

Histamine deficiency in gene-targeted mice strongly reduces antigen-induced airway hyper-responsiveness, eosinophilia and allergen-specific IgE

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Keywords: chemokine, inflammation, knockout, mast cell, T_H1/T_H2 cell

Abstract

Histamine is an important mediator released from activated mast cells provoked by allergen and has a substantial role in the pathophysiology of asthma. However, several lines of evidence indicate that histamine could also have important functions in the regulation of basic cell biological processes. We have used histidine decarboxylase gene-targeted (HDC-KO) mice, lacking histamine, to investigate the effect of histamine deficiency in an animal model of asthma. Our previous investigations revealed that HDC-KO mice had fewer mast cells with reduced granular content and defective degranulation characteristics. Ovalbumin (OVA)-sensitized and challenged HDC-KO mice had significantly reduced airway hyper-responsiveness, lung inflammation, bronchoalveolar lavage eosinophilia, and OVA-specific IgE compared with congenic wild-type littermates treated in the same way. Comparing the expression profiles of cytokines, the levels of IL-1 α , IL-1 β , IL-4, IL-5, IL-6 and IFN- γ were significantly lower in the HDC-KO mice in asthmatic late phase, indicating a significantly altered immune response to OVA provocation and challenge. Evaluation of chemokine gene expression revealed that OVA treatment caused elevation of both T_H1- and T_H2-type chemokines in wild-type mice, while the chemokine expression was polarized toward a T_H1 response in HDC-KO mice. According to our results we can suggest that the possible causes of the reduced asthma symptoms in the HDC-KO mice may be the imperfect mast and eosinophil cell system, and an altered immune response to OVA provocation and challenge.

Introduction

Allergic asthma is a complex disease associated with airway hyper-responsiveness (AHR) and chronic airway inflammation. The inflammatory response is characterized by the appearance of activated eosinophils, mast cells, and T and B cells in bronchial biopsies and in the bronchoalveolar lavage (BAL) fluid of asthmatic patients. Allergic asthma is also known to be accompanied by an increase in allergen-specific IgE and a cytokine profile in BAL that is representative of a T_H2-mediated event (1).

Histamine is an important mediator released from activated mast cells provoked by allergen, and has a substantial role in the pathophysiology of asthma through its ability to stimulate smooth muscle cell contraction, vasodilatation, increased venular permeability and further mucus secretion. Plasma histamine concentrations are elevated during the early and late responses to inhaled allergens, and may also increase during spontaneous acute asthma episodes (2). Studies have shown that antihistamines can prevent the development of asthma in specifically sensitized infants with atopic dermatitis

and controlling allergic rhinitis with antihistamines has a small, indirect effect in improving asthma symptoms (3). In contrast, in severe persistent asthma, histamine H1 receptor antagonists have no significant clinical effect (4,5). Although the biological role of histamine in allergic diseases has been extensively studied with pharmacological approaches using specific receptor agonists and antagonists or using histamine synthesis inhibitors (6,7), its exact role in asthma is far from clear.

Histamine is produced by the enzyme histidine decarboxylase (HDC). Although the highest activity of HDC and histamine content were detected in mast cells and basophils, the main sources of histamine in immunological reactions, the expression of HDC and histamine was observed in nearly all tissues studied. These findings raise the possibility that in addition to being a primary mediator in allergic and inflammatory processes, histamine could also have important functions in the regulation of basic cell biological processes (8). Histamine was found to exert its actions in auto-, para- and endocrine ways through four types of histamine receptors (H1, H2, H3 and H4), signaling via different signal-transduction pathways (9,10). 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. Although the histamine synthesis blocker α -fluoromethyl histidine significantly decreases the level of histamine in various organs, it is difficult to achieve complete and long-lasting elimination of histamine *in vivo* in this manner.

On this basis, establishing knockout mice with defective histamine synthesis was thought to be important and, therefore, we introduced a mutation into the HDC gene that resulted in mice without HDC activity (11).

In our present study we investigated the effect of histamine deficiency on the development of allergic airway disease induced by ovalbumin (OVA) sensitization and challenge, using HDC gene knockout (HDC-KO) and congenic wild-type mice.

Methods

Animals

The strategy to generate HDC-KO mice has been described previously (11). HDC^{-/-} CD1 mice were backcrossed onto the BALB/c background over eight generations. Female mice were 6–8 weeks of age at the beginning of the sensitization. Most mice were maintained on an OVA- and histamine-free diet. To study the effects of exogenous histamine, the experiments were also carried out with mice maintained on a diet containing histamine. The wild-type and HDC-KO mice were littermates of HDC^{+/-} × HDC^{+/-} crosses.

The study was approved by an Institutional Review Committee.

Sensitization and airway challenge

Sensitization and airway challenge were carried out as described by others (12). Briefly, groups of mice (seven to 10 mice per group) were sensitized by i.p. injection of 20 μ g OVA (Sigma) emulsified in 2.25 mg aluminum hydroxide in a

total volume of 100 μ l PBS on days 1 and 14. Mice were challenged (20 min) via the airways with OVA (1% in PBS) for 3 days (days 28–30) using ultrasonic nebulization. Control mice were sensitized and challenged with PBS without OVA and aluminum hydroxide. AHR was assessed on day 31. On day 32 mice were provoked with OVA (5%), and BAL and tissues were obtained after 5 h for further analysis.

Determination of airway responsiveness

Airway responsiveness to inhaled methacholine (MCh; Sigma) in conscious, spontaneously breathing animals was measured by recording respiratory pressure curves by whole-body plethysmography (Buxco Electronics). Aerosolized PBS or MCh in increasing concentrations (3–50 mg/ml) was nebulized through an inlet of the main chamber for 12 s, and readings were taken and averaged for 5 min following each nebulization. Enhanced pause (Penh) was expressed as a fold increase above PBS challenge baseline values. Penh was shown to closely correlate with airway resistance as measured by traditional invasive techniques using ventilated animals (13).

BAL

Five hours after the last provocation (day 32) lungs were lavaged via the tracheal tube with 3 × 0.6 ml PBS. The resulting BAL fluids were collected and immediately centrifuged (700 g, 5 min, 4°C) and cells were washed and resuspended in 5 ml PBS. Total leukocyte numbers were measured (Coulter Counter). Resuspended BAL fluid (500 μ l) was cytocentrifuged and differential cell counts were performed by counting at least 300 cells. The cells were stained by the Pappenheim method and differentiated by standard hematological procedures.

