

Histamine 4 Receptor Activation Induces Recruitment of FoxP3⁺ T Cells and Inhibits Allergic Asthma in a Murine Model¹

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Histamine has an important role in regulation of immune response which is mediated by differential expression of four distinct receptors, H1R–H4R. H1R and HR2 have previously been shown to be involved with modulation of lung inflammation. H4R is also expressed on inflammatory cells; therefore, we investigated the potential role of H4R in development of allergic asthma in a murine model. We determined that the H4R agonist 4-methylhistamine when delivered intratracheally before Ag challenge mitigated airway hyperreactivity and inflammation. This was associated with an increase in IL-10 and IFN- γ , but not TGF- β or IL-16, as well as a decrease in IL-13 in the bronchoalveolar lavage fluid. We also observed that H4R agonist instillation resulted in accumulation of FoxP3⁺ T cells suggesting a direct effect on T regulatory cell recruitment. To investigate this further, we determined the in vitro effect of H4R stimulation on human T cell migration. The H4R agonist induced a 2- to 3-fold increase in T cell migration, similar to that seen for H1R agonists. Cells transmigrating to the H4R agonist, but not H1R, were skewed toward a CD4 cell expressing CD25 and intracellular FoxP3. H4R-responsive cells suppressed proliferation of autologous T cells, an effect that was dependent on IL-10 production. We conclude that H4R stimulation enriches for a regulatory T cell with potent suppressive activity for proliferation. These findings identify a novel function for H4R and suggest a potential therapeutic approach to attenuation of asthmatic inflammation. *The Journal of Immunology*, 2007, 178: 8081–8089.

Histamine release in the lungs is a well-recognized feature of allergic asthma and is associated with a cascade of events, including airway constriction, mucus secretion, vascular leak, and recruitment of immune cells. These proinflammatory effects are mainly the results of histamine H1R receptor activation on a variety of resident lung cells. Despite this, clinical usefulness of specific histamine H1R receptor antagonists, in use for over 50 years, are limited to atopic nasal, conjunctival, and skin disease. Four classes of histamine receptor have now been described and it has emerged that the effects of histamine depend upon target cell and receptor subtype expression (1, 2). Importantly, histamine has been found to play a role in modulation of the immune response through differential expression and activation of these receptors. For example, the net effect of signaling via the histamine H2 receptor on cells of hemopoietic origin, including both Th1- and Th2-type immunocytes, is suppression of inflammation (3–5). These effects are attributed to stimulation of regulatory dendritic and Th2 cells to produce IL-10, which augments the suppressive activity of TGF- β on T cells (6–10).

The fourth histamine receptor, H4R, has recently been described (11) and like the other three receptors, H4R is a G protein-coupled receptor (11, 12). Unlike the other receptor subtypes, histamine H4 receptors are principally expressed on hemopoietic cells including splenocytes, thymus, dendritic cells, and lymphocytes (11–13). In the lung, H4R is present in low amounts where it appears to be expressed primarily on immune cells and bronchial epithelium (14). The functional significance of histamine H4R expression on these cells is not known but studies using selective H4R agonists have found that activation induces chemotaxis of mast cells and eosinophils (15–18). We have also previously reported that stimulation of H4R on human T cells results in secretion of the CD4⁺ T cell chemoattractant IL-16 (14). This effect on IL-16 secretion is similar to what is observed following selective H2R stimulation (14). As H2R stimulation can result in suppression of inflammation and studies by Little et al. (19) demonstrate that elevated levels of IL-16 in the lungs of mice result in attenuation of asthmatic inflammation, we investigated the potential effects of selective H4R stimulation in a murine model of allergic asthma.

In this study, we demonstrate that intratracheal administration of a selective H4R agonist into the lungs of asthmatic mice results in inhibition of both airway resistance and airway inflammation. This effect appears to be mediated through the recruitment of CD25⁺FoxP3⁺ T regulatory cells. In vitro assays confirm a direct effect on FoxP3⁺ cells with IL-10-induced suppressive effects on autologous T cell proliferation. These findings suggest a novel immunotherapeutic approach for allergic asthma using a locally delivered selective H4R agonist.

Materials and Methods

In vivo experiments

BALB/c mice were used in all vivo studies. Intratracheal instillations were conducted on anesthetized mice using 10 μ g of the histamine

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Received for publication February 1, 2007. Accepted for publication April 4, 2007.

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¹ This work was supported in part by Grants HL32802 and AI35680 from the National Institutes of Health.

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receptor agonists histamine-trifluoromethyl-toluidide derivative (HTMT)³ or 4-methylhistamine (4MH; both from Sigma-Aldrich) in 100 μ l of saline. For induction of the asthma model, the mice were sensitized by i.p. injections of an OVA protein (10 μ g) in alum as previously described (19) on days 1 and 14. Airway challenge, initiated on day 28, was accomplished by aerosolized OVA (10 μ g/ml in saline) for 30 min on 6 consecutive days. For measurement of airway resistance, mice were anesthetized with an i.p. injection of 0.1 ml/10 g body weight of a solution of 0.4 mg/ml xylazine and 8 mg/ml ketamine in PBS. When adequate sedation has been reached, determined by a firm squeeze of the foot pad, a tracheotomy was performed and an 18G tracheal cannula was inserted. The mouse was then placed on the FlexiVent mechanical ventilator (Scireq Scientific Respiratory Equipment) and ventilated at 300 breaths/min with positive-end expiratory pressure set at 3 cmH₂O. Measurement of airway resistance in response to increasing concentrations of aerosolized methacholine is obtained through periodic computer-generated "snapshot 150" forced-maneuver interruptions in ventilation. Data are then presented as resistance change from baseline (cmH₂O per milliliter per second). Following these measurements, mice were removed from the ventilator and bronchoalveolar lavage (BAL) was performed with 1.0 ml of 1 $\times$ PBS. The mice were then sacrificed by abdominal aortic transection, after which the lungs were perfused via the instillation of 3 ml of cold saline into the right ventricle, before retrieval of the lungs for digestion. In some mice, lungs were filled with 4% paraformaldehyde via intratracheal instillation before removal for histological preparation. All experiments were performed with the approval of the Boston University School of Medicine Institutional Animal Care and Use Committee committee.

