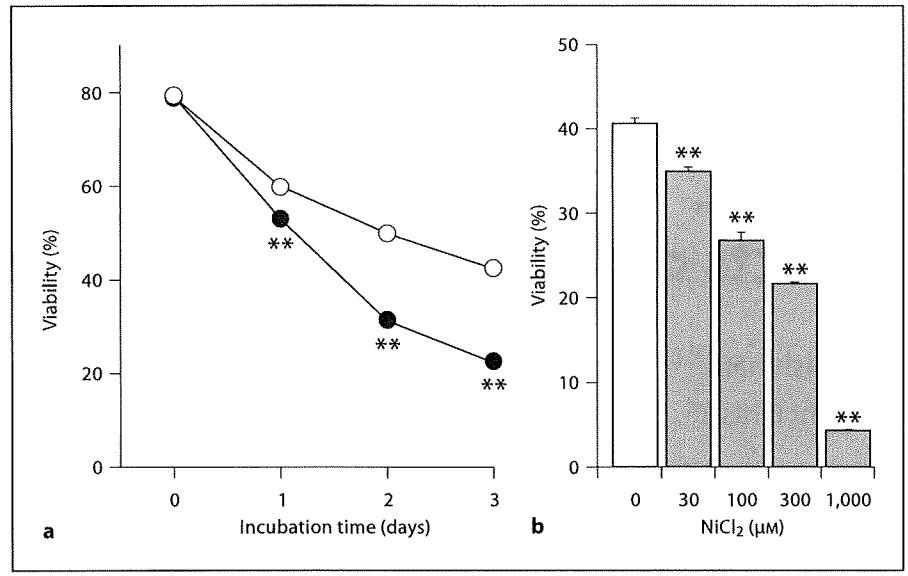


**Fig. 1.** Effects of nickel chloride on the viability of eosinophils. Eosinophils were incubated at 37°C (**a**) for the periods indicated in medium in the absence (○) or presence of nickel chloride (300 μM; ●) and (**b**) for 3 days in the presence of the indicated concentrations of nickel chloride. After the incubation, to determine the viability of eosinophils, the cells were stained with 7-AAD and analyzed by flow cytometer. Values are the means from 4 samples with SEM. SEM is within each symbol. Statistical significance: ** $p<0.01$ vs. corresponding control.

ed in each well of 24-well plates at a density of $1 \times 10^6$ cells in 1 ml of 10% FBS-RPMI-1640 medium containing various kinds of metals. The metals compounds used in this experiment were nickel chloride, cobalt chloride, manganese chloride, nickel sulfate, zinc sulfate, and aluminum sulfate (Wako, Osaka, Japan). These metals were dissolved in water and added to the medium. The concentration of the vehicle in the medium was adjusted to 0.1% (v/v).

*Flow Cytometry*

After the incubation of eosinophils for various periods in the presence of metal compounds, the cells were stained with 7-aminoactinomycin D (Sigma, St. Louis, Mo., USA) for analysis of viability, or with FITC-labeled annexin V and propidium iodide (PI) (Sigma) for analysis of apoptosis for 5 min at room temperature. To detect binding of nickel ions to eosinophils, the cells were incubated in medium containing nickel chloride for 5 min at room temperature, washed with PBS three times, and further incubated with Newport Green DCF (Invitrogen, Carlsbad, Calif., USA) for 5 min at room temperature. After washing with PBS, the fluorescence of Newport Green binding on nickel ions and eosinophils was analyzed using a flow cytometer (FACScan; Beckton Dickinson, Franklin Lakes, N.J., USA).

*Statistical Analysis*

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons.

## Results and Discussion

When rat eosinophils were incubated for 1, 2 and 3 days at 37°C, their viability was decreased time-dependently (fig. 1a). Nickel chloride further decreased the viability of cells at each day (fig. 1a). Upon treatment with nickel chloride at 30–1,000 μM, the viability of cells at 3 days was decreased by nickel chloride in a concentration-dependent manner (fig. 1b). To clarify whether the decrease in the viability of eosinophils by nickel chloride was due to the induction of apoptosis, we examined the annexin $V^+$ $PI^-$ population in eosinophils incubated for 1 day with medium containing 300 μM of nickel chloride. As shown in figure 2, nickel chloride increased the percentage of annexin $V^+$ $PI^-$ eosinophils. These findings suggest that nickel chloride decreases the viability of eosinophils through the induction of apoptosis. Next, we analyzed whether other metal compounds at 300 μM induce eosinophil apoptosis. Nickel sulfate induced apoptosis at almost the same level as nickel chloride, while that induced by cobalt chloride was moderate and that induced by zinc sulfate was weak compared with induction by nickel chloride and nickel sulfate (fig. 2). These results suggest that nickel ions, cobalt ions and zinc ions induce the apoptosis of eosinophils. It has been reported that nickel, cobalt and zinc are allergenic, and it is well known that nickel and cobalt cause allergy [1]. In contrast, manganese chloride inhibited apoptosis (fig. 2). Manganese is an essential element for all types of tissue, and plays important roles in the free radical defense system as manganese superoxide dismutase (MnSOD), which protects mitochondria from damage by superoxide radicals [8, 9]. Eosinophils have been reported to undergo apoptosis by reactive oxygen species (ROS) [10], and MnSOD is involved in the inhibition of eosinophil apoptosis [11].

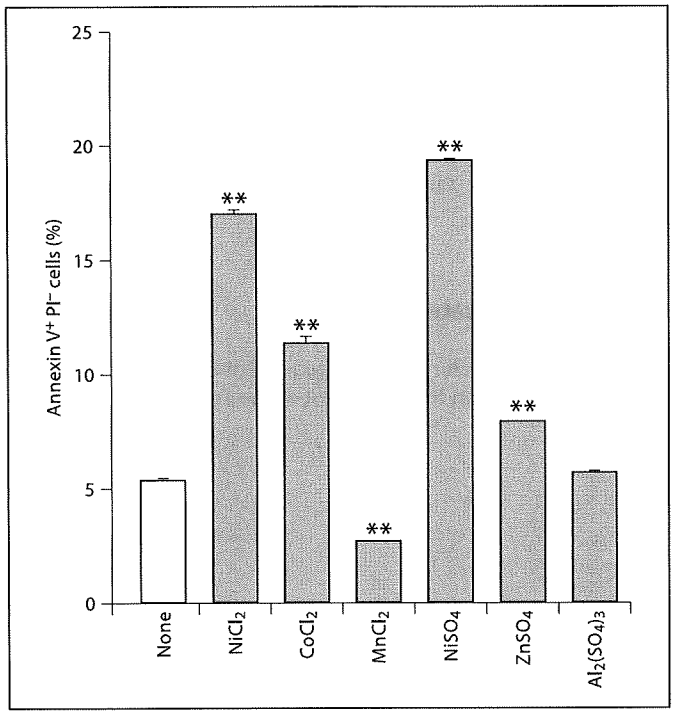


**Fig. 2.** Effects of various metal compounds on eosinophil apoptosis. Eosinophils were incubated at 37°C for 1 day in medium in the presence of 300 μM of the indicated metal compounds. After the incubation, the cells were stained with FITC-labeled annexin V and PI and analyzed by flow cytometer. Values are the means from 4 samples with SEM. Statistical significance: ** $p < 0.01$ vs. corresponding control.

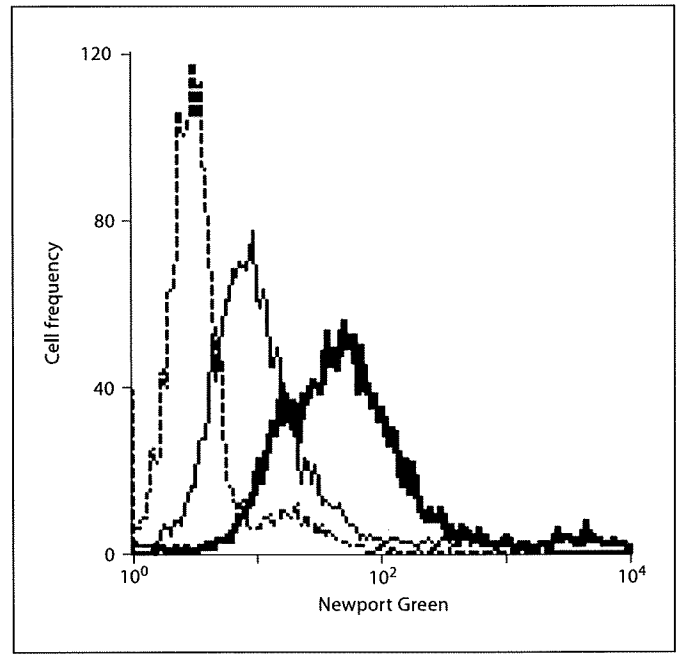


**Fig. 3.** Binding of nickel ions to eosinophils. Eosinophils were incubated in the presence (bold line) or absence (thin line) of 300 μM of nickel chloride for 5 min and then with 1 μM of Newport Green for 5 min at room temperature. After washing with PBS, the binding of nickel ions to eosinophils was analyzed by flow cytometer. The dashed line represents the fluorescence of cells incubated in the absence of nickel chloride and Newport Green. The experiment shown is representative of 4 independent experiments.

Therefore, it is possible that manganese inhibits eosinophils apoptosis via MnSOD. Taken together, our findings suggest that the metal specificity of the induction of eosinophil apoptosis is correlated with allergenicity.

We analyzed whether nickel ions bind to eosinophils, and detected an increase of fluorescence of Newport Green, which is a cell-impermeable fluoropore for nickel ions, on eosinophils by flow cytometry (fig. 3). Pretreatment of eosinophils with nickel chloride enhanced the fluorescence intensity, suggesting that nickel ions bind to the surface of eosinophils (fig. 3). It has been reported that nickel ions bind to numerous proteins including cell surface molecules, major histocompatibility complex (MHC) class I and MHC class II [12]. Therefore, it is possible that nickel ions bind not only to the molecules on the cell surface as shown in figure 3, but also to intracellular molecules in eosinophils.

It has been reported that nickel ions induce the production of ROS and prostaglandins, expression of Fas ligand, and various cytokines, such as IL-8, TNF-α and TGF-β, via activation of signal transduction molecules including mitogen-activated protein kinases [13–19]. In these molecules, ROS, Fas ligand and TGF-β are reported to induce apoptosis of eosinophils. Therefore, it is suggested that eosinophils undergo apoptosis by nickel ions through the production of these molecules. In contrast, infiltration of eosinophils toward inflammatory sites is induced by nickel in patients with contact allergy [3] and bronchial asthma [6], and it is thought that eosinophils play important roles in the pathogenesis of allergies. However, this study indicated that eosinophil viability is decreased by nickel ions (fig. 1b). In patients, nickel ionized by sweat on the surface of the skin induces the expression of various cytokines at the inflammatory sites [2, 13–19]. IL-8, which is a chemotactic factor for eosinophils, produced by nickel ions from epithelial cells [16] might be involved in the infiltration of eosinophils into the sites. Eosinophils might also produce inflammatory mediators such as cytokines and lipids involved in the pathogenesis of allergies on exposure to nickel ions. Thus,

it is possible that simultaneous signaling for the expression of various inflammatory mediators from eosinophils and that for the induction of apoptosis in eosinophils might be involved in the inflammation and allergy induced by nickel in vivo. Therefore, eosinophils might play important roles for the nickel-induced allergy as effector cells.

In the study of metal allergy, T cells have been focused on because metal allergy is recognized as being a type IV allergy. However, nickel ions were found to act on eosinophils in the present study. Because it has been unknown whether nickel ions induce the expression of mediators from eosinophils, further investigation is necessary for the implication of eosinophils in metal allergy.

In conclusion, we suggest that nickel may exhibit a modifying activity on the function of eosinophils in allergy.