Histochemistry

One half of the lung was fixed in 10% neutral buffered formalin and paraffin embedded. Sections (4 μ m) were cut onto microscope slides and stained with hematoxylin & eosin according to standard protocols. Slides were examined in a blinded fashion with an Olympus microscope and photographed with an Olympus camera. Numbers of eosinophils in the lung tissues were counted at ×400 magnification counting four different fields of view per animal. Numbers were expressed per field of view. The other half of the lung was used for gene expression analysis. BAL and histology were not performed on the same lung.

Measurement of OVA-specific IgE antibody

Blood was taken 5 h after the last provocation. Serum levels of OVA-specific IgE were measured by ELISA. Briefly, OVA conjugated with biotin was applied to 96-well plates coated with streptavidin. After incubation with serum samples, horseradish peroxidase-conjugated anti-mIgE antibody was added. The OVA-specific IgE titers of the samples were related to pooled standards that were generated in the laboratory and expressed as ELISA units (EU)/ml.

Table 1. Chemokines analyzed in this study and their localization on the GEArray membranes

Genes		GenBank accession no.	GEA location
Abbreviation	Description/full name		
6-Ckine, Exodus-2	secondary lymphoid-tissue chemokine	AF001980	A1, B1
Eotaxin		U26426	C1, D1
Eotaxin-2		AF244367	E1, F1
Fractalkine		AF071549	A2, B2
Gro1		J04596	C2, D2
I-309		AF05928	E2, F2
IP-10	IFN- γ -inducible protein 10	M33266	A3, B3
Lymph	lymphotactin	NM_008510	C3, D3
MCP-1 (Scya2)	monocyte chemoattractant protein-1	NM_011333	E3, F3
MCP-2	monocyte chemoattractant protein-2	AB023418	A4, B4
MCP-3	monocyte chemoattractant protein-3	S71251	C4, D4
MCP-5	monocyte chemoattractant protein-5	NM_011331	E4, F4
MIG	monokine induced by γ interferon	NM_008599	A5, B5
MIP-1 α	macrophage inflammatory protein-1 α	X12531	C5, D5
MIP-1 β	macrophage inflammatory protein-1 β	AF128219	E5, F5
MIP-1r	macrophage inflammatory protein-1r	U49513	A6, B6
MIP-2	macrophage inflammatory protein-2	NM_009140	C6, D6
MIP-3 α	macrophage inflammatory protein-3 α	AJ222694	E6, F6
PF4	platelet factor 4	AB017491	A7, B7
RANTES (Scya5)	regulated on activation normal T cell expressed and secreted	AF128187	C7, D7
SDF-1 α	stromal derived factor-1 α	L12029	E7, F7
SDF-2	stromal derived factor-2	NM_009143	A8, B8
TECK	thymus-expressed chemokine	U86357	C8, D8
β -actin		M12481	G3, G4
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	M32599	G5, G6, G7E8, F8, G8
PUC18	bacterial plasmid		G1, G2

Measurement of gene expression

Total RNA from the lungs was extracted by a single-step method using TRIzol reagents (Invitrogen) 5 h after the last OVA provocation.

Chemokine gene expression was determined by the radioactive mouse chemokines GEArray kit (Super Array) as recommended by the supplier. Each kit contains 23 chemokine genes, two housekeeping genes and pUC18 plasmid as a negative control (Table 1). Gene expression levels of IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IFN- γ , tumor necrosis factor (TNF)- α , IL-1R, IL-4R, IL5-R, IL-6R, IL-9R, IL-10R, IL-12R, CCR1, CCR2, CCR3, CCR4, CCR5, CCR8 and CXCR3 were determined by the similar ChoiceArray kit (Super Array). The relative expression level of each gene was determined by comparing the signal intensity of each gene in the arrays after normalization to the signal of the housekeeping gene GAPDH on the same membrane. The signal intensities on the developed X-ray films were evaluated by Kodak EDAS290 and Kodak 1D image analysis software (Eastman Kodak).

Protein measurement

Five hours after the last provocation (day 32) lungs were isolated and the snap-frozen lung lobes were suspended in 2 ml PBS and homogenized for 20 s. Next, 0.1 ml FBS was added as a protein stabilizer. The homogenate was centrifuged at 3000 *g* for 20 min, and then the supernatant was collected, aliquoted and frozen at -80°C before protein assays. IL-4 and IFN- γ proteins were measured in the whole-lung aqueous extracts by ELISA using commercial reagents (R & D Systems). Next, total lung protein concentration was

determined and then cytokine levels were normalized to mg lung protein after subtraction of the FBS protein component.

Statistical analysis

Data were analyzed using the MedCalc program. ANOVA was used to determine the levels of difference between all groups for airway responsiveness, and unpaired Student's *t*-test for differences in the cellular composition and IgE levels. For analyzing the results of the GEArray kit, a gene was regarded as significantly differently expressed between two groups of mice if it fulfilled two criteria: (i) the difference in the mean relative signal intensity between two groups was at least 2-fold and (ii) the mean relative signal intensity between two groups was significantly different as determined by an unpaired Student's *t*-test.

Confidence intervals were calculated at the 95% level. The *P* values for significance were set at 0.05. Values for all measurements were expressed as the mean $\pm$ SEM.

Results

AHR is reduced in HDC-KO mice

To determine the effect of lack of endogenous histamine on the development of AHR, OVA-sensitized (days 1 and 14) wild-type and HDC-KO mice were challenged on 3 consecutive days (days 28, 29 and 30) with OVA aerosol, and Penh was measured in response to increasing concentrations of inhaled MCh on day 31. Control mice were sensitized and challenged with PBS without OVA and aluminum hydroxide. As shown in Fig. 1, sensitization and repeated airway challenge with

allergen of wild-type BALB/c mice increased airway responsiveness to aerosolized MCh with a left shift in the dose-response curve. The difference in the fold increase in Penh

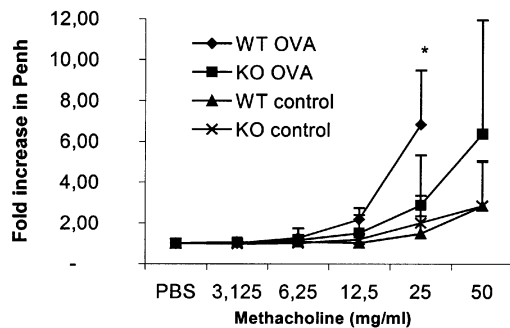


Fig. 1. Effect of histamine deficiency on AHR. AHR was measured 24 h after the final OVA challenge using the Buxco system. Control mice were sensitized and challenged with PBS without OVA and aluminum hydroxide. Aerosolized PBS or MCh in increasing concentrations (3–50 mg/ml) was nebulized through an inlet of the main chamber for 12 s, and readings were taken and averaged for 5 min following each nebulization. Penh is expressed as a fold increase above PBS challenge baseline values. Values are expressed as mean $\pm$ SEM; $n = 7$ –10 per group. *Significant differences ($P < 0.001$) between OVA-treated wild-type mice versus controls and OVA-treated HDC-KO mice.

was significant at 25 mg/ml MCh ($P < 0.001$; OVA- versus PBS-treated wild-type mice).

