-1 was performed. In vitro-generated WT and CD69-deficient DO11.10 Tg Th2 cells were transferred into unprimed BALB/c mice and the mice were exposed to OVA challenge with two cycles of inhalation (Fig. 6*A*). As expected, the induction of eosinophilic infiltration was dramatically impaired in the mice transferred with CD69-deficient Th2 cells accompanied with the decreased donor-derived $KJ1^+CD4^+$ Th2 cells in the lung (Fig. 6, *B* and *C*). The expression of VCAM-1, assessed by immunofluorescent staining analysis as seen in Fig. 6*D*, was substantially induced in the lung of mice with WT Th2 cell transfer, but it was marginal at best in the mice with CD69-deficient Th2 cell transfer. These

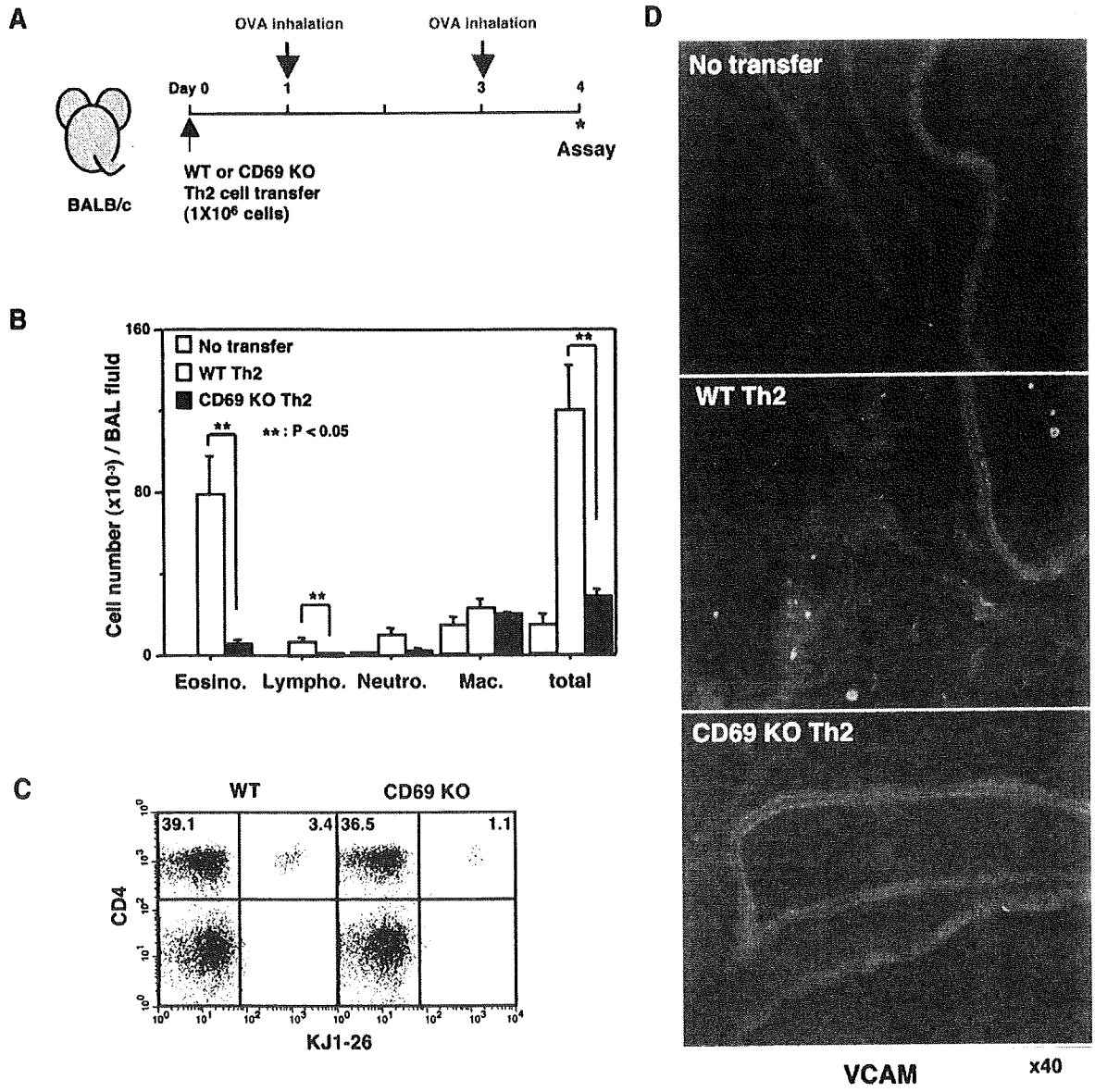


FIGURE 6. Reduced VCAM-1 induction and Th2 cell migration in the asthmatic lung of mice that received CD69-deficient Th2 cells. *A*, A schematic overview of the study protocol for the induction of asthma. OVA-specific DO11.10 Tg CD69-deficient Th2 cells (1×10^6) were transferred into BALB/c mice. The recipient mice were exposed to OVA challenges by inhalation 1 and 3 days after cell transfer. *B*, The cells in the BAL fluid from WT mice that did not receive cells (no transfer), WT mice that received WT Th2 cells (WT Th2), or CD69-deficient Th2 cells (CD69KO Th2) were analyzed. The absolute cell numbers of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with SDs. **, $p < 0.05$ (between WT transfer and CD69KO transfer). *C*, Representative CD4/KJ1-26 profiles of the lung leukocytes from the mice that received WT or CD69-deficient Th2 cells. *D*, Fluorescence microscopic analysis of the lung was performed using Abs for VCAM-1 (red). Blue represents the tissue autofluorescence.

results suggest that VCAM-1 is involved in the CD69-dependent migration of Th2 cells into the asthmatic lung.

In vivo treatment with anti-CD69 mAb inhibited the induction and the maintenance of OVA-induced airway inflammation and AHR

To explore the therapeutic effect of the administration of anti-CD69 mAb during allergic airway inflammation, WT BALB/c mice were immunized with OVA on days 0 and 7 and then treated with anti-CD69 mAb or control Ab 1 day before the first airway challenge with OVA (Fig. 7*A*). The examination of infiltrated cells in the BAL fluid revealed a marked decrease in the number of infiltrated eosinophils in the BAL fluid (Fig. 7*B*). Similarly, infiltrated eosinophils in the perivascular and peribronchiolar regions of the lung were lower in the mAb-treated animals (Fig. 7*C*). The treatment with anti-CD69 mAb resulted in a reduction of the extent of AHR (Fig. 7*D*) and milder mucus hyperproduction in the airways of mice treated with anti-CD69 mAb in comparison to the mice receiving control Abs (Fig. 7, *E* and *F*). These results suggest that the development of OVA-induced airway inflammation and AHR could be inhibited by treatment with anti-CD69 mAb.