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## Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

## References


1 Garner LA: Contact dermatitis to metals. Dermatol Ther 2004;17:321–327.
2 Nestle FO, Speidel H, Speidel MO: Metallurgy: high nickel release from 1- and 2-euro coins. Nature 2002;419:132.
3 Lerche A, Bisgaard H, Christensen JD, Venge P, Dahl R, Søndergaard J: Lactoferrin, myeloperoxidase, lysozyme and eosinophil cationic protein in exudate in delayed type hypersensitivity. Allergy 1988;43:139–145.
4 Barata LT, Ying S, Meng Q, Barkans J, Rajakulasingam K, Durham SR, Kay AB: IL-4- and IL-5-positive T lymphocytes, eosinophils, and mast cells in allergen-induced late-phase cutaneous reactions in atopic subjects. J Allergy Clin Immunol 1998;101:222–230.
5 Schatz M, Wasserman S, Patterson R: The eosinophil and the lung. Arch Intern Med 1982;142:1515–1519.
6 Gül Ü, Cakmak SK, Olcay I, Kilic A, Gönül M: Nickel sensitivity in asthma patients. J Asthma 2007;44:383–384.
7 Ishihara K, Satoh I, Nittoh T, Kanaya T, Okazaki H, Suzuki T, Koyama T, Sakamoto T, Ide T, Ohuchi K: Preparation of recombinant rat interleukin-5 by baculovirus expression system and analysis of its biological activities. Biochim Biophys Acta 1999;1451:48–58.
8 Szentmihályi K, Vinkler P, Fodor J, Balla J, Lakatos B: The role of manganese in the human organism. Orv Hetil 2006;147:2027–2030.
9 Epperly MW, Bernarding M, Gretton J, Jefferson M, Nie S, Greenberger JS: Overexpression of the transgene for manganese superoxide dismutase (MnSOD) in 32D cl 3 cells prevents apoptosis induction by TNF-α, IL-3 withdrawal, and ionizing radiation. Exp Hematol 2003;31:465–474.
10 Wedi B, Straede J, Wieland B, Kapp A: Eosinophil apoptosis is mediated by stimulators of cellular oxidative metabolisms and inhibited by antioxidants: involvement of a thiol-sensitive redox regulation in eosinophil cell death. Blood 1999;94:2365–2373.
11 Gardai SJ, Hoontrakoon R, Goddard CD, Day BJ, Chang LY, Henson PM, Bratton DL: Oxidant-mediated mitochondrial injury in eosinophil apoptosis: enhancement by glucocorticoids and inhibition by granulocyte-macrophage colony-stimulating factor. J Immunol 2003;170:556–566.
12 Czarnobilska E, Obtulowicz K, Wsolek K, Pietowska J, Spiewak R: Mechanisms of nickel allergy. Przegl Lek 2007;64:502–505.
13 Andrew AS, Klei LR, Barchowsky A: Nickel requires hypoxia-inducible factor-1α, not redox signaling, to induce plasminogen activator inhibitor-1. Am J Physiol 2001;281:L607–L615.
14 Lerche A, Bisgaard H, Kassis V, Christensen JD, Søndergaard J: Prostaglandin $E_1$ and prostaglandin $F_{2\alpha}$ in exudate in nickel allergy. Acta Derm Venereol 1989;69:253–256.
15 Kim K, Lee SH, Seo YR, Perkins SN, Kasprzak KS: Nickel(II)-induced apoptosis in murine T cell hybridoma cells is associated with increased fas ligand expression. Toxicol Appl Pharmacol 2002;185:41–47.
16 Barchowsky A, Soucy NV, O'Hara KA, Hwa J, Noreault TL, Andrew AS: A novel pathway for nickel-induced interleukin-8 expression. J Biol Chem 2002;277:24225–24231.
17 Taira M, Sasaki M, Kimura S, Araki Y: Dose-dependent effects of Ni (II) ions on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6), superoxide dismutase and free radical NO by murine macrophage-like RAW264 cells with or without LPS stimulation. J Mater Sci Mater Med 2008;19:2173–2178.
18 Wesselkamper SC, Case LM, Henning LN, Borchers MT, Tichelaar JW, Mason JM, Dragin N, Medvedovic M, Sartor MA, Tomlinson CR, Leikauf GD: Gene expression changes during the development of acute lung injury: role of transforming growth factor-β. Am J Respir Crit Care Med 2005;172:1399–1411.
19 Tessier DM, Pascal LE: Activation of MAP kinases by hexavalent chromium, manganese and nickel in human lung epithelial cells. Toxicol Lett 2006;167:114–121.

Cutting Edge

THE JOURNAL OF IMMUNOLOGY

# Cutting Edge: Histamine Receptor H4 Activation Positively Regulates In Vivo IL-4 and IFN-γ Production by Invariant NKT Cells[1]

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**Histamine (HA) is a biogenic amine with multiple activities in the immune system. In this study we demonstrate that histamine-free histidine decarboxylase-deficient ($HDC^{-/-}$) mice present a numerical and functional deficit in invariant NK T (iNKT) cells as evidenced by a drastic decrease of IL-4 and IFN-γ production. This deficiency was established both by measuring cytokine levels in the serum and intracellularly among gated iNKT cells. It resulted from the lack of HA, because a single injection of this amine into $HDC^{-/-}$ mice sufficed to restore normal IL-4 and IFN-γ production. HA-induced functional recovery was mediated mainly through the H4 histamine receptor (H4R), as assessed by its abrogation after a single injection of a selective H4R antagonist and the demonstration of a similar iNKT cell deficit in $H4R^{-/-}$ mice. Our findings identify a novel function of HA through its H4R and suggest that it might become instrumental in modulating iNKT cell functions.** *The Journal of Immunology,* **2009, 182: 1233–1236.**

Histamine (HA)[3] is one of the most versatile biogenic amines with multiple physiological functions in the CNS, the intestinal tract, and inflammatory reactions. More recently, a number of studies have established that besides its most obvious contribution to allergic reactions, HA also exerts more subtle regulatory functions influencing the orientation of the immune response, thus rekindling interest in this field of investigation (1–4). It has been assumed until lately that these immunomodulatory effects were mediated mainly through classical HA receptors of the H1 and H2 subtypes (5, 6). However, this explanation has since been complicated by the identification of organic cation transporter 3, OCT3, as a means through which HA can be taken up by murine basophils and exert a negative feedback on their HA, IL-4, IL-6, and IL-13 production (7), as well as by the identification of an additional HA receptor, HA receptor subtype 4 (H4R) expressed mainly in hematopoietic and immunocompetent cells (8, 9). The most clearly established activities of H4R consist in the recruitment and activation of cells involved in inflammatory responses such as eosinophils, mast cells, neutrophils, conventional T lymphocytes, and dendritic cells (10–13). However, its functional expression in the immunoregulatory invariant NK T (iNKT) cells has not been investigated so far.

iNKT cells constitute a distinctive population of mature T lymphocytes positively selected by the nonpolymorphic MHC class-I-like molecule CD1d. They coexpress a highly restricted TCR repertoire composed of a single invariant Vα14Jα18 chain in mice and a Vα24Jα18 chain in humans, preferentially paired with a limited TCR Vβ-chain repertoire that specifically recognizes glycolipids (14–16). iNKT cells are implicated in the control of several immune responses, most likely because of their capacity to promptly produce several cytokines (14–20) such as IL-4 and IFN-γ. In the present study we demonstrate that HA participates in this functional tuning to ensure optimal IL-4- and IFN-γ production by iNKT cells.

## Materials and Methods

### *Animals*

Male C57BL/6J mice (7–9 wk old) were purchased from Janvier. Histidine decarboxylase (HDC)-deficient ($HDC^{-/-}$) and $H4R^{-/-}$ mice, backcrossed 12 and 10 times to C57BL/6J mice, respectively (21, 22), were bred in our own facilities. $HDC^{-/-}$ mice received a histamine-low diet (SAFE Scientific Animal Food and Engineering) to avoid exogenous uptake. Animal experiments were performed according to the French institutional committee.

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[3] Abbreviations used in this paper: HA, histamine; α-GalCer, α-galactosylceramide; H4R, HA receptor subtype 4; HDC, histidine decarboxylase; iNKT, invariant NK T (cell); MNC, mononuclear cell.

*In vivo treatment*

Mice received a single i.p. administration of 2 μg of α-galactosylceramide (α-GalCer; Alexis) 90 min before sacrifice. In some experiments, mice were injected i.p. 1 h before α-GalCer administration with a single dose of HA (Sigma-Aldrich) (20 mg/kg) with or without the H4R antagonist JNJ 7777120 (23) (20 mg/kg) administered i.p. 1 h before HA injection.

*Cell preparation*

Lymphocytes were isolated from the spleen using a homogenizer. Mononuclear cells (MNC) were separated from hepatocytes and cellular debris by way of a 35% isotonic Percoll density gradient (Amersham Biosciences). Liver and spleen MNC were depleted of RBC using red cell lysis buffer (8.3 mg/ml $NH_4Cl$, 1 mg/ml $KHCO_3$, and 3.72 μg/ml EDTA).

*Flow cytometry*

Splenocytes and liver MNC were preincubated with mAbs against FcγR (clone 2.4G2 culture supernatant), washed, and incubated with CD1d-α-GalCer tetramer-allophycocyanin or control tetramers, anti-CD4 PerCP-Cy-5.5, anti-TCRβ-FITC, anti-IL-4-PE, anti-IFN-γ-PE, or isotype control (BD Pharmingen) as described (20). In some experiments an anti-H4R (clone Y-19; Santa Cruz Biotechnology) was used according to the manufacturer's instructions. Cells were analyzed on a FACSCanto II (BD Biosciences) flow cytometer using FACSDiva software.

*Determination of cytokines*

IL-4 and IFN-γ were measured by ELISA as described (20).

*Statistical analysis*

The nonparametric test t was used to calculate significance levels for all measurements. Values of $p < 0.05$ were considered statistically significant.

# Results and Discussion

*IL-4 and IFN-γ production by iNKT cells is decreased in HA-free HDC$^{-/-}$ mice*

Prompt production of IL-4 and IFN-γ in response to TCR cross-linking constitutes a typical feature of iNKT cells. We measured these cytokines to establish whether exogenous HA participated in their modulation. To this end, we injected wild-type and histamine-free HDC$^{-/-}$ mice (deficient for the HA-forming enzyme HDC) with α-GalCer, a glycolipid widely used as a specific activator of iNKT cells, to determine its capacity to specifically activate and promptly induce these cytokines. We found that both IL-4 and IFN-γ levels generated after a single injection were significantly lower in the serum of HDC$^{-/-}$ mice than in wild-type controls (Fig. 1, *A* and *B*). This decrease could result either from a lower incidence or a functional defect of iNKT cells. Indeed, we found that CD1d/α-GalCer tetramer$^+$ cells were effectively reduced in spleen and liver of HDC$^{-/-}$ mice, both in terms of cell counts and percentage (Fig. 1, *C–E*).

The lower iNKT cell counts in histamine-free mice do not exclude the presence of functional deficiencies in the remaining cells, promoting us to analyze cytokine production in single cells by intracellular staining. It turned out that among gated iNKT cells the percentage that was actually positive for IL-4 and IFN-γ cells after injection of α-GalCer was strikingly reduced in HDC$^{-/-}$ mice compared with controls (Fig. 2, *A* and *B*). These data clearly show that iNKT cells are both numerically and functionally impaired in histamine-deficient mice.

*HA injection restores the IL-4- and IFN-γ-producing capacity of iNKT cells*

To confirm the implication of HA in the cytokine-producing capacity of iNKT cells, HDC$^{-/-}$ mice were treated with HA 1 h before α-GalCer stimulation. Remarkably, a single injection of HA was sufficient to restore the seric levels of IL-4 and IFN-γ in HDC$^{-/-}$ mice (Fig. 1, *A* and *B*). Even though this treatment did not enhance the percentage or the absolute number of iNKT cells significantly (data not shown), it did increase the proportion of IL-4$^+$ and IFN-γ$^+$ cells among gated iNKT lymphocytes (Fig. 2), consistent with the restored seric cytokine levels. It can therefore be concluded that HA is capable of upregulating both IL-4 and IFN-γ production by iNKT cells activated in vivo.

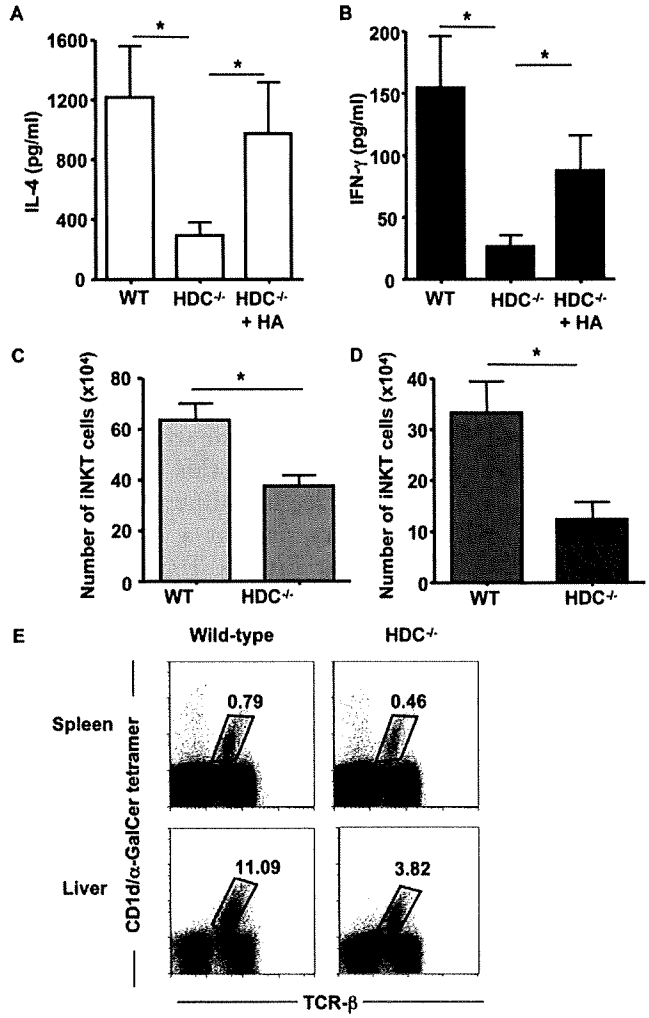


**FIGURE 1.** Marked decrease in cytokine production by iNKT cells in HDC$^{-/-}$ mice. Wild-type (WT) and HDC$^{-/-}$ mice were injected with α-GalCer. A group of HDC$^{-/-}$ mice were treated with HA 1 h before α-GalCer injection (HDC$^{-/-}$ + HA). Ninety minutes later, IL-4 (*A*) and IFN-γ (*B*) were measured in the serum. No cytokine was detected without α-GalCer stimulation (data not shown). The total number (*C* and *D*) and the percentage (*E*) of iNKT cells were analyzed in both spleen (*C* and *E*) and liver (*D* and *E*) from untreated wild-type or HDC$^{-/-}$ mice. Data represent the mean ± SEM from 8–20 mice (*A* to *D*). *, $p < 0.05$. Representative FACS profiles showing the percentage of CD1d/α-GalCer$^+$TCRβ$^+$ cells (*E*) in wild-type vs HDC$^{-/-}$ mice.