Although the dose-response curve of the OVA-treated HDC-KO mice fell between those of controls and OVA-treated wild-type mice, the difference between OVA- and PBS-treated control HDC-KO mice did not reach the level of significance at any dose of MCh, but the fold increase in Penh was significantly lower at 25 mg/ml MCh concentration as compared to the OVA-treated wild-type mice ($P < 0.001$).

Within groups, there was no significant difference between mice maintained on a histamine-free diet and mice maintained on a diet containing histamine in any investigated parameters (data not shown).

Our data demonstrate that OVA-treated HDC-KO mice have significantly reduced AHR compared with wild-type mice treated in the same way.

Reduced inflammatory response in the lungs of HDC-KO mice

The development of inflammation in the lungs of mice was assessed by means of histologic examination of hematoxylin & eosin-stained sections of lung tissue. Lungs were isolated on day 32, 5 h after the last OVA or PBS provocation. As shown in Fig. 2(A), marked wall edema and inflammatory influx into the peribronchial and perivascular tissue were observed in lung

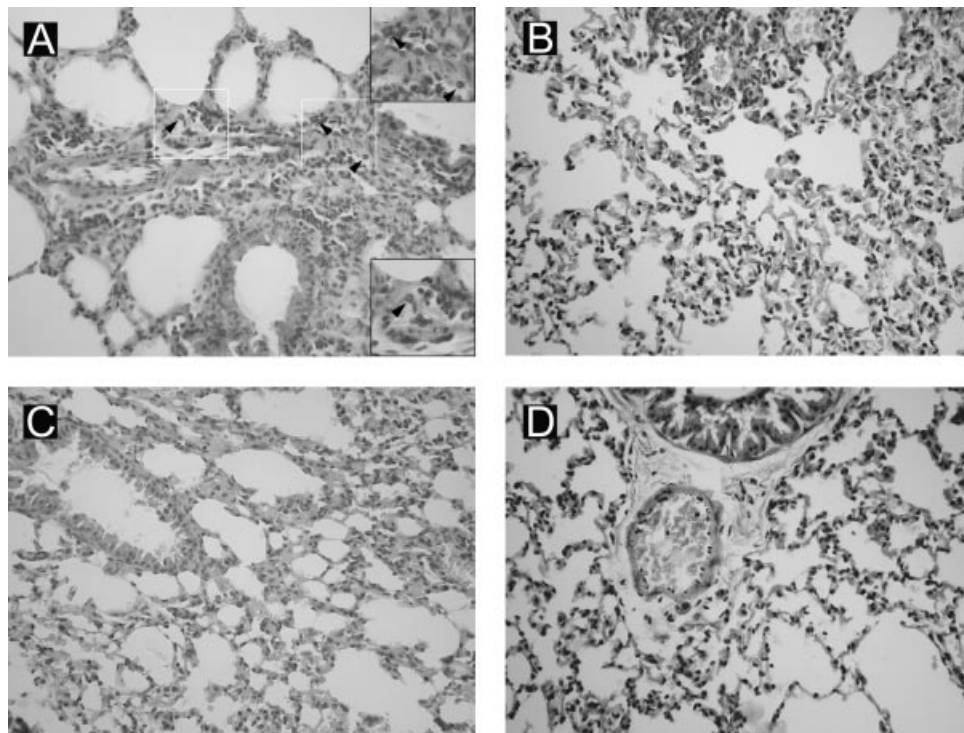


Fig. 2. Histological analysis of lungs of OVA- or PBS-treated wild-type and HDC-KO mice. Original magnification: $\times 400$. Lungs were isolated on day 32, 5 h after the last OVA or PBS provocation, and were fixed in 10% neutral buffered formalin and paraffin embedded. Sections (4 μ m) were cut onto microscope slides and stained with hematoxylin & eosin according to standard protocols. Slides were examined in a blinded fashion with an Olympus microscope and photographed with an Olympus camera. Numbers of eosinophils in the lung tissues were counted at $\times 400$ magnification, counting four different fields of view per animal. (A) Inflammatory influx into the peribronchial and perivascular tissue can be seen in lung tissue from wild-type mice treated with OVA. Number of eosinophils = 11.6 ± 3.8 /field of view. Original magnification in insets: $\times 800$. Arrows indicate eosinophils. (B) No marked lung inflammation in HDC-KO mice treated with OVA. Number of eosinophils = 1.0 ± 1.7 /field of view ($P < 0.001$ versus OVA-treated wild-type mice). (C and D) Lung sections of wild-type and HDC-KO control mice respectively. No eosinophils were detectable in these lung tissues.

