

ORIGINAL ARTICLE

United airways: circulating Th2 effector cells in an allergic rhinitis model are responsible for promoting lower airways inflammation

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Summary

Background Allergic rhinitis (AR) and asthma often coexist and are referred to as 'united airways' disease. However, the molecular and cellular pathways that are crucially involved in the interaction between upper and lower airways remain to be identified.

Objective We sought to assess whether and how AR exacerbates lower airway inflammation upon allergen challenge in mice.

Methods We previously developed an intranasal ovalbumin (OVA)-driven AR model, characterized by nasal eosinophilic inflammation, enhanced serum levels of OVA-specific IgE and Th2 cytokine production in cervical lymph nodes. In OVA-sensitized mice with or without AR, a lower airway challenge was given, and after 24 h, lower airway inflammation was analysed.

Results We found that AR mice were more susceptible to eosinophilic inflammation following a lower airway OVA challenge than OVA-sensitized controls. AR mice manifested increased numbers of eosinophils in bronchoalveolar lavage fluid and increased inter-cellular adhesion molecule-1 (ICAM-1) expression on lung endothelium, when compared with OVA-sensitized controls. Depletion of T cells in OVA-challenged AR mice completely abrogated all hallmarks of lower airway inflammation, including enhanced IL-5 and tissue eosinophilia. Conversely, adoptive transfer of Th2 effector cells in naïve animals induced lower airway eosinophilic inflammation after challenge with OVA. Blocking T cell recirculation during AR development by the sphingosine-1 analogue FTY720 also prevented lower airway inflammation including ICAM-1 expression in AR mice upon a single lower airway challenge.

Conclusion Our mouse model of 'united airways' disease supports epidemiological and clinical data that AR has a significant impact on lower airway inflammation. Circulating Th2 effector cells are responsible for lung priming in AR mice, most likely through up-regulation of ICAM-1.

Keywords allergic rhinitis, asthma, ICAM-1, mouse model, Th2 cells

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Introduction

Allergic rhinitis (AR) and allergic asthma are two features of allergy of the respiratory tract. Accumulating data emerged over the last decades that support a connection between upper and lower airway inflammation, often referred to as 'united airways' or 'one airway – one disease' [1, 2]. Airway allergy is a Th2 cell-driven disease that leads to airway hyper-reactivity in both the nose and the lungs and is characterized by airway inflammation with eosinophilic cells and mast cells, as well as histological changes of the epithelium and thickening of the

basal membrane [3, 4]. A close relationship between upper and lower airways is supported by epidemiological data [5, 6]. The prevalence of AR reaches 10%–20% in primary schoolchildren (from age 3 to 5 years) and increases up to 30% in teenagers. AR usually occurs in over 75% of patients with allergic asthma and in an even higher proportion of patients with non-allergic asthma [7]. Asthma patients with only lower airway symptoms are only sporadically seen [8]. AR patients are at a risk for development of concomitant asthma, and one in three AR patients may progress to develop asthma within 10 years [5]. In some cases, the presence of upper airway

inflammation like sinus disease or nasal polyposis renders the clinical course of asthma more severe and treatment more cumbersome [9]. Patient studies suggest that appropriate treatment of upper airway inflammation reduces lower airway symptomatology and improves asthma control [2, 10].

Various mechanisms have been proposed that could explain the nose–lung interaction, including neurological cross-talk and circulation of inflammatory cells or mediators between upper and lower airways [11]. Indeed, cells, cytokines and chemokines from the nose are drained by the systemic circulation and can subsequently affect tissues at a distance. Th2 effector cells which secrete cytokines IL-4, IL-5, and IL-13, play an important role in the initiation and perpetuation of allergic airways disease [12]. In AR, Th2 cells can be detected in blood, suggesting that these cells continuously circulate in the body [12]. Moreover, pathophysiological abnormalities in AR patients with lower airway inflammation are similar to those found in allergic asthma patients [8]. It has been reported that AR patients develop lower airway symptoms after nasal allergen provocation [13]. Conversely, upon segmental broncho-provocation, AR patients show nasal symptoms and an influx of inflammatory cells, including IL-5⁺ effector T cells, eosinophils and basophils, adhesion molecule up-regulation, mast cell degranulation and increased IL-5 levels in the serum [13, 14]. In view of the marked pathophysiological and histological similarities of these two disease processes, and the fact that both are controlled by Th2 cells, the high degree of concordance between asthma and AR is not surprising.