*Cytokine production by iNKT cells is impaired in H4 receptor-deficient mice*

Knowing that histamine exerts its biological effect through four specific receptors and that the most recently discovered H4 subtype is preferentially expressed in hematopoietic cells, we used mice in which the corresponding gene had been disrupted (H4R$^{-/-}$) to assess their IL-4 and IFN-γ production following

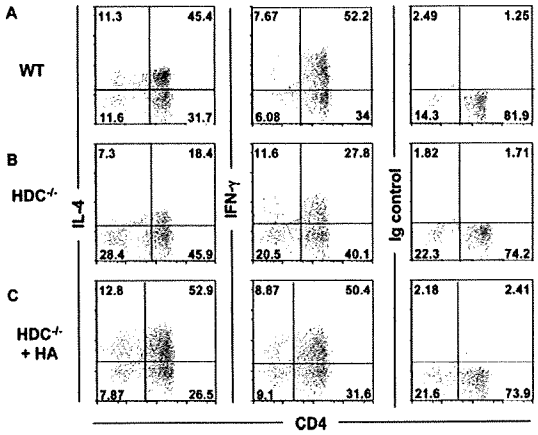


**FIGURE 2.** Impaired IL-4 and IFN-γ production by in vivo α-GalCer-stimulated iNKT cells from HDC$^{-/-}$ mice Representative FACS profiles showing the percentage of IL-4, IFN-γ, and isotype control (Ig control) positive cells among gated CD1d/α-GalCer tetramer$^{+}$TCRβ$^{+}$ iNKT splenocytes from α-GalCer treated wild-type (WT) (*A*) or HDC$^{-/-}$ mice (*B*). In the later group, HDC$^{-/-}$ mice were treated with HA (HDC$^{-/-}$ + HA) before α-GalCer injection (*C*).

α-GalCer injection. We found that H4R$^{-/-}$ mice, which presented no significant modification in the absolute number of iNKT cells ($5.2 \times 10^5 \pm 0.9 \times 10^5$ vs $6.7 \times 10^5 \pm 1.2 \times 10^5$ splenic iNKT cells from wild-type and H4R$^{-/-}$ mice, respectively), generated significantly fewer circulating cytokines than wild-type controls (Fig. 3, *A* and *B*), suggesting that the positive effect of HA on these biological activities was mediated through H4R activation.

*In vivo treatment of HDC$^{-/-}$ mice with a H4R antagonist abrogates the restoration of iNKT cell functions in response to HA*

To prove that H4Rs were required for the up-regulation of IL-4 and IFN-γ production by iNKT cells, we blocked their binding sites with the highly selective H4 antagonist JNJ7777120 injected 1 h before HA into HDC$^{-/-}$ mice. In this case HA failed to restore a normal IL-4 and IFN-γ production, as shown in Fig. 3, *C* and *D*. In further support of this result, we show that iNKT cells express H4R (Fig. 3*E*), leading us to conclude that HA modulates iNKT cell functions through this receptor subtype.

The contribution of iNKT cells to immune responses is complex because of their capacity to produce both IFN-γ and IL-4, thereby supporting Th1 or Th2 responses, respectively. We and others have reported that iNKT cells can aggravate asthma through their Th2 cytokine profile (18, 24). Similarly, HA plays a major role in atopic diseases, namely in allergic asthma, because its release in the airways is one of the typical features of this pathology that triggers a cascade of events, including airway constriction, mucus secretion, vascular leak, and recruitment of immune cells. Our present data suggest an additional means for HA to enhance the severity of asthma by promoting optimal IL-4 production by iNKT cells. Consistent with this assumption, it has recently been reported that asthmatic mice treated with the JNJ7777120 antagonist used herein develop less airway inflammation than untreated controls (11).

Taken together, our data reveal a new role of histamine through H4R activation in the functional modulation of the immunoregulatory iNKT cell population and provide additional evidence for the complex influence of the microenvironment on iNKT cell functions.

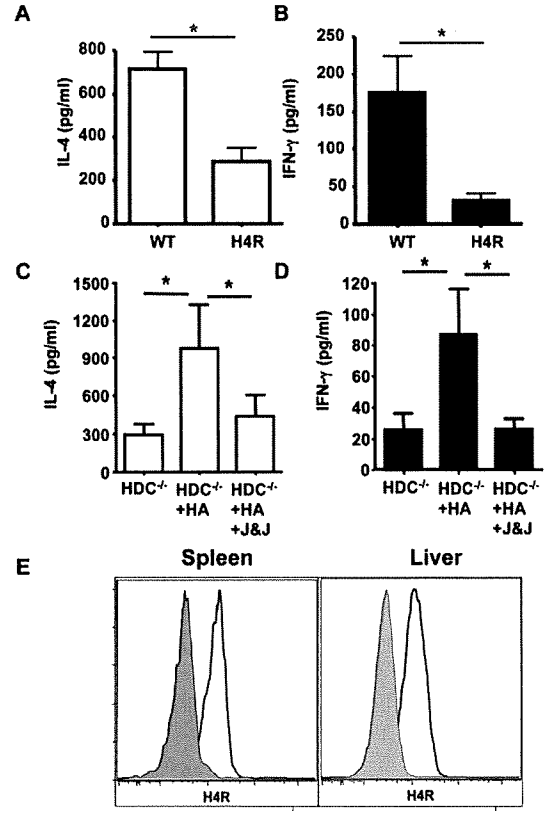


**FIGURE 3.** HA can modulate iNKT cell functions through H4R. *A* and *B*, Wild-type (WT) and H4R$^{-/-}$ mice were injected with α-GalCer. Ninety minutes later, IL-4 (*A*) and IFN-γ (*B*) were measured in the serum. *C* and *D*, HDC$^{-/-}$ mice were treated or not with the selective H4R antagonist JNJ7777120 (J&J), followed by HA and then α-GalCer injection as described in *Materials and Methods*. Ninety minutes after the last injection, IL-4 (*C*) and IFN-γ (*D*) were measured in the serum. Data represent the mean ± SEM from 6–10 mice; *, $p < 0.05$. *E*, Representative FACS profiles showing the expression of H4R (empty histogram) compared with control (filled histogram) among gated CD1d/α-GalCer$^{+}$TCRβ$^{+}$ splenocytes (*left panel*) or liver MNC (*right panel*) recovered from wild-type mice.

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## Disclosures

The authors have no financial conflict of interest.

## References

1. Schneider, E., M. Rolli-Derkinderen, M. Arock, and M. Dy. 2002. Trends in histamine research: new functions during immune responses and hematopoiesis. *Trends Immunol.* 23: 255–263.
2. Akdis, C. A., and K. Blaser. 2003. Histamine in the immune regulation of allergic inflammation. *J. Allergy Clin. Immunol.* 112: 15–22.
3. Dy, M., and E. Schneider. 2004. Histamine-cytokine connection in immunity and hematopoiesis. *Cytokine Growth Factor Rev.* 15: 393–410.
4. Beghdadi, W., A. Porcherie, B. S. Schneider, D. Dubayle, R. Peronet, M. Huerre, T. Watanabe, H. Ohtsu, J. Louis, and S. Mécheri. 2008. Inhibition of histamine-mediated signaling confers significant protection against severe malaria in mouse models of disease. *J. Exp. Med.* 205: 395–408.
5. Jutel, M., T. Watanabe, S. Klunker, M. Akdis, O. A. Thomet, J. Malolepszy, T. Zak-Nejmark, R. Koga, T. Kobayashi, K. Blaser, and C. A. Akdis. 2001. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* 413: 420–425.

6. Kharmate, G., Z. Liu, E. Patterson, and M. M. Khan. 2007. Histamine affects STAT6 phosphorylation via its effects on IL-4 secretion: role of H1 receptors in the regulation of IL-4 production. *Int. Immunopharmacol.* 7: 277–286.
7. Schneider, E., F. Machavoine, J. M. Pleau, A. F. Bertron, R. L. Thurmond, H. Ohtsu, T. Watanabe, A. H. Schinkel, and M. Dy. 2005. Organic cation transporter 3 modulates murine basophil functions by controlling intracellular histamine levels. *J. Exp. Med.* 202: 387–393.
8. Nguyen, T., D. A. Shapiro, S. R. George, V. Setola, D. K. Lee, R. Cheng, L. Rauser, S. P. Lee, K. R. Lynch, B. L. Roth, and B. F. O'Dowd. 2001. Discovery of a novel member of the histamine receptor family. *Mol. Pharmacol.* 59: 427–433.
9. Coge, F., S. P. Guenin, H. Rique, J. A. Boutin, and J. P. Galizzi. 2001. Structure and expression of the human histamine H4-receptor gene. *Biochem. Biophys. Res. Commun.* 284: 301–309.
10. Ling, P., K. Ngo, S. Nguyen, R. L. Thurmond, J. P. Edwards, L. Karlsson, and W. P. Fung-Leung. 2004. Histamine H4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation. *Br. J. Pharmacol.* 142: 161–171.
11. Dunford, P. J., N. O'Donnell, J. P. Riley, K. N. Williams, L. Karlsson, and R. L. Thurmond. 2006. The histamine H4 receptor mediates allergic airway inflammation by regulating the activation of $CD4^+$ T cells. *J. Immunol.* 176: 7062–7070.
12. de Esch, I. J., R. L. Thurmond, A. Jongejan, and R. Leurs. 2005. The histamine H4 receptor as a new therapeutic target for inflammation. *Trends Pharmacol. Sci.* 26: 462–469.
13. Gutzmer, R., C. Diestel, S. Mommert, B. Kother, H. Stark, M. Wittmann, and T. Werfel. 2005. Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *J. Immunol.* 174: 5224–5232.
14. Lisbonne, M., and M. C. Leite-de-Moraes. 2003. Invariant Vα14 NKT lymphocytes: a double-edged immuno-regulatory T cell population. *Eur. Cytokine Network* 14: 4–14.
15. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877–900.
16. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu. Rev. Immunol.* 25: 297–336.
17. Campos, R. A., M. Szczepanik, M. Lisbonne, A. Itakura, M. C. Leite-de-Moraes, and P. W. Askenase. 2006. Invariant NKT cells rapidly activated via immunization with diverse contact antigens collaborate in vitro with B-1 cells to initiate contact sensitivity. *J. Immunol.* 177: 3686–3694.
18. Lisbonne, M., S. Diem, A. de Castro Keller, J. Lefort, L. M. Araujo, P. Hachem, J. M. Fourneau, S. Sidobre, M. Kronenberg, M. Taniguchi, et al. 2003. Cutting edge: invariant Vα14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J. Immunol.* 171: 1637–1641.
19. Leite-de-Moraes, M. C., M. Lisbonne, A. Arnould, F. Machavoine, A. Herbelin, M. Dy, and E. Schneider. 2002. Ligand-activated natural killer T lymphocytes promptly produce IL-3 and GM-CSF in vivo: relevance to peripheral myeloid recruitment. *Eur. J. Immunol.* 32: 1897–1904.
20. Michel, M.-L., A. Castro Keller, C. Paget, M. Fujio, F. Trottein, P. B. Savage, C. H. Wong, E. Schneider, M. Dy, and M. C. Leite-de-Moraes. 2007. Identification of an IL-17-producing $NK1.1^{neg}$ iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 204: 995–1001.
21. Ohtsu, H., S. Tanaka, T. Terui, Y. Hori, Y. Makabe-Kobayashi, G. Pejler, E. Tchougounova, L. Hellman, M. Gertsenstein, N. Hirasawa, et al. 2001. Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS Lett.* 502: 53–56.
22. Hofstra, C. L., P. J. Desai, R. L. Thurmond, and W. P. Fung-Leung. 2003. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J Pharmacol. Exp. Ther.* 305: 1212–1221.
23. Thurmond, R. L., P. J. Desai, P. J. Dunford, W. P. Fung-Leung, C. L. Hofstra, W. Jiang, S. Nguyen, J. P. Riley, S. Sun, K. N. Williams, et al. 2004. A potent and selective histamine H4 receptor antagonist with anti-inflammatory properties. *J. Pharmacol. Exp. Ther.* 309: 404–413.
24. Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582–588.

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ORIGINAL ARTICLE

# Enhanced Goblet Cell Hyperplasia in HDC Knockout Mice with Allergic Airway Inflammation

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## ABSTRACT

**Background:** Histamine is known to have immunoregulatory roles in allergic reactions through histamine receptor 1 (H1R), H2R, H3R and H4R. However, its role in goblet cell hyperplasia in the airways of asthma patients is yet to be clarified.

**Objective:** This study was designed to examine the role of histamine in goblet cell hyperplasia using histamine-deficient mice (*Hdc-/-* mice) with allergic airway inflammation.

**Methods:** Wild-type and *Hdc-/-* C57BL/6 mice were sensitized with ovalbumin (OVA). After a 2-week exposure to OVA, goblet cell hyperplasia was evaluated. Cell differentials and cytokines in BALF were analyzed. The mRNA levels of MUC5AC and Gob-5 gene were determined quantitatively.