Isolation of nylon wool nonadherent human T cells

Primary human mononuclear cells were generated from the peripheral blood of healthy volunteers by Hypaque Ficoll (Amersham Biosciences) density centrifugation of mononuclear cell buffy coat. Following a wash step in M199 containing 0.4% BSA and penicillin/streptomycin, the T cells were isolated by nylon wool (Polysciences) adherence and the resulting nylon wool nonadherent T cells (NWNAT) were routinely >95% CD3⁺ by flow cytometry assessment. The cells were rested overnight in M199 supplemented with 4% BSA, HEPES buffer, and penicillin/streptomycin before conducting chemotaxis assays. In some experiments, cells were further purified into CD4⁺ T cells by negative selection using a magnetic bead isolation kit (DynaL Biotech) before addition to the chemotaxis Transwell. The kit contains Abs to CD8, CD11b, CD16/32, CD45R, and Ter-119, which depletes B cells, NK cells, monocytes/macrophages, dendritic cells, CD8⁺ T cells, erythrocytes, and granulocytes. Following depletion, we routinely find the resultant cell population to be >90% CD4⁺CD3⁺.

Chemotaxis assays

The chemotactic activity of histamine, the histamine receptor agonist HTMT derivative, dimaprit, R(-)- α -methylhistamine, 4MH (all Sigma-Aldrich) and MIP-1 β or RANTES (R&D Systems) were assessed by measuring lymphocyte migration through an 8- μ m pore polycarbonate filter in 6- or 12-well Transwell chambers (Corning Costar). Briefly, 1.0×10^6 freshly isolated peripheral blood human CD4⁺ T cells suspended in at least 300 μ l of complete medium (M199 from Sigma-Aldrich, containing 200 U/ml penicillin, 200 μ g/ml streptomycin, 2 mM glutamine, 20 mM HEPES, and 10% FBS) were added to the top chamber. Histamine or histamine agonists at concentration ranges from 10^{-8} to 10^{-2} M in 600 μ l of complete medium were added to the lower chamber; MIP-1 β and RANTES at 50 ng/ml were used as control chemoattractants. After 2 h of incubation at 37°C with 5% CO₂, the chambers were placed at 4°C for 30 min to facilitate detachment of cells attached on the bottom of the filter. Nonmigrating cells were then aspirated from the top chamber and migrating cells were aspirated from the bottom chamber. Cell counts were then performed on each sample using a hemocytometer. All counts were performed in duplicate using a 1/10 dilution of cells. The chemotactic activity was expressed as the number of cells counted in either the lower or upper wells. As the migration to medium alone was significantly less than in the presence of a chemoattractant, control cell migration was conducted in three to four separate wells and pooled to assure sufficient cell yields for migrating cells. In some experiments, for use in suppression assays, migratory cells were further divided into CD25⁺ and negative populations using a commercially available magnetic bead isolation kit (DynaL Biotech).

Flow cytometric analysis

CD4, CD25, and CTLA4 expression was analyzed using FITC or PE fluorescently conjugated Abs (R&D Systems); intracellular staining for FoxP3 was then performed using the PCH101 mAb (eBioscience). Briefly, cells were recovered from the top and bottom chambers of the Transwell, washed, and resuspended in staining buffer containing PBS with 0.1% sodium azide and 1% BSA. Typically, from a starting population of 1×10^6 cells placed in the upper well, 10–17% of these ($1\text{--}1.7 \times 10^5$ cells) were retrieved from lower wells containing medium alone (negative control) following the 2-h incubation. This increased to 30–50% migration ($3\text{--}5 \times 10^5$ cells) in the presence of a chemoattractant. Staining was performed on 2×10^5 cells from each chamber, with pooling of lower wells from negative control. The appropriate cell surface Ab was added for 30 min at 4°C; the cells were then washed, fixed, and permeabilized before intracellular FoxP3 staining using the FoxP3 staining kit (eBioscience). Samples were analyzed using a FACScan flow cytometer (BD Biosciences), 10,000 cells were collected and data were processed using CellQuest software (BD Biosciences). In other experiments, mice lungs were first minced and then subjected to enzyme digestion with solution containing dispase II (2.4 U/ml), collagenase A (0.1%), and CaCl₂ (2.5 mM). At the end of 60 min, the resultant lung slurry was filtered through a 70- μ m nylon filter to retrieve lung cells. CD4⁺ lymphocytes were further purified from this cell mixture by negative selection using a commercial kit (DynaL Biotech), before staining for the above regulatory T (Treg) cell markers. The Student *t* test was used for statistical analysis and statistical significance was established at 5% confidence.

Proliferation assays

A standard lymphocyte proliferation assay was used to assess the proliferative capacity of migratory and nonmigratory cells to anti-CD3 stimulation. Briefly, $1\text{--}3 \times 10^5$ cells/well were cultured in anti-CD3 (1 μ g/well; R&D Systems) coated round-bottom 96-well plates (Corning Costar) for 4 days in a humidified 37°C, 5% CO₂ incubator. To assess cell proliferation, [³H]thymidine was added to the cultures at 1 μ Ci/well for the final 24 h of culture. Cells were harvested onto glass-fiber filters and [³H]thymidine incorporation was assessed using a β -scintillation counter.

Suppressor assays and ELISA

The suppressive capacity of migratory cells or their supernatants was assessed by their ability to inhibit anti-CD3-activated autologous human T cell proliferation. Migrating and nonmigrating cells from each Transwell condition were added at ratios of 1:1 to 1:100 to a constant number of freshly isolated T cells (3×10^5 /well). Cell proliferation in these assays was determined after 4 days of culture by the addition of [³H]thymidine at 1 μ Ci/well to the wells for the final 24 h of culture. The cells were then harvested onto glass-fiber filters and [³H]thymidine incorporation was assessed on a β -scintillation counter. In additional experiments, to investigate the mechanism of migratory cell-induced suppression, migratory cells (0.5 to 1×10^6 /well) were kept in culture for 48 h at which time supernatants were collected for use in suppression assays as described above. In similar culture conditions, the cytokine levels of TGF- β , IL-10, IL-16, IL-5, and IFN- γ were assessed from the cell supernatants using commercially available ELISA kits (BD Biosciences).

Quantitative real-time RT-PCR for FoxP3 expression

RNA was extracted from cells and reverse transcribed using AMV reverse transcriptase and a cDNA synthesis kit (Promega). Real-time PCR of the cDNA preparations was then performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the following thermal cycling parameters: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. FoxP3 gene expression was detected using the Hs00203958_m1 assay (Applied Biosystems) containing primers that generate an amplicon of 64 bp from the NM_014009.2 transcript of the human FoxP3 gene and a FAM dye-labeled TaqMan MGB probe. FoxP3 mRNA expression in each sample was calculated using the relative quantitation method, with human β -actin mRNA from the same sample used as endogenous control.