Furthermore, when anti-CD69 mAb or control mAb was administered after the last OVA inhalation (on day 16), an examination of infiltration of inflammatory cells, including eosinophils and lymphocytes in the BAL fluid 1, 3, and 5 days after the last OVA inhalation (Fig. 7*G*), revealed that infiltration of inflammatory cells

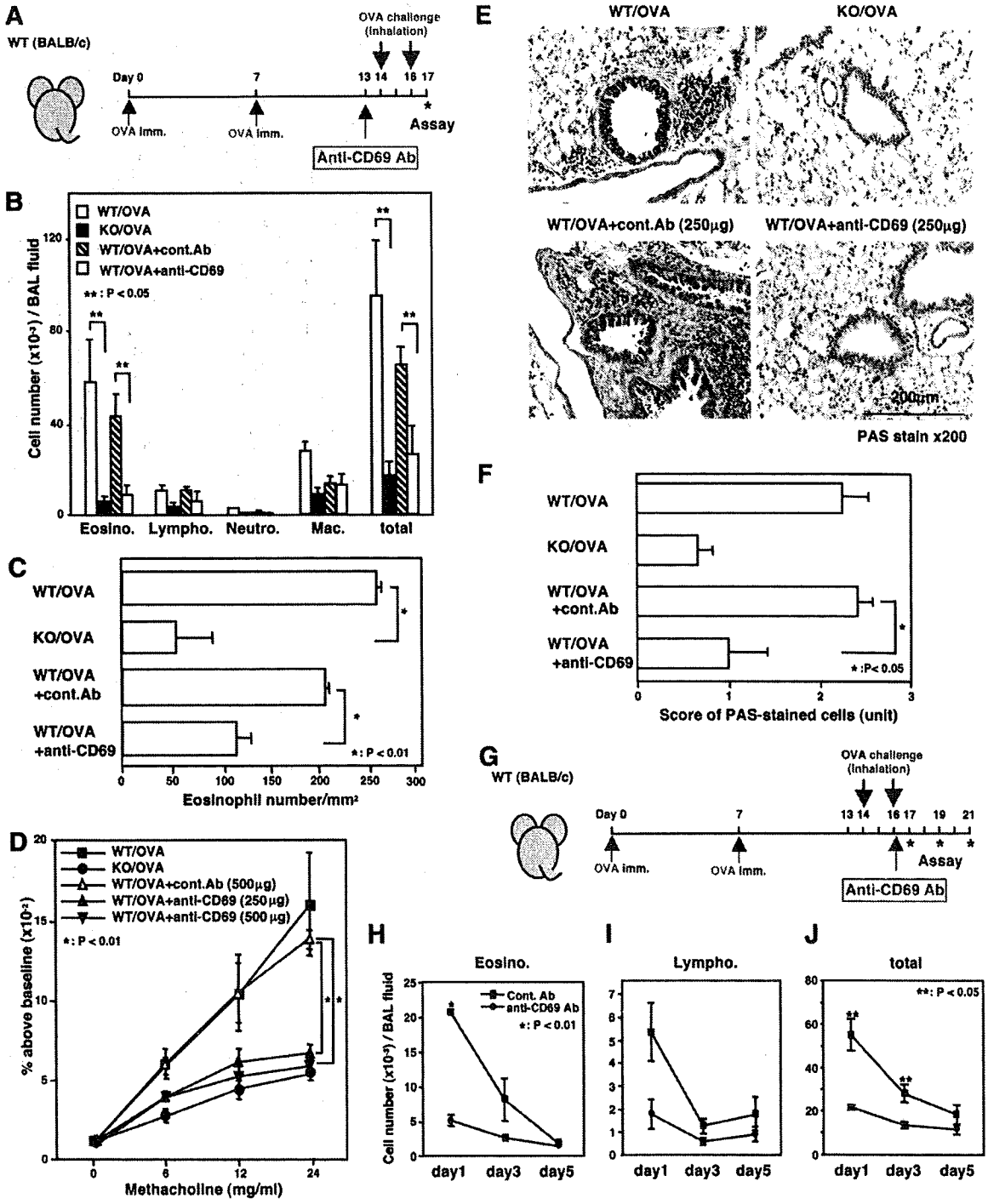


FIGURE 7. Effect of in vivo treatment with anti-CD69 mAb on OVA-induced airway inflammation and AHR. *A*, Six-week-old WT mice were sensitized with OVA-alum and were subsequently challenged with OVA on days 14 and 16. The mice were treated with anti-CD69 mAb or control hamster IgG 24 h before the first airway challenge with aerosolized OVA. *B*, WT BALB/c mice were treated with anti-CD69 mAb or control hamster IgG 24 h before the first airway challenge with aerosolized OVA. One day after the last challenge, the mice were sacrificed and the BAL fluid was collected. The absolute cell number of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. Four mice from each group were used in this experiment. **, $p < 0.05$ (between WT/OVA and KO/OVA groups and between control Ab- (cont.Ab) and anti-CD69-treated groups). *C*, The lung specimens were fixed and stained with Luna. The results of peribronchiolar eosinophilic infiltrates are shown with SDs. *, $p < 0.01$. *D*, OVA-induced AHR was induced as in Fig. 1. WT BALB/c mice were treated with anti-CD69 mAb or control hamster IgG 24 h before the first airway challenge with aerosolized OVA. Mean values of the percent above baseline are shown with the SEM ($n = 4$). Five independent experiments were performed with similar results. *, $p < 0.01$ (between cont.Ab- and anti-CD69-treated groups). *E*, The lung specimens were fixed and stained with PAS. A representative staining pattern from each group is shown. Original magnification, ×200. *F*, The numerical scores of PAS-stained cells. *G*, The OVA-sensitized and challenged mice were treated with anti-CD69 mAb or control hamster IgG just after the last OVA inhalation, and the BAL fluid was collected on days 17, 19, and 21. *H–J*, Anti-CD69 mAb was administered just after the last OVA inhalation (on day 16) and the BAL fluid was collected 1, 3, and 5 days later. The numbers of infiltrated eosinophils (*H*), lymphocytes (*I*), and total leukocytes (*J*) are shown with the SEM (five mice in each group). *, $p < 0.01$ and **, $p < 0.05$.

