

The leukocyte activation antigen CD69 limits allergic asthma and skin contact hypersensitivity

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Background: Allergic diseases have a major health care impact in industrialized countries. The development of these diseases is influenced by exposure to allergen and to immunological and genetic factors. However, the molecular mechanisms underlying the inflammatory response that triggers allergy are not well defined. **Objective:** We have investigated the role of the leukocyte activation antigen CD69 in the regulation of two allergic diseases, asthma and contact dermatitis.

Methods: Analysis of two models of allergic diseases in CD69 knockout and wild-type mice: ovalbumin-induced allergic airway inflammation (BALB/c genetic background) and contact hypersensitivity to oxazolone (C57BL/6J genetic background).

Results: CD69 deficiency dramatically enhanced the inflammatory response in the ovalbumin-induced asthma model of antigen-induced airway allergy. CD69 knockout mice showed exacerbated pulmonary eosinophil recruitment, high vascular cell adhesion molecule 1 expression levels in lung vasculature, and enhanced T_H2 and T_H17 cytokines in the bronchoalveolar space and lung tissue. In the hapten-induced cutaneous contact hypersensitivity model, both CD69 deficiency and treatment with anti-CD69 mAb increased inflammation. Treatment with contact allergens induced enhanced T_H1 and T_H17 responses in CD69 deficient mice, and neutralizing anti-IL-17 antibodies reduced skin inflammation. In both experimental systems, adoptive transfer of lymph node cells from CD69 knockout mice increased the inflammatory response in recipient mice.

Conclusion: These results demonstrate that the early activation receptor CD69 is an intrinsic modulator of immune allergic

processes through the negative regulation of allergen-induced T-cell effector responses. (*J Allergy Clin Immunol* 2010;■■■:■■■-■■■.)

Key words: Allergic airway inflammation, contact hypersensitivity, inflammation, CD69, T helper responses

The prevalence of allergic diseases has increased dramatically over the past two decades to the extent that these diseases have reached epidemic proportions, and associated health care expenditures in industrialized countries are very high.¹ Allergy is a complex disease influenced by interaction of genetic and environmental factors, such as exposure to allergens, infections and air pollution.^{2,3} Studies using new immunologic and molecular approaches have provided new insights into the nature of this inflammatory response and its relationship to the physiologic alterations characteristic of the disorder. However, the molecular mechanisms underlying the inflammatory response that triggers allergic reactions have not yet been fully elucidated.^{4,5}

Asthma and contact dermatitis, two of the most common allergic diseases, respectively affecting lungs and skin, have been shown to be related or at least to share some molecular mechanisms leading to their development.^{6,7} These diseases have been extensively studied in mouse models of ovalbumin (OVA)-induced airway inflammation for allergic asthma and contact hypersensitivity (CHS) response to allergens for contact dermatitis. Allergic asthma is considered a typical T_H2-mediated inflammatory disease¹; however, recent studies have identified an important contribution by T_H17 lymphocytes.⁸⁻¹² Functional studies showed that IL-17 is a potent inducer of the migration and activation of inflammatory cells that perpetuate chronic airway inflammation.^{8,13,14} In CHS, the main effector population was formerly considered to be hapten-specific effector CD8⁺ T cells secreting IFN- γ Cytotoxic T cells type 1 (Tc1), with IL-4-secreting CD4⁺ T cells playing a regulatory role.^{15,16} CHS is reduced in IL-17-deficient mice,^{9,17} and IL-17-producing CD8⁺ T cells (Tc17 cells) are generated during the hapten sensitization phase and are required for the inflammatory response.⁹ Moreover, recent work has established that Tc17 lymphocytes, in addition to Tc1 cells, play an important role in CHS.^{18,19} These data clearly indicate that chronic activation of IL-17⁺ T cells is an important factor in the pathogenesis of allergic diseases such as asthma and contact dermatitis. Nevertheless, the mechanisms that regulate the activity of IL-17⁺ T cells in these processes have not been fully defined.

One potential regulator of immune inflammatory responses is the homodimeric leukocyte transmembrane protein CD69. CD69 is transiently expressed upon cell activation *in vitro*²⁰⁻²² and is

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Supported by the Spanish Ministry of Science and Innovation (SAF2008-06235 to F.S.-M.; SAF2008-02719 to P.M.), Instituto de Salud Carlos III—Spanish Ministry of Science and Innovation (grant PI06/0937 to M.G.), and MEICA from Genoma España Foundation, INSINET from Comunidad Autónoma de Madrid, and FONCICYT-C002-2009-1ALA/127249 to F.S.-M. P.M. is an investigator from Spanish Ministry of Science and Innovation “Ramón y Cajal Program” (RYC-2006). Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III is supported by the Ministry of Science and Innovation and the Pro Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III Foundation.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication December 18, 2009; revised April 21, 2010; accepted for publication May 10, 2010.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2010.05.010

Abbreviations used

BAL:	Bronchoalveolar lavage
CHS:	Contact hypersensitivity
CIA:	Collagen-induced arthritis
DLN:	Draining lymph node
H&E:	Hematoxylin/eosin
KO:	Knockout
OTII:	C57BL/6-Tg(Tcr α Tcr β)425Cbn/J mice expressing a TCR specific for peptide 323-339 of OVA in the context of I-A ^b
OTKO:	OTII mice backcrossed with CD69-deficient mice in the C57BL/6 background
OVA:	Ovalbumin
OXZ:	Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one)
S1P:	Sphingosine-1-phosphate
S1P ₁ :	Sphingosine-1-phosphate receptor 1
S1P ₃ :	Sphingosine-1-phosphate receptor 3
VCAM-1:	Vascular cell adhesion molecule 1
WT:	Wild-type

detected *in vivo* on small subsets of T and B cells in peripheral lymphoid tissues.²³ CD69 is expressed in leukocyte infiltrates in various chronic inflammatory processes (such as rheumatoid arthritis and chronic viral hepatitis)^{24,25} and autoimmune disorders (such as SLE, insulin-dependent diabetes mellitus, and autoimmune thyroiditis).^{26,27} Although antigen-dependent T-cell activation and proliferation do not appear to be altered in CD69-deficient lymphocytes,²⁸ CD69 knockout (KO) mice develop an exacerbated form of collagen-induced arthritis (CIA) characterized by a diminished local synthesis of TGF- β .²⁹ These and other studies³⁰ led us to postulate that CD69 is a negative regulator of the immune response and that this molecule might therefore modulate *in vivo* inflammatory responses in pathologic settings.³¹ This idea is supported by the observation that systemic treatment with specific anti-CD69 mAbs exacerbates CIA in mice.³² In contrast, CD69 does not affect other inflammatory processes, such as neutrophil-dependent acute arthritis induced by anticollagen type II mAbs,³³ or atheroma plaque formation in apoE^{-/-} apolipoprotein E-null mice (apoE^{-/-}).³⁴ Detailed investigation into the role of CD69 in the development of additional inflammatory processes is necessary to characterize precisely its regulatory function.

To gain insight into the potential role of CD69 in the control of allergic asthma and contact dermatitis, we investigated the effect of CD69 deficiency in mouse models of OVA-induced allergic airway inflammation and oxazolone CHS. We show that CD69 deficiency exacerbates the inflammatory response in both models and is associated with increased T_H2 and T_H17 effector responses in OVA-induced allergic airway inflammation and Tc1 and Tc17 effector responses in CHS. These results indicate that CD69 modulates allergic processes by negatively regulating allergen-induced T-cell effector responses.

METHODS**Mice**

CD69-deficient mice were generated as described²⁸ and backcrossed for more than 12 generations to the BALB/c and C57BL/6J genetic background. C57BL/6-Tg(Tcr α Tcr β)425Cbn/J mice expressing a T-cell receptor specific for peptide 323-339 of OVA in the context of I-A^b (OTII mice) were purchased from the Jackson Laboratory (stock number 004194). OTII mice were

backcrossed with CD69-deficient mice in the C57BL/6 background (OTKO mice). Either male or female sex-matched mice of the C57BL/6J background between 10 and 12 weeks old were used for *in vivo* CHS experiments. For *in vivo* OVA-induced asthma experiments, we used females 10 to 12 weeks old that were either littermates or age-matched offspring of these littermates in the BALB/c background. Mice were kept in pathogen-free conditions at the Animal Unit of the School of Medicine, Universidad Autónoma de Madrid. Experimental procedures were approved by the Committee for Research Ethics of the Universidad Autónoma de Madrid and conducted under the supervision of the Universidad Autónoma de Madrid Head of Animal Welfare and Health in accordance with Spanish and European guidelines.