Results

The H4R agonist 4MH reduces airway hyperreactivity and inflammation in a mouse model of allergic inflammation

The model used for sensitizing and challenging mice for the induction of allergic asthma is shown in Fig. 1A. OVA-sensitized mice were challenged with aerosolized OVA on 6 consecutive

³ Abbreviations used in this paper: HTMT, histamine-trifluoromethyl-toluidide; 4MH, 4-methylhistamine; BAL, bronchoalveolar lavage; NWNAT, nylon wool nonadherent T cell; Treg, regulatory T; AHR, airway hyperreactivity.

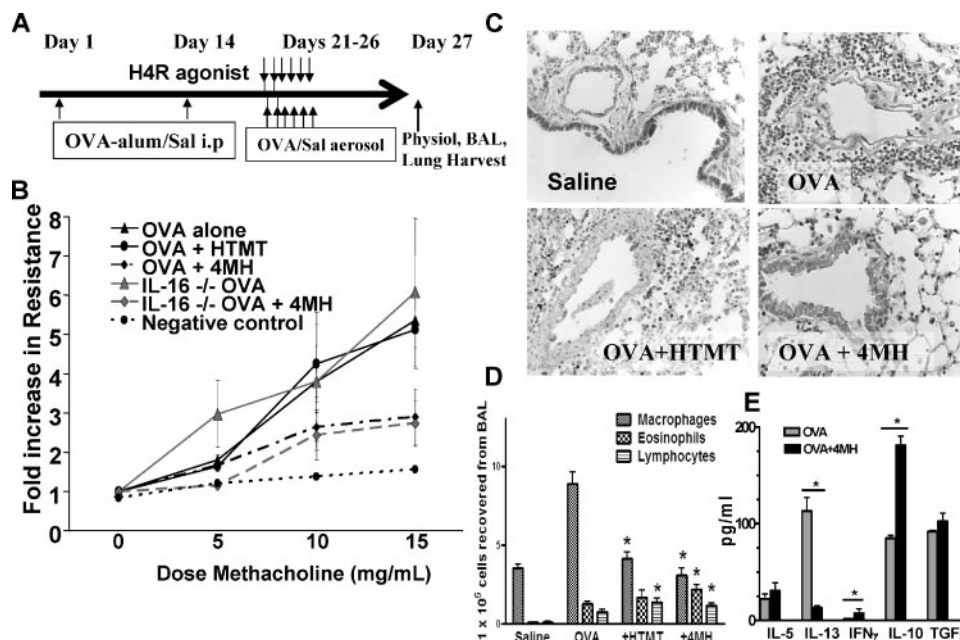


FIGURE 1. A, Experimental protocol. Wild-type (WT) and IL-16^{-/-} (knockout (KO)) mice were sensitized to OVA and then on 6 consecutive days received histamine receptor agonists (10 μ g in 100 μ l saline) or saline alone by the intratracheal route 30 min before each aerosolized OVA challenge. B, WT and IL-16^{-/-} mice were assessed for changes in airway physiology following exposure to HTMT, 4MH, or saline as described in A. Assessment of physiology was determined by measuring airway resistance using the FlexiVent apparatus with increasing concentrations of methacholine. The data are expressed as fold increase above baseline for each mouse. There was a statistical difference between values for OVA + 4MH compared with OVA alone or OVA + HTMT, $p < 0.05$. There was no difference between WT and IL-16^{-/-} mice treated with 4MH. C, Following airway physiology assessment, some lungs were harvested, processed for histology, and stained with H&E to determine inflammation. Representative sections are shown. D, Following airway physiology, some lungs were lavaged with 1 ml of PBS. Cytospins were performed and cell counts and differentials conducted. E, The BAL fluid from OVA or OVA + 4MH-treated mice was also assessed for levels of cytokines IL-13, IL-5, IFN- γ , TGF- β , and IL-10 for each group. Graphs represent the average of four separate experiments with five animals per group for each experiment. *, Statistically different from numbers obtained with OVA alone, $p < 0.05$.

days before assessment of airway physiology. This model results in increased airway hyperreactivity to aerosolized methacholine as well as histological evidence of airway inflammation. To test the effects of H4R stimulation, 30 min before OVA challenge on each of the 6 days, the mice received intratracheal instillation of either saline, an H1R (HTMT) or H4R (4MH) agonist. On day 27 of the model, physiology was assessed for airway resistance. As shown in Fig. 1B, at both the 5 and 10 mg/ml dose of methacholine, mice receiving H1R agonist demonstrated increased airway resistance that was similar to the positive control mice receiving OVA alone. Mice receiving the H4R agonist demonstrated significantly less airway resistance at both the 10 and 15 mg/ml dose. This data indicate that H4R stimulation in the lung reduces airway resistance. To determine the potential secondary effect through the induction of IL-16, the experiment was then conducted in the IL-16^{-/-} mouse. As shown in Fig. 1B, there was no difference in airway resistance between the wild-type mouse and the IL-16^{-/-} mouse following treatment with the H4R agonist 4MH, suggesting that under these conditions the effects of IL-16 were not contributory. In addition, ELISA assessment of BAL fluid from H4R agonist-treated mice indicated undetectable levels of IL-16 (data not shown), suggesting that either the concentration of H4R agonist was not sufficient to induce secretion of IL-16 or that production of IL-16 was insufficient for ELISA detection as well as for detection of a biological effect in the model.

To determine the effects of H4R stimulation on development of inflammation, BAL fluid was analyzed from all mice in each group for cell counts and cell differentials as well as expression of cytokines. Lung tissue was processed for histological staining and lung sections were homogenized to evaluate immune cell infil-

trates. As shown in Fig. 1C, OVA challenge in sensitized mice induced the expected increase in airway hyperplasia, as well as perivascular and peribronchial inflammation. The inflammation was not markedly different in mice also challenged with the H1R agonist. However, in mice treated with H4R agonist the overall inflammatory response appeared to be reduced (Fig. 1C).

To more accurately quantitate changes in cellular infiltrates, cell counts and differentials were performed from the BAL fluid. As anticipated, cell differentials indicated a significant increase in T cells and eosinophils induced in the OVA model as compared with saline control (Fig. 1D). Interestingly, the number of T lymphocytes increased significantly with both the H1R and H4R agonist treatment. The number of eosinophils also increased in the OVA-H4R agonist-challenged mice as compared with OVA treated alone. This effect was not surprising as H4R agonists have been reported to induce eosinophil chemoattraction (17). The H1R agonist had no additional effect on eosinophil recruitment (Fig. 1D). An unexpected finding was the reduction in monocytes/macrophages with both the HTMT and 4MH treatment. The changes in lung-associated T cells were then assessed in lung homogenates following tissue digest and negative selection for CD4⁺ cells. As shown in Fig. 1D, the addition of H4R agonist induced a significant increase in detectable CD4⁺ T lymphocytes which were comparable to that seen in the BAL fluid.