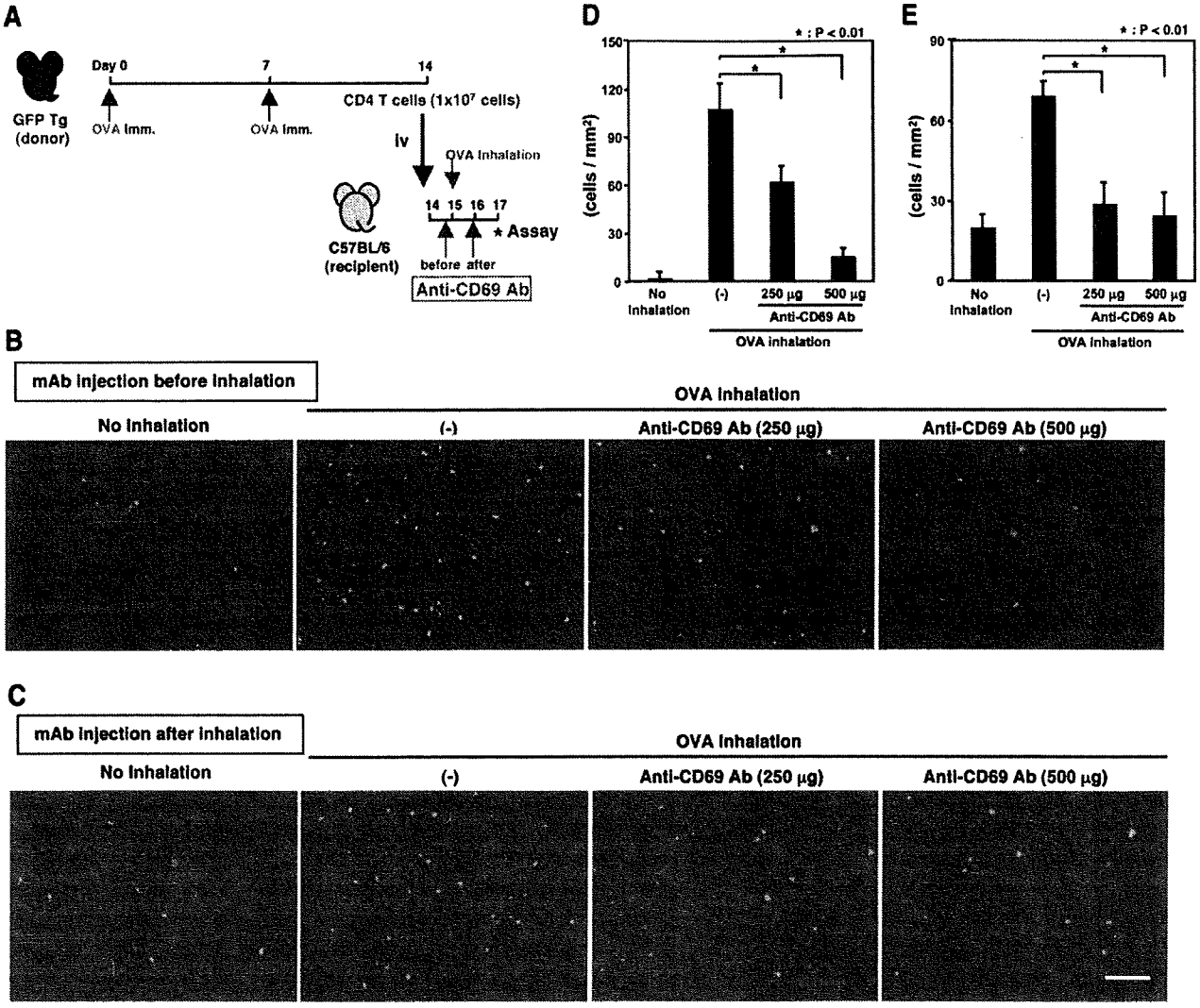


FIGURE 8. Effect of in vivo treatment with anti-CD69 mAb on CD4 T cell migration in the asthmatic lung. *A*, A schematic overview of the study protocol. GFP Tg mice were immunized with OVA on days 0 and 7. Splenic $CD4^+$ T cells (1×10^7) from OVA-sensitized GFP Tg mice were purified and injected into normal C57BL/6 mice on day 14. The recipient mice were exposed to airway challenge with aerosolized OVA on day 15. Anti-CD69 mAb (250 or 500 μg) was administered 1 h before OVA inhalation or 1 day after OVA inhalation. Mice were analyzed on day 17. *B* and *C*, Color-coded images of CD4 T cells. GFP^+ CD4 T cells in the excised lung were monitored 2 days after OVA inhalation on day 17 using the M205FA fluorescence microscope equipped with the VB-7010 CCD color camera system. Bar, 100 μm. *D* and *E*, The numbers represent the absolute cell number of CD4 T cells/mm^2 with SD, $n = 20$ fields). *, $p < 0.01$.

including eosinophils and lymphocytes decreased gradually in the control mAb-injected groups, whereas inflammatory cell infiltration was already substantially lower on day 1 in the groups receiving anti-CD69 mAb (Fig. 7, *H–J*). These results indicate that the recovery from airway inflammation occurred more quickly even when anti-CD69 mAb was administered after the onset of airway inflammation. Thus, a therapeutic effect of anti-CD69 mAb is suggested from this allergic asthma model.

In vivo treatment with anti-CD69 mAb inhibited the migration of OVA-primed Th2 cells

Finally, to evaluate the extent of CD4 T cell migration in the inflamed lung of mice treated with anti-CD69 mAb, GFP Tg mice with a C57BL/6 background were immunized on days 0 and 7 and then splenic CD4 T cells were transferred into normal C57BL/6 mice. One day after cell transfer, the mice were challenged with OVA by inhalation. Anti-CD69 mAb was injected 1 h before inhalation or 1 day after inhalation (Fig. 8*A*). As can be seen in Fig. 8, *B* and *D*, the migration of OVA-primed CD4 T cells was inhibited in a dose-dependent manner when the mice were treated with anti-CD69 mAb before inhalation. The migration of OVA-primed CD4 T cells tested on day 17 was also inhibited when the mice were treated with anti-CD69 mAb 1 day after inhalation (Fig. 8, *C* and *E*). These results indicate that the treatment with anti-CD69 mAb resulted in the inhibition of CD4 T cell migration in the inflamed lung, further supporting the therapeutic effect of anti-CD69 mAb treatment.

Discussion

Murine models of allergic airway inflammation have been used to dissect the underlying pathogenesis of asthma. In this study, we used an OVA-induced allergic asthma model and CD69-deficient mice to demonstrate that CD69 on CD4 Th2 cells

plays an important role in the development of Ag-induced Th2-driven eosinophilic airway inflammation and AHR (Fig. 3). The attenuated airway inflammation in CD69-deficient mice appeared to be due largely to the reduced migration of Th2 cells into the inflamed lung (Fig. 5). The involvement of VCAM-1 expression was suggested (Fig. 6). In addition, a therapeutic effect of the administration of anti-CD69 mAb was revealed (Figs. 7 and 8), indicating that CD69 could be a new target for mAb treatment of asthmatic patients.