OVA-induced allergic airway disease

CD69 KO and wild-type (WT) mice (10-12 weeks old) were immunized as described.³⁵ Briefly, on day 0, OVA (15 μ g; Sigma, St Louis, Mo) in 200 μ L alum (Pierce, Rockford, Ill) was intraperitoneally injected (i.p.) into sensitized mice. The injection was repeated on day 5 and, on day 12, mice were challenged with aerosolized 0.5% OVA in PBS (two 60-minute challenges 4 hours apart). Control animals were exposed to inhaled PBS. In another set of experiments, BALB/c WT mice were intraperitoneally injected with 300 μ g anti-CD69 mAb (2.2) or isotype control mAb (antihuman CD11a, TS1/11) on days 0, 5, and 12. Mice were killed on day 14, 40 hours after aerosol OVA challenge. For histologic analysis, lungs were inflated through the trachea with 50% Tissue Tek-freezing medium (Sakura) in PBS and processed for sectioning. For cytokine profiling, the right caudal lung lobe was removed, embedded, frozen in liquid nitrogen and stored at -80°C; lung homogenates were prepared from frozen tissue with tissue protein extraction reagent (T-PER; Pierce) as described.³⁶ Cytokine concentrations were measured with the FlowCytomix Mouse T_H1/T_H2 10plex kit (Bender MedSystems GmbH, Vienna, Austria).

Sensitization and elicitation of CHS

Contact hypersensitivity was induced by painting shaved mouse abdomen skin with 50 μ L 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; OXZ; Sigma) in ethanol, and hind footpads with 10 μ L each. After 5 days, mice were challenged with 10 μ L 1% OXZ on each side of the right ear. The left ear was painted with vehicle as a control. Ear thickness was measured every day for 4 days. Net ear swelling was calculated by subtracting the thickness of the vehicle-treated ear from the thickness of the hapten-challenged ear. For analysis of the *in vivo* T_H17 response, inguinal lymph nodes LNs were removed from mice 5 days after sensitization of abdominal skin, single-cell suspensions were prepared, and intracellular staining of activated cells for IL-17 was performed as described. For secondary sensitization, mice sensitized and challenged as described and analyzed for CHS were resensitized 1 month after challenge. Five days after resensitization, draining lymph node (DLN) cells were analyzed for cytokine expression. To check the role of IL-17 in the CHS response, mice were intraperitoneally injected at the time of challenge with 200 μ g anti-IL-17 neutralizing mAb (R&D Systems, Minneapolis, Minn); rat IgG_{2A} was used as an isotype-matched control (R&D Systems). Net ear swelling was measured 48 hours after OXZ treatment and the relative percentage reduction in ear swelling with anti-IL-17 mAb was calculated with respect to the inflammation obtained with the isotype control. To block CD69 effects, mice were intraperitoneally injected at the time of sensitization and at the time of challenge with 300 μ g anti-CD69 mAb 2.2; mAb 2.8 was used as a mouse IgG₁ isotype-matched control.³² Immediately after mAb treatment, mice were challenged with OXZ and CHS was measured.

RESULTS**CD69 deficiency exacerbates allergic airway inflammation**

We assessed the development of OVA-induced allergic airway disease in CD69 KO and WT animals. Both groups were sensitized twice with OVA-alum and then challenged with aerosolized antigen or PBS. Forty-eight hours after airway exposure to

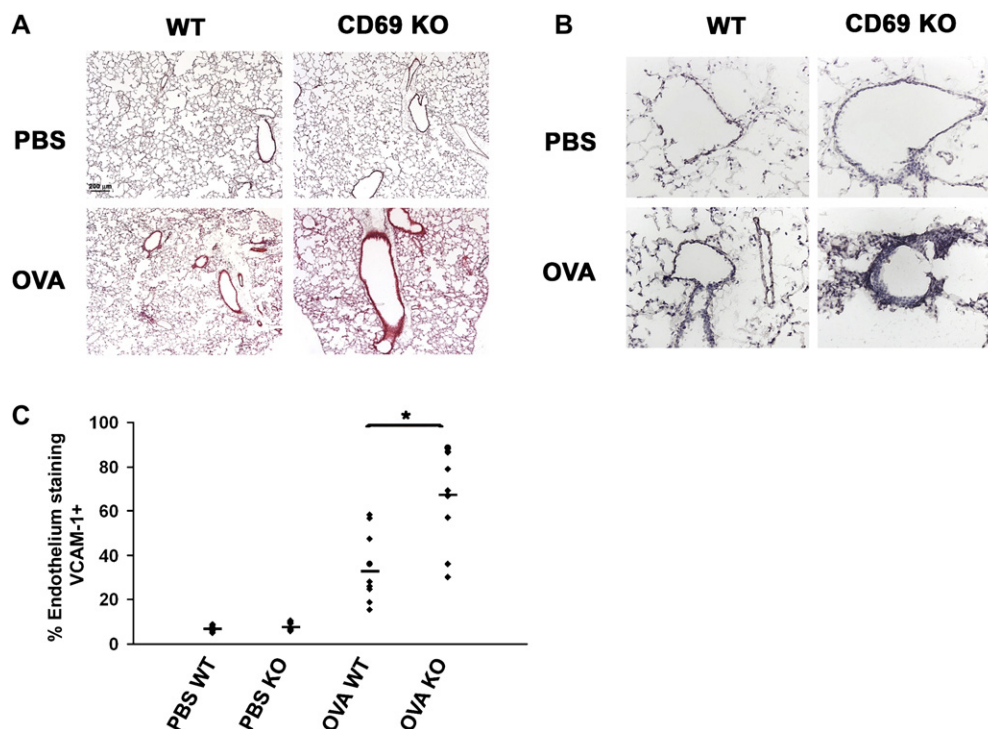


FIG 1. OVA-induced allergic airway inflammation is enhanced in CD69 KO mice. WT and CD69 KO mice were sensitized to OVA, and airway inflammation was induced by aerosol OVA challenge; control animals were challenged with PBS (see Methods). Lung sections were stained with H&E (A) or anti-VCAM-1 antibody revealed with a peroxidase-conjugated secondary (B). C, Percentages of bronchial epithelium staining for VCAM-1. Horizontal lines denote median values (* $P < .01$).

antigen, the lungs were examined histologically by hematoxylin/eosin (H&E) staining for eosinophilic infiltration. The lungs of CD69 KO mice showed an exacerbated inflammatory response, with massive perivascular and peribronchial infiltration (Fig 1, A). The lungs of CD69 KO mice also showed a higher expression of vascular cell adhesion molecule 1 (VCAM-1; Fig 1, B) as well as increased mucin expression (data not shown). The total number of leukocytes recovered from bronchoalveolar lavage (BAL) was much greater in CD69 KO mice compared with WT (Fig 2, A), and lung recruitment of eosinophils was dramatically increased in CD69-deficient mice (Fig 2, C and D). Moreover, BALB/c WT mice were treated with the anti-CD69 mAb 2.2, which has been shown to downregulate CD69 expression *in vivo* and increases the inflammatory response in CIA.³² Treatment of mice with mAb 2.2 induced higher numbers of eosinophils in BALs compared with control OVA-treated mice or mice treated with an isotype control antibody (Fig 2, B). Therefore, antibody-induced CD69 downregulation in this system increases the inflammatory response, mimicking the results obtained with CD69-deficient mice.

To further demonstrate the role of CD69 in the regulation of T_H -cell responses, we transferred OTII or OTKO mice with T_H2 plus T_H17 cells obtained *ex vivo* from the OVA_p-restimulated lymph nodes of OTII-immunized or OTKO-immunized mice. Analysis of leukocyte recruitment in BALs showed that mice injected with OTKO T_H2/T_H17 cells, independently of their genotype, had increased leukocyte counts (neutrophils, eosinophils, and macrophages) compared with mice transferred with OTII cells (Fig 2, E). These results unequivocally demonstrate that CD69 exerts its regulatory function in the T_H compartment.

CD69 deficiency increases airway levels of T_H2 and T_H17 cytokines

The bronchoalveolar space of CD69-deficient animals was significantly enriched in the T_H2 cytokines IL-4, IL-5, and IL-13 (Fig 3, A). Interestingly, we also found high levels of IL-17A in BAL (Fig 3, B), as well as eotaxin and OVA-peptide-specific IgE (Fig 3, C).