To further address the apparent contradiction between H4R-induced lower airway hyperreactivity (AHR) with increased eosinophil and T cells, cytokines obtained from the BAL fluid were assessed. As shown in Fig. 1E, H4R treatment induced a significant reduction in IL-13 detected in the BAL fluid, but this was not representative of all Th2 cytokines as IL-5 levels were slightly

Table I. Profile of cells recovered on BAL from mice following intratracheal histamine receptor agonists^a

Condition	Total Cells ($\times 10^5$) ^b	(% Cells)		
		Macrophages	Eosinophils	Lymphocytes
Saline	4.1 $\pm$ 0.3	3.54 $\pm$ 0.25 (86%)	0.09 $\pm$ 0.01 (2%)	0.11 $\pm$ 0.01 (3%)
HTMT ^c	5.9 $\pm$ 0.5	4.87 $\pm$ 0.22 (83%)	0.15 $\pm$ 0.08 (3%)	0.61 $\pm$ 0.06* (10%)
4MH ^d	5.1 $\pm$ 0.4	3.07 $\pm$ 0.06* (60%)	1.27 $\pm$ 0.14* (25%)	0.55 $\pm$ 0.12* (11%)

^a Cell counts and percentage of total cells following histamine receptor agonist instillation on 5 consecutive days.

^b Counts shown are mean $\pm$ SD cells ($\times 10^5$). *, Statistically different from saline values, $p < 0.05$. The numbers were obtained from three different experiments with $n = 4$ /group/experiment.

^c HTMT, an H1R agonist, 10 μ g in 100 μ l of saline.

^d 4MH, an H4R agonist, 10 μ g in 100 μ l of saline.

elevated. IFN- γ levels remained low but were significantly increased above control levels. Interestingly, levels of IL-10 in mice treated with 4MH were approximately twice those of control (mean of 183 $\pm$ 47 pg/ml vs 86 $\pm$ 16 pg/ml, $p = 0.02$), while there was no statistical difference in TGF- β levels between the groups (Fig. 1E).

The H4R agonist 4MH recruits FoxP3⁺ cells to the mouse lung

The observations that H4R agonist treatment resulted in reduced AHR with increased eosinophils and T cells, but lower levels of IL-13, indicated the presence of a suppressive effect. This effect was independent of the generation of IL-16 and therefore suggested the possibility of a direct *in vivo* effect by the H4R agonist on T cell recruitment. To investigate this, 6- to 8-wk-old BALB/c mice received daily intratracheal injections of 4MH (10 μ g in 100 μ l of 1 $\times$ PBS), HTMT (10 μ g in 100 μ l of 1 $\times$ PBS) or 100 μ l of 1 $\times$ PBS alone on 5 successive days. On day 6, BAL was performed with 1.0 ml of 1 $\times$ PBS, with an average recovery rate of 80%. Cellular assessment of the BAL fluid indicated a significant increase in the number of eosinophils in mice that received 4MH (25 vs 2% in control) (Table I). There was also an increase in the number of BAL lymphocytes (11 vs 3% for the control). A comparable increase in T cells was observed following HTMT instillation, with no discernable effect seen for eosinophils. Lungs were then removed and cells recovered after lung digest. Cells were stained for CD4 and CD25 expression with analysis by flow cytometry. As shown in Fig. 2, both H1R and H4R agonists induced an influx of CD4⁺ T cells into the lungs. There was a significant

increase in the percentage of CD4⁺CD25⁺ T cells from 6.7 $\pm$ 1.3% in control mice, to 18.3 $\pm$ 3.2% in the lungs of mice that had received 4MH ($p < 0.05$, Fig. 2A). Instillation of H1R agonist also induced a rise in CD4⁺CD25⁺ T cells to 11.2 $\pm$ 2.1%.

Because H4R agonist treatment resulted in a suppressive effect on OVA-induced AHR and inflammation, the T regulatory marker FoxP3 was also assessed. To best identify FoxP3 staining, CD4-positive cells were isolated from whole lung digest by negative selection and stained for CD25 and intracellular FoxP3 expression (Fig. 2B). Lungs from control mice contained 1.5% CD25⁺FoxP3⁺ cells. This was unchanged in the H1R-stimulated lungs, despite a significant increase in CD25⁺ cells (Fig. 2, B and C). However, there was a 3-fold increase in the percentage of CD25⁺FoxP3⁺ cells in the CD4 population of mice exposed to the H4R agonist 4MH compared with control mice or mice exposed to H1R agonist. Approximately 87% of the FoxP3⁺ cells were contained within the CD25^{high} population, consistent with a Treg phenotype.

Histamine induces T cell migration

The *in vivo* data suggested that histamine receptors H1R and H4R could transduce a migratory signal in T cells. To more fully define this effect, and to better characterize the responding cell population, *in vitro* migration assays were conducted using human lymphocytes. Human cells were used due to the requirement for larger numbers of cells to conduct the migration assays, not easily obtainable using murine cells. Freshly isolated T cells, prepared from the peripheral blood of healthy human volunteers were placed in

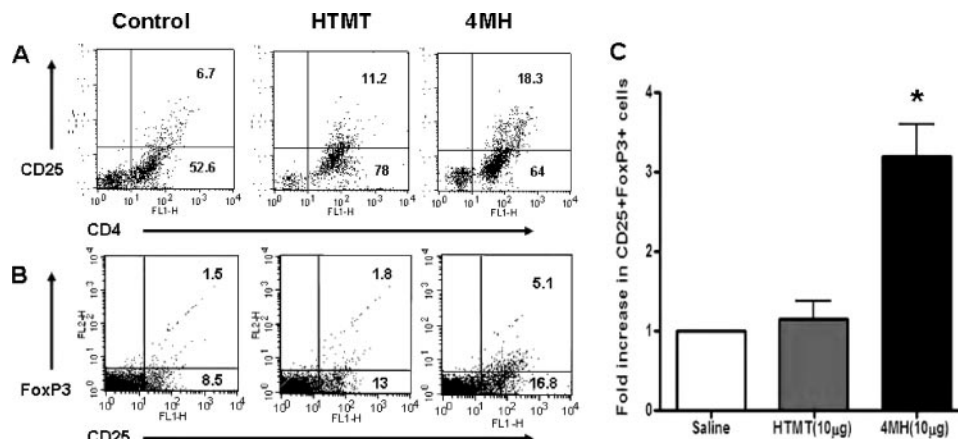


FIGURE 2. Agonists for histamine-1 (HTMT) or H4 (4MH) receptors (10 μ g in 100 μ l saline for both) or saline alone (Ctrl) were delivered by intratracheal injection to 12-wk-old BALB/c mice daily for 5 consecutive days before BAL and lung digestion. **A**, Representative scatter plots gated on lymphocytes of CD4 and CD25 staining cells. **B**, CD4⁺ cells were extracted by negative selection from whole lung digest and then stained for CD25 and intracellular FoxP3 expression. Representative scatter plots are shown. **C**, A histogram demonstrating the average $\pm$ SD for CD25⁺FoxP3⁺ cells detected in control, H1R, or H4R instilled mice. The data have been generated from three separate experiments with three mice in each group. *, Statistically different from saline controls, $p < 0.05$.