The deficit in the effector Th2 cell differentiation and Th2 cytokine expression was modest in CD69-deficient CD4 T cells (Fig. 2, *D* and *E*). However, the expression of IL-5, IL-13, and eotaxin 2 in the CD69-deficient CD4 T cells in the BAL fluid was substantially reduced (Fig. 2*A*), and Th2 cytokine expression (IL-4, IL-5, and IL-13) in the lung CD4 T cells was very low compared with that of WT (Fig. 2*B*). Airway inflammation and AHR were attenuated substantially in the absence of CD69 on CD4 T cells (Figs. 3 and 4). Using an imaging system to detect migration of CD4 T cells in the lung, we found that the migration of CD69-deficient CD4 T cells was severely inhibited (Fig. 5). Thus, the alteration in the development of airway inflammation and AHR in CD69-deficient mice appears to be due mainly to the impaired migration of CD69-deficient Th2 cells into the inflamed lung.

It is known that the expression of VCAM-1 on endothelial cells is induced in the inflamed lung and that VCAM-1 expression is critical for the recruitment of allergen-specific T cells and eosinophils during allergic airway inflammation (32, 33). VCAM-1/VLA-4 interaction was shown to be important for the recruitment of T cells in the inflamed tissues (32). IL-13 has been recognized as a molecule that induces VCAM-1 expression in endothelial cells during allergic inflammation (5, 34). Several small molecule inhibitors of the interaction of VLA-4/VCAM-1 have been reported to attenuate allergic inflammation (35, 36). We detected a substantially reduced expression of IL-13 in the infiltrated CD4 T cells in the BAL fluid and the lung tissue in CD69-deficient mice (Fig. 2, *A* and *B*). Marginal induction of the expression of VCAM-1 in the lung of mice that received CD69-deficient Th2 cells was observed (Fig. 6). Thus, the effect on the migration of CD69-deficient Th2 cells appears to be at least in part due to the failure to induce VCAM-1 expression in the asthmatic lung. However, more precise molecular mechanisms underlying the CD69-dependent Th2 cell migration into the inflamed lung will require further investigation.

Although the ligand for CD69 has not been identified, a possible scenario is that a putative ligand may be induced and expressed on the inflamed lung tissues. Activated Th2 cells expressing CD69 then may migrate into the lung tissue and remain at the inflammatory site efficiently via the CD69/CD69 ligand interaction. CD69-deficient CD4 T cells, in contrast, may not be retained at the inflammatory site very efficiently because of the lack of this interaction.

The regulatory roles of CD69 in an anti-collagen Ab-induced arthritis model (16) and a collagen-induced arthritis model (15) have been reported. In an anti-collagen Ab-induced arthritis model, a critical role for CD69 in neutrophil function at the effector phase was indicated. In a collagen-induced arthritis model, it is suggested that CD69 is a negative regulator of arthritis through TGF-β synthesis during the induction phase. In our mouse model of OVA-induced asthma, the expression levels of TGF-β in the BAL fluid and in the activated spleen cells in vitro were similar between WT and CD69-deficient mice (T. Nakayama, unpublished observation). Therefore, the CD69-mediated induction of TGF-β synthesis appeared not to be involved in the regulation of Th2 cell differentiation and/or the regulation of Th2-driven allergic airway inflammation. Thus, CD69 can play different roles in the initiation of inflammatory immune responses. CD69 is also expressed on activated B cells. Although B cell development appeared to be mildly affected in CD69-deficient mice, normal proliferative responses of peripheral B cells were detected (37). We also observed normal IgG1 and IgE production in CD69-deficient B cell cultures after LPS and IL-4 stimulation (T. Nakayama, unpublished observation). Thus, the function of B cells appeared to be less dependent on CD69, suggesting a redundant mechanism during B cell activation.

It was recently reported that CD69 is physically associated with spingosine 1-phosphate receptor 1 ($S1P_1$) and inhibits the S1P/$S1P_1$-mediated egress of the cells from the lymphoid organs (17). To address the involvement of $S1P_1$-mediated regulation of CD4 T cell egress in the CD69-dependent airway inflammation, we used FTY720, which acts on $S1P_1$ to inhibit the egress of lymphocytes from the lymph nodes. The numbers of CD4 and CD8 T cells in the peripheral blood, spleen, and lymph nodes were equivalent between WT and CD69-deficient mice even after OVA immunization and challenge, and their numbers in the peripheral blood were reduced equivalently when FTY720 was administered (T. Nakayama unpublished observation). We observed reduced airway inflammation and AHR in CD69-deficient mice (Fig. 1). Although FTY720 injection reduced the extent of OVA-induced eosinophilic airway inflammation and AHR in WT mice, no effect was observed in CD69-deficient mice (T. Nakayama unpublished observation). Moreover, CD69-mediated inhibition of $S1P_1$ function appeared to be observed only when CD69 up-regulation is induced with IFN-α/β (17). No significant amount of IFN-α/β was detected in the BAL fluid in our OVA-induced airway inflammation model (T. Nakayama, unpublished observation). Thus, in our allergic airway inflammation model, S1P/$S1P_1$-mediated regulation of lymphocyte egress from the lymph nodes does not appear to account for the change in the airway inflammation induced by CD69-deficient Th2 cells.

We used an imaging system to follow Th2 cell migration into the lung with a fluorescent microscope and CD4 T cells labeled with a fluorescent dye (Fig. 5) or GFP Tg T cells (Fig. 8). We detected infiltrated CD4 T cells at the near surface area of the asthmatic lung. Migration of CD4 T cells into the parenchyma or through the endothelial cells of the lung vessels has been reported (33). The imaging system used here is useful for the quantitative detection of the migrated lymphocytes into the asthmatic lung, since the number of lymphocytes including Th2 cells is relatively small among inflammatory cells such as eosinophils.

In a model of Th1-induced airway inflammation characterized by the neutrophilic infiltration, CD69-deficient Th1 cells also showed reduced levels of airway inflammation compared with WT (T. Nakayama, unpublished observation). Although the molecular and cellular mechanisms involved in the Th1-induced airway inflammation is distinct from that of Th2-cell induced eosinophilic inflammation, this result indicates that CD69 on Th1 cells also plays an important role in the induction of airway inflammation.

This study demonstrated the therapeutic effect of the administration of anti-CD69 mAb (Figs. 7 and 8). The anti-CD69 Ab treatment via the airway also showed a significant inhibitory effect (our unpublished observation). The ligand for CD69 has not been identified, but it is possible that anti-CD69 mAb blocks the interaction of CD69 and putative CD69 ligands, resulting in the reduced Th2 cell migration and attenuated airway inflammation. Although a very early study suggested that the anti-CD69 mAb if cross-linked induced the enhancement of Con A-induced proliferation of T cells in vitro (38), we have not detected any dramatic effect on T cell numbers or the phenotypes of CD4 T cells in the lung or spleen of

mice receiving anti-CD69 mAb (T. Nakayama unpublished observation). In any event, it is notable that a potent therapeutic effect of the anti-CD69 mAb administration was observed even after the onset of airway inflammation.