Recent reports indicate that during allergic lung inflammation, IL-17 can be produced by alveolar macrophages and bronchial epithelial cells as well as by infiltrating inflammatory cells.^{8,37,38} Although lung resident hematopoietic cells are not altered in the absence of CD69 (Fig 3, D), the levels of IL-17 and IL-4 were significantly higher in lung homogenates from CD69-deficient mice, whereas other cytokines, such as GM-CSF, occurred at similar concentrations in WT and CD69 KO mice (Fig 3, E). These results indicate that lung resident hematopoietic cells are highly activated in immunized CD69-deficient mice.

Expression of sphingosine-1-phosphate receptor 1 is upregulated on CD69-deficient T_H17 cells

Sphingosine-1-phosphate (S1P) is an important mediator of airway inflammation.³⁹⁻⁴³ Interestingly, CD69 negatively regulates the expression of sphingosine-1-phosphate receptor 1 (S1P₁),⁴⁴ and the expression of S1P₁ favors the differentiation of T_H17 cells by unknown mechanisms.⁴⁵ We analyzed the expression of S1P₁ receptor in T_H2 and T_H17 differentiated cells from CD69-deficient and WT cells. CD69 KO T_H17 cells showed an almost 2-fold higher surface membrane expression of S1P₁ than WT cells; sphingosine-1-phosphate receptor 3 (S1P₃) levels

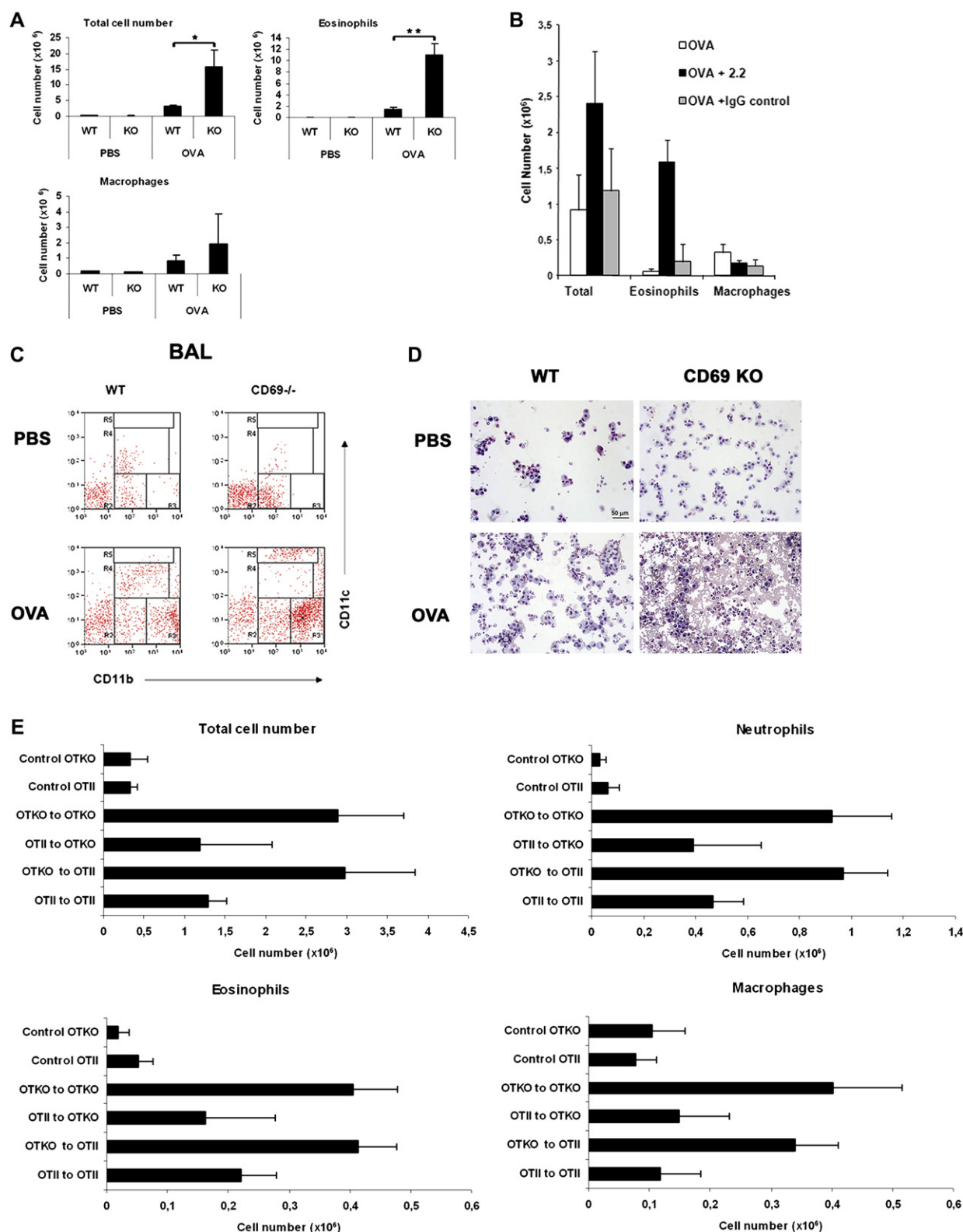


FIG 2. Exacerbated eosinophil recruitment in CD69-deficient mice. **A**, Absolute numbers of total cells, eosinophils and macrophages in BAL. Results are means (SDs) from 10 mice per genotype. Bonferroni P values from 1-way ANOVA (* $P < .01$; ** $P < .001$). A representative experiment of a total of 4 is shown. **B**, BAL cell counts of OVA-WT or mice treated with anti-CD69 mAbs (2.2, black bars) or with an isotype control IgG (gray bars). Results are means (SDs) from 6 mice per genotype. **C**, BAL cells were recovered, stained for CD11c and CD11b and analyzed by flow cytometry. **D**, Cytospin BAL samples from WT and CD69 KO mice stained with Wright-Giemsa. **E**, OTII or OTKO mice were adoptively transferred with T_H2 plus T_H17 cells obtained *ex vivo* from OVAp-restimulated lymph nodes of OTII-immunized or OTKO-immunized mice. Absolute numbers of the indicated leukocyte population were analyzed in BALs. Results are means (SDs) of 10 mice per genotype.