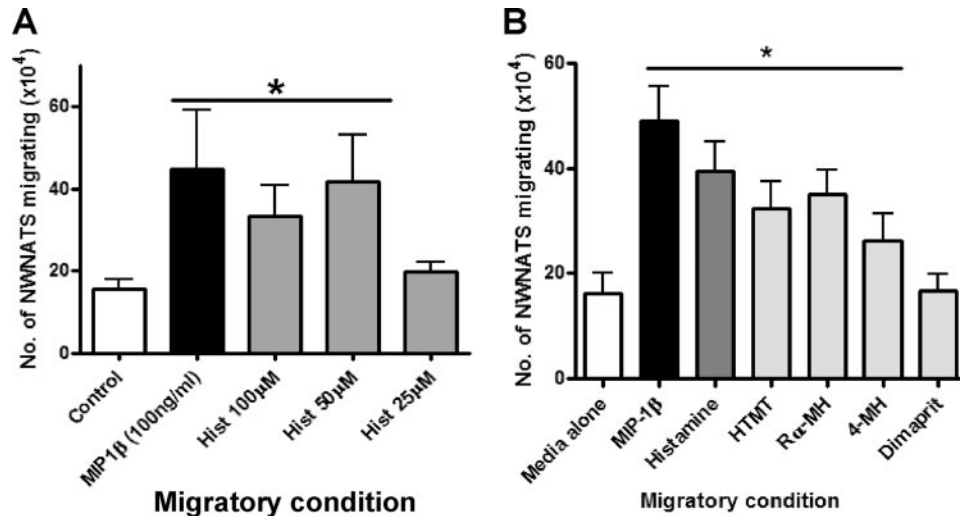


FIGURE 3. Human T cells were subjected to the chemotaxis assay as described in *Materials and Methods*. One $\times 10^6$ freshly isolated T cells were placed in the upper wells of Transwell chambers with the stimulants placed in the lower chamber. **A**, Migration was initially assessed following stimulation with histamine at concentrations of 1 mM to 25 μ M, MIP-1 β (100 ng/ml), or control medium. **B**, Human T cells were stimulated for Transwell migration with selective histamine receptor agonists: HTMT (H1R), dimaprit (H2R), R α -MH and 4MH (H4R agonists) (all were used at 50 μ M), MIP-1 β (100 ng/ml), or control medium. Following a 2-h incubation, cells were harvested from both upper (nonresponsive) and lower (migratory) wells and counted. For these studies, an average of 17.4×10^4 cells $\pm 2 \times 10^4$ were counted under control migration. The graphs display the average $\pm$ SD from four independent experiments performed in triplicate. *, Statistically different from control cell migration, $p < 0.05$.

the upper wells of Transwell chemotaxis chambers. To initially investigate the migratory response, medium alone (negative control), histamine (100 μ M to 25 μ M), or MIP-1 β (100 ng/ml, positive control) were used as the stimulants. As shown in Fig. 3A, histamine, at a concentration range from 100 to 50 μ M, induced migration of T cells that was 2- to 2.5-fold above control and comparable to that seen with MIP-1 β . Using the same system, we then investigated the relative contributions by H1R (10–100 μ M) and H4R (10–100 μ M) agonists, as well as the potential for H2R (10–100 μ M) induced migration. It should be noted that we have previously demonstrated that T lymphocytes do not express HR3 (14). Significant migration of T cells above control levels was observed for the H1R agonist HTMT (3- to 4-fold increase). H4R agonists 4MH and R α -MH also induced a migratory response that was 2- to 3-fold above control migration (Fig. 3B). In contrast, there was no migration of cells to the selective H2R agonist dimaprit (Fig. 3B). The addition of anti-IL-16 Ab to the lower well, in combination with the selective H1R or H4R agonists, had no effect on total cell migration (data not shown). This indicates, similar to the *in vivo* findings, that histamine agonist are stimulating a direct effect on T cell migration and under these conditions there is no contribution by IL-16 to the migratory response.

Cells migratory to H4R agonist are enriched for T cells with a regulatory phenotype expressing FoxP3

As the *in vivo* model suggested that H4R-recruited cells to the lungs were suppressive on the inflammatory response, the potential that H4R induced migration in a T regulatory cell subset was investigated. Using the two most selective histamine agonists that induced T cell migration (HTMT for H1R and 4MH for H4R) the responding cell population was initially characterized for CD4 vs CD8 expression. As shown in Fig. 4, neither stimulant had a selective effect on T cell subsets as the percentage of CD4 and CD8 cells was comparable to control cell migration. We next stained for expression of CD25, the α -chain of the IL-2R, to evaluate for the activation level of the cells. We found a 2-fold increase in CD25 $^+$ CD4 $^+$ T cells in the population migratory to the H4R ag-

onist 4MH, while H1R-responsive cells demonstrated a 1.5-fold increase (Fig. 4A). This increase in CD25 $^+$ cells following H4R stimulation was a result of increased migration rather than induction of CD25 expression as incubation of T cells with the H4R agonist for up to 24 h did not induce detectable changes in CD25 expression (data not shown). As CD25 is considered to be marker of regulatory T cells, we subsequently performed intracellular staining for FoxP3 expression. As shown in Fig. 4A, ~2% of the migratory cells under control conditions were FoxP3 $^+$. This is the same percentage as detected in the T cells before migration. Therefore, migration alone has no selective effect of FoxP3 $^+$ cells. There was also no change following migration induced by H1R agonist (Fig. 4, A and B). There was, however, a significant increase in FoxP3 $^+$ cells responding to the H4R agonist ($2.6 \pm 0.87\%$ for control migration vs $11.2 \pm 2.1\%$, $p < 0.005$). This effect on FoxP3 expression was not detected in the nonresponsive cell population (Fig. 4A). Therefore, despite H1R and H4R ligands inducing comparable overall increase in T cell migration, only stimulation by the H4R agonist affected the number of migratory FoxP3 $^+$ cells.