**Results:** The number of eosinophils in BALF increased in both the sensitized wild-type mice and *Hdc-/-* mice with OVA inhalation. In addition, the numbers of alveolar macrophages and lymphocytes in BALF increased significantly in the sensitized *Hdc-/-* mice with OVA inhalation compared to the wild-type mice under the same conditions. The concentrations of Interleukin-4 (IL-4), IL-5, IL-13, Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2 in the BALF all increased significantly in both groups compared to those exposed to saline. In particular, the concentration of TNF-α in the *Hdc-/-* mice exposed to OVA was significantly higher than that in the wild-type mice under the same conditions. The mRNA levels of Gob-5 and MUC5AC, and the ratio of the goblet cells in the airway epithelium significantly increased in *Hdc-/-* mice exposed to OVA compared to wild-type mice.

**Conclusions:** These results suggested that histamine may play a regulatory role in goblet cell hyperplasia in allergic airway inflammation.

## KEY WORDS

airway remodeling, asthma, Gob-5, MUC5AC, TNF-α

## *INTRODUCTION*

Goblet cell hyperplasia and mucus overproduction are important features of bronchial asthma.[1,2] Although goblet cell hyperplasia with mucus hypersecretion has been reported to be associated with the development of airway hyperresponsiveness and the increase of severity and mortality in bronchial asthma[1-4], the mechanisms responsible for goblet cell hyperplasia are not completely understood.

Histamine is known to be a strong chemical mediator that stimulates secretion from goblet cells. Bryce *et al.* recently reported that the airway inflammatory response and goblet cell hyperplasia to allergic stimuli diminished in H1 receptor (H1R) knock-out mice, suggesting that histamine may be involved in the immunomodulation in airway allergic reaction including Th1 and Th2 cytokine production and goblet cell hy-

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perplasia.[5]

It has been reported that histamine can modulate T-cell-mediated immune responses.[6] Mast cells and basophils, which have been considered mainly to be effector cells for IgE-mediated reactions, can modulate the immune response through secreted histamine. Jutel *et al.* demonstrated, using H1R and histamine H2 receptor (H2R) knock-out mice, that histamine augmented TH1 response T lymphocytes through H1R and suppressed both TH1 and TH2 responses through H2R.[7] In addition, histamine H4 receptor (H4R) has recently been identified and an H4R inhibitor has been demonstrated to have a suppressive effect on allergic airway inflammation, including eosinophil and mast cell infiltration in murine asthma models.[8-11] In this context, histamine may have various roles in allergic airway inflammation through H1R, H2R and H4R in immune cells including T lymphocytes and dendritic cells.

To date, goblet cell hyperplasia has been thought to be induced after epithelial damage by numerous stimuli, such as endogenous oxidants induced by neutrophil elastase,[12] or exogenous oxidants produced by cigarette smoke[13] or diesel engine emissions.[14] On the other hand, it has been demonstrated that the TH2 lymphocyte-derived cytokines interleukin-4 (IL-4), IL-5, IL-9, and IL-13 induce goblet cell hyperplasia in animal models and *in vitro* studies.[15-19]

According to previous reports, several genes are up-regulated in association with goblet cell hyperplasia. Among them, MUC genes encode various mucin glycoproteins which are primary secretory proteins consisting of a family of genes (ie, MUC-1, MUC-2 and MUC4).[20,21] MUC5AC is the predominant MUC gene expressed in the airway.[21] It has been demonstrated that the level of MUC5AC gene expression increases in both animal models of asthma and tissue samples from patients with asthma.[22-24]

In addition, the overexpression of the Gob-5 gene has been reported in airway epithelia with exacerbated AHR, goblet cell hyperplasia, mucus overproduction, and eosinophil infiltration in a murine asthma model.[25] Gob-5 gene in the mouse corresponds to CLCA1 gene in humans, which regulates calcium activated chloride conductance. It has been shown to induce mucin gene expression (MUC5AC) in the human mucoepidermoid cell line NCI-H292 and its expression was demonstrated to increase in patients with asthma.[25-27]

Histamine-deficient mice are now available by disrupting *L*-histidine decarboxylase gene (HDC knockout mice: *Hdc-/-*).[28] Histamine exerts its various biological actions via H1, H2, H3 and H4. Due to the overlapping, and sometimes antagonistic function of the receptors in the presence of endogenous histamine, receptor blocking alone cannot achieve complete elimination of the histamine system. It is, therefore, reasonable to evaluate the total role of histamine with *Hdc-/-* mice.

An analysis of cytokine production and cell differentials in BALF in sensitized *Hdc-/-* mice with allergic airway inflammation has been previously reported, however, these investigated only the acute airway responses in murine asthma models with a short exposure to allergens.[29,30] There have been few data about airway remodeling that included goblet cell hyperplasia in histamine-deficient mice.

In this study, we designed an experimental asthma model with a relatively long duration of allergen exposure to *Hdc-/-* mice to elucidate the roles of histamine in airway remodeling, focusing especially on goblet cell hyperplasia.

## METHODS

### ANIMALS

HDC knockout (*Hdc-/-*) mice were generated by Ohtsu *et al.*[28]. In the present study, we used 6 to 8-week-old female *Hdc-/-* mice backcrossed for six generations with C57BL/6 mice, and used age/gender-matched C57BL/6N mice (Japan SLC, Shizuoka, Japan) as controls. The mice used for this study were generated by breeding homozygous *Hdc-/-* mice in the facilities of Tohoku University (Sendai, Japan). Both HDC-/- and wild-type mice were kept on a normal diet containing <0.3 mg of histamine/g of food. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animals Science in 1987.

### IMMUNIZATION AND AEROSOLIZATION PROTOCOL

The mice were sensitized according to the methods described in a previous study.[31] In brief, mice were sensitized on days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 ml aluminum hydroxide-precipitated antigen containing 8 μg OVA (Sigma Chemical Co., St. Louis, MO) adsorbed overnight at 4℃ to 4 mg of aluminium hydroxide (Wako Chemical Co., Tokyo, Japan) in phosphate-buffered saline (PBS). Twelve days after the second immunization, the wild-type mice and *Hdc-/-* mice were divided into 3 groups respectively, each consisting of 6 animals. Each of the wild-type mice groups and one of the *Hdc-/-* mice groups were killed for analysis as controls without inhalation. To perform chronic exposure of OVA, 2 groups of each of the wild mice and *Hdc-/-* mice were placed in a plastic chamber (10 cm × 15 cm × 25 cm) and exposed to aerosolized OVA (5 mg/ml in 0.9% saline) for 1 hour every other day for 14 days. The other 2 groups of the wild-type mice and *Hdc-/-* mice were exposed only to 0.9% saline every other day for 14 days. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer

(Devilbiss) (Sunrise Medical HHG, Inc., Somerset, PA, USA) at a flow rate of 5–7 liter/minute.

## COLLECTION AND MEASUREMENT OF SPECIMENS

After being exposed to aerosolized saline or OVA every other day for 2 weeks, each group of mice was killed on the 14th day, 24 hours after final inhalation, and bronchoalveolar lavage fluid (BALF) and lung tissues were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD, USA). The lungs were lavaged twice with 0.5 ml PBS, and up to 0.8 ml of the instilled fluid was consistently recovered. The recovered fluid was centrifuged (300 × *g* for 6 minutes) and the cells were resuspended in 0.5 ml PBS. The total number of cells was counted using an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made from each sample containing 10,000 cells by cytospin (Cytocentrifuge, Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard error of the mean (SEM) for each treated group.

After centrifugation, supernatants were stored at -80℃ for the measurement of cytokines. After harvesting BALF, lungs were fixed with formaldehyde and were embedded in paraffin. These 3-μm-thick sections were stained with hematoxylin eosin (HE) and periodic acid-Schiff (PAS). In addition, other parts of the left lungs were stored at -80℃ for RNA extraction.

## MEASUREMENT OF SERUM CONCENTRATION OF IgE

Serum IgE levels were determined using the commercially available ELISA kit (Yamasa, Chiba, Japan).

## HISTOLOGICAL MEASUREMENT OF GOBLET CELL NUMBER

The 3 mm paraffin sections of the lungs described above were stained with PAS for evaluation of goblet cells. Goblet cell hyperplasia was determined by counting the number of PAS positive cells in more than 5 large (diameter >200 μm) and more than 10 small bronchi (diameter <150 μm) per lung under microscopy. The results were expressed as percentages of PAS positive cells per total epithelial cells. In addition, lung sections were stained with HE.

## ISOLATION OF TOTAL RNA AND REAL-TIME QUANTITATIVE PCR

Total RNA from the whole lobes of the left lungs was obtained using ISOGEN (Wako Pure Chemicals, Osaka, Japan), quantified by spectrophotometry. The quality of the obtained RNA was confirmed by agarose gel electrophoresis.

To quantify the mRNA of Gob-5, MUC5AC, and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) expression in the murine lungs, quantitative PCR was carried out using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) as previously described.[32]

Oligonucleotide PCR primer pairs and fluorogenic probes for murine MUC5AC and Gob-5 were designed from the published sequences using Primer Express software (Perkin-Elmer) (sense primer: 5'-ACTGTTACTATGCGATGTGTAGCCA-3', antisense primer; 5'-GAGGAAACACATTGCACCGA-3'; Taqman probe: 5'-[FAM] ACTGCCACCTGTCACTGGGCGG [TAMRA]-3') for MUC5AC and (sense primer: 5'-AGGGCATCGTCATCGCC-3'; antisense primer: 5'-TCCTTTATGTGTTGAATGAGGGC-3'; Taqman probe: 5'-[FAM] AGACCACGACGTGCCGGAAGATG [TAMRA]-3') for Gob-5.[33] Primers and the labeled probe (VIC) for rodent GAPDH were purchased from Perkin-Elmer Applied Biosystems. 100 ng of RNA dissolved in 10 μl of water from each aliquot of murine lung tissue was denatured at 90℃ for 90 seconds. Each RNA sample (100 ng/10 μl of water) was mixed in 40 μl of buffer containing the following reagents for the one-step RT-PCR reaction: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 5 mM $MgCl_2$, 100 nM sense primer, 100 nM antisense primer, 0.3 mM deoxynucleoside triphosphate (Boehringer), 0.4 U/μl RNase inhibitor (Promega), 0.4 U/μl Moloney murine leukemia virus RT (Perkin Elmer), 0.0025 U/μl *Taq* Gold Polymerase (Perkin Elmer), and 100 nM Taqman probe, as described above. The fragment of mRNA for Gob-5, MUC5AC and GAPDH was reversely transcribed into cDNA (30 minutes at 48℃) and amplified by PCR for 40 cycles (15 seconds at 95℃ and 1 minute at 60℃). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (C), which is predictive of the quantity of an input target fragment.[34] The standard curve was obtained between the fluorescence emission signals and C by means of duplicated serial dilutions of each quantified cDNA fragment which contained the targeted sequences. The expression of MUC5AC and Gob-5 mRNA was normalized to the constitutive expression of GAPDH mRNA.

## CYTOKINE ASSAY

Concentrations of IL-4, IL-5, Interferon-g (IFN-g), tumor necrosis factor-a (TNF-α) and IL-2 in BALF were determined with cytokine Bead Array inflammatory kits using flow cytometry according to the manufacturer's instructions (BD PharMingen, San Diego, CA,

**Table 1** Total cell number and cell differential in BALF of the wild-type and *Hdc*-/- mice after saline and OVA inhalation

| | C57BL/6: wild saline, 14th day | C57BL/6: wild OVA, 14th day | C57BL/6: *Hdc*-/- saline, 14th day | C57BL/6: *Hdc*-/- OVA, 14th day |
|---|---|---|---|---|
| Total cells (x$10^4$/ml) | 2.29 ± 0.64 ($n$ = 6) | 28.2 ± 5.1 ($n$ = 6) | 3.60 ± 0.21 ($n$ = 6) | 62.1 ± 7.0* ($n$ = 6) |
| Alveolar macrophages | 2.16 ± 0.06 (94.2 ± 2.5%) | 2.09 ± 0.56 (7.40 ± 2.0%) | 3.43 ± 0.11 (95.2 ± 3.0%) | 23.5 ± 3.73* (37.9 ± 6.0%) |
| Lymphocytes | 0.13 ± 0.06 (5.75 ± 2.5%) | 0.45 ± 0.25 (1.6 ± 0.9%) | 0.15 ± 0.09 (4.20 ± 2.6%) | 10.8 ± 4.04* (17.4 ± 6.5%) |
| Eosinophils | < 0.001 (< 0.1%) | 25.7 ± 0.50 (91.0 ± 1.8%) | 0.22 ± 0.01 (0.6 ± 0.4%) | 27.8 ± 6.40 (44.8 ± 10.3%) |
| Neutrophils | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) |
| Epithelial cells | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) | < 0.01 (< 0.1%) |

The absolute number of cells in BALF on 14th day after saline and OVA inhalation in mice sensitized with OVA was calculated. The numbers in parentheses indicated % in the total cells in BALF. BALF was collected as described in "Methods". Values were expressed as the means ± SEM of 6 mice. * $p < 0.01$ relative to the respective value of wild-type mice with OVA inhalation.

USA). The concentration of IL-13 in BALF was measured using ELISA (RayBio, Norcross, GA, USA).

### STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. Multiple comparisons of mean data among the groups were analyzed by the Mann-Whitney U test. Probability values of less than 0.05 were considered to indicate a statistically significant difference.