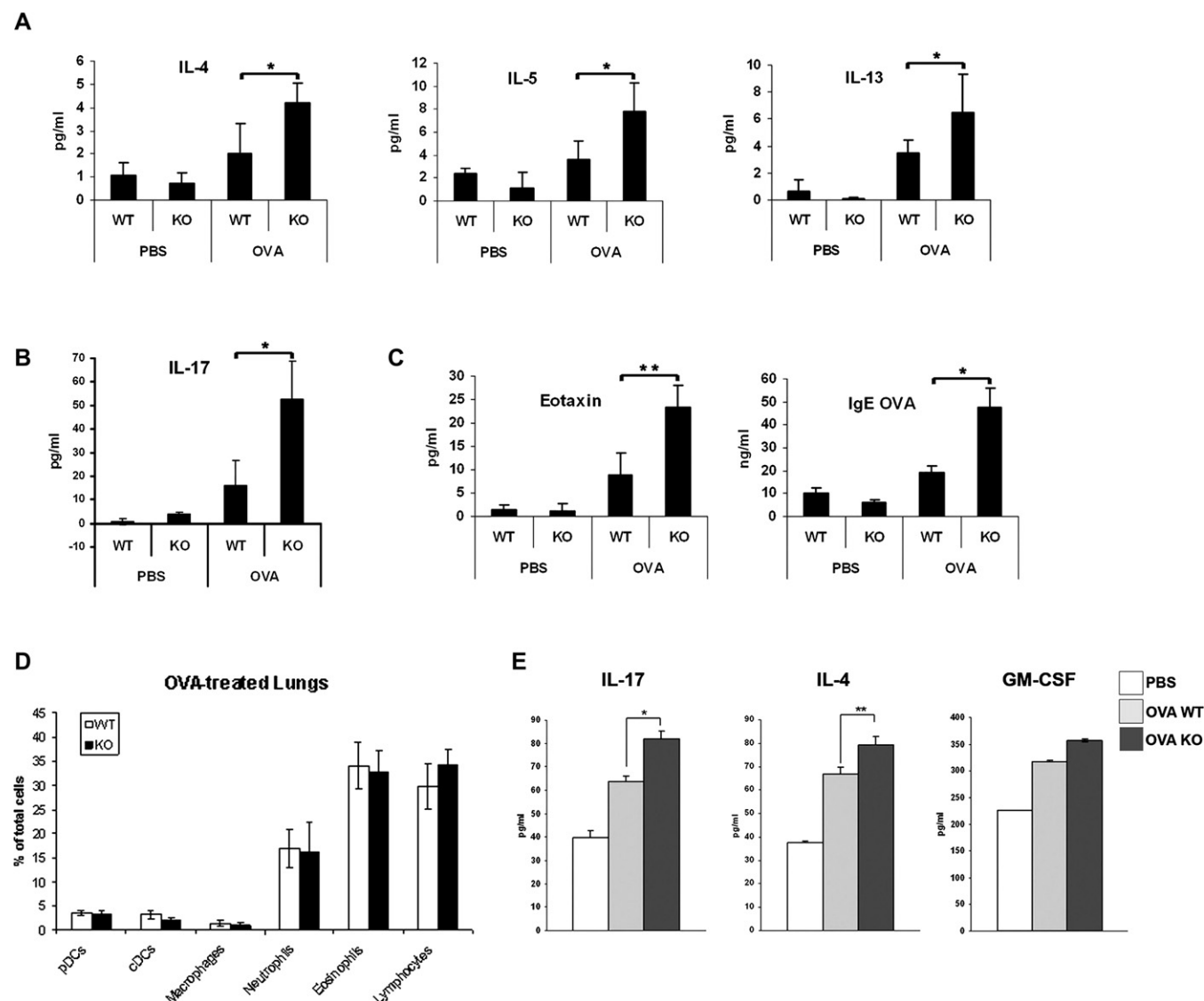


FIG 3. CD69 deficiency increases the levels of T_H2 and T_H17 cytokines in the lungs of OVA-immunized mice. BAL levels of the cytokines IL-4, IL-5, and IL-13 (**A**) and IL-17 (**B**) and of eotaxin and OVA-specific IgE (**C**) measured by ELISA. Bars represent means (SDs) of 4 (**B**) or 6 (**A** and **C**) mice per genotype. Bonferroni P values from 1-way ANOVA (* $P < .05$; ** $P < .01$). Data are representative of 2 (**B**) or 4 (**A** and **C**) independent experiments. Lungs of WT or CD69 KO mice were collected 48 hours after OVA inhalation. Cells were recovered after lung digestion and stained for CD11c and Gr-1. The percentages of lymphocytes, neutrophils, macrophages, eosinophils, plasmacytoid DCs (pDCs) and conventional DCs (cDCs) were analyzed by fluorescence-activated cell sorting (**D**). Bars represent means (SDs) of 6 mice per genotype. **E**, Protein expression of IL-17, IL-4, and GM-CSF, measured by fluorescence-activated cell sorting after suspension bead array, in lung homogenates of WT and CD69 KO mice sensitized and challenged (aerosol) with OVA to induce airway inflammation; control animals were sensitized with OVA and challenged with PBS. Bars represent means (SDs) of 10 mice per genotype. Bonferroni P values from 1-way ANOVA (* $P < .003$; ** $P < .0001$).

were similar in the 2 cell types (Fig 4, *A* and *B*). S1P₁ mRNA expression was likewise higher in CD69 KO T_H17 cells, whereas S1P₃ mRNA levels were unaltered (Fig 4, *C*). We also analyzed S1P₁ expression in OTII T_H17 differentiation cultures in the presence of the anti-CD69 mAb 2.2. Although CD69 was downregulated on the membrane of OTII T cells in these cultures, we did not find any increase in S1P₁ expression (see this article's Fig E1 in the Online Repository at www.jacionline.org). We next analyzed S1P₁ levels on the membrane of CD4⁺ T cells in the mediastinal

lymph node after the induction of asthma *in vivo* with transferred T_H2/T_H17 cells as in Fig 2, *E*. In line with our *in vitro* results with OTKO T_H17 cells, mediastinal CD4⁺ T cells from OTKO mice or from mice transferred with T_H2/T_H17 -OTKO cells showed an enhanced S1P₁ expression compared with cells from OTII mice or OTII mice transferred with OTII T cells (Fig 4, *D*). These data strongly suggest that CD69 deficiency in T cells results in increased S1P₁ expression *in vitro* and *in vivo* and might critically influence the severity of the airway inflammation.

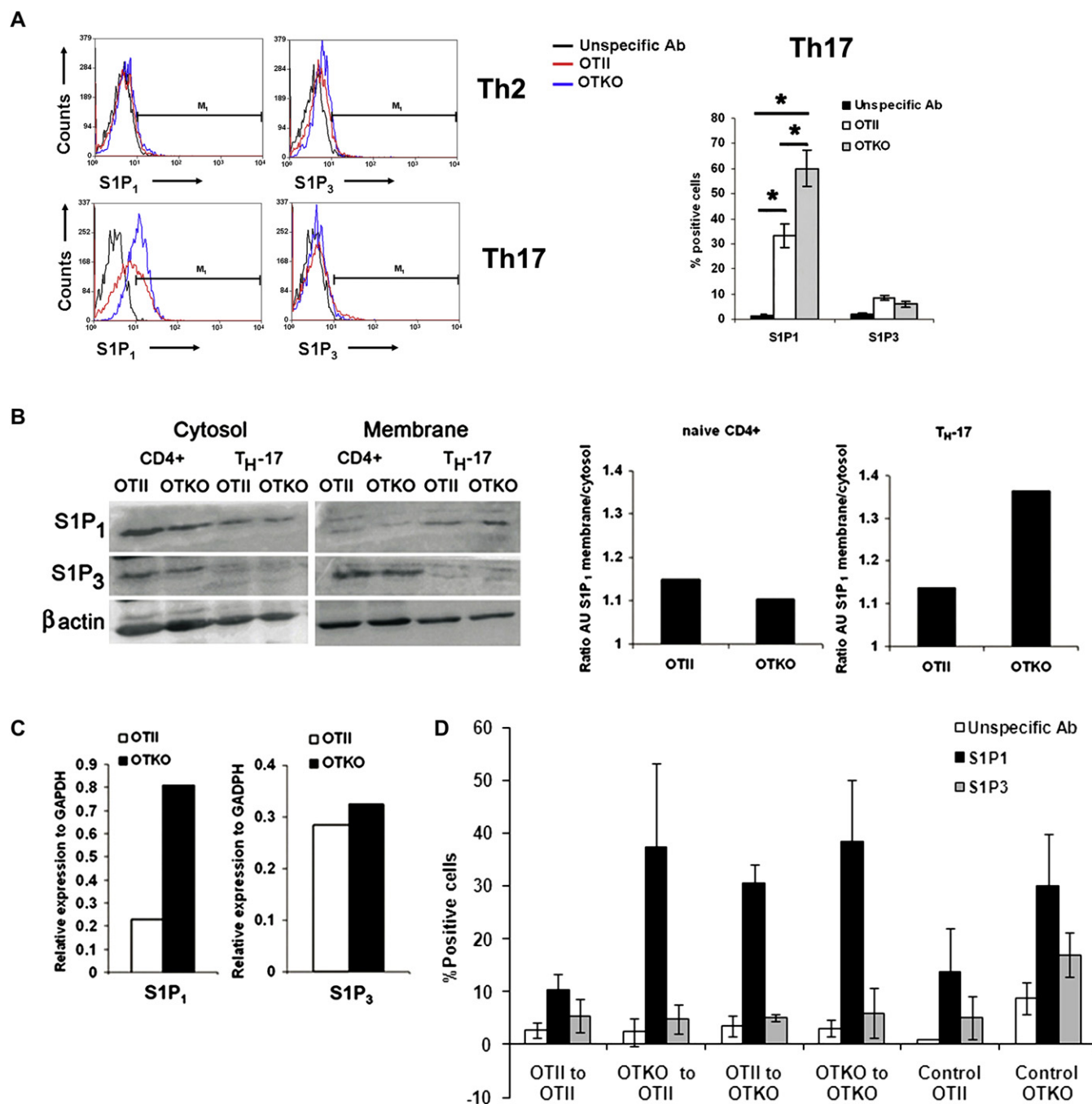


FIG 4. CD69 deficiency upregulates S1P₁ expression on T_H17 cells. **A**, Fluorescence-activated cell sorting analysis of membrane levels of S1P₁ and S1P₃ in OTII and OTKO T_H2 and T_H17 cells. An irrelevant polyclonal antibody (black line) was used as a control. The bar chart shows the percentages of S1P₁ and S1P₃-positive cells; data are means $\pm$ SDs from 2 independent experiments. **B**, Western analysis of S1P₁ and S1P₃ in membrane and cytosol extracts of naive CD4⁺ and T_H17 T cells from OTII and OTKO mice. The bar charts show densitometry analysis of the ratios of membrane versus cytosolic content of S1P₁ in naive and T_H17 cells. AU, Arbitrary unit. **C**, Quantitative RT-PCR analysis of S1P receptor mRNA expression. Results were normalized to Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. Data are representative of 3 experiments. **D**, OTII and OTKO mice were adoptively transferred with T_H2 plus T_H17 cells obtained from OVA-immunized OTII or OTKO mice and were exposed to inhaled OVA. The levels of S1P₁ and S1P₃ from OVA-p-primed mediastinal lymph node CD4⁺ T cells were analyzed by flow cytometry. Bars represent means (SDs) of 5 mice per group. Tukey HSD (Honestly Significant Difference) *P* values from 1-way ANOVA (**P* < .05; ***P* < .01).