Histamine and H4 agonists do not induce FoxP3 expression in T cells

The lack of induction of FoxP3 in H4R-nonresponsive cells suggested that the observed increase in the FoxP3 $^+$ CD25 $^+$ cells responsive to the H4R agonist was due to a selective migratory response as opposed to *de novo* induction of this transcription factor. To investigate this further, freshly isolated human T cells were cultured in the presence or absence of 4MH (50 μ M) for times ranging from 30 min to 24 h. The cells were harvested, RNA was extracted, and FoxP3 mRNA was quantified using real-time RT-PCR. We found no evidence of inducible expression of FoxP3 under these conditions (Fig. 4C), indicating that the enrichment of FoxP3 $^+$ cells was a result of selective migration.

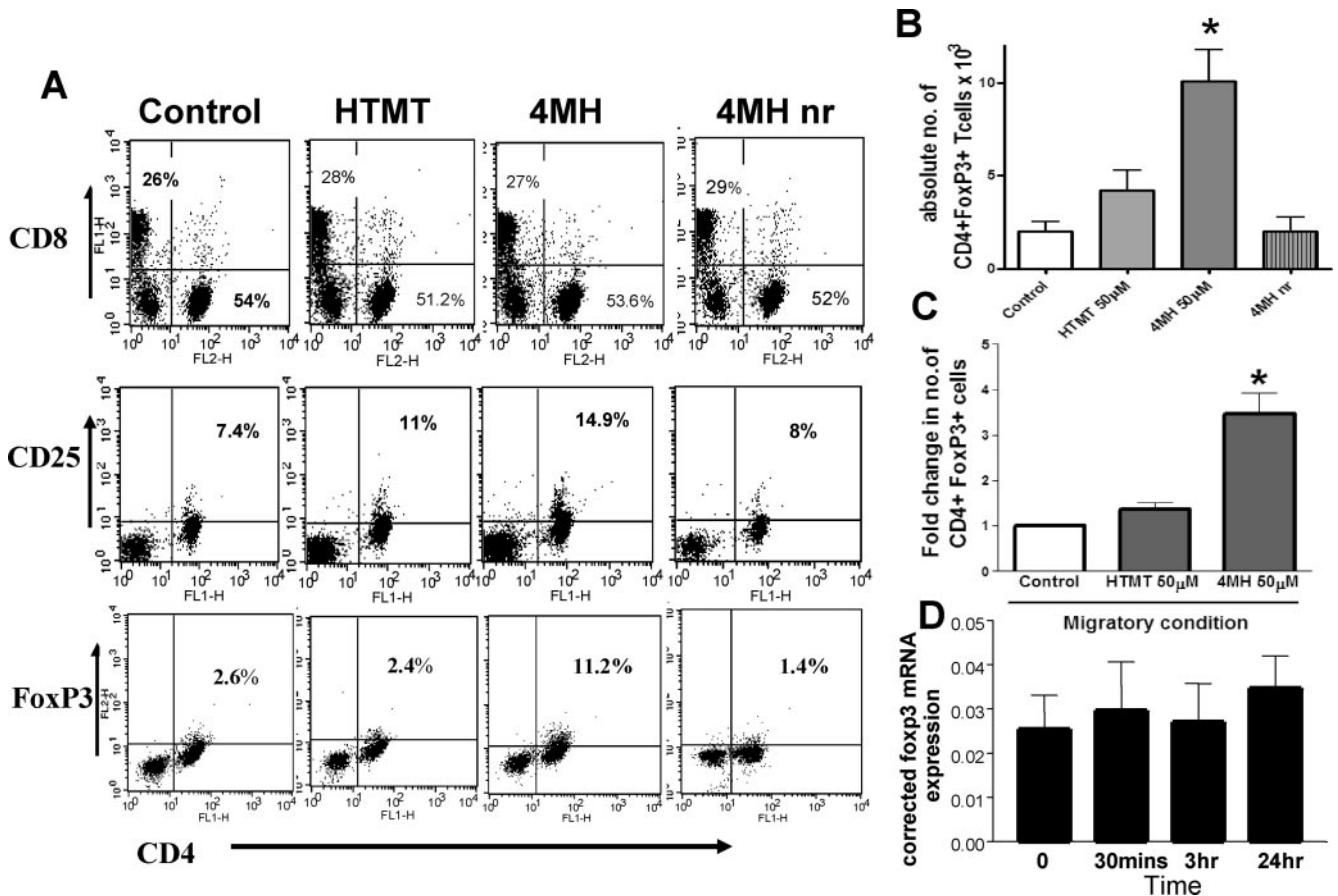


FIGURE 4. Cells migratory under control conditions or to the H1R agonist HTMT and the H4R agonist 4MH were collected from the lower wells of Transwell chambers, stained for cell surface markers, and analyzed by flow cytometry. Cell staining for 4MH-responsive cells (lower chamber) was compared with nonresponsive (nr) cells that were collected from the top chamber. *A*, Representative scatter plots demonstrating CD4/CD8 staining (*top row*), CD4/CD25 (*middle row*), and CD4/FoxP3 (*bottom row*) are shown. *B*, The histogram depicts the averaged values $\pm$ SD for CD4⁺FoxP3⁺ cell staining from five separate experiments performed in triplicate induced under control conditions or stimulated with HTMT or 4MH (50 μ M for both). *, Statistically different from control values at $p < 0.05$. *C*, The fold increase in FoxP3⁺ cells following HTMT or 4MH stimulation. *D*, Freshly isolated T cells were cultured with 4MH (50 μ M) for up to 24 h before assessing for FoxP3 mRNA by quantitative real-time PCR. The data represent the average from three separate experiments.