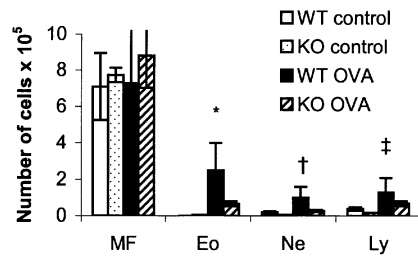


Fig. 3. Cell numbers in the BAL in groups of mice. BAL was performed on day 32, 5 h after the last OVA or PBS challenge; MF: macrophages; Eo: eosinophils; Ne: neutrophils; Ly: lymphocytes. Values are expressed as mean $\pm$ SEM; $n = 7$ per group. *Significant differences between OVA-treated wild-type and PBS-treated wild-type mice ($P < 0.001$) and OVA-treated wild-type and HDC-KO mice ($P < 0.05$). †Significant differences between OVA-treated wild-type and PBS-treated wild-type mice ($P < 0.05$) and OVA-treated wild-type and HDC-KO mice ($P < 0.05$). ‡Significant difference between OVA-treated wild-type and PBS-treated wild-type mice ($P < 0.05$).

tissue from wild-type mice sensitized/challenged with OVA. Evaluation of the infiltrates revealed high numbers of eosinophils, lymphocytes and plasma cells in the peribronchial and perivascular regions. In contrast, HDC-KO mice sensitized and challenged with OVA did not exhibit marked lung inflammation; they were almost indistinguishable from the control mice. In some HDC-KO mice a few patchy areas of a low degree of inflammation were noted in which eosinophils were barely detectable (Fig. 2B). There was a significant difference between eosinophil numbers in the lung tissues of OVA-treated wild-type mice ($11.6 \pm 3.8/\text{field of view}$) and those of in OVA-treated HDC-KO mice ($1.0 \pm 1.7/\text{field of view}$; $P < 0.001$).

No inflammation was observed in control lungs from wild-type and HDC-KO mice sensitized/challenged with PBS (Fig. 2C and D).

Reduced numbers of eosinophils and neutrophils in the BAL of HDC-KO mice

To assess the influx of leukocytes into the airway lumen, BAL was performed on day 32, 5 h after the last OVA or PBS challenge. Figure 3 shows the cellular composition of the BAL in each group of mice. The total cell numbers were higher in both groups of OVA-treated mice compared with the control groups ($0.8 \pm 0.6 \times 10^6$ versus $1.2 \pm 0.8 \times 10^6 \times 10^6$ cells/ml BAL in wild-type control and OVA-treated mice respectively, and $0.8 \pm 0.4 \times 10^6$ versus $1.1 \pm 0.4 \times 10^6 \times 10^6$ cells/ml BAL in HDC-KO control and OVA-treated mice respectively).

In all groups, macrophages were the predominant cell type in the BAL. OVA sensitization and challenge caused significant increase in the numbers of eosinophils ($P < 0.001$), neutrophils ($P < 0.05$) and lymphocytes ($P < 0.05$) in wild-type mice, but not in the HDC-KO mice ($P > 0.05$). The numbers of eosinophils and neutrophils were significantly higher in the wild-type mice than in the HDC-KO mice ($P < 0.05$). The lymphocyte numbers were also lower in OVA-treated HDC-KO mice than in OVA-treated wild-type mice, but the differences did not reach the level of significance.

These results obtained from the lungs and BAL fluids indicate that the lack of endogenous histamine significantly reduces the eosinophil and neutrophil infiltration into the airways of OVA sensitized-challenged HDC-KO mice, with a more dramatic effect on the cellular composition of the lungs than that of the BAL.

Reduced OVA-specific IgE in HDC-KO mice

Serum levels of OVA-specific IgE levels were measured 5 h after the last OVA/PBS provocation, on day 32. Sensitization and challenge with OVA resulted in significantly increased serum levels of anti-OVA IgE in wild-type mice. In sensitized/challenged HDC-KO mice the serum levels of anti-OVA IgE were significantly lower compared with the wild-type groups (0.72 ± 0.31 versus 0.05 ± 0.08 EU in OVA-treated wild-type and HDC-KO mice respectively; $P < 0.001$). The serum levels of OVA-specific IgE levels were below the limit of detection in both control groups.

These findings demonstrate that HDC-KO mice do not mount normal IgE responses after sensitization/challenge with OVA.

The mRNA expression profile of the cytokine/chemokine system differs significantly between HDC-KO and wild-type mice

To evaluate the differences in the mRNA expression of the cytokine/chemokine system during the asthmatic late-phase reaction in the different groups of mice, total RNA from the lungs was extracted 5 h after the last OVA/PBS provocation (day 32), and the expression levels of 46 genes were determined by the radioactive mouse chemokines GEArray (Fig. 4 and Table 1) and ChoiceArray kits (see Methods).

Altogether, 18 chemokines could be detected in the lung of mice studied. The mouse chemokine GEArray kit detected no mRNA of five chemokines in the lungs of any mice studied: MIP-3 α , MCP-5, MIP-1 β , TECK and Gro1.

Of the detectable 18 chemokines, 10 showed significant up- or down-regulation when comparing their signal intensities after PBS and OVA sensitization/challenge. Figure 5(A and B) shows the fold differences of the normalized mean signal intensities of these 10 chemokines in wild-type and HDC-KO mice respectively. The fold differences are positive when the signal is stronger in the OVA-treated mice and negative when the signal is stronger in the PBS-treated mice. Eotaxin-2 was detected only in the OVA-treated wild-type mice. In OVA-treated wild-type mice, five chemokines showed significant up-regulation (eotaxin, eotaxin-2, IP-10, MCP-1 and MIG) and five showed down-regulation (6-Ckine, MIP-1 α , PF4, SDF-1 α and SDF-2). In the OVA-treated HDC-KO mice, six chemokine genes showed up-regulation (IP-10, MCP-1, MIG, MIP-1 α , PF4 and SDF-2) and two showed down-regulation (6-Ckine and SDF-1 α) in the asthmatic late-phase reaction. Figure 5(C) shows the comparison of the relative chemokine levels in the lung of OVA-treated wild-type and HDC-KO mice. Altogether, the expression levels of five chemokines differed significantly between the two groups of mice. The eosinophil chemoattractant eotaxin and eotaxin-2 were detected only in the lung of the wild-type mice. The relative expression level of the MCP-3, which has also an eosinophil chemoattractant activity, was on average 2.4-fold higher in the lungs of the wild-type group. The