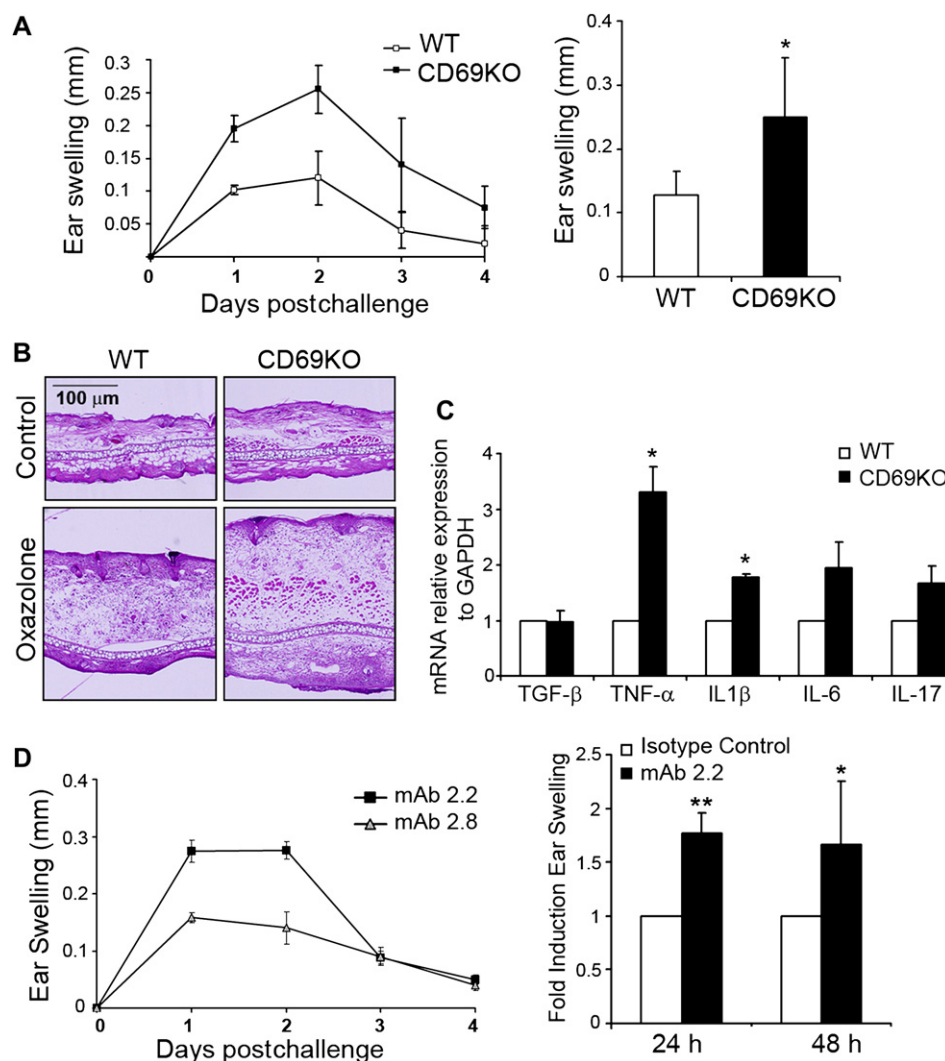


FIG 5. CD69 deficiency enhances the contact hypersensitivity response to oxazolone in mice. **A, Left panel,** Time course of ear swelling of treated WT and CD69 KO mice (means $\pm$ SDs; $n = 6$). **Right panel,** Ear swelling at the peak of the CHS response (means $\pm$ SDs; $n = 36$). **B,** H&E stained 5 μ m sections of ears collected 48 hours after OXZ challenge. Bar = 100 μ m. **C,** RT-PCR analysis of cytokine expression in inflamed ear tissue at the peak of inflammation. Results were normalized to *GAPDH* expression. Bars represent means $\pm$ SEMs; $n = 4$ to 12 mice. **D,** CHS response in WT mice treated with either anti-CD69 mAb 2.2 or isotype-matched control mAb 2.8. **Left panel,** Time course of ear swelling (means $\pm$ SDs; $n = 5$). **Right panel,** Net ear swelling at the peak of the CHS response (means $\pm$ SDs; $n = 24$). * $P < .01$; ** $P < .001$ (Student *t* test).

CD69 deficiency enhances CHS to oxazolone

To determine the role of CD69 in the regulation of CHS responses, WT and CD69-deficient mice were sensitized by application of OXZ to abdominal skin and rechallenged after 5 days by application to ear skin. Although the time course of CHS was similar in WT and CD69 KO mice (Fig 5, A, left panel), the extent of ear swelling was significantly greater in CD69-deficient mice. Ear swelling at the peak of the response (24-48 hours) was consistently enhanced in CD69-deficient mice (Fig 5, A, right panel), with gross enlargement of tissue shown by histologic analysis of H&E-stained frozen sections (Fig 5, B). Moreover, treatment with anti-CD69 mAb 2.2 enhanced ear swelling responses to OXZ, closely paralleling the effect observed in CD69-deficient mice (Fig 5, D). To characterize better the increased inflammation in CD69 KO mice, we analyzed the expression of a panel of inflammatory and immunomodulatory

cytokines by quantitative RT-PCR (see this article's Table E1 in the Online Repository) of RNA taken from inflamed ears at the peak of response. Expression of the proinflammatory cytokines IL-1 β and TNF- α was increased in CD69 KO mice, whereas the immunosuppressive cytokine TGF- β remained unchanged (Fig 5, C). These data indicate that CD69 deficiency results in an increased inflammatory response to the contact allergen OXZ.

Increased T-cell effector response to hapten mediates enhanced CHS responses in CD69-deficient mice

To test the effect of CD69 deficiency on the generation of T-cell effector responses, mice were sensitized with OXZ (primary sensitization) with or without resensitization after 1 month (secondary sensitization). In response to *in vitro* stimulation with anti-

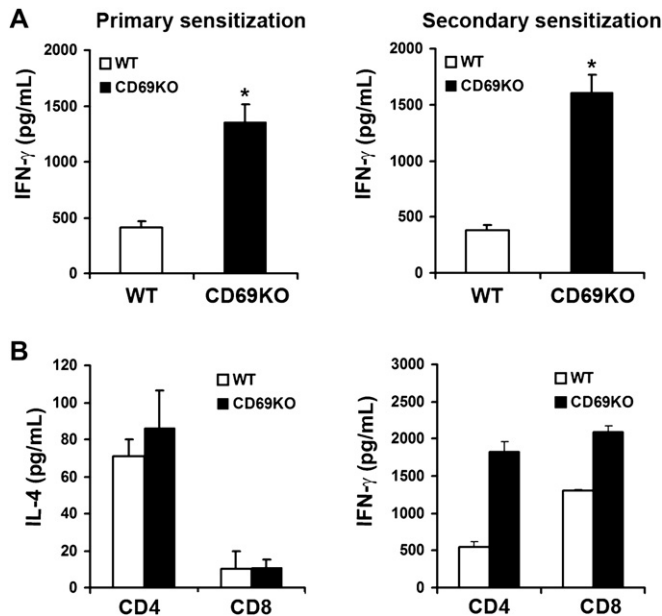


FIG 6. IFN- γ T-cell response induced after oxazolone sensitization is enhanced in CD69 KO mice. **A**, ELISA analysis of IFN- γ secreted by DLN cells from OXZ-sensitized mice activated by plate-bound anti CD3 mAb. **B**, ELISA analysis of IL-4 (left panel) or IFN- γ (right panel) secreted by CD4 $^{+}$ and CD8 $^{+}$ T cells purified from cell suspensions of DLNs from OXZ sensitized mice and activated by phorbol 12-myristate 13-acetate plus ionomycin.