Cells migratory to H4R agonist suppress proliferation of autologous T cells through release of IL-10

There is a strong correlation between expression of FoxP3 and suppressor activity; however, this association is not definitive. Therefore, suppression assays were conducted to establish that H4R-induced migration selectively enriched for T regulatory cells. These assays investigated the ability of responding human T cells (NWNAT) to suppress the proliferative response of autologous T cells following stimulation by anti-CD3. In these experiments, we used total migratory cell populations from the bottom wells of the Transwell chamber, added back at different ratios to 3×10^5 autologous T cells. As shown in Fig. 5A, cells collected from control wells as well as HTMT (H1R) stimulated wells did not alter proliferation following anti-CD3 stimulation as compared with the response of autologous cells alone. There was no effect at either the 1:1 or 1:10 ratio of migratory cells to autologous T cells. In contrast, a dose response of H4R-induced migrating cells indicated that at both the 1:1 and 1:2 ratios, proliferation was suppressed by 50%. This effect was not seen at ratios of 1:10 or 1:100 (Fig. 5A), nor was it observed for cells remaining in the upper well of the Transwell after the 2-h time course (4MH nonresponsive cells, Fig. 5A). To demonstrate that the suppressive activity was attributable to the CD25⁺ cells, which were mostly FoxP3⁺, these cells were

deleted by positive selection and magnetic bead extraction. The resultant CD25[−] cell population was added to autologous cells at a ratio of 1:1. These cells were unable to induce any suppressive effect (Fig. 5A). These data indicate that H4R stimulation induces an enhanced migratory cell population of CD25⁺ T cells in which the majority are functional FoxP3⁺ Treg cells.

Treg cells have a number of proposed mechanisms for inducing suppression. One mechanism is through the production of the regulatory cytokines IL-10 and TGF- β . In addition, the immunomodulatory cytokine IL-16 is released from T cells in response to histamine (14). We looked at the profile of these cytokines by ELISA in the supernatants of cells migratory to the H4R agonist that had been stimulated with anti-CD3 Ab for 72 h. Ten $\times 10^4$ cells were used in each culture. After 72 h, there was an increase in production of IL-10 (11.2 ± 1.7 pg/ml) for H4R migration vs undetectable levels for control cells (Fig. 5B). TGF- β levels were undetectable in all conditions and levels of IL-16 were not increased by H4R stimulation.

These data suggest that IL-10 may be involved in facilitating the suppressive effect induced by the H4R-responsive Treg cells. To address this, proliferation assays were conducted in which autologous T cells were resuspended in supernatants from cultures containing migratory control cells, H1R migratory cells, or H4R migratory cells.

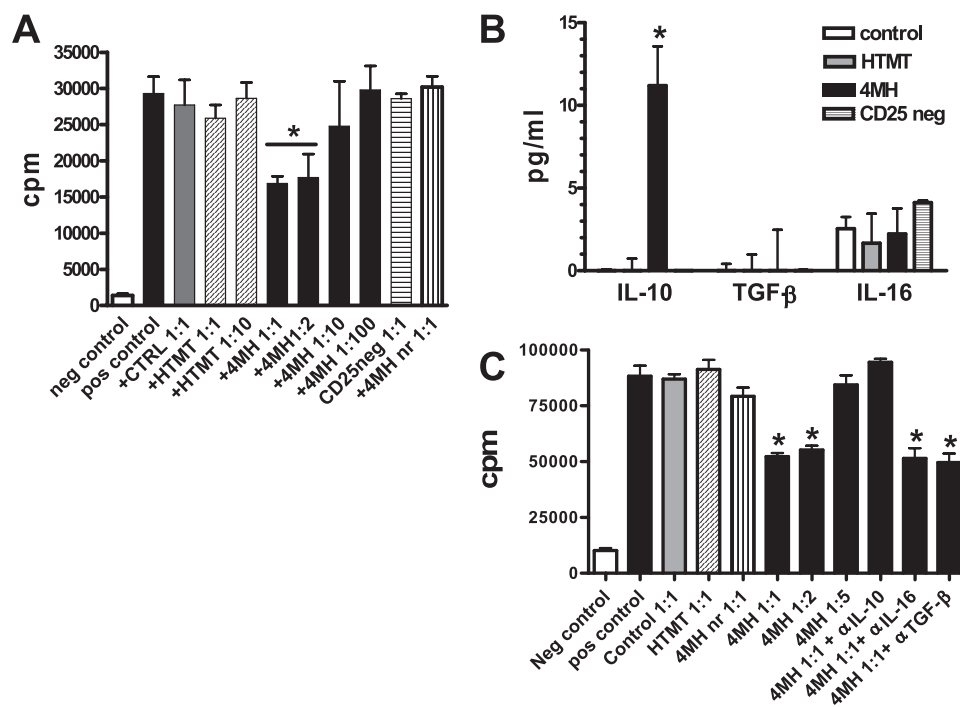


FIGURE 5. Human T cells harvested from the top and bottom chambers following migration to control buffer, HTMT, or 4MH were cocultured with autologous cells in the presence of anti-CD3 Ab. Proliferation was determined after 72 h by [^{3} H] incorporation as measured by β scintillation counting and cytokines were assessed by ELISA. **A**, The proliferative responses of autologous cells mixed at the designated ratios with migratory cells following HTMT or 4MH stimulation, or under control conditions. CD25-depleted cells following 4MH-induced migration, and nonresponsive cells to 4MH-induced migration, were also assessed for effects on proliferation (*last two columns*). **B**, Cytokine profile recovered from supernatants of migratory cells following 48 h of culture with 1 μ g/ml anti-CD3 Ab. Cytokines were assessed by commercial ELISA kits. **C**, Migratory cells from control wells or following stimulation with either HTMT or 4MH; or cells nonresponsive to 4MH (4MH nr) were cultured on anti-CD3-coated plates for 48 h before harvesting the supernatant. The supernatants from each culture condition were added to cultures of autologous T cells at the designated ratio with a final volume of 500 μ l. In some wells, Abs to IL-10, TGF- β , or IL-16 were also added. The data represent the average values $\pm$ SD from four separate experiments. *, Statistically different from control conditions at $p < 0.05$.

The supernatants were added at a 1:1 ratio with medium containing the autologous cells. Abs to TGF- β (5 μ g/ml), IL-10 (5 μ g/ml), or IL-16 (5 μ g/ml) were also added to the cultures. The addition of either anti-TGF- β or IL-16 had no effect on altering suppression induced by the H4R-responsive cells (Fig. 5C). The TGF- β and IL-16 Abs were also added together in culture with no detectable effects on suppression (data not shown). In contrast, Abs to IL-10 completely blocked the suppressive effect (Fig. 5C). This indicates that the suppressor activity induced by the H4R-responsive Treg cells was mediated by secretion of IL-10. This data would be consistent with the elevated levels of IL-10 detected in the BAL fluid following treatment with the H4R agonist in the OVA model (shown in Fig. 2). Although many groups have reported on the suppressive effects of IL-10 for T cell proliferation (20, 21), we wanted to confirm this in our culture system. A dose response of rIL-10 was added to parallel cultures of autologous cells as was used for the supernatant studies described above. As expected, the IL-10 at concentrations >10 pg/ml suppressed the proliferative response induced by anti-CD3 Ab (data not shown).