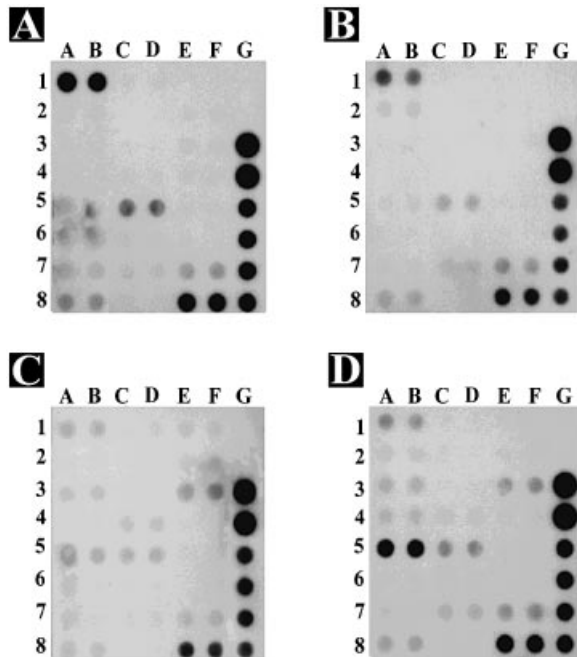


Fig. 4. Analysis of differential expression levels of chemokines in lung tissues after various treatments by the radioactive mouse chemokines GEMArray kit. Total RNA from the lungs was extracted 5 h after the last OVA provocation on day 32. Names and localization of the genes on the membrane are presented in Table 1. Normalization and comparison of the signal intensities of the corresponding spots were performed as described in Methods; $n = 7$ per group. (A) Wild-type control. (B) HDC-KO control. (C) Wild-type OVA-treated. (D) HDC-KO OVA-treated.

levels of MIG and the ubiquitously expressed SDF-2 were significantly higher in the HDC-KO mice.

Of the investigated 10 cytokine, seven cytokine receptor and seven chemokine receptor genes, seven cytokines (IL-1 α , IL-1 β , IL-4, IL-5, IL-6, TNF- α and IFN- γ), three cytokine receptors (IL-1R, IL-9R and IL-10R) and a chemokine receptor (CCR1) showed significant up-regulation in OVA-treated wild-type mice, and three cytokines (IL-1 β , IL-4 and TNF- α) and a cytokine receptor (IL-10R) in OVA-treated HDC-KO mice (Fig. 6A and B). Interestingly, the low expression levels of IL-12 in PBS-treated wild-type and HDC-KO mice were reduced to an undetectable level in both OVA-treated groups.

Figure 6(C) shows the comparison of the relative expression levels of these genes in the lung of OVA-treated wild-type versus HDC-KO mice. Altogether, the expression levels of seven genes differed significantly between the two groups of mice. IL-5 and IL-6 were detected only in the OVA-treated wild-type mice. The relative expression levels of IL-1 α , IL-1 β , IL-4 and IFN- γ were significantly higher in the lungs of the wild-type group, while the level of IL-10 was significantly higher in the HDC-KO mice. Notably, the levels of both IL-4 and IL-4R, the two most important players in the T_H2-type cytokine response and IgE production, were significantly higher in the OVA-treated wild-type mice.

These results indicate that OVA sensitization and challenge considerably modulate the expression of several genes of the cytokine/chemokine system in both groups of animals, but the

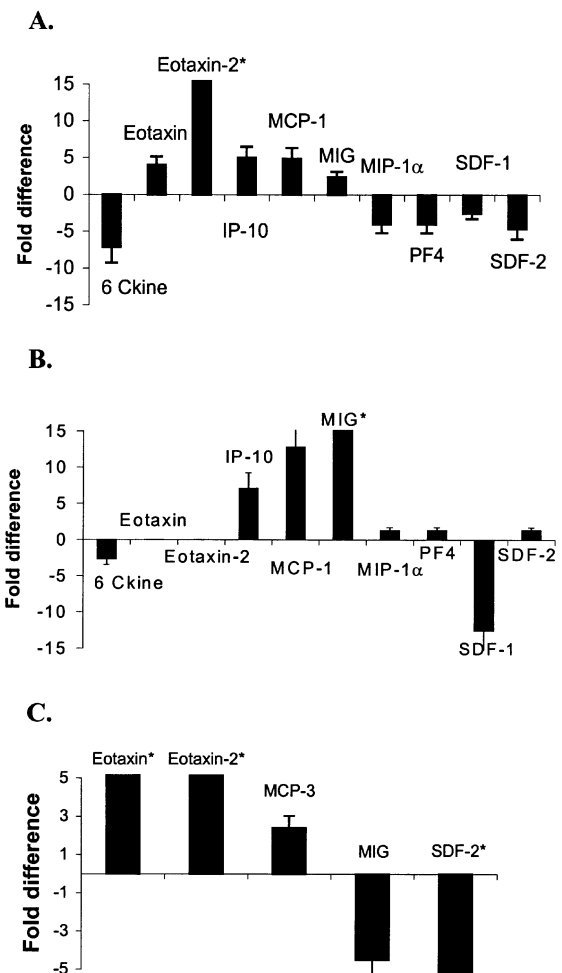


Fig. 5. (A) Fold differences in OVA-treated versus in control wild-type mice. *Eotaxin-2 was detected only in the OVA-treated mice. (B) Fold differences in OVA-treated versus in control HDC-KO mice. *MIG expression was 43.6 ± 7.8 -fold higher in the OVA-treated mice. (C) Comparison of the relative chemokine levels in the lung of OVA-treated wild-type and HDC-KO mice. The fold differences are positive when the signal was stronger in the wild-type mice and negative when the signal was stronger in the HDC-KO mice. *Eotaxin and eotaxin-2 were detected only in the lung of wild-type mice. SDF-2 expression was 18.3 ± 5.4 -fold higher in the HDC-KO mice.

expression profiles of these genes differ significantly between HDC-KO and wild-type mice.

Protein expression profiles are similar to the mRNA levels

As relative mRNA expression may not necessarily reflect levels of translated protein, we assessed levels of IFN- γ and IL-4 in the lung of the different groups of animals. Figure 7 shows the levels of IFN- γ and IL-4 detected in lung aqueous extracts during the asthmatic late-phase reaction in the different groups of mice expressed as pg/mg lung protein. Similar to the mRNA, both levels of IFN- γ ($P = 0.02$) and IL-4 ($P = 0.04$) were significantly higher in the OVA-treated wild-type mice than in the OVA-treated HDC-KO mice. While the levels of IFN- γ did not differ between the PBS- and OVA-

treated HDC-KO mice, the level of IL-4 was significantly higher in the OVA-treated than in the PBS-treated HDC-KO mice ($P < 0.001$). OVA treatment induced significant elevation of

both IFN- γ ($P = 0.02$) and IL-4 ($P = 0.004$) in the lung of wild-type mice. It is noteworthy that the IL-4 level was significantly higher in the control wild-type mice than in the control HDC-KO mice ($P = 0.02$).