CD3 antibody, DLN cells from CD69-deficient mice secreted higher levels of the T $_{H}$ 1-type effector cytokine IFN- γ than WT cells after both primary and secondary sensitization (Fig 6, A). The ratio of CD4 $^{+}$ to CD8 $^{+}$ cells in the total lymph node population was not altered in CD69 KO mice (data not shown). To identify the T-cell subpopulations responsible for IFN- γ secretion, CD4 $^{+}$ and CD8 $^{+}$ T cells purified from draining lymph nodes of OXZ-sensitized mice were stimulated *in vitro* with phorbol 12-myristate 13-acetate plus ionomycin, and IFN- γ production was analyzed by ELISA. CD4 $^{+}$ and CD8 $^{+}$ populations from CD69-deficient mice both produced higher levels of IFN- γ than their WT equivalents. Levels of IL-4 production by CD4 $^{+}$ and CD8 $^{+}$ cells were similar in WT and CD69 KO mice (Fig 6). These results indicate that OXZ sensitization of CD69-deficient mice generates a higher CD8 $^{+}$ Tc1 response, previously recognized as the main effector population in CHS, and a higher CD4 $^{+}$ T $_{H}$ 1 response, usually low in normal CHS.

Hapten-specific IL-17-producing T cells have recently been reported as important effector cells in CHS.^{17,18} To analyze the effect of CD69 deficiency on the generation of IL-17-producing T cells after allergen challenge, total DLN cells from OXZ-sensitized WT and CD69 KO mice were activated *in vitro*, and IL-17 production was analyzed by flow cytometry. OXZ-sensitized CD69 KO mice generated a higher percentage of IL-17 $^{+}$ T cells than WT animals in both the CD4 $^{+}$ -cell and the CD8 $^{+}$ -cell populations (Fig 7, A), suggesting increased differentiation of IL17 $^{+}$ T cells in CD69 KO mice. Parallel quantitative RT-PCR analysis of immunoregulatory cytokine expression detected increased expression of IL-17, TGF- β and IL-6 in CD69-deficient mice (Fig 7, B). TGF- β and IL-6 have been implicated in the generation of IL-17-producing effector T cells from naive

precursors in response to T-cell activation,^{46,47} and this finding therefore suggests that CD69 deficiency increases differentiation of IL-17-positive T cells after allergen challenge. Blockade of IL-17 with a neutralizing anti-IL-17 antibody strongly reduced OXZ-induced ear swelling in WT and CD69 KO mice (Fig 7, C), demonstrating that the CHS response is highly dependent on IL-17.

To test whether the increased T-cell effector response could account for the augmented CHS response found in CD69-deficient mice, we performed adoptive transfer experiments. Total DLN cells from sensitized mice were transferred to unsensitized mice. The recipient mice were then challenged and the CHS response measured. WT recipients injected with CD69 KO cells showed an increased response compared with WT cells (Fig 7, D and E). Conversely, in WT or CD69 KO recipients injected with WT cells, no differences were observed. Taken together, these results indicate that CD69 regulates CHS mainly during the sensitization phase through the regulation of hapten-specific effector T cells.

DISCUSSION

The current study establishes that CD69 negatively regulates the inflammatory response in two disease models of allergy, antigen-induced asthma and CHS. Although asthma has classically been considered a T $_{H}$ 2-lymphocyte-mediated process, the specific immunologic mechanisms that trigger inflammation and allergic asthma *in vivo* are not well defined.¹³ Our study of OVA-induced allergic airway inflammation shows that the exacerbated response in the absence of CD69 is associated with increased T $_{H}$ 2 and T $_{H}$ 17-mediated immune responses. There is mounting evidence in mouse models that T $_{H}$ 17 cells play an important role in enhancing antigen-induced cell-mediated airway inflammation.^{9,46,47} Moreover, allergic asthma in human beings is associated with increased airway expression of the IL-17F isoform located on chromosome 6p, which has been linked to asthma in multiple genome scans.^{2,48} It is therefore conceivable that the increased T $_{H}$ 2 and T $_{H}$ 17 cytokine expression reported here might together be responsible for the increased airway inflammation found in the absence of CD69.

A recent study reported that CD69 deficiency inhibits the development of asthma,⁴⁹ showing an effect opposite to our results. The authors propose that reduced migration of T $_{H}$ 2 effector cells to lung tissue accounts for the reduced inflammation, whereas we found increased T $_{H}$ 2 and T $_{H}$ 17 responses and a higher number of leukocytes in BAL. In our model of CD69 deficiency, T $_{H}$ 17 cells differentiated *in vitro* express higher levels of S1P $_{1}$ than WT equivalents, and this might translate into enhanced migration to the lymph or to inflamed tissues, where the levels of S1P are known to be elevated.⁵⁰ Moreover, S1P levels have been shown to be increased in the BAL of patients with asthma,^{39,40} and recent studies with mice deficient for sphingosine kinase 1 show that the sphingosine kinase 1/S1P pathway is essential for the development of acute lung inflammation,⁵¹ and therefore for OVA-induced airway inflammation. In our system, CD69 might therefore regulate the immune response not only by limiting T $_{H}$ 2 and T $_{H}$ 17 responses in the lungs but also by controlling their rate of migration to the inflamed tissue by regulating the S1P/S1P $_{1}$ pathway. Because the experimental asthma models used in both studies are similar, the results are difficult to reconcile. The differences observed might be a result of

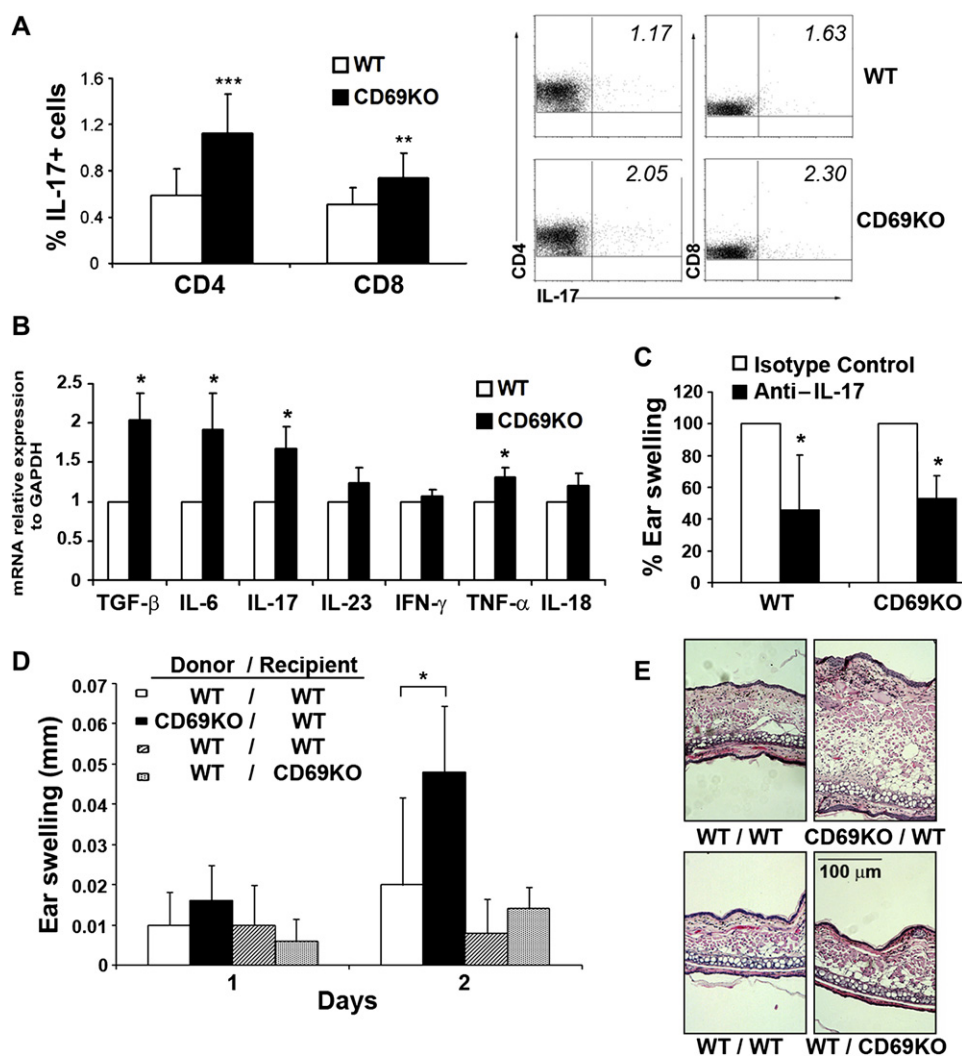


FIG 7. Enhanced effector T-cell response induced after oxazolone sensitization increase CHS in CD69 KO mice. **A**, Flow cytometry analysis of intracellular IL-17 in activated CD4⁺ and CD8⁺ T cells from sensitized mice. Representative dot plots (right panel). Percentages of IL17⁺ cells (left panel). Means $\pm$ SDs; n = 18. **B**, RT-PCR analysis of cytokine expression in DLNs from sensitized mice. Results were normalized to GAPDH expression. (means $\pm$ SEMs; n = 8 to 17 mice). **C**, Percentage ear swelling reduction 48 hours after challenge in mice treated with anti-IL-17 neutralizing mAb (IL-17) or isotype matched control mAb. Means $\pm$ SDs; n = 5. Adoptive transfer: DLN cells from sensitized WT or CD69 KO mice were injected intravenously into either WT or CD69 KO naive mice that were later challenged with OXZ. See additional information on Methods in the Online Repository at www.jacionline.org. **D**, Net ear swelling at the indicated times (means $\pm$ SDs; n = 5). **E**, H&E-stained 5- μ m sections of ears collected 48 hours after OXZ challenge. Bar = 100 μ m. * P < .01; ** P < .005 (Student t test).