Discussion

The vasoactive amine histamine is released by mast cells and basophils in the lungs following allergen challenge and induces a variety of effects including vascular leak, smooth muscle contraction, and elevated immune responses. As part of the immune response, histamine stimulation of T cells results in secretion of IL-16; a CD4 ligand which mediates inflammation in a number of different disease states (22). Histamine-induced release of IL-16 is facilitated through interaction with histamine receptor subclasses, H2R and H4R (14).

Because IL-16 has been shown to inhibit inflammation and AHR in a murine model of allergic asthma (19), we initially investigated whether H4R-induced release of IL-16 would result in the same inhibitory effect. Treatment with H4R agonist did result in attenuation of both overall airway inflammation and AHR; surprisingly, however, this effect was independent of IL-16 as there was no difference in effectiveness between wild-type mice and IL-16 knockout mice. Treatment with both H1R and H4R agonists resulted in an increase in T cells with an unexpected loss of macrophages. The histamine-induced mechanism for reduced macrophage recruitment has not been determined; however, it likely represents a secondary effect as lung CD14 $^{+}$ monocytes lack the H4R (14). Airway instillation of the H4R agonist did induce an increase in T cells as well as the expected increase in eosinophils in the lungs of these mice. Analysis of the BAL fluid indicated that there was a significant decrease in IL-13 with an increase in both IFN- γ and IL-10. These observations suggested an induced suppressor effect of H4R and led us to investigate the potential direct effect of H4R agonist stimulation on the motility of T cell subsets. Interestingly, while IL-13 was inhibited by H4R agonist treatment, IL-5 levels were unchanged. This may suggest that IL-5 production in this model is non-T cell derived and may result from the recruited eosinophils or resident mast cells.

Histamine binds to four different receptor subtypes (H1R–H4R) and with the advent of selective histamine receptor agonists it has become clear that different functions can be ascribed to each of the four receptors. In the present study, we show that histamine is a lymphocyte chemoattractant factor in vivo as well as in vitro,

which can mediate migration through either the H1R or H4R receptors, but not via interaction with H2R. Although both receptors can induce a migratory signal, the responding cell populations are phenotypically different for each receptor. Stimulation of H1R has previously been shown to induce migration predominantly in Th2 cells (23). Although we did not phenotype the cells responsive to H1R stimulation, our data would support a selective Th2 effect that contributed to the inflammatory response. The present study does indicate that ligation of H4R with the selective H4R agonist 4MH, but not H1R ligation by HTMT, enriches for a Treg cell population. These cells express high levels of CD25 and the Treg-associated transcription factor, FoxP3. These cells function as suppressor cells with inhibitory activity on autologous T cell proliferation. Suppression was mediated by secreted factors rather than through a contact-dependent mechanism as supernatant alone from cells migratory to 4MH retained the ability to suppress T cell proliferation to a similar level as cocultures with the Treg cells. The suppressive effect was completely dependent on IL-10 and independent of either TGF- β or IL-16. Although these studies focused on preferential recruitment of Treg cells mediated by 4MH, a percentage of the cells were not FoxP3⁺ classical Treg cells. The contribution of these cells to the regulatory process of immune function may also represent a critical component and phenotypic analyses of these cells are currently ongoing.

Regulatory T cells, defined by their ability to control T cell proliferation *in vitro* and actively suppress the immune response, are currently the subject of intense investigation in allergic disease (24–28). Although numerous classifications exist, two broad categories can be considered: naturally occurring, thymically derived CD4⁺CD25⁺FoxP3 (natural Tregs) and Ag-specific CD25⁺ Tregs that are induced in the periphery, have a variety of phenotypes and appear to function *in vivo* by the secretion of IL-10 and/or TGF- β (26). Our data would suggest that H4R stimulation results in recruitment of the inducible Treg population; however, 4MH alone is insufficient to induce expansion of these cells. There is also mounting evidence that deficiency in number or function of Treg cells contributes to common allergic disease and asthma (25–31). Inhibition of proliferation by CD4⁺CD25⁺ T cells from atopic compared with normal donors is reported to be reduced (28) and glucocorticoids, the most effective treatment for allergy, induces Treg cell activation (29).

In mice, transfer of allergen-specific CD4⁺CD25⁺ T cells has been shown to resolve AHR and inflammation (30). In our animal experiments, we cannot definitively conclude that the relationship between recruitment of FoxP3-positive cells and reduction in AHR and airway inflammation in response to local delivery of a H4R agonist is directly causal. It is possible that the H4R agonist is having effects on airway epithelium or nerve cells independent of Treg cell recruitment. However, it is likely that the protective activity is mediated by inhibition of T cell-driven up-regulation of airway immune response. In support of this, a recent study found that chronic local allergen exposure was associated with the induction of airway mucosal Treg cells resulting in functional silencing of Th2 cells and the prevention of airway hyperresponsiveness (31).

In these studies, we used selective agonists of histamine receptors in preference to histamine receptor antagonists, on which much of the literature of the physiological effects of histamine is based. The H4R agonist used in the current study, 4MH, was recently identified as the first potent, high-affinity H4R ligand (K_i = 50 nM) that has a >100-fold selectivity for the histamine H4R over the other histamine receptor subtypes (32). Consistent with our findings, a recent study identified that mice in the OVA asthma model treated with a selective H4R antagonist, JNJ777120, ex-

hibited decreased eosinophil and T cell recruitment into the lung, as detected from BAL fluid staining (33). Interestingly, some of the findings using the H4R antagonist were similar to those we observed here with a H4R agonist, such as a decrease in Th2 cytokine presence in the BAL fluid. The differences in the two studies may reside in the route of administration. Their data were generated using oral gavage administration which would result in elevated systemic levels of H4R antagonist while we used direct local administration of the H4R agonist which would result in a concentration gradient within the lung allowing for directed migration of the Treg cells, likely not possible with elevated systemic levels. Both studies are very encouraging, however, in demonstrating that the H4R may be a pivotal receptor for modulating the asthmatic response in the lung as it has been shown in other models of inflammation (34).

As manipulation of Treg cells is a potential target in allergic disease, knowledge of factors that may recruit these cells is important. To date, Treg cells have been found to express the chemokine receptors CCR4 and CCR8 (35) which respond to the chemokines macrophage-derived chemokine/CCL22, thymus and activation-regulated chemokine/CCL17, I-309/CCL1, and to viral MIP-I (35). Our results demonstrate that ligation of H4R is another mechanism for recruiting FoxP3⁺ Treg cells into the lungs and therefore represents another potential therapeutic target.

Disclosures

The authors have no financial conflict of interest.

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