## *RESULTS*

### CELL DIFFERENTIALS IN BALF OF *Hdc*-/- MICE

Repetitive exposure to OVA for 2 weeks induced a marked increase of the total cell numbers in BALF in both wild-type and *Hdc*-/- mice sensitized with OVA, compared with those from the wild-type mice group and mice sensitized with OVA and exposed to saline (Table 1). The total cell numbers in BALF from the *Hdc*-/- mice sensitized with OVA and then exposed to OVA increased significantly more than in wild-type mice under the same conditions. The absolute numbers of alveolar macrophages, lymphocytes and eosinophils in *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared to mice sensitized with OVA and exposed to saline. In particular, the absolute numbers of alveolar macrophages and lymphocytes in *Hdc*-/- mice sensitized with and exposed to OVA were significantly higher than those of wild-type mice under the same condition. Eosinophils were the predominant cells in BALF from the wild-type mice exposed to OVA and the ratio of eosinophils in BALF was higher compared with that of *Hdc*-/- mice. However, there was no significant difference in the absolute cell numbers of eosinophils in these 2 groups.

### CYTOKINES IN BALF OF *Hdc*-/- MICE

TNF-α, IL-4, IL-5, IL-13, INF-γ and IL-2 in BALF were measured. The concentrations of TNF-a in BALF of both wild (15.08 ± 0.58 pg/ml: mean ± SEM) and *Hdc*-/- mice (25.30 ± 4.23 pg/ml) exposed to OVA increased markedly compared to those of wild-type (2.28 ± 0.40 pg/ml) and *Hdc*-/- mice (2.27 ± 0.40 pg/ml) exposed to saline (Fig. 1A). The concentration of TNF-α in the *Hdc*-/- mice exposed to OVA was significantly higher than that of the wild-type mice under the same conditions.

The concentrations of IL-4 in BALF from both wild-type and *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared to wild and *Hdc*-/- mice sensitized with OVA and exposed to saline. The concentration of IL-4 in the *Hdc*-/- mice exposed to OVA was significantly lower than that of wild mice under the same conditions (Fig. 1B).

The concentrations of IL-2, IL-5, IL-13 and INF-γ in BALF from both wild-type and *Hdc*-/- mice sensitized with and exposed to OVA increased significantly compared with that of wild-type and *Hdc*-/- mice sensitized with OVA and exposed to saline. No statistically significant difference in these cytokines between the wild-type and *Hdc*-/- mice exposed to OVA (Fig. 1C, D, E, F).

### SERUM IgE OF *Hdc*-/- MICE

The concentrations of serum IgE in both wild-type (2133.6 ± 370.3 ng/ml) and *Hdc*-/- mice (998.9 ± 254.0 ng/ml) exposed to OVA were markedly higher than those exposed to saline (wild-type exposed to saline: 416.1 ± 25.1 ng/ml, *Hdc*-/- mice exposed to saline: 371.7 ± 17.2 ng/ml) and without exposure (wild-type: 390.1 ± 32.5; *Hdc*-/-: 328.1 ± 14.8) (Fig. 2). The concentration of serum IgE in the *Hdc*-/- mice exposed to OVA was significantly lower than that of the wild-type mice under the same conditions.

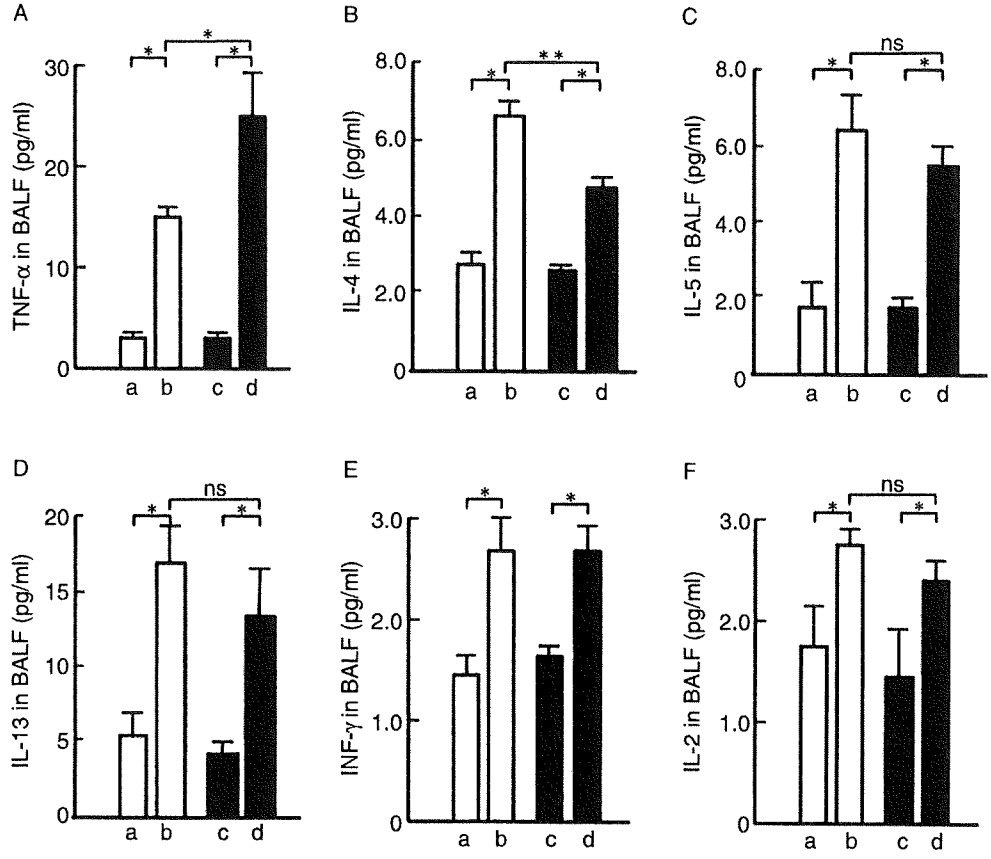


**Fig. 1** **A, B, C, D, E, F**: Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **a** and **c**: saline inhalation; Column **b** and **d**: OVA inhalation, **A**: TNF-α, **B**: IL-4, **C**: IL-5, **D**: IL-13, **E**: INF-γ, **F**: IL-2 concentration in BALF. $*p < 0.01$, $**p < 0.05$.

## Gob-5 GENE EXPRESSION IN THE LUNG OF *Hdc-/-* MICE

Levels of Gob-5 mRNA in lung tissue of both wild-type and *Hdc-/-* mice sensitized with and exposed to OVA increased significantly compared with those of wild-type and *Hdc-/-* mice sensitized with OVA and exposed to saline. The level of Gob-5 mRNA in the *Hdc-/-* mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 3).

## MUC5AC GENE EXPRESSION IN THE LUNG OF *Hdc-/-* MICE

Levels of MUC5AC mRNA in lung tissue of both wild-type and *Hdc-/-* mice sensitized with OVA and exposed to OVA increased markedly compared with those of wild and *Hdc-/-* mice sensitized with OVA exposed to saline. The level of MUC5AC mRNA in the *Hdc-/-* mice exposed to OVA was significantly higher than that of wild-type mice under the same conditions (Fig. 4).

## GOBLET CELLS OF LARGE AND SMALL AIRWAYS

PAS staining demonstrated an increase in the number of goblet cells in the epithelium in large and small airways of both wild-type and *Hdc-/-* mice sensitized with OVA (Fig. 5A a–f, B a–f). Goblet cell hyperplasia was more prominent in the *Hdc-/-* mice exposed to OVA than in the wild-type mice under the same conditions (Fig. 5A c, A f, B c, B f). Repetitive saline inhalation induced a mild increase in the number of goblet cells in large and small airways of both wild-type and *Hdc-/-* mice (Fig. 5A b, A e, B b, B e).

## QUANTIFICATION OF GOBLET CELLS IN LARGE AND SMALL AIRWAYS

The ratios of goblet cells in the epithelium in large and small airways, of both wild-type and *Hdc-/-* mice sensitized with and exposed to OVA, increased markedly compared with those of the wild-type and *Hdc-/-* mice sensitized with OVA and exposed to saline (Fig. 6A, B). The ratios of goblet cells in the epithelium in large and small airways of the *Hdc-/-* mice exposed to OVA were significantly higher than that of wild-type mice under the same condition.

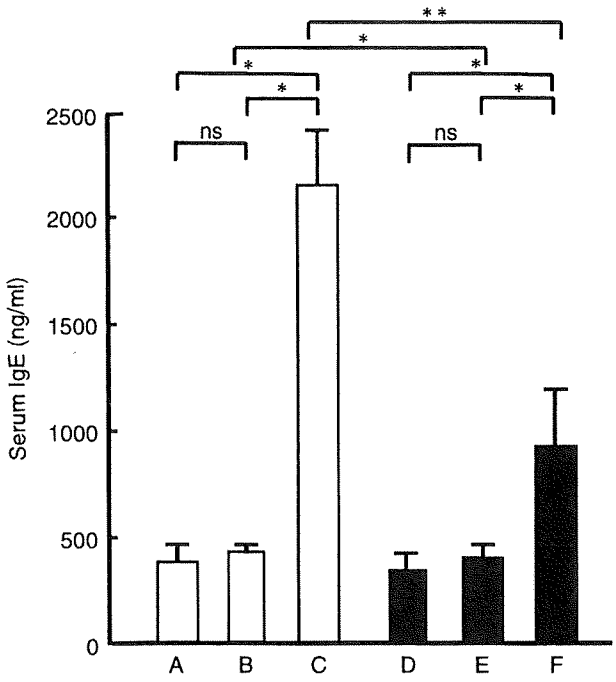


**Fig. 2** IgE concentration in serum. Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **A** and **D**: before inhalation; Column **B** and **E**: saline inhalation; Column **C** and **F**: OVA inhalation. $*p < 0.01$, $**p < 0.05$, ns: not significant.

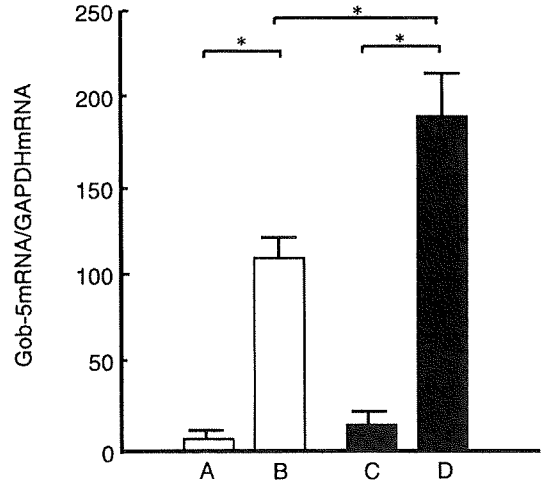


**Fig. 3** Gob-5 gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **A** and **C**: saline inhalation; Column **B** and **D**: OVA inhalation. $*p < 0.01$.

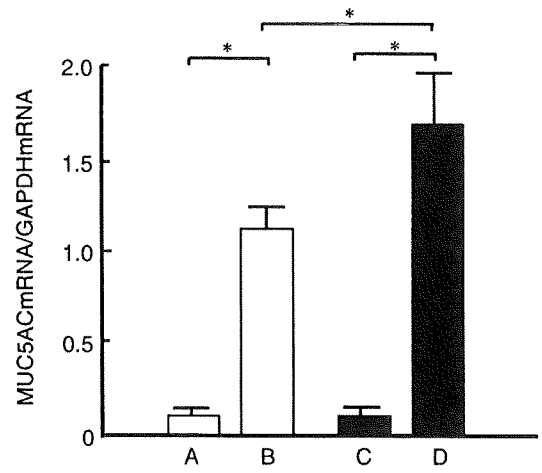


**Fig. 4** MUC5AC gene expression in the lung. Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **A** and **C**: saline inhalation; Column **B** and **D**: OVA inhalation. $*p < 0.01$.

## *DISCUSSION*

In this study, we demonstrated that goblet cell hyperplasia was enhanced in *Hdc-/-* mice sensitized with OVA after repetitive OVA exposure for 14 days. We also demonstrated a striking difference in differential cytological features in BALF between 2 murine asthma models with *Hdc-/-* mice, representing the acute allergic airway response with a short period of OVA exposure previously reported[29,30] and a relatively chronic allergic airway response shown in the present study. We found significant increases of alveolar macrophages and lymphocytes in BALF of *Hdc-/-* mice with repetitive exposure to OVA for 2 weeks compared with the wild-type mice under the same conditions. The concentration of TNF-a in BALF in the *Hdc-/-* mice after repetitive OVA exposure was significantly higher than that of the wild-type mice under the same conditions.