Previously the protein levels of MCP-1 and MIP-2 in HDC-KO and wild-type mice were also found to be similar to the relative mRNA levels of these chemokines (14).

In addition, Qiu *et al.* compared the gene and protein expression levels of nine chemokines in mouse lungs in T_H1 and T_H2 cytokine-mediated granulomas, and concluded that semiquantitative mRNA analysis was a reasonably accurate reflection of local chemokine synthesis (15).

Discussion

The role of histamine in allergic diseases and inflammatory processes as a primary mediator has long been established (16). The present study demonstrates that histamine could also have important roles in basic immunological processes, since lack of endogenous histamine had multiple effects on allergen-induced inflammatory reactions. OVA-sensitized and challenged HDC-KO mice lacking endogenous histamine had significantly reduced AHR, lung inflammation, serum OVA-specific IgE, BAL eosinophilia and an altered gene expression profile compared with wild-type mice treated in the same way. The results also suggest the more important role of endogenous histamine compared to the exogenous histamine in the regulation of biologic processes, since a diet containing histamine did not cause any significant alteration in any investigated parameters, although previously histamine could be detected in different tissues only in mice maintained on diet containing histamine (11).

Although data indicate that histamine has a major influence on the immunological processes through selective enhancement and inhibition of a range of cytokines and chemokines, these mainly *in vitro* results are rather controversial, and depend on the different experimental conditions and cell types used (17), e.g. histamine reduces the production of IL-2 and IFN- γ , but there are also data about IFN- γ inducing effects of histamine (18). In several cell types histamine increases IL-6 mRNA secretion through H1 receptors, while decreases it in others through H2 receptors. In a recent report, histamine alone failed to induce the maturation of dendritic cells, but it greatly influenced the expression of multiple cytokines and chemokines during lipopolysaccharide-driven maturation (19). Histamine either totally or partially blocked the lipopolysaccharide-mediated induction of IL-12, IL-1 α , IL-1 β , IL-18, IL-6, IP-10, MIP-3 α and RANTES through H2 receptors; in contrast, histamine increased the expression levels of IL-8 and IL-10. As a result, histamine-matured dendritic cells polarized naive CD4 $^+$ T cells toward a T_H2 phenotype, as compared with dendritic cells that had matured in the absence of histamine (19). In other experiments, histamine induced CC chemokine (MCP and RANTES) production (20). On the other hand, several cytokines and chemokines were shown to modulate the production and release of histamine, e.g. IL-3, IL-18, MCP, RANTES, MIP-1 α and eotaxin appear to induce or enhance histamine secretion by mast cells or basophils, suggesting the possibilities of (local) prolonged inflammatory circuits (17,20).

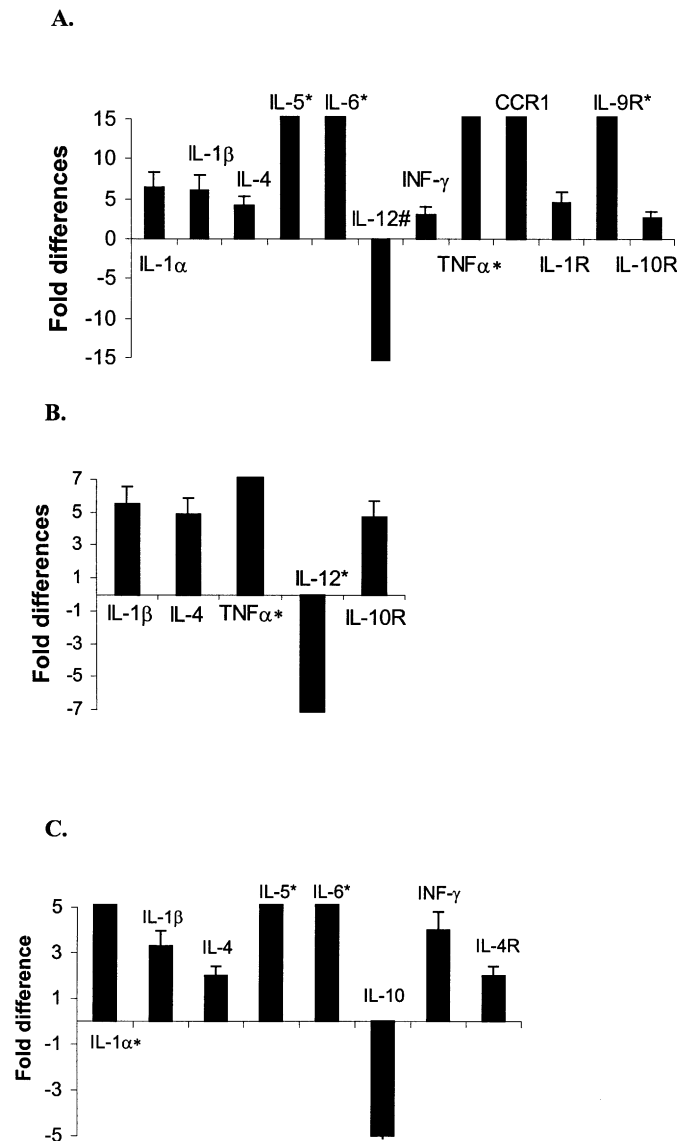


Fig. 6. Fold differences of the normalized mean signal intensities of genes expressed in the lungs in OVA-treated wild-type and HDC-KO mice relative to those of in control mice. Only the significantly differently expressed genes are presented. The fold differences are positive when the signal was stronger in the OVA-treated mice and negative when the signal was stronger in the control mice. Values are expressed as mean $\pm$ SEM; $n = 7$ per group. (A) Fold differences in OVA-treated versus in control wild-type mice. *IL-5, IL-6 and TNF- α were detected only in the lung of OVA-treated mice, IL-12 only in the control mice. (B) Fold differences in OVA-treated versus in control HDC-KO mice. *TNF- α was detected only in the OVA-treated mice, IL-12 only in the control mice. (C) Comparison of the relative levels of different genes in the lung of OVA-treated wild-type versus HDC-KO mice. The fold differences are positive when the signal was stronger in the wild-type mice and negative when the signal was stronger in the HDC-KO mice. Values are expressed as mean $\pm$ SEM; $n = 7$ per group. *IL-5 and IL-6 were detected only in the lung of wild-type mice; IL-1 α expression was 6.8 ± 2.4 -fold higher in wild-type mice.