intrinsic differences between the two strains of CD69 KO mice. We previously reported the use of the neutralizing anti-CD69 mAb 2.2 to downregulate the *in vivo* expression of CD69.³² The effect of this antibody on the immune and inflammatory response consistently mimics the CD69 KO phenotype in different experimental models—for example, tumor rejection,³⁰ CIA,³² acute arthritis induced by anticollagen type II antibodies,³³ and asthma and CHS to allergens (current report). Our current results with this antibody strongly suggest that the enhanced inflammatory response in our CD69 KO strains is a result of loss of CD69 expression and not additional genetic alterations.

In hapten-induced CHS, loss of CD69 expression by treatment with antibody 2.2 or targeted gene deletion also results in an augmented inflammatory response. Hapten-specific effector T

cells secreting IFN- γ were formerly considered the main effector population.^{15,16} However, CHS is reduced in IL-17^{-/-} mice⁹ and in WT mice treated with anti-IL-17 neutralizing antibodies,¹⁷ and recent studies show that both IL-17 and IFN- γ produced by hapten-specific effector cells are important for the CHS response.^{18,19} In our experiments, the exacerbated CHS reaction of CD69 KO mice was associated with an increase in the generation, after allergen sensitization, of both IFN- γ ⁺ and IL-17⁺ T cells. Interestingly, antibody-mediated neutralization of IL-17 strongly reduced hypersensitivity responses in WT and CD69 KO mice, highlighting the importance of IL-17⁺ cells in this inflammatory system. Moreover, adoptive transfer experiments show that cells from CD69 KO mice enhanced allergic asthma and CHS responses in naive recipient mice, indicating that

CD69 deficiency exerts its effect in the sensitization phase. Taken together, these data strongly suggest that CD69 regulates inflammation in CHS through the regulation of hapten-specific T-cell effector responses.

Previous reports established exacerbated CIA inflammatory response in CD69 KO mice²⁹ and led to the proposal that CD69 is a negative regulator of inflammatory responses³¹ and the suggestion that CD69 might regulate T-cell effector differentiation. The results presented here identify a previously unknown function of CD69 as a regulator of allergic diseases through the regulation of T effector–cell responses. CIA inflammatory responses are mediated by T-cell effectors, mainly T_H17 cells, and our results show that both T_H17 and Tc17 responses are regulated by CD69 in airway inflammation and CHS. Together these observations strongly suggest that CD69 might regulate inflammation in pathologic processes mediated by effector T cells, especially T_H17 or Tc17. Although much further research is needed into the regulation and function of CD69, this study increases our knowledge of the origin and progression of asthma and contact dermatitis and reveals an important regulatory role for this molecule in allergic diseases, enabling the development of new targeted therapies.

We thank Simon Bartlett for comments and critical reading of the article.

Key messages

- CD69 regulates the immune response to antigen-induced airway inflammation by limiting T_H2 and T_H17 responses in the lungs.
- In hapten-induced CHS, loss of CD69 expression by treatment with antibody or targeted gene deletion results in augmented skin inflammatory response.
- CD69 modulates allergic processes by negatively regulating allergen induced T-cell effector responses.

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METHODS

T-cell isolation

Five days after sensitization of abdominal skin, CD4⁺ and CD8⁺ T cells were obtained from single-cell suspensions of inguinal LN. The cell suspensions were incubated with biotinylated anti-CD8 or anti-CD4 antibody and subsequently with Streptavidin Microbeads (MACS; Miltenyi Biotec). CD4⁺ and CD8⁺ T cells were positively selected in an auto-MACS Pro Separator (Miltenyi Biotec) according to the manufacturer's instructions.

T_H2-cell and T_H17-cell differentiation cultures

Naive CD4⁺ T cells were obtained from single-cell suspensions of spleen and mesenteric lymph node. The cell suspensions were incubated with biotinylated antibodies against CD8, CD16, CD19, CD24, CD117, MHC class II (I-A^b), and CD11b, and subsequently with Streptavidin Microbeads. CD4⁺ T cells were negatively selected in an auto-MACS Pro Separator according to the manufacturer's instructions. The cells were then labeled with antibodies to CD4, CD25, and CD62L and analyzed by flow cytometry to confirm naive status. Naive CD4⁺ T cells (10⁶/mL) were cultured in the presence of irradiated antigen-presenting cells (T-cell-depleted splenocytes) and OVA peptide 323-339 (OVAp; 10 µg/mL) plus a cytokine or antibody combination appropriate for differentiation of the desired T-cell lineage: IL-2 (10 ng/mL), anti-IFN-γ (4 µg/mL), and recombinant murine, rIL-4 (10 ng/mL) for T_H2; and anti-IFN-γ (4 µg/mL), anti-IL-4 (4 µg/mL), rIL-6 (10 ng/mL), rIL-23 (10 ng/mL), and recombinant human, rhTGF-β1 (5 ng/mL) for T_H17. After culture, the cells were extensively washed and restimulated. No differences in viability were observed between CD69^{+/+} and CD69 KO T_H2 or T_H17 cells.

Intracellular staining and flow cytometry

Inguinal LNs were removed from mice 5 days after sensitization of abdominal skin, and single-cell suspensions were prepared. Cells were extensively washed and then restimulated for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate and 750 ng/mL ionomycin plus brefeldin A (1 µg/mL). Cells were stained with anti-CD4 or anti-CD8 antibodies and then fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin; cells were then stained with anti-IL-17 antibody (BD Pharmingen) and analyzed by flow cytometry (FACSCanto II; BD Pharmingen). For analysis of BAL, subpopulations of viable leukocytes were gated based on their Forward Scatter/Side Scatter (FSC/SSC) parameters; different populations of BAL leukocytes were identified by their expression of CD11b, Gr-1, B220, and CD11c. We analyzed these populations by using the previously described gating systems^{E1,E2} to distinguish between monocytes (R2; CD11b^{int} CD11c⁻ Gr-1^{int} B220⁻), eosinophils (R3; CD11b^{high} CD11c⁻ Gr-1^{int} B220⁻), plasmacytoid DCs (R4; CD11b⁺ CD11c^{int} Gr-1⁺ B220⁺), and conventional DCs (R5; CD11b⁺ CD11c⁺ Gr-1⁻ B220⁻).

Histology and immunohistochemistry

For histologic analysis of inflamed tissue in CHS, the ears of treated mice were excised 48 hours after hapten challenge, fixed with 10% formalin, and embedded in paraffin, and 5-µm sections were stained with H&E.

For quantitative analysis of VCAM-1, we used the scoring system previously described^{E3} in which the staining of adhesion molecules is expressed as the percentage of the total length of the epithelium or basement membrane.

Cytokine assays

Bronchoalveolar lavage (BAL) were collected from immunized mice, and IL-17, eotaxin, and IgE-OVA concentrations were measured with ELISA kits (R&D Systems). Supernatants from lung homogenates were collected and cytokine concentrations measured with the FlowCytomix Mouse Th1/Th2 10plex kit (Bender MedSystems GmbH, Vienna, Austria), followed by flow cytometry in a BD FACSCanto II cytometer (BD Biosciences). The following cytokines were measured: GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-

10, IL-17, and TNF-α. Data were analyzed with FlowCytomix Pro 2.2 Software (Bender MedSystems GmbH, Vienna, Austria).

Five days after sensitization of abdominal skin or 5 days after secondary OXZ sensitization, total LN cell suspensions or CD4⁺ and CD8⁺ T cells were stimulated for 24 hours with anti-CD3 (10 µg/mL). Supernatants were collected from stimulated cells and IFN-γ and IL-4 concentrations measured by ELISA (OptEIA ELISA Sets; BD-Pharmingen).