Histamine has been recognized as a chemical mediator playing a central role in allergic reactions including mucosal edema, mucous gland secretion, smooth muscle contraction, etc. Recent studies have demonstrated that histamine plays a critical role in immunomodulation by acting on different types of histamine receptors such as H1R, H2R and H4R on the surface of immune cells and inflammatory cells.[6-11] Using mutant mice lacking H1R and H2R, Jutel *et al.* demonstrated that histamine enhances TH1-type responses by triggering H1R, whereas both TH1- and TH2-type responses were negatively regulated by H2R. Indeed, the deletion of H1R resulted in suppression of IFN-γ, IL-4 and IL-13, and the deletion of H2R showed an up-regulation of both TH1 and TH2 cytokines (IL-4 and IL-13).[7] Bryce *et al.* recently demonstrated the interesting opposite effects of H1R inhibition on cultured T cells *in vitro* and the airway response with allergic inflammation *in vivo* using a murine asthma model.[5] In their report, allergen-stimulated splenic T cells from sensitized H1R knock-

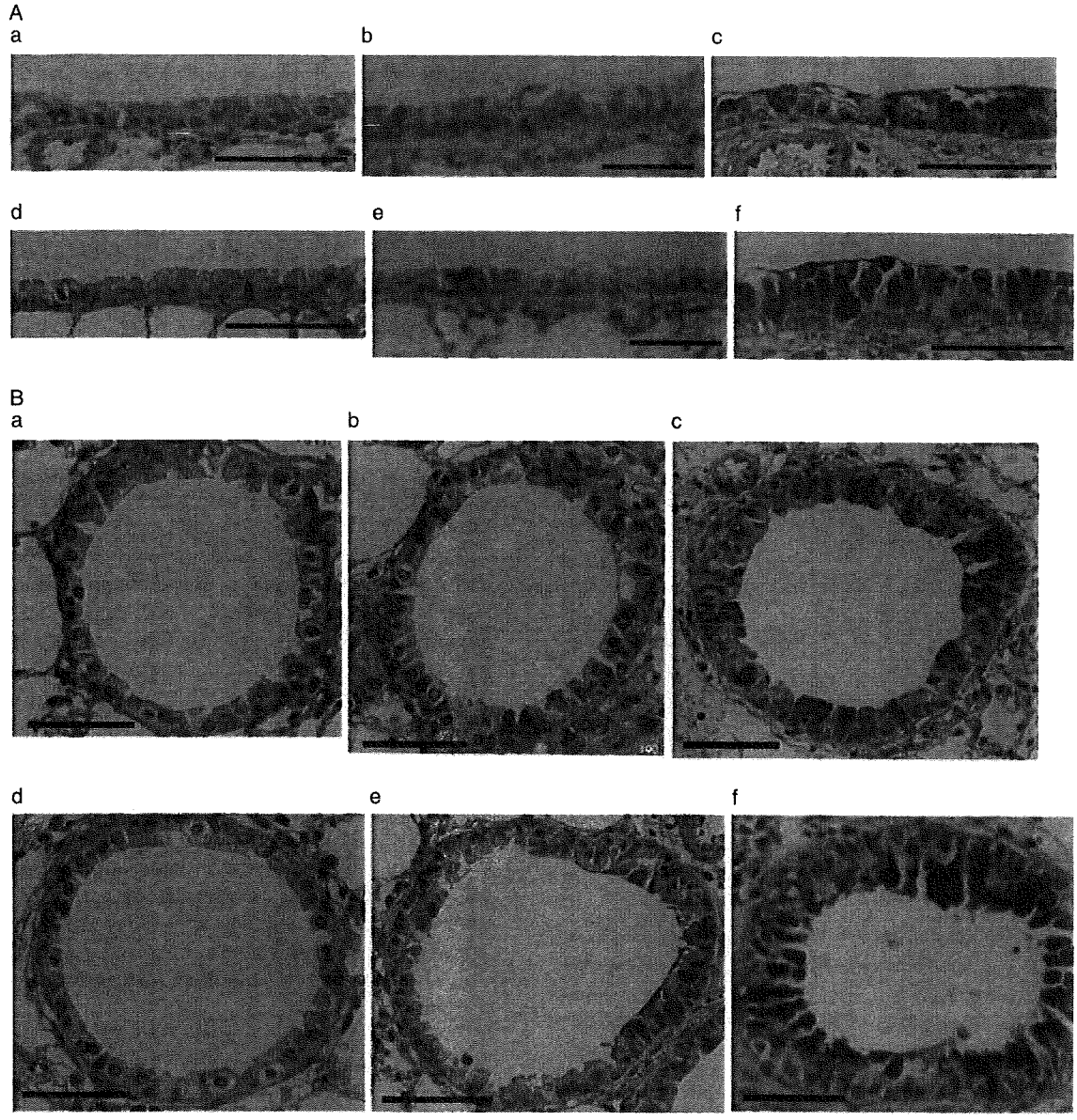


**Fig. 5** **A**: Goblet cells in the large airways, **B**: Goblet cells in the small airways. The goblet cells in the epithelium were stained with PAS. **a**, **b** and **c**: wild-type mice; **d**, **e** and **f**: *Hdc-/-* mice; **a** and **d**: before inhalation; **b** and **e**: saline inhalation; **c** and **f**: OVA inhalation. black bars indicate 50μm.

out mice exhibited enhanced TH2 cytokine production. However, in contrast, allergen-challenged H1R knockout mice exhibited diminished lung TH2 cytokine mRNA levels, airway inflammation, goblet cell metaplasia, and airway hyperresponsiveness (AHR).

The present study demonstrated that the concentration of IL-4 in BALF was significantly lower in the *Hdc-/-* mice than in the wild-type mice after exposure to OVA, which was consistent with the experiment of the *Hdc-/-* mice by Kozma *et al.*,[30] despite differences in the duration of OVA exposure. The concentrations of IL-2, IL-5, IL-13 and INF-γ showed no significant differences between the *Hdc-/-* mice and the wild-type mice. The lower level of IL-4 in the *Hdc-/-* mice was consistent with the lower level of serum IgE in the *Hdc-/-* mice after the exposure to OVA. These changes may be explained at least in part by the suppressed recruitment of activated TH2 cells into the lung due to the blockade of H1R in T lymphocytes, as suggested by Bryce *et al.*[5]

H1R, H2R and H4R are expressed in monocytes, macrophages and dendritic cells, and histamine plays a regulatory role in expression of cytokines and adhesion molecules in these cells.[35] Mazzoni *et al.* demonstrated that histamine inhibited INF-a and released TNF-a from activated plasmacytoid dendritic cells

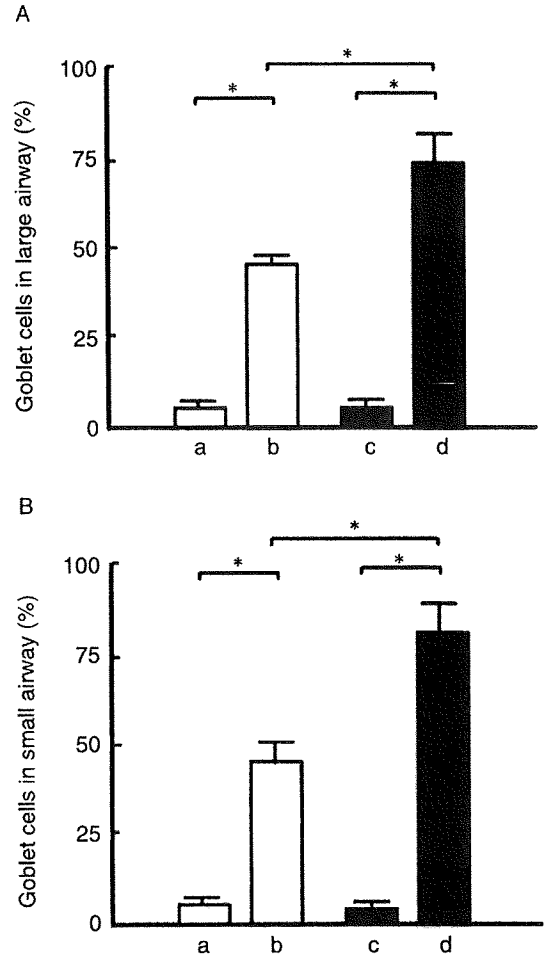


**Fig. 6** **A**: Goblet cell ratio in the large airways, **B**: Goblet cell ratio in the small airways. Outlined column: wild-type mice; Solid column: *Hdc-/-* mice; Column **a** and **c**: saline inhalation, Column **b** and **d**: OVA inhalation. $*p < 0.01$.

through H2R.[36] Histamine also inhibited lipopolysaccharide-induced TNF-α production by down-regulating intercellular adhesion molecules (ICAM)-1 in human monocytes through H2R.[37] Alveolar macrophages are known to be a major source of TNF-α in the lung, and the increased production of TNF-α by alveolar macrophages in patients with asthma has been reported.[38,39] The present study demonstrated an increase in the TNF-α concentration in BALF in the *Hdc-/-* mice exposed to OVA compared to the wild-type mice under the same condition. This result suggested that histamine may play a role in suppressing TNF-α production through H2R in allergic airway inflammation in mice.

Busse *et al.* have recently demonstrated that chronic exposure of TNF-α to the airway induced goblet cell hyperplasia.[40] Taking these findings into consideration, we speculate that the depletion of histamine in the *Hdc-/-* mice induced the up-regulation of TNF-α production, resulting in the increase of goblet cells in the airways with allergic inflammation.

In association with the increase of goblet cells, higher levels of Gob-5 and MUC5ac mRNA were found in the *Hdc-/-* mice exposed to OVA. Previous reports have suggested that the expression of the Gob-5 gene, which corresponds to hCLCA1 in human beings, would be one of the first steps in mucus cell metaplasia and hyperplasia by inducing mucin gene expression.[25] MUC5ac gene, a member of the mucin gene family, has been shown to be expressed exclusively in the airway and associated with the hyperplasia of goblet cells. In addition to IL-4, IL-5 and IL-13, TNF-α was reported to increase the expression of the Gob-5 and MUC5ac genes.[41] These findings suggested that the increased production of TNF-α in the BALF of the *Hdc-/-* mice exposed to OVA may be, at least in part, involved in the increase of mRNA levels of the Gob-5 and MUC5ac genes, leading to the increased number of goblet cells in the airway.

Antigen challenge of sensitized mice or human asthmatic subjects results in increased TNF-α expression in BALF, peripheral blood, and tissue biopsy specimens.[42-46] TNF-α production is partly regulated by histamine via H2R in macrophages and dendritic cells, as described above. Although we had no direct evidence to evaluate the role of TNF-α in goblet cell hyperplasia, the results of our study suggested that the increased level of TNF-α might contribute to enhance goblet cell hyperplasia and that histamine may be involved in the negative regulation of TNF-α production in allergic airway inflammation *via* the histamine receptors of inflammatory cells, including macrophages. To confirm the above, further studies are needed, such as experiments using mice in which the TNF-α activity is blocked with antibody or gene disruption.

There was a discrepancy between the TNF-α levels and the macrophage counts. The concentration of TNF-α in the knock-out mice was twice that in the wild-type mice, and the number of alveolar macrophages in *Hdc-/-* mice was ten times that in the wild-type mice. We have no data to explain this, however, to answer this question, it would be necessary to evaluate the capacity of TNF-α production by alveolar macrophages in *Hdc-/-* mice and wild mice. It would also be necessary to evaluate the time course of the concentration of TNF-α and the number of alveolar macrophages to understand their relationship.

We demonstrated the increased number of lymphocytes in BALF of *Hdc-/-* mice. It has been reported by Jutel *et al.* that histamine stimulated the Th1 response through the H1 receptor. On the other hand, the activities of both Th1 and Th2 lymphocytes were inhibited by histamine through the H2 receptor.[7] Elimination of the inhibitory actions on lymphocytes by histamine through the H2 receptor in *Hdc-/-* mice may contribute to the increased number of lymphocytes in BALF.

The number of lymphocytes in the BALF of *Hdc-/-* mice was twenty times that of wild-type mice, although no significant difference in the concentration of IL-2 in the BALF was found between knock-out and wild-type mice. The number of cells and cytokine levels in BALF are not always concordant in the time courses of *in vivo* experiments. The level of IL-2 in BALF peaked within 1 week during exposure to OVA (unpublished data). However, in this study we examined the cytokine levels and the cell differentials in BALF only on the 14th day. To explain the discrepancy between the IL-2 level and the number of lymphocytes in BALF in the study, it would have been helpful to evaluate the time course of IL-2 concentration and the number of lymphocytes in BALF.

Although we designed a 2- week exposure program to evaluate the extent of goblet cell hyperplasia in the *Hdc-/-* mice, we did not examine airway hyperresponsiveness (AHR). However, we found no difference in AHR between the *Hdc-/-* and the wild mice in our preliminary study as was reported in the previous acute model (unpublished data).[29]

In the present study, we did not measure the anti-OVA specific IgE in serum. Although the levels of serum OVA-specific IgE and total IgE are not always consistent, the procedures for sensitization with OVA and exposure to OVA were directly associated with the increase of IgE in serum. Despite the fact that the levels of serum OVA-specific IgE were different from the level of total IgE in serum, the level of total IgE in serum reflected strongly the levels of serum OVA-specific IgE in our study.

Anti-histamine drugs available for therapy consist of H1 and H2 blockers. H1 blockers have not been recommended for therapy for asthma, but their anti-allergic actions have been recognized. According to the results of this study, the H2 blocker may have the possibility of stimulating goblet cell hyperplasia in patients with allergic asthma. It will be necessary to evaluate the effects of H2 blockers in a clinical study in the future.

In conclusion, we demonstrated enhanced goblet cell hyperplasia in the airway of OVA sensitized histamine-deficient mice. The results of our study suggested that histamine may play a significant role in goblet cell hyperplasia in the airway with allergic inflammation.