In summary, our results indicate that CD69 on CD4 T cells plays a critical role in the development of allergen-induced eosinophilic inflammation and AHR by effecting efficient migration of activated Th2 cells into the asthmatic lung. Moreover, our mAb administration experiments revealed that CD69 could consequently be a possible therapeutic target for the treatment of asthmatic patients.

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Disclosures

The authors have no financial conflict of interest.

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SHORT COMMUNICATION

Mast cell regulation of epithelial TSLP expression plays an important role in the development of allergic rhinitis

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Epithelial cell-derived thymic stromal lymphopoietin (TSLP) is a master switch for asthma or atopic dermatitis by inducing a dendritic cell-mediated Th2-type allergic inflammation. Allergic rhinitis is also pathologically characterized by Th2-type allergic inflammation. This study demonstrates that mast cells regulate the epithelial TSLP expression in allergic rhinitis. TSLP expression was found to be up-regulated predominantly in the nasal epithelium in the ovalbumin (OVA)-sensitized and -nasally challenged mouse model of allergic rhinitis, which was abolished in mast cell-deficient WBB6F1-W/W^v in comparison with control WBB6F1-+/+ mice. Similarly, the epithelial TSLP expression was reduced in Fc receptor γ chain (FcγR)-deficient mice, where the high-affinity IgE receptor (FcεRI) is not expressed on mast cells, in comparison with control C57BL/6 mice. Furthermore, the administration of neutralizing TSLP antibody during the challenge phase of OVA inhibited the development of allergic rhinitis. These results suggest that the direct stimulation of epithelial cells by antigens alone may not be sufficient to induce TSLP expression in the nasal epithelium, and that mast cell regulation of epithelial TSLP expression, possibly *via* FcεRI, plays an important role in the development of allergic rhinitis.

Key words: Allergic rhinitis · Epithelial cells · Mast cells · TSLP

Introduction

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine, which binds to a TSLP receptor (TSLPR) consisting of the IL-7 receptor α-chain (IL-7Rα) and a common γ receptor-like chain (TSLPR-γ) [1, 2]. TSLP was originally identified as a factor derived from a thymic stromal cell line that could support the growth of a mouse B cell line[3]. However, recently, TSLP, derived from epithelial cells, has been shown to be capable of activating CD11c$^+$ myeloid DC to up-regulate costimulatory molecules, leading to the differentiation of CD4$^+$ T cells into Th2 cells. Therefore, it plays a key role in the development of allergic diseases such as asthma or atopic dermatitis [1, 2, 4]. For instance, transgenic mice expressing TSLP in keratinocytes or in lung epithelial cells have been shown to develop atopic dermatitis- or asthma-like inflammation in the skin or the lung, respectively, while TSLPR null mice failed to develop an inflammatory lung response to inhaled antigen[5–7]. In humans, TSLP has been shown to be expressed by keratinocytes in atopic dermatitis and by bronchial epithelial cells in asthmatic airways[8, 9].

In contrast to the pro-allergic action of TSLP, regulation of TSLP expression in epithelial cells has not been fully elucidated. Previous studies suggest that TSLP expression in mouse keratinocytes is regulated by vitamin D and retinoic acid signaling [10]. Most recently, TNF-α, IL-1β, and ligands for Toll-like receptors

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such as LPS were shown to up-regulate TSLP expression *via* NF-κB pathway in cultured human bronchial epithelial cells and keratinocytes[11–13].

Mast cells play a critical role in allergic inflammation including asthma and allergic rhinitis *via* multiple mechanisms [14–16]. Mast cells in patients with allergic rhinitis and asthma produce Th2-type cytokines, induce IgE synthesis in B cells, and can autoactivate themselves *via* the mast cell-IgE-high affinity receptor (FcεRI) cascade [17]. In addition, mast cells up-regulate the production of a variety of cytokines/chemokines in epithelial cells and fibroblasts, and induce the recruitment of basophils, T cells, and eosinophils into sites of allergic inflammation as well as their own intraepithelial accumulation. However, it remains unclear whether mast cells contribute to the epithelial TSLP expression at the site of allergic inflammation.

In this study, a direct assessment of the roles of mast cells on epithelial TSLP expression was conducted using a mouse model of allergic rhinitis and two types of genetically mast cell-deficient mice (WBB6F$_1$-*KitW/W-v* mice and C57BL/6-*KitW-sh/W-sh* mice).

Results and discussion

TSLP expression in nasal epithelium requires mast cells and FcγR in a mouse model of allergic rhinitis

WBB6F1-+/+ mice with a mutation of c-kit are deficient for mast cells [18] and FcγR-deficient mice showed no expression of FcR (FcγRI, FcγRIII, and FcεRI) [19] because the expression of FcR requires a homodimer of the subunit FcγR for surface expression and signal transduction in the mouse [20]. C57BL/6 and WBB6F1-+/+ mice challenged with OVA showed an infiltration of leukocytes in the nasal submucosa, whereas WBB6F1-W/W^v mice and FcγR-deficient mice challenged with OVA did not show any infiltration of leukocytes into the nasal submucosa, as previously described [21, 22] (data not shown). Immunohistochemical staining with anti-TSLP antibody revealed little positive immunoreactivity of TSLP in the nasal mucosa obtained from the PBS-challenged C57BL/6 mice, but the positive immunoreactivity of TSLP increased predominantly in the nasal epithelium after the induction of allergic rhinitis in C57BL/6 and WBB6F1-+/+ mice (Fig. 1 and 2). In contrast, the increase of TSLP-positive cells in the nasal epithelium after the induction of allergic rhinitis was absent in WBB6F1-W/W^v mice and FcγR-deficient mice (Fig. 2). Immunohistochemical staining with control IgG antibody showed negative staining in the nasal mucosa after the induction of allergic rhinitis in C57BL/6 mice (Fig. 1). Therefore, TSLP expression is induced in the nasal epithelium in a mouse model of allergic rhinitis and the presence of mast cells and FcγR is necessary for the allergen-induced epithelial TSLP expression.

To further clarify the roles of mast cells and FcγR in mast cells in epithelial TSLP expression in a mouse model of allergic rhinitis, mast cell-deficient C57BL/6-*KitW-sh/W-sh* mice [23, 24] were reconstituted with BM-derived mast cells (BMMC) derived from wild-type C57BL/6 mice or FcγR-deficient mice with C57BL/6 genetic background. As shown in Fig. 2, an increase in the number of TSLP-positive cells in the nasal epithelium after the induction of allergic rhinitis was observed in the C57BL/6-*KitW-sh/W-sh* mice reconstituted with wild-type BMMC, but not with FcγR-deficient BMMC. Therefore, mast cells and FcγR in mast cells plays a critical role in the epithelial TSLP expression in a mouse model of allergic rhinitis.