Cytokine expression by real-time PCR

Ears or LNs were harvested, frozen in liquid nitrogen, and minced. Tissue was homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland). RNA was isolated with Ultraspec RNA reagent (Biotecx, Houston, Tex), with 2 phenol-chloroform-isoamyl alcohol extractions and Deoxyribonuclease I (DNase I) treatment. RNA was precipitated at 4°C with ice-cold ammonium acetate, and the ethanol-washed pellet was resuspended in 0.02 mL water. RNA was stored at -70°C. Two micrograms DNaseI-treated RNA were reverse-transcribed to cDNA with ImProm-II Reverse Transcriptase (Promega GmbH, Mannheim, Germany), and gene expression was quantified by SYBR Green real-time PCR (Roche Diagnostics GmbH, Penzberg, Germany) in a DNA Lightcycler rapid thermal cycler (Roche, Mannheim, Germany). Results for each cytokine were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and measured in parallel in each sample. The primers generated products of about 200 bp; the sequences of cytokine specific primer pairs are provided in Table E1.

Adoptive transfer experiments

For the adoptive transfer experiments of asthma, OTII and OTKO mice were intraperitoneally injected with 50 µg OVA in 2 mg alum, and 3 days later, spleens and mesenteric lymph nodes were removed for CD4⁺ T cells isolation and were cultured for T_H2 or T_H17 differentiation for 3 days. T_H2 and T_H17 cells were collected, washed, and intravenously injected (5 × 10⁶ T_H2 and 4 × 10⁶ T_H17) into OTII or OTKO recipient mice. Beginning 24 hours later, the recipients were exposed to inhaled 1% OVA in PBS for 7 days (4 consecutive days exposed, 2 days rested, and 3 consecutive days exposed) for 20 minutes daily. Mice were killed 24 hours after the final OVA exposure, and BALs analysis was performed.

For adoptive transfer experiments of CHS, donor mice were sensitized by application of 50 µL 3% OXZ to shaved abdominal skin. On day 5, lymph node cells were taken from the inguinal lymph nodes of donor mice. Pooled LN cells (1 × 10⁷) were injected into the tail vein of naive recipient mice. Immediately after injection, mice were challenged with 1% OXZ, and ear swelling was determined 24 and 48 hours later.

Statistical analysis

P values were calculated with the Student *t* test, and values below .05 were considered significant. Means of the experimental groups were compared by using 1-way ANOVA. To account for multiple comparisons, the Bonferroni and Tukey posttest were used to compare selected pairs of means and all pairs of means, respectively.

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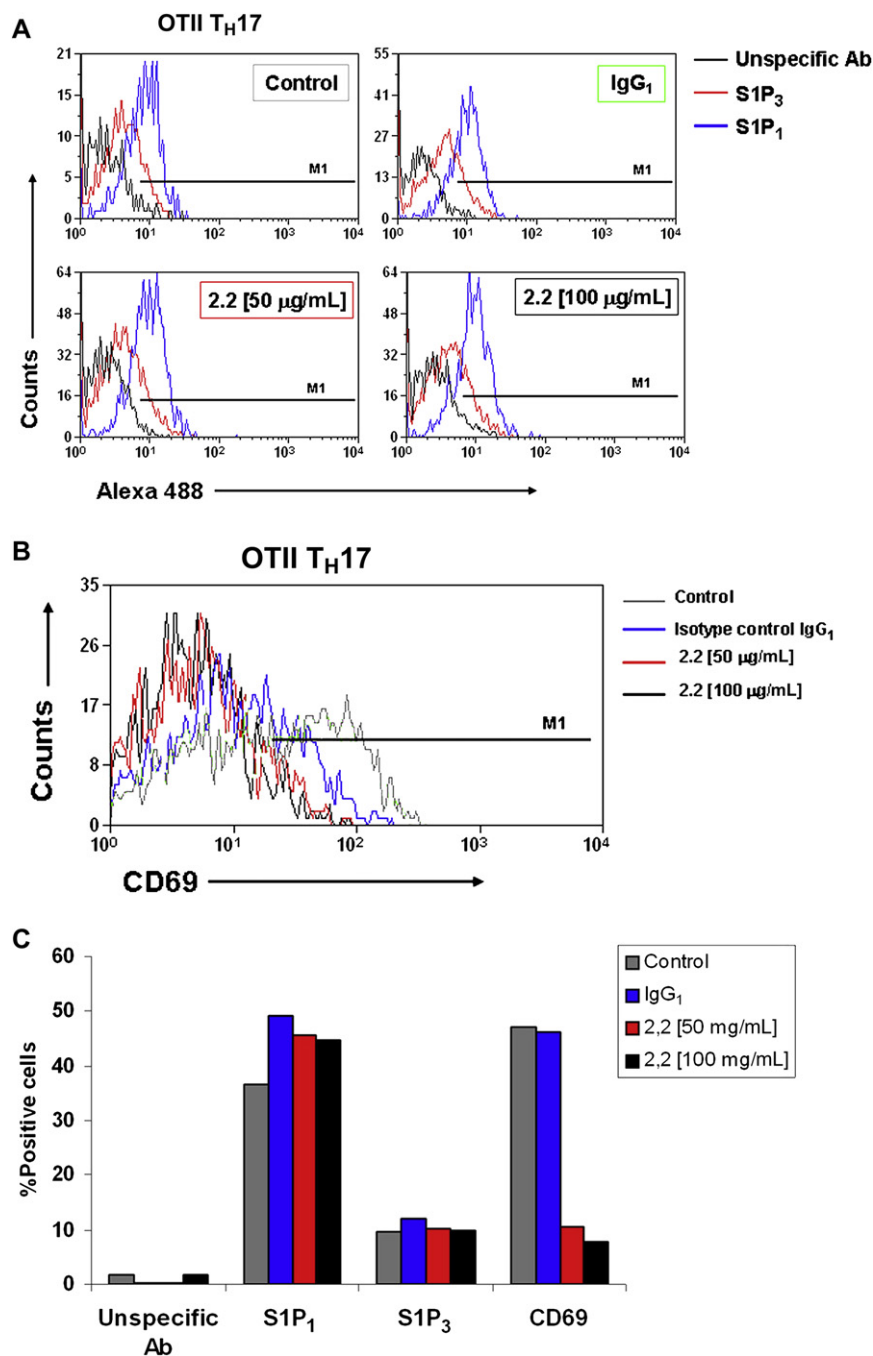


FIG E1. S1P1 expression in OTII Th17 differentiation cultures in the presence of anti-CD69 mAb. **A**, FACS analysis of membrane levels of S1P1 (blue line) and S1P3 (red line) in OTII Th17 cells cultured in the presence of anti-CD69 mAb 2.2 at the indicated concentrations or with an isotype control Ab (Unspecific Ab). **B**, FACS analysis of CD69 expression levels in the membrane of OTII Th17 cells cultured as in **A** or without Abs (Control, grey line). **C**, FACS analysis of the percentage of positive cells for S1P1, S1P3, CD69 or an isotype control (Unspecific Ab) in OTII Th17 cells cultured as in **B**.

TABLE E1. Specific sequences of the primers used in quantitative real-time RT-PCR analyses

Cytokines	Forward (5'-3')	Reverse (3'-5')
TNF- α	GCA AGC TTC GCT CTT CTG TCT ACT GAA CTT CGG	GCT CTA GAA TGA GAT AGC AAA TCG GCT GAC GG
IFN- γ	TGG CTC TGC AGG ATT TTC ATG	TCA AGT GGC ATA GAT GTG GAA GAA
TGF- β 1	CCG AAG CGG ACT ACT AT	GTA ACG CCA GGA ATT GT
IL-1 β	TCA CGC AGG CAG TAT C	GTC GTT GCT TCG TTC T
IL-6	GCG ACT TCC ATC CAG TTG CC	TTC TGC AAG TGC ATC ATC G
IL-17A	CTC CAG AAG GCC CTC AGA CTA C	GGG TCT TCA TTG CGG TGG
IL-23 p19	AGC GGG ACA TAT GAA TCT ACT AAG AGA	GTC CTA GTA GGG AGG TGT GAA GTT G
IL-18	AAT GGC TGC CAT GTC A	TCC GTA TTA CTG CGG TTG
S1P ₁	GTG TAG ACC CAG AGT CCT GCG	AGC TTT TCC TTG GCT GGA GAG
S1P ₃	GGA GCC CCT AGA CGG GAG	CCG ACT GCG GGA AGA GTG T
GAPDH	GAA GGT GAA GGTCGG AGT C	GAA GAT GGT GAT GGG ATT TC

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.