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## REFERENCES

1. Laitinen A, Laitinen LA. Pathology of asthma. *Allergy Proc* 1994;**15**:323-8.
2. Shimura S, Andoh Y, Haraguchi M, Shirato K. Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. *Eur Respir J* 1996; **9**:1395-401.
3. Carroll N, Carello S, Cooke C, James A. Airway structure and inflammatory cells in fatal attacks of asthma. *Eur Respir J* 1996;**9**:709-15.
4. Aikawa T, Shimura S, Sasaki H *et al.* Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest* 1992;**101**:916-21.
5. Bryce PJ, Mathias CB, Harrison KL *et al.* The H1 histamine receptor regulates allergic lung responses. *J Clin Invest* 2006;**116**:1624-32.
6. Siegel JN, Schwartz A, Askenase PW, Gershon RK. T-cell suppression and contrasuppression induced by histamine H2 and H1 receptor agonists, respectively. *Proc Natl Acad Sci U S A* 1982;**79**:5052-6.
7. Jutel M, Watanabe T, Klunker S *et al.* Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature* 2001;**413**:420-5.
8. Oda T, Matsumoto S, Masuho Y *et al.* cDNA cloning and characterization of porcine histamine H4 receptor. *Biochim Biophys Acta* 2002;**1575**:135-8.
9. Nakayama T, Kato Y, Hieshima K *et al.* Liver-expressed chemokine/CC chemokine ligand 16 attracts eosinophils by interacting with histamine H4 receptor. *J Immunol* 2004;**173**:2078-83.
10. Ling P, Ngo K, Nguyen S *et al.* Histamine H4 receptor mediates eosinophil chemotaxis with cell shape change and adhesion molecule upregulation. *Br J Pharmacol* 2004;**142**:161-71.
11. Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J Pharmacol Exp Ther* 2003; **305**:1212-21.
12. Voynow JA, Fischer BM, Malarkey DE *et al.* Neutrophil elastase induces mucus cell metaplasia in mouse lung. *Am J Physiol Lung Cell Mol Physiol* 2004;**287**:L1293-302.
13. Harkema JR, Hotchkiss JA. Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways. *Toxicol Lett* 1993;**68**:251-63.
14. Ichinose T, Takano H, Miyabara Y *et al.* Enhancement of antigen-induced eosinophilic inflammation in the airways of mast-cell deficient mice by diesel exhaust particles. *Toxicology* 2002;**180**:293-301.
15. Dabbagh K, Takeyama K, Lee HM *et al.* IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *J Immunol* 1999;**162**:6233-7.
16. Longphre M, Li D, Gallup M *et al.* Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest* 1999;**104**:1375-82.
17. Temann UA, Prasad B, Gallup MW *et al.* A novel role for murine IL-4 *in vivo*: induction of MUC5AC gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol* 1997;**16**:471-8.
18. Zhu Z, Homer RJ, Wang Z *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999;**103**:779-88.
19. Trifilieff A, Fujitani Y, Coyle AJ *et al.* IL-5 deficiency abol-

ishes aspects of airway remodelling in a murine model of lung inflammation. *Clin Exp Allergy* 2001;**31**:934-42.
20. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 2006;**86**:245-78.
21. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. *Chest* 2002;**122**:320S-6.
22. Groneberg DA, Eynott PR, Lim S *et al.* Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology* 2002;**40**:367-73.
23. Ordonez CL, Khashayar R, Wong HH *et al.* Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* 2001;**163**:517-23.
24. Zuhdi Alimam M, Piazza FM, Selby DM *et al.* Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. *Am J Respir Cell Mol Biol* 2000;**22**:253-60.
25. Nakanishi A, Morita S, Iwashita H *et al.* Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci U S A* 2001;**98**:5175-80.
26. O'Neill L. Gob genes, mucus and asthma. *Trends Immunol* 2001;**22**:353.
27. Zhou Y, Shapiro M, Dong Q *et al.* A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction. *Novartis Found Symp* 2002; **248**: 150-65; discussion 165-70, 277-82.
28. Ohtsu H, Tanaka S, Terui T *et al.* Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS Lett* 2001;**502**:53-6.
29. Koarai A, Ichinose M, Ishigaki-Suzuki S *et al.* Disruption of L-histidine decarboxylase reduces airway eosinophilia but not hyperresponsiveness. *Am J Respir Crit Care Med* 2003;**167**:758-63.
30. Kozma GT, Losonczy G, Keszei M *et al.* Histamine deficiency in gene-targeted mice strongly reduces antigen-induced airway hyper-responsiveness, eosinophilia and allergen-specific IgE. *Int Immunol* 2003;**15**:963-73.
31. Ohkawara Y, Lei XF, Stampfli MR *et al.* Cytokine and eosinophil responses in the lung, peripheral blood and bone marrow compartments in a murine model of allergen-induced airways inflammation. *Am J Respir Cell Mol Biol* 1997;**16**:510-20.
32. Martell M, Gomez J, Esteban JI *et al.* High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* 1999;**37**:327-32.
33. Ryseck RP, Macdonald-Bravo H, Mattei MG, Bravo R. Structure, mapping, and expression of fisp-12, a growth factor-inducible gene encoding a secreted cysteine-rich protein. *J Cell Growth Differ* 1991;**2**:225-33.
34. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;**6**:986-94.
35. Gutzmer R, Diestel C, Mommert S *et al.* Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *J Immunol* 2005;**174**:5224-32.
36. Mazzoni A, Leifer CA, Mullen GE *et al.* Cutting edge: histamine inhibits IFN-alpha release from plasmacytoid dendritic cells. *J Immunol* 2003;**170**:2269-73.
37. Morichika T, Takahashi HK, Iwagaki H *et al.* Histamine inhibits lipopolysaccharide-induced tumor necrosis factor-α production in an intercellular adhesion molecule-1- and B7.1-dependent manner. *J Pharmacol Exp Ther* 2003;**304**: 624-33.
38. Azevedo I, de Blic J, Dumarey CH *et al.* Increased spontaneous release of tumour necrosis factor-α by alveolar macrophages from wheezy infants. *Eur Respir J* 1997;**10**: 1767-73.
39. Broide DH, Lotz M, Cuomo AJ *et al.* Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992;**89**: 958-67.
40. Busse PJ, Zhang TF, Srivastava K *et al.* Chronic exposure to TNF-alpha increases airway mucus gene expression *in vivo*. *J Allergy Clin Immunol* 2005;**116**:1256-63.
41. Hauber HP, Daigneault P, Frenkiel S *et al.* Niflumic acid and MSI-2216 reduce TNF-α-induced mucin expression in human airway mucosa. *J Allergy Clin Immunol* 2005;**115**: 266-71.
42. Gosset P, Tsicopoulos A, Wallaert B *et al.* Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J Allergy Clin Immunol* 1991;**88**:561-71.
43. Gosset P, Tsicopoulos A, Wallaert B *et al.* Tumor necrosis factor-α and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation. *Am Rev Respir Dis* 1992;**146**:768-74.
44. Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA. Elevated release of tumor necrosis factor-α and interferon-γ by bronchoalveolar leukocytes from patients with bronchial asthma. *Am Rev Respir Dis* 1993;**147**:291-5.
45. Lukacs NW, Strieter RM, Chensue SW *et al.* TNF-α mediates recruitment of neutrophils and eosinophils during airway inflammation. *J Immunol* 1995;**154**:5411-7.
46. Krishnaswamy G, Liu MC, Su SN *et al.* Analysis of cytokine transcripts in the bronchoalveolar lavage cells of patients with asthma. *Am J Respir Cell Mol Biol* 1993;**9**: 279-86.

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# Roxithromycin inhibits chemokine-induced chemotaxis of Th1 and Th2 cells but regulatory T cells

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ABSTRACT

*Background:* Roxithromycin (RXM), a 14-member macrolide antibiotic, has a variety of bioregulatory functions such as anti-inflammatory effects, anti-oxidant effects, and modulation of immune responses.
*Objectives:* In this study, we analyzed the effect of RXM on chemokine-induced chemotaxis of Th1, Th2, and regulatory T (Treg) cells established from three normal human peripheral blood lymphocytes by the reported methods.
*Methods and results:* Incubation with 10 μM RXM for 18 h did not alter the expression profile of CXCR3 on Th1 cells and CCR4 on Th2 and Treg cells. However, upon RXM preincubation, the migration of Th1 cells to IP-10 and Th2 cells to TARC was partially suppressed, although RXM did not influence Treg cell migration. Erythromycin and clarithromycin at the same concentration did not exert such effects. F-actin polymerization and $Ca^{++}$ influx induced by IP-10 and TARC in Th1 and Th2 cells, respectively, was down-regulated by RXM pretreatment.
*Conclusion:* These results imply that RXM exhibits bioregulatory function by influencing chemotaxis of Th1 and Th2 cells while leaving Treg cell migration unaffected.


## 1. Introduction

Macrolides were originally exploited as antibiotics against *Staphylococcus*, *Streptococcus pneumoniae*, *Moraxella*, and *Propionibacterium acnes* [1]. Some macrolides have a variety of bioregulatory functions such as anti-inflammatory and anti-oxidant effects, and modulation of immune responses including immunosuppression [2,3]. The 14-member macrolide roxithromycin (RXM) inhibits T cell proliferation responses, cytokine production by T cells and macrophages [4,5,6], and accessory functions of professional and non-professional antigen presenting cells [7,8]. In fact, RXM exerts beneficial effects on various skin diseases such as psoriasis [9,10], atopic dermatitis [7], prurigo, and eosinophilic pustule folliculitis [10] partly through these immunomodulatory activities.

The interaction between chemokines and chemokine receptors is crucial in cell trafficking such as steady-state circulation, inflammation, and tumor metastasis [11]. Among chemokines, IFN-γ-inducible protein 10 (IP-10/CXCL10) is a T helper (Th)1 chemokine with affinity to CXC chemokine receptor 3 (CXCR3) on Th1 cells [12]. On the other hand, thymus and activation-regulated chemokine (TARC/CCL17) is known as a Th 2 chemokine that binds to CC chemokine receptor 4 (CCR4) on Th2 cells. It has been reported that RXM influences production of chemokines and expression of chemokine receptors in relation to skin immunity [13,14]. In order to further clarify these issues, we assessed the effect of RXM on TARC and IP-10-induced chemotaxis of established Th1, Th2, and regulatory T (Treg) cells.

## 2. Materials and methods

### 2.1. Study participants

Two healthy males (age, 46 and 38 years) and a female (33 years) were enrolled in this study after written informed consent was obtained. The study was approved by the ethical committee of the Hamamatsu University School of Medicine, and conducted according to the Declaration of Helsinki principles.

### 2.2. Reagents and kits

RXM, erythromycin (EM), and clarithromycin (CAM) were obtained from Wako Pure Chemical Industries (Osaka, Japan); $CD4^+$ T Cell Isolation Kit II and CD45RO microbeads from Miltenyi Biotec (Auburn, CA); staphylococcal enterotoxin B (SEB), anti-human interleukin (IL)-4 monoclonal antibody (mAb), anti-human IL-12 mAb, recombinant (r)IL-2, phycoerythrin (PE)-labeled mouse IgG1 anti-human CD183 (CXCR3) mAb, PE-labeled mouse IgG1 anti-human CCR4 mAb, fluorescein isothiocyanate (FITC)-labeled mouse IgG1 anti-human CD4 mAb, FITC-labeled mouse IgG1 anti-human CD25 mAb, peridinin chlorophyll protein-labeled mouse

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IgG1 anti-human CD4 mAb, Cytoperm solution, and Perm/Wash buffer from BD Bioscience (San Jose, CA); FITC-conjugated anti-human interferon (IFN)-γ mAb, PE-conjugated anti-human IL-4 mAb, Goldiplug, and Cytofix/Cytoperm Plus Kit from BD Pharmingen (San Diego, CA); human rIL-12, and human rIL-4 from PeproTech (Rocky Hill, NJ); phytohemagglutinin (PHA), human rTGF-β thalidomide, dimethylsulfoxide (DMSO), FITC-conjugated phalloidin, and Fluo-3AM from Sigma–Aldrich (St. Louis, MO); 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) from Molecular Probes (Eugene, OR); and CD3/CD28 molecules from Dynal Biotec (Oslo, Norway).

### 2.3. Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation with Ficoll-Paque PLUS in LeucoSep (Greiner Bio-One, Frickenhausen, Germany) from three volunteers. $CD4^+$ T cells were prepared from PBMC with a $CD4^+$ T Cell Isolation Kit II by negative selection. The remaining $CD4^-$ cells were used as antigen-presenting cells (APC) to establish Th1, Th2, and Treg cells. $CD45RA^+CD4^+$ cells were purified from $CD4^+$ T cells with the CD45RO microbeads.

### 2.4. Cell culture

Culture medium was RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, $5 \times 10^{-5}$ M 2-mercaptethanol, $10^{-5}$ M sodium pyruvate, 25 mM HEPES, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin from Gibco-BRL (Carlsbad, CA); human TARC and IP-10 from R&D systems (Minneapolis, MN). PBMC ($5 \times 10^6$ cells/well) in culture medium were incubated in 12-well tissue culture plates at 37 °C in 5% $CO_2$ in air.

### 2.5. Generation of Th1, Th2 and Treg cells

All type of cells was established in serum-free medium in the presence of APC and various stimulants according to the reported methods [15–17]. Each cell type was come from PBMCs of three volunteers. In brief, SEB, an anti-human IL-4 antibody, rIL-12, and rIL-2 were employed to establish Th1 cells [15]. For establishment of Th2 cells, PHA, thalidomide, anti-IL-12 antibody, rIL-4, and rIL-2 were used [16]. Treg cell were established with SEB and human rTGF-β [17].

### 2.6. Immunofluorescence staining and flow cytometric analysis

For cell surface staining, cells were doubly stained with PE-labeled and FITC-labeled mAb in PBS for 30 min at room temperature. For intracytoplasmic cytokine staining, 1 μl of Goldiplug was added to each well during the last 6 h of culture according to the manufacturer's protocol. The cells were then reacted with 100 μl of CytoFix for 15 min at 4 °C, washed with phosphate-buffered saline containing 0.1% saponin, and stained with FITC-conjugated anti-human IFN-γ mAb and PE-conjugated anti-human IL-4 mAb for 30 min, followed by reaction with peridinin chlorophyll protein-labeled mouse IgG1 anti-human CD4 mAb. After washing in PBS, 10,000 cells were analyzed on a FACSCalibur (Becton Dickinson). Mean fluorescence intensity (MFI) was calculated on a log scale. Cells stained with isotype-matched mAbs served as the control.

