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CD69 Controls the Pathogenesis of Allergic Airway Inflammation

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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) 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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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 (XM4-12), PE-conjugated anti-IL-4 Ab (11B11), PE-conjugated anti-Ki-126 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 bronchoalveolar 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 cytospin-fractioned onto slides by a Cytospin® (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 CO₂ 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. To describe the number of infiltrated mononuclear cells in the perivascular and peribronchial regions was determined by direct counting of 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%; 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 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; Buco 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; Buco 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.

**Adaptive transfer of CD4 T cells**

Splenitic CD4 T cells of OVA-sensitized WT or CD69-deficient mice were purified using a CD4⁺ T cell isolation kit (Miltenyi Biotec) and AutoMACS sorters (Miltenyi Biotec), yielding a purity of >98%. These cells were administered i.v. through the tail vein to OVA-sensitized CD69-deficient mice (1 × 10⁷ 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.

**Adaptive transfer of CD69-overexpressed Th2 cells**

Naïve 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 DO11.10 (mCD69) gene was introduced into a retrovirus vector containing the mCD69 gene (29). On day 5, the cultured cells were harvested and one or three million cells (K11⁺ Th2 cells) were injected i.v. into normal BALB/c mice.

**In vitro Th2 cell differentiation cultures**

DO11.10 Tg CD4⁺CD8⁻CD4 T cells (1.5 × 10⁷) 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 on days 0 and 7. Splenic CD4 T cells were purified using a CD4 T cell isolation kit and a MACS sorter. These CD4 T cells were administered 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 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 at 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 1 day 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. 1A). 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. 1B. 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. 1C). 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. 1F).

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. 1G), and the level of AHR was dramatically reduced (Fig. 1H) 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. 1I). 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. 2A). 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. 2B). 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. 2C). 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. 2D). 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. 2E). 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. 3A). As shown in Fig. 3B, 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. 3C) 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. 3D). 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 TCRαβ 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. 4A). The overexpression of CD69 was confirmed (Fig. 4B). The CD69-overexpressed Th2 cells (3 × 10⁶) 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. 4C, 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 < 0.01. 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.
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 × 10⁷) and SNARF-1-labeled CD69KO T cells (2 × 10⁷)
were mixed and i.v. transferred into normal syngeneic C57BL/6 mice. One day later, the recipient mice were challenged by OVA inhalation (Fig. 5A). 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 (CD69KO) 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. 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. 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. 1A. The absolute cell numbers of eosinophils (Eosino.), lymphocytes (Lympho.), neutrophils (Neutro.), and macrophages (Mac.) are shown with the SEM. *p < 0.01. Three independent experiments with titration of transferred cells were performed and similar results were obtained.

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. 1A. 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. 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. 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, A, and KO/OVA + OVAp.KO CD4T; C). 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.
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. 5C). 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-deficient 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. 6A). 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. 6D, 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
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. 7A). The examination of infiltrated cells in the BAL fluid revealed a marked decrease in the number of infiltrated eosinophils in the BAL fluid (Fig. 7B). Similarly, infiltrated eosinophils in the perivascular and peri-bronchiolar regions of the lung were lower in the mAb-treated animals (Fig. 7C). The treatment with anti-CD69 mAb resulted in a reduction of the extent of AHR (Fig. 7D) 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. 7G), 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.
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. 8A). 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. 2A), 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. 2B). 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/CDC69 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 (S1P1) and inhibits the S1P1-mediated egress of the cells from the lymphoid organs (17). To address the involvement of S1P1-mediated regulation of CD4 T cell egress in the CD69-dependent airway inflammation, we used FTY720, which acts on S1P1, 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 S1P1 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/SIP1-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 neutrophil 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.

References
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