TSLP is critical for the development of allergic rhinitis

To determine whether TSLP, expressed in the nasal epithelium, plays a critical role for the development of the mouse model of allergic rhinitis, the effects of TSLP neutralizing antibody on the development of allergic rhinitis were clinically and pathologically evaluated by periodic acid-Schiff (PAS) staining to demonstrate goblet cells.

C57BL/6 mice treated with control IgG antibody during the challenge phase with OVA showed a significant increase in the nasal rub counts (Fig. 3). The mice also showed an infiltration of leukocytes in the nasal submucosa, and the number of PAS-positive cells in the nasal epithelium (goblet cells) and submucosa (gland cells) also increased in these mice (Fig. 3 and data not shown). In contrast, the mice treated with anti-TSLP neutralizing antibody showed a significant reduction in the frequency of nasal rubs with little infiltration of leukocytes in the nasal submucosa, and a decreased number of PAS-positive cells in the nasal epithelium and submuosa (Fig. 3 and data not shown). Interestingly, immunohistochemical staining revealed the TSLP levels in the nasal epithelium to be relatively up-regulated after treatment with anti-TSLP antibody (Fig. 3). To exclude the possibility that a trivial deposition of anti-TSLP antibody on the tissue may affect the TSLP staining, the nasal mucosa specimens obtained from C57BL/6 mice treated with anti-TSLP antibody during PBS challenge were stained with anti-TSLP antibody and only slight immunoreactivity for TSLP was confirmed in these tissue specimens (data not shown). These results indicated that the blockade of TSLP activity inhibited the development of the mouse model of allergic rhinitis.

The current results suggest that the direct stimulation of epithelial cells by antigens alone may not be sufficient to induce TSLP expression in the nasal epithelium because the deficiency of mast cells and FcγR in mast cells abolished epithelial TSLP expression regardless of the stimulation with the antigen (OVA) on the nose. This is consistent with recent *in vitro* studies, which suggest that TSLP expression in epithelial cells requires proinflammatory cytokines such as TNF-α [11–13]. Therefore, it is possible that mast cell-derived inflammatory cytokines including TNF-α generated at the site of allergic rhinitis may play an important role for the induction of TSLP expression in the nasal epithelium. Because the mast cell release of TNF-α or other proinflammatory cytokines can occur immediately following the antigen stimulation in the nose of patients with allergic rhinitis [25] and FcγR was also shown to be critical for the epithelial TSLP expression (Fig. 2), we therefore speculate that mast cells (possibly

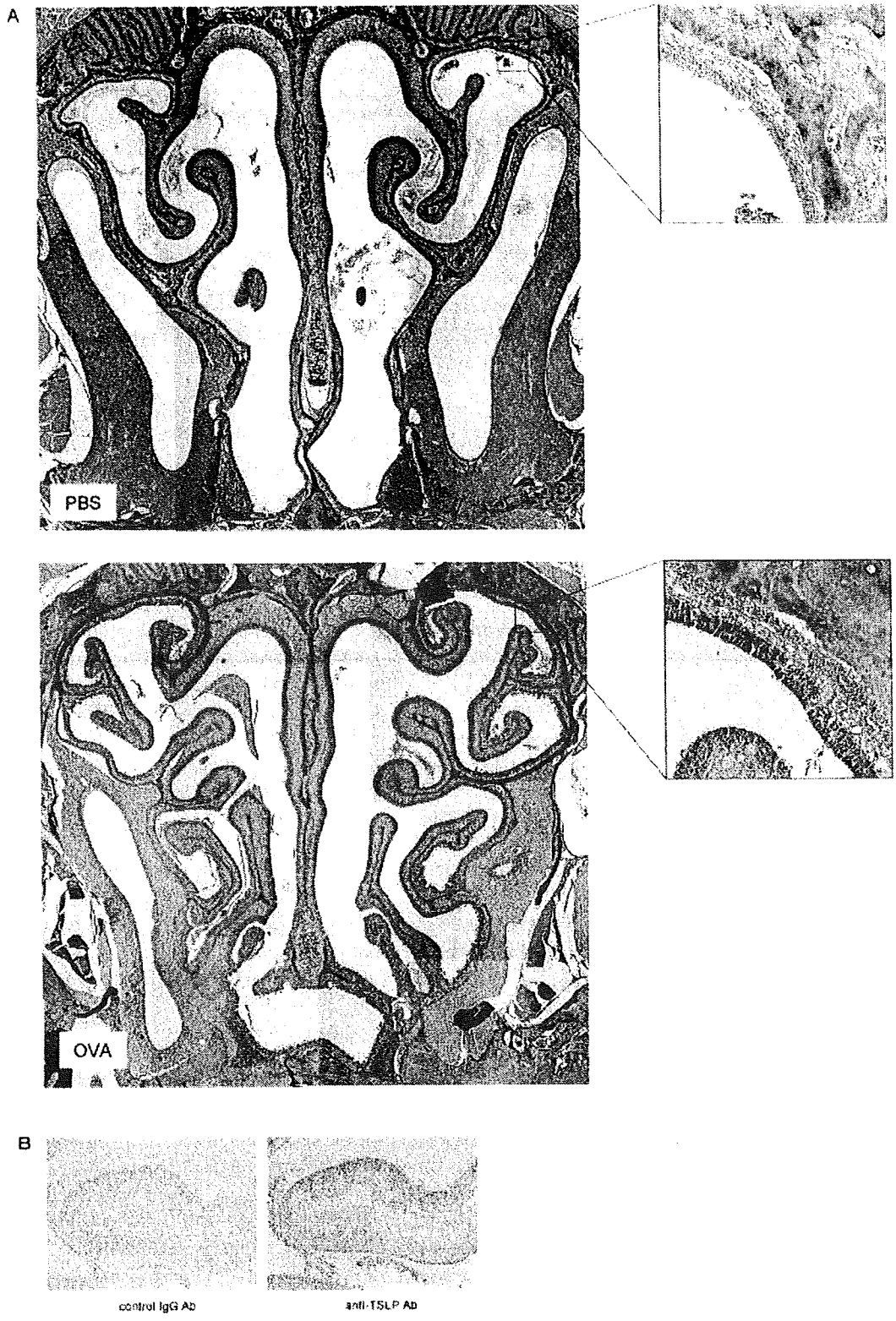

Figure 1. Repeated nasal exposure to OVA in OVA-sensitized C57BL/6 mice induces TSLP expression in the nasal epithelium. (A) Representative pictures of TSLP expression in the nasal mucosa of OVA-sensitized C57BL/6 mice repeatedly challenged with OVA (OVA) or control PBS (PBS) are shown. The curved lines indicate positive staining area. (B) Representative pictures of the nasal mucosa specimens stained with control IgG antibody (control Ab) or anti-TSLP antibody (anti-TSLP Ab) after the induction of allergic rhinitis.