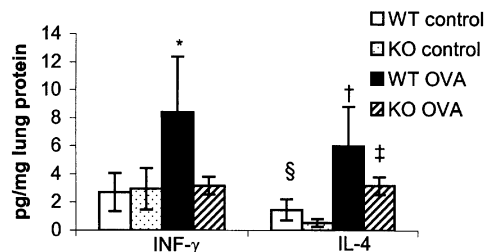


Fig. 7. Levels of IFN- γ and IL-4 expressed in the lung during the asthmatic late-phase reaction (day 32) in the different groups of mice. The lungs were isolated 5 h after the last provocation (day 32). The proteins were measured in whole-lung aqueous extracts by ELISA. Cytokine levels were normalized to mg lung protein after subtraction of the FBS used as a protein stabilizer. Values are expressed as mean $\pm$ SEM; $n = 7$ per group. *Significant differences between OVA-treated wild-type versus control wild-type mice and OVA-treated wild-type versus OVA-treated HDC-KO mice ($P = 0.02$). †Significant differences between OVA-treated versus control wild-type mice ($P = 0.004$) and OVA-treated wild-type versus OVA-treated HDC-KO mice ($P = 0.04$). ‡Significant differences between OVA-treated versus control HDC-KO mice ($P < 0.001$). §Significant differences between control wild-type versus control HDC-KO mice ($P = 0.02$).

Although HDC-KO mice were been found to be apparently healthy and developed normally, they have several interesting phenotypes. Among others, our previous investigations revealed that HDC-KO mice had a major reduction in the number of mast cells and their precursors. In addition, the HDC-KO mice-derived mast cells had reduced granular content and defective degranulation characteristics (11,21).

Mast cells and their released products are widely believed to contribute to the development of allergic asthma. IgE-dependent activation of mast cells can induce these cells to release a panel of preformed or newly synthesized mediators, which can result in acute-phase allergic reactions in the lung (2). In addition, data indicate that mast cells can represent an important local amplifier of antigen-dependent chronic inflammation in asthma as well (22). The defective and reduced numbers of mast cells in the HDC-KO mice may contribute to the mild symptoms of the OVA-treated animals, but presumably are not the main cause of them, since OVA sensitization and challenge of mast cell-deficient mice resulted in AHR, serum OVA-specific IgE, BAL and lung eosinophilia indistinguishable from their congenic wild-type littermates, suggesting that mast cells are not necessary for these phenotypes (23). It must be noted, however, that the mast cell-deficient W/W^v mice used in these experiments had a C57BL/6 background, which had some genetic deficiencies (e.g. a point mutation in the mast cell protease 7 gene) and are probably resistant to the induction of mast cell-dependent AHR (24). In our experiments we used BALB/c mice in which the important role of mast cells in the development of asthma has been proven (25). On the other hand, the OVA-treated HDC-KO mice had significantly reduced levels of serum OVA-specific IgE compared with wild-type mice. IgE, bound to high-affinity IgE receptors (Fc ϵ RI), triggers the activation of mast cells following cross-linking with specific antigen. The OVA-treated HDC-KO mice have both defective and fewer mast cells, and low antigen-specific serum IgE levels, suggesting that in these

animals this pathway to the asthmatic phenotype is damaged. However, it is also true that features of chronic asthma can be induced in mice by IgE and mast cell-independent mechanisms (23,26).

The imbalance in the T_H cell response to allergens plays an important role in the development of allergic asthma, leading to a predominant T_H2 -type cytokine response, thus resulting in increased IL-4, IL-5 and IL-13 production (27). IL-4 and IL-13 are the main isotype switch factors leading to increased production of IgE by B lymphocytes in response to allergen (27). Comparing the expression profiles of genes in the cytokine/chemokine system, the low levels of IL-1 α , IL-1 β , IL-4, IL-5, IL-6 and IFN- γ in the HDC-KO mice indicate a significantly weakened, or at least altered, immune response to OVA provocation and challenge. The reduced expression of IL-1 α , IL-1 β and IL-6, which regulate the production of acute-phase proteins involved in local and systemic inflammation, indicates attenuated inflammation of the lung of the OVA-treated HDC-KO mice. The low level of IL-6 in the HDC-KO mice is in good correlation with our earlier findings that the inducibility of IL-6 is significantly reduced in histamine-deficient mice (28). Although the level of IL-4 was increased in response to OVA treatment, its concentration was significantly lower than that of wild-type mice. In addition, the expression of the IL-4R gene was also lower in the lung of OVA-treated HDC-KO mice. The low levels of these two genes might be responsible for the reduced IgE responses after sensitization and challenge with OVA. Alternatively, the altered antigen presentation, found in the HDC-KO mice, might also be responsible for this phenomenon (our unpublished observation).

Eosinophils are the major infiltrating cells in the airways of allergic asthmatics and are thought to be the major effector cells in the pathogenesis of allergen-induced AHR (1). However, the exact role of eosinophils in the disease is still controversial, since an attempt to establish a casual link between major basic protein, the main effector granule proteins of the eosinophils, and AHR has been unsuccessful (29). In a more recent work, Foster *et al.* suggested that the eosinophil contribution is through its feedback effect on T cells (30). The T_H2 cytokine IL-5 is known to regulate the growth, differentiation, activation and survival of eosinophils. In animal models blocking IL-5 inhibited the allergen-induced infiltration of eosinophils into the lung (31). There was no detectable IL-5 in the lung of OVA-sensitized and challenged HDC-KO mice, which might explain the reduced eosinophil numbers in the BAL and lung tissue. In addition, endogenous histamine is known to induce expression of P-selectin (32). Also, P-selectin has been reported to be an adhesion molecule expressed in lung vascular endothelium that is necessary for the rolling of eosinophils that leads to tissue infiltration (33). Earlier it had been shown that, in contrast to the wild-type mice, P-selectin was not up-regulated in the lung after allergen challenge in HDC-KO mice (34). This phenomenon might also contribute to the reduced eosinophil numbers in BAL and lung tissue.

The significant differences in the cellular composition of BAL and lung tissue of OVA-treated wild-type and HDC-KO mice may also be a result of the characteristic shift of differentiation patterns toward a macrophage precursor predominance in the bone marrow, found previously in the HDC-KO mice (21).