### 2.7. Cell proliferation assay

$CD4^+$ T cells were labeled with 5 μM CFSE in DMSO for 15 min at 37 °C as previously described [18], stimulated with CD3/CD28 for 4 days, and analyzed by flow cytometry [18]. Cells once divided showed half the CSFE intensity of parental cells shown as the multipeak histogram of several divisions. In order to examine the suppressive effect of Treg cells on lymphocytic proliferation, autologous Treg cells were added to $CD4^+$ T cells ranging from 0.05 to 1.0 for 24 h, and the cell mixtures were subjected to CFSE analysis. Control culture continued with Treg cells.

### 2.8. Incubation of cells with macrolide

Cells were cultured for 8 h in the presence or absence of either 10 μM RXM, EM, or CAM. Cell viability after incubation with macrolides was >90% as judged by dye exclusion.

### 2.9. Real-time horizontal chemotaxis assay

Time-lapse images of cell migration during chemotaxis were observed directly with an optically accessible horizontal chemotaxis apparatus TAXIScan *via* a CCD camera (EZ-TAXIScan; GE Healthcare, Tokyo, Japan) as described [19]. The apparatus consisted of front and back chambers containing cells and a chemoattractant, respectively, which were connected by a microchannel. A 1-μl suspension of cells ($5 \times 10^6$ cells/ml) was placed in one compartment, and 1 μl of either TARC at 25 mg/ml or IP-10 at 10 mg/ml was injected into the other compartment to initiate chemotaxis under the concentration gradient in the channel. Data were analyzed using the Image J software (NIH, Bethesda, MD) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie, Orsay, France; http://rsb.info.nih.gov/ij/plugins/manual-tracking.html).

### 2.10. F-actin polymerization

Phalloidin has been found to bind only to polymeric and oligomeric forms of actin and not to monomeric actin. Therefore, the level of polymerized actin was determined by staining cells with phalloidin as described before. Cells were permeabilized in a Cytofix/Cytoperm solution for 20 min, washed in Perm/Wash buffer for 10 min, and incubated with 5 mg/ml FITC-conjugated phalloidin for 30 min. All procedures were done at 4 °C. Cells were analyzed on a FACSCalibur, and the level of actin-polymerization was expressed by MFI. Because MFI varied among each cell type, the percentage of fluctuation was calculated by the ratio before and after treatment with chemokines as follows: %MFI = MFI of treated cells/MFI of control, non-treated cells.

### 2.11. Calcium influx

Cells were incubated with 0.8 mM Fluo-3AM/DMSO in RPMI supplemented with 5% FCS for 30 min at 37 °C as described [20]. Fluorescence intensity was continuously measured on a FACSCalibur for 50 s after incubation with Fluo-3AM/DMSO. Chemokine was added into cell suspensions at 10 s after starting the measurement of $Ca^{++}$ concentrations. For quantitative evaluation, the variation of intracellular $Ca^{++}$ concentrations was calculated according to the following equation: $(F - F_{min})/(F_{max} - F) \times 100$, in which $F$ was MFI of chemokine-treated cells, $F_{min}$ was MFI of cells incubated with 6 mM EGTA to chelate $Ca^{++}$, and $F_{max}$ was MFI of cells incubated with 700 mM ionophore in DMSO [21].

### 2.12. Statistical analysis

All values were expressed as means ± standard deviation (SD). Data were analyzed with Student's $t$-test. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Immunological characteristics of established cells

More than 80% of Th1 cells expressed CD4 and CXCR3 and produced intracellular IFN-γ (Fig. 1A and B), while 75% of Th2 cells were positive for CD4 and CCR4, and 12% synthesized IL-4 (Fig. 1C and D). Since Th2 cells established according to the reported method [16] express relatively low levels of IL -4 and no IFN-γ, we used this cell line. Around 90% of Treg cells expressed CD4, CCR4, and CD25 (Fig. 1E and F), and none produced intracellular IFN-γ or IL-4 (Fig. 1G). The CSFE assay revealed that Treg cells suppressed proliferation of autologous $CD4^+$ T cells in response to immobilized CD3/CD28 in a dose-dependent manner (Fig. 2).

### 3.2. RXM down-regulates the migration of Th1 and Th2 cells, but not of Treg cells, toward IP-10 and TARC without influencing chemokine receptor expression

The migration of Th1, Th2, and Treg cells toward IP-10 or TARC was observed on a TAXIScan for 60 min. Non-treated control Th1 and Th2 cells ran toward IP-10 and TARC, respectively (Fig. 3). On the other hand, RXM-treated cells did not migrate toward the corresponding chemokine. Both non-treated control and RXM-treated Treg cells exhibited comparable chemotaxis to TARC. RXM did not affect chemokine receptor expression such as CXCR3 on Th1 cells, CCR4 on Th2 cells (Fig. 4A–D), or CCR4 on Treg cells (data not shown) as revealed by flow cytometry. Preincubation with CAM and EM did not influence the migration pattern of Th1, Th2, or Treg cells toward corresponding chemokines (data not shown).

### 3.3. F-actin polymerization to chemokines in Th1, Th2, and Treg cells

Actin polymerization is an early event that controls cell migration and reorganization of the actin cytoskeleton [22]. In Th1 cells, the baseline MFI of phalloidin was 503.7 ± 13.4. MFI increased by 106.2 ± 0.46% with IP-10 compared to control ($p < 0.05$). MFI significantly decreased by 92.23 ± 2.6% with RXM

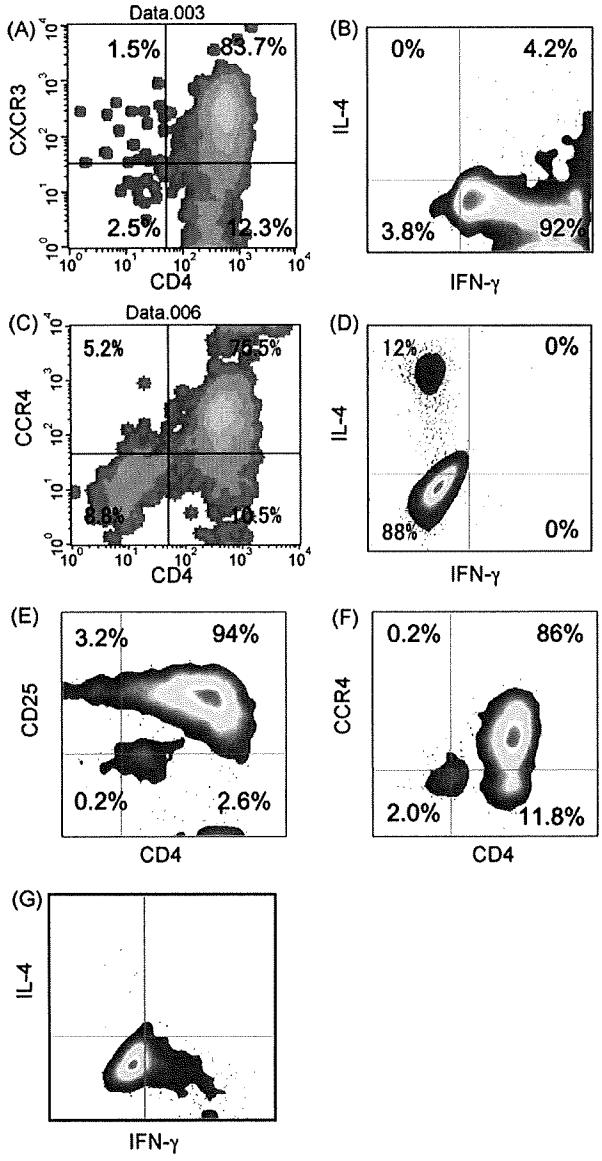


**Fig. 1.** Surface phenotypes and intracellular cytokine expression on established Th1, Th2, and Treg cells. (A) Expression of CD4 and CXCR3 on Th1 cells; (B) intracellular IL-4 and IFN-γ expression in Th1 cells; (C) expression of CD4 and CCR4 on Th2-t cells; (D) intracellular IL-4 and IFN-γ expression on Th2-t cells; (E) expression of CD4 and CD25 on Treg-h cells; (F) expression of CD4 and CCR4 on Treg-h cells; (G) intracellular IL-4 and IFN-γ expression in Treg-h cells.

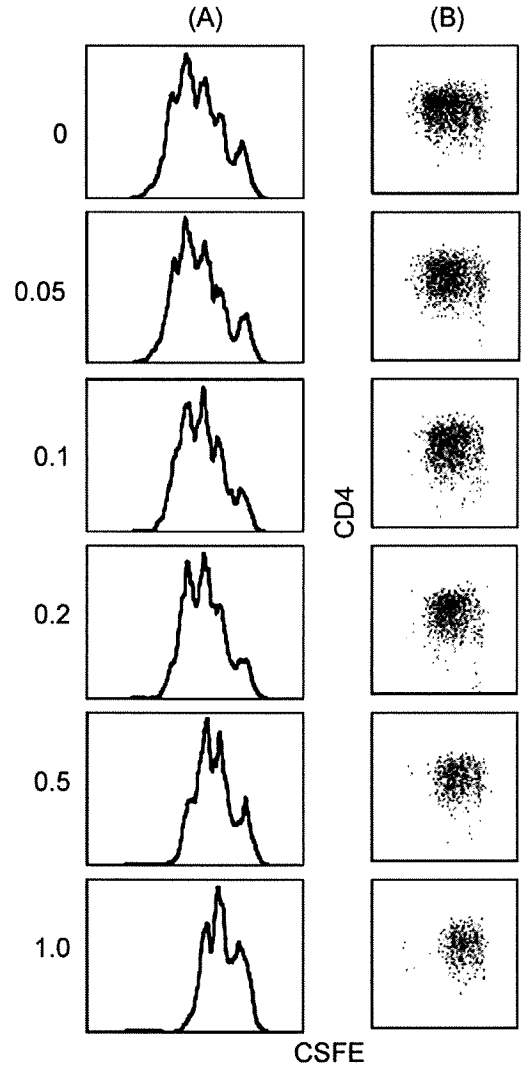


**Fig. 2.** CFSE analysis of Treg cell division. Tregs were coincubated with $CD4^+$ T cells for 24 h, and then labeled with 5 μM CFSE for 15 min at 37 °C. To prepare CFSE-labeled $CD4^+$ T cells, an equal volume of 10 mM CFSE was added such that the final concentration was 5 mM and the mixture was incubated for 10 min at 37 °C. After labeling, $CD4^+$ T cells were stimulated by CD3 (2C11) molecules. 3 and 4 days after stimulating with CD3 molecules, $CD4^+$ T cells were analyzed on a FACSCaliber.

pretreatment compared to non-treatment ($p < 0.05$). The MFI also significantly decreased by 92.0 ± 5.5% in cells pretreated with RXM and subsequently incubated with IP-10 compared to non-treated cells ($p < 0.05$) (Fig. 5A). In Th2 cells, the baseline of phalloidin MFI was 352.6 ± 23.0, and MFI increased by 107.5 ± 3.3% with TARC compared to control ($p < 0.05$). MFI significantly decreased by 94.5 ± 2.23% with RXM pretreatment compared to non-treatment ($p < 0.05$). The MFI significantly increased by 104.8 ± 2.9% in cells pretreated with RXM and subsequently incubated with TARC compared to non-treated cells ($p < 0.05$). This MFI was significantly lower than that of TARC-treated cells ($p < 0.05$) (Fig. 5A). In Treg cells, the baseline of MFI was 72.2 ± 8.9, and MIF did not change with TARC (MFI = 101.2 ± 0.2%), RXM (MFI = 100.8 ± 0.02%), or RXM + TARC (99.9 ± 2.2%) compared to non-treatment (Fig. 5A). Therefore, RXM hampered chemokine-induced F-actin polymerization in Th1 and Th2 cells but not in Treg cells. The representative data is shown in Fig. 5B and C.

### 3.4. Calcium influx

In the control Th1 cells, the baseline value of $Ca^{++}$ influx was 164.3 ± 2.2, and the $Ca^{++}$ influx (as expressed by %MFI ± SD) increased by 12 ± 2.5% ($p < 0.01$) by the addition of IP-10 compared to the influx before IP-10 addition (baseline $Ca^{++}$ influx) (Fig. 6A). In RXM-treated Th1 cells, the baseline $Ca^{++}$ influx was 158.9 ± 3.03, and it increased by 0.53 ± 0.04% upon IP-10 addition compared to baseline; this was lower than in non-treated cells. In the control Th2 cells, the baseline value of $Ca^{++}$ influx was 238.0 ± 18.8, and TARC increased it by 81.3 ± 7.28%. In RXM-treated Th2 cells, the baseline $Ca^{++}$ influx was 230.5, and TARC increased it by 55.9 ± 7.71%; this was significantly lower than in non-treated Th2 cells ($p < 0.01$) (Fig. 6B). In non-treated and RXM-treated Treg cells, the baseline value of $Ca^{++}$ influx was 315.5 ± 53.2 and 333.1 ± 73.5, respectively. RXM did not influence $Ca^{++}$ influx following the addition of TARC in non-treated and RXM-treated cells (Fig. 6C). These results indicate that RXM significantly suppresses chemokine-induced $Ca^{++}$ influx in Th1 and Th2 cells, but not in Treg cells. The representative data is shown in Fig. 6D.