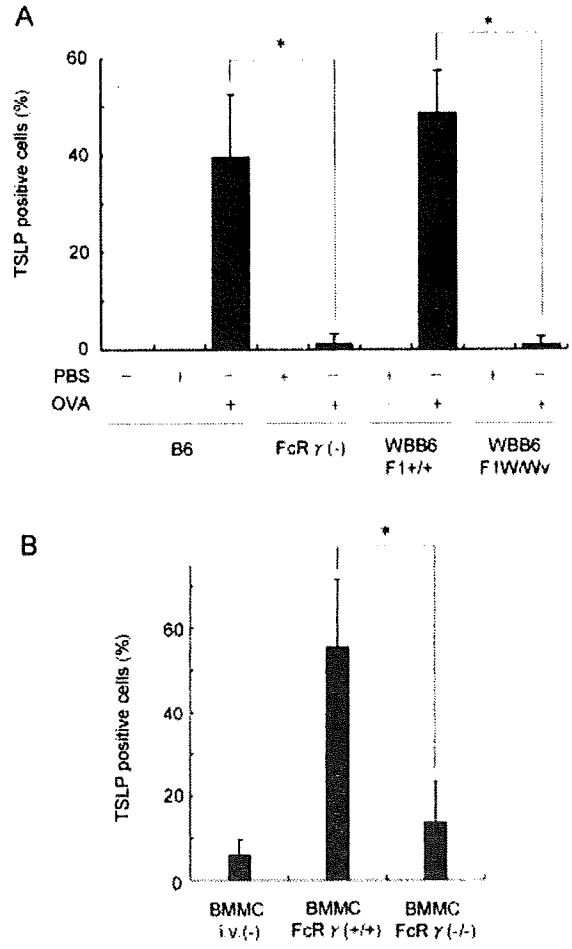

Figure 2. Deficiency of mast cells and FcγR abolishes TSLP expression in the nasal epithelium during allergic rhinitis. (A) A quantitative analysis of TSLP expression in the nasal epithelium during allergic rhinitis using C57BL/6, FcγR-deficient, WBB6F1-+/+, WBB6F1-W/W^v mice as described in the text. (B) A quantitative analysis of TSLP expression in the nasal epithelium during allergic rhinitis using C57BL/6-KitW-sh/W-sh mice reconstituted with wild-type BMMC (BMMC FcγR(+/+)) or with FcγR-deficient BMMC (BMMC FcγR(-/-)), as described in the text. BMMC i.v.(-): C57BL/6-KitW-sh/W-sh mice without the transfer of BMMC. Values represent the mean + SD of eight mice in each group. *$p<0.05$.

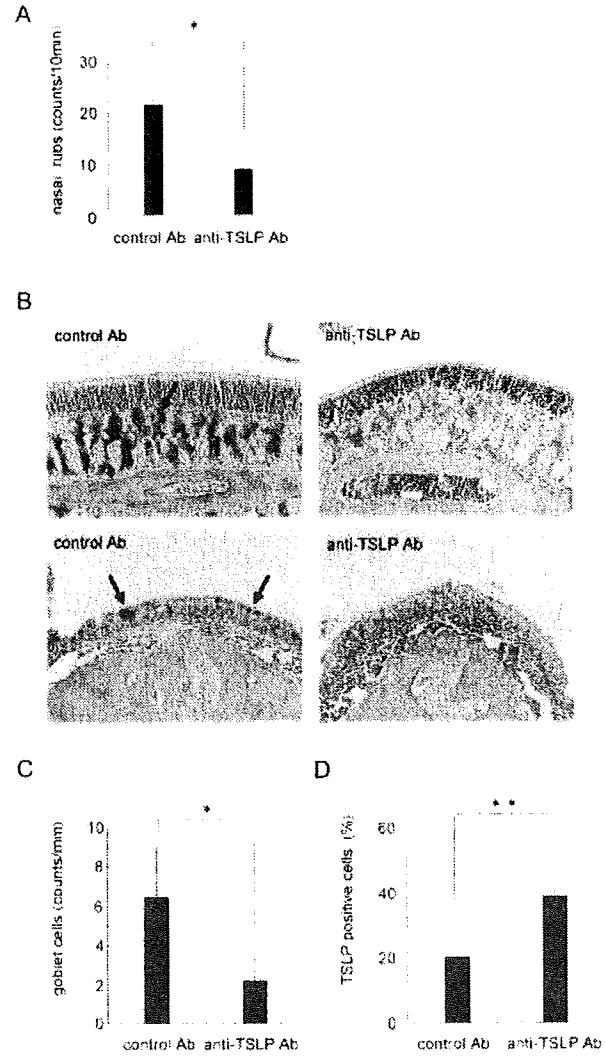

Figure 3. Anti-TSLP neutralizing antibody inhibits the development of allergic rhinitis. The OVA-sensitized mice were intranasally challenged with 100 μg OVA in 10 μL PBS twice per day for 1 week (total 14 times/week). The mice were challenged intranasally with PBS in a similar manner for the negative control. During the OVA challenge, rat anti-mouse TSLP neutralizing antibody or the isotype control rat IgG2a (15 mg/kg per mouse) was administered intraperitoneally every other day. (A) Appearance of nasal symptom (nasal rubs). The frequency of nasal rubs was counted for 10 min after the last intranasal challenge with OVA. Control Ab: mice treated with control IgG antibody, anti-TSLP Ab: mice treated with anti-TSLP antibody. (B) Representative PAS staining of the nasal mucosa obtained from C57BL/6 mice treated with anti-TSLP antibody (anti-TSLP Ab) or control IgG (control Ab). Upper panels: submucosa; lower panels: epithelial area. Note the decreased number of PAS-positive cells in the nasal submucosa and epithelium. The arrows indicate the PAS-positive cells. (C) A quantitative analysis of the epithelial PAS-positive cells. (D) A quantitative analysis of the epithelial TSLP-positive cells. Values represent the mean + SD of eight mice in each group. Both * and ** represent $p<0.05$.

via FcεRI-mediated release of TNF-α) might play a critical role in the initial phase of allergic rhinitis through the induction of epithelial TSLP expression.

Conversely, TSLP has been shown to stimulate the production of high levels of Th2 cytokines by human mast cells synergistically with IL-1 and TNF-α [12]. Therefore, it is possible that there is an important interaction between epithelial cells and mast cells *via* TSLP and proinflammatory cytokines for the development of allergic rhinitis. Mast cells may induce TSLP expression in the nasal epithelium following the allergen challenge, and then epithelial-derived TSLP could stimulate mast cells to produce Th2 cytokines to further augment allergic inflammation.