Of the investigated cytokines, only the expression of IL-10 was significantly higher in the OVA-treated HDC-KO mice. Although IL-10 belongs to the group of cytokines produced by T_H2 cells, it has been considered an inhibitory factor for allergic responses and T_H2 cytokine production (35). Thus, exogenous recombinant IL-10 suppresses IL-5 production by CD4 T cells and inhibits airway eosinophilic inflammation induced by allergen (36). Thus, the high levels of IL-10 in OVA-treated HDC-KO mice might contribute to the low levels of IL-5 and the reduced airway eosinophil inflammation.

Although the immunologic background of the animals and the design of the experiments greatly influences the cellular pathways leading to AHR, it seems to be clear that there are two main effector cell types in these processes: the IgE-mediated mast cells and the IL-5-triggered eosinophils (22,37). As HDC-KO mice have fewer and defective mast cells, low levels of OVA-specific IgE, and the eosinophil infiltration was reduced in the lung of the OVA-treated HDC-KO mice, it is not unexpected that the allergen-induced AHR was also significantly attenuated compared with the wild-type animals. Recently, several research groups developed protocols to produce polarized models of T cell-mediated type 1 and 2 responses, and tested the hypothesis that there would be patterns of chemokine expression characteristic of type 1 versus 2 responses (15,38). According to Qiu *et al.* who investigated the expression pattern of 24 chemokines in mouse lung, among the chemokines studied in this paper, eotaxin and MCP-3 showed a predominant expression in the type 2 response, IP-10, MIG and MIP-1 α dominated in the type 1 responses, while MCP-1 did not display definite polarization. SDF-1 α was found to be expressed constitutively by lung tissue, and showed no significant change after sensitization and challenge (15). Although eotaxin-2 was not studied with regard to its eosinophil chemoattractant activity, it could also be regarded as a type 2 chemokine. 6-Ckine or secondary lymphoid chemokine (also known as Exodus-2), SDF-2 and PF4 have not been characterized in this respect. However, considering that 6-Ckine is expressed constitutively in lymphoid organs and SDF-2 is ubiquitous in all organs studied, they may not be characterized as type 1 or 2 chemokines (39). PF4 is released during acute vascular injury, or chronic disease by activated platelets, has quite heterogeneous activities and with our present knowledge cannot be classified in this regard (40).

According to our results, OVA sensitization and challenge in the late-phase reaction caused elevation of both T_H1 (IP-10 and MIG) and T_H2 (eotaxin and eotaxin-2) chemokines in wild-type mice, while in the OVA-treated HDC-KO mice the chemokine expression was polarized towards a type 1 response (elevation of the type 1 IP-10, MIG and MIP-1 α , but no elevation of type 2 chemokines). In addition, as well as eotaxin and eotaxin-2, the level of the type 2 MCP-3 was also significantly higher in the OVA-treated wild-type mice, while the level of the type 1 MIG was significantly higher in the OVA-treated HDC-KO mice. Among the chemokines examined above, eotaxin and eotaxin-2 are the most selective regarding the chemoattraction of eosinophils. In addition, their lung mRNA levels correlated well with the accumulation of eosinophils in the wild-type mice.

The mRNA level of MCP-1, which is primarily involved in the recruitment of mononuclear phagocytes, was elevated in both groups of OVA-treated animals. MCP-1 administration to normal mice induced a significant increase in airway resistance, which was dose dependent. This ability of MCP-1 to induce a hyper-reactive response was associated with increased levels of histamine. It has been suggested that MCP-1 can activate basophils and mast cells to release histamine (41). The inability of the MCP-1 in the HDC-KO mice to cause histamine release might contribute to the reduced AHR in the OVA-treated HDC-KO mice. On the other hand, it has been shown that MCP-1 is able to induce chemotaxis of eosinophils (42,43). It is possible that the eosinophils in the BAL of the OVA-treated HDC-KO mice were recruited by the MCP-1.

Earlier, a potential role for IP-10 in maintaining the default T_H1 -type responses to environmental antigens had been suggested, which is a response that may play an active role in preventing atopic diseases (44). Our results show that it is also highly expressed in the lung in the asthmatic late phase, which is in good correlation with a recent report showing an active involvement of IP-10 in asthma (45).

Recently, Koarai *et al.* published their results of OVA-induced allergic airway eosinophil recruitment and hyper-responsiveness in HDC-KO mice, strain 129Sv (34). Similar to our results, the eosinophil recruitment in the lung was significantly reduced in the HDC-KO mice, although the AHR was not suppressed. The differences in the results of the AHR can be explained by the different strains and protocols used. Koarai *et al.* used 129Sv inbred mice, which after sensitization have significantly lower AHR than the BALB/c mice used in our experiments (25). Furthermore, it is also well known that changes in the protocol can significantly influence the outcome of the experiments.

Naturally, the molecular, cellular and phenotypic characteristics in the HDC-KO mice cannot be attributed to the direct effect of endogenous histamine deficiency during the OVA treatment. As previous results suggest, histamine influences several pathways during embryogenesis, e.g. during the development of the immune system, and also later, e.g. during the hemopoiesis and cell differentiation in the bone marrow (21). The gross consequence is an altered immune response in the HDC-KO mice because of the superimposed effect of several modified characteristics, e.g. mast cell and eosinophil deficiency, altered antigen presentation, reduced IL-6 inducibility, etc. (11,21,28,34).

In summary, our findings demonstrate that histamine, in addition to its role in immediate hypersensitivity, has a major influence on basic immunological processes. HDC-KO mice, lacking endogenous histamine, responded to antigen stimuli very differently than congenic wild-type mice. OVA-sensitized and challenged HDC-KO mice had significantly reduced AHR, lung inflammation, serum OVA-specific IgE, BAL eosinophilia, and altered cytokine and chemokine gene expression profiles compared with wild-type mice treated in the same way. The possible causes of these reduced asthma symptoms may be the imperfect mast and eosinophil cell system, and an altered immune response to allergen provocation and challenge in the HDC-KO mice.

Acknowledgements

OTKA (National Scientific Research Fund): T032349; T031887; 042609; Hungarian Ministry of Welfare: ETT 134/2001, 300/2000, 16/2000; János Bolyai Research Grant.

Abbreviations

AHR	airway hyper-responsiveness
BAL	bronchoalveolar lavage
EU	ELISA unit
HDC	histidine decarboxylase
KO	knockout
MCh	methacholine
OVA	ovalbumin
TNF	tumor necrosis factor

Abbreviated chemokines are listed in Table 1.

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