Treatment of the mice with anti-TSLP neutralizing antibody inhibited the development of allergic rhinitis (Fig. 3). These results

are consistent with the previous *in vivo* studies using mouse models of asthma and atopic dermatitis [5–7]. Thus, the current results also support the importance of TSLP in allergic inflammation. In addition, the findings that treatment with anti-TSLP neutralizing antibody up-regulated TSLP expression in the nasal epithelium (Fig. 3) may suggest the presence of negative feedback regulation in the epithelial TSLP expression.

Concluding remarks

In summary, this study provides significant evidence that mast cells play a critical role in TSLP expression in the nasal epithelium in allergic rhinitis and subsequent development of the disease. FcεRI, expressed on mast cells, is likely to be involved in the epithelial TSLP expression. To our knowledge, this is the first study suggesting TSLP to play a potential role in allergic rhinitis.

Materials and methods

Mice

Female 4–6 wk C57BL/6 mice, WBB6F1-+/+, and WBB6F1-W/W^v mice [18] were purchased from Japan SLC (Tokyo, Japan), FcγR-chain deficient mice (C57BL/6 background) and mast cell-deficient KitW-sh/W-sh mice on the C57BL/6 background were previously described [19, 23, 24], and were bred under specific pathogen-free conditions.

Allergic rhinitis model

An allergic rhinitis model was established as previously described with some modifications [26]. Briefly, the mice were actively immunized i.p. with 10 μg OVA (Sigma Aldrich, St. Louis, MO) in 4 mg aluminum hydroxide on days 1 and 7. Starting on day 14, they were challenged intranasally with 100 μg OVA in 10 μL PBS twice per day for 1 week (total 14 times/week). The mice were challenged intranasally with PBS in a similar manner for the negative control. For some experiments, rat anti-mouse TSLP neutralizing antibody or control rat IgG2a (15 mg/kg per mouse, R&D Inc., Minneapolis, MN) were administered intraperitoneally every other day, starting on day 14 until sacrifice. The dosage of the antibody (15 mg/kg) was based on previous experiments [27]. The animal experiments were approved by the Institutional Review Board of the University of Yamanashi.

Histological examination

At 12 h after the final nasal challenge, mice were killed with carbon dioxide. The heads were removed, fixed, and decalcified. Coronal nasal sections were visualized by staining with hematoxylin and eosin (H&E) or PAS/hematoxylin (to demonstrate goblet cells). For immunohistochemistry, the coronal nasal sections were deparaffinized and stained with anti-TSLP antibody or control IgG antibody (Santa Cruz Biotechnology Inc.) through the use of peroxidase-based Vectastain ABC kits with DAB substrate (Vector Laboratories, Burlingame, CA).

Nasal symptom

Nasal rubs were observed for 10 min after the last intranasal challenge using a video recorder and the frequency of the nasal rubs were counted by investigators who were blind to the treatment protocol.

Assessment and quantification of histological examination

The length of the positively TSLP-stained epithelial layer in the total nasal epithelial lining of the coronal sections was measured and expressed as percentage of the total length of the total nasal epithelial lining of the coronal sections (% of the total length). The number of PAS-positive cells in the total nasal epithelial lining of the coronal sections was counted microscopically in a blinded manner and it was expressed as the number per the total length of the total nasal epithelial lining of the coronal sections (number/mm). Two or four specimens of the TSLP- or PAS-stained coronal sections from one mouse were selected. The mean score was counted, and then the final mean scores were calculated from eight animals.

Reconstitution of KitW-sh/W-sh mice with BMMC

BMMC were generated from the femoral BM cells of mice and maintained in the presence of 10% pokeweed mitogen-stimulated spleen-conditioned medium as a source of mast cell growth factors as previously described [28]. Mast cell deficiency of KitW-sh/W-sh was reconstituted by the intravenous injection, 4 weeks after starting the culture, of 2×10^6 BMMC derived from C57BL/6 or FcγR-chain deficient mice, as previously described [23, 24]. The mice were used for experiments 5 wk after the injection of BMMC. The reconstitution of the mast cells was confirmed by toluidine blue staining of the nasal mucosa.

Data analysis

Data are represented as the mean + SD. The statistical analysis was performed by unpaired Student's *t*-test. A value of $p<0.05$ was considered to be significant.

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Abbreviations: **BMMC:** bone marrow-derived mast cells · **FcεR:** Fc receptor ε chain · **FcγR:** Fc receptor γ chain · **PAS:** periodic acid-Schiff · **TSLP:** thymic stromal lymphopoietin

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Question

アレルギー性鼻炎の民間療法とは？

アレルギー性鼻炎にどのような民間療法がありますか？

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Answer

アレルギー性鼻炎の民間療法について，以前筆者は薬剤師会の情報センターに依頼し情報を収集しました．その結果，健康食品，水，食物，入浴剤，器具，漢方薬，針治療，超音波療法など数多くの民間療法があることがわかりました[1]．この中で，漢方薬の小青竜湯については，プラセボを対照とした臨床試験でその有用性が証明されています．しかしながら，調査した民間療法のほとんどはEBMに基づく評価はなされていません．

次に，種々雑多な民間療法のなかで実際にアレルギー性鼻炎患者により多く利用されている民間療法について述べます．われわれは，スギ・ヒノキ科花粉症シーズン中に，山梨県内の耳鼻咽喉科診療所を受診した花粉症患者を対象に，2000年と2006年に民間療法に関するアンケートによる実態調査を実施しました[2]．まず2000年の上位5つを順に挙げると，市販の漢方薬，甜茶，鼻スチーム療法，鼻内洗浄，クロレラ，でした．2006年では，甜茶，**プロバイオティクス**，にがり，シソ系（シソジュース，シソ油など），鼻内洗浄，の順となっていました．2006年上位に登場したプロバイオティクス，にがりなどは6年前には認められなかったものです．このように，民間療法として多く利用されるものについてはその時代の流行を反映したものの影響があるのかもしれません．民間療法に対する患者の主観的評価については**図**に示した通りです．鼻スチーム療法や鼻内洗浄では40%以上の患者で効果を実感していますが，残りは10～30%程度です．

近年，特にプロバイオティクスに関して乳酸菌やビフィズス菌による抗アレルギー作用が研究され注目を浴びていることも最近の傾向を反映したものでしょう．動物実験による作用機序の解明など科学的根拠を示す報告もいくつか散見され，プラセボを対照とした臨床試験の施行によりプロバイオティクスの有用性を科学的に証明しようという試みもなされています[3～5]．これらはもちろん医薬品と同等のレベルにあるものではありませんが，アレルギー性鼻炎の症状緩和あるいは発症の予防として補助的に使用することにより，アレルギー性鼻炎患者のQOLに貢献できると考えられます．

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