In the present study, we used our recently described mouse model for ovalbumin (OVA)-driven AR to assess the mechanisms of interaction between upper and lower airways. [15]. In this AR model, mice are sensitized via the peritoneal route and show airway eosinophilic inflammation in the upper, but not in the lower airways. We have previously reported that cervical lymph nodes (LN), which drain the upper airways, contained Th2 cytokine-producing T cells, while the lower airway draining mediastinal LN were almost completely unresponsive, illustrating that we induced a compartmentalized allergic Th2 response in the nose only [15]. We sought to assess whether and how the presence of AR in mice exacerbates lower airway inflammation upon allergen challenge. We found that AR mice were more susceptible to the induction of lung tissue eosinophilia by a single OVA allergen challenge in the lower airways than OVA-sensitized control mice without AR. Moreover, we could show the crucial role of T cells by: (I) T cell depletion; (II) blocking T cell migration; and (III) adoptive transfer of Th2 effector cells. Circulating Th2 effector cells are essentially involved in the priming of the lower airways in AR mice, explaining the enhancement of airway inflammation upon local allergen challenge to the lungs.

Material and methods

Mice

Six- to 8-week-old Balb/c female mice were obtained from Harlan (Zeist, the Netherlands) DO11. 10 (Balb/c strain) transgenic animals were obtained from ErasmusMC EDC and housed under SPF conditions at the animal care facility of the Erasmus MC Rotterdam, under approval of the local animal ethics and welfare committee.

A mouse model of allergic rhinitis

To induce AR, we first immunized mice with OVA (10 µg OVA, grade V, Sigma, Zwijndrecht, the Netherlands) emulsified in 2 mg AL(OH)₃ (OVA/alum) intraperitoneally (i.p.), followed by a booster of OVA/alum at day 7. Ten days later, awake-sensitized mice were challenged by instillation of a 10 µL droplet of either OVA (1 mg/mL; AR group) or phosphate-buffered saline (PBS) (sensitized group) per nostril, using a micropipette, on 3 successive days in a week for 3 consecutive weeks [15].

Challenge of the lower airways with ovalbumin antigen

Twenty-four hours after the last intranasal (i.n.) application of either OVA or PBS, awake mice were challenged with either OVA or PBS through a half-an-hour aerosol exposure. Alternatively, in case of bypassing the upper airways, anaesthetized mice received an intratracheal (i.t.) injection of OVA in a volume of 80 µL. Mice were killed, bled, bronchoalveolar lavage (BAL) were performed using 3 × 1 mL of saline solution and the nasal palate containing nasal mucosa tissue and the lungs were snap frozen in freezing solution (TissueTek, Zoeterwoude, the Netherlands). The cervical, mediastinal and axillary LN were collected as described previously [15].

Treatment regimens

Hamster antibodies against CD3 or isotype control (100 µg i.p.) were given 2 days and 1 day before the lower airway challenge. Treatment with FTY720 6 µg i.p. (Cayman chemicals, Bio-connect, Huissen, the Netherlands) was given during the i.n. challenge period three times per week, ending on the day before the last i.n. challenge. The lower airway challenge was given after an FTY720 wash-out period of 13 days and was checked by analysing blood lymphocyte counts, indicating a complete washout.

In vitro lymph node cell restimulation with ovalbumin

Cervical, mediastinal and axillary LN single-cell suspensions (1 × 10⁶/mL) were prepared by pressing the LN through a 100 µm cell strainer. Cells were stimulated with 10 µg/mL

OVA in RPMI supplemented with 5% fetal calf serum, gentamycin and β -mercaptoethanol for 4 days. Supernatants from LN cell suspensions were harvested and stored for cytokine quantification by ELISA.

Enzyme-linked immunosorbent assay

Total and OVA-specific IgE were determined by ELISA as described previously [15]. Concentrations were expressed as relative values, compared with serum taken from OVA/Alum-immunized mice exposed to OVA aerosol three times, which was arbitrarily set to 100 U/mL [16]. Cytokine ELISA systems for IFN- γ , IL-4, IL-5 and IL-13 were from BD PharMingen (BD Immunocytometry Systems, San Jose, CA, USA).

Bronchoalveolar lavage fluid

BAL were performed three times with 1 mL of Ca^{2+} - and Mg^{2+} -free PBS, containing 10 mM EDTA. Cells were collected for cellular differentiation by flow cytometry, as described previously [17]. Briefly, cells were stained with antibodies specific for MHC class II, CD11c, CD3, CD19 and CCR3. Acquisitions and analyses were performed on a FACSCalibur flow cytometer using CellQuest (BD Immunocytometry Systems) and FlowJo (Treestar, Costa Mesa, CA, USA) software. Supernatants of BAL fluid were stored for ELISA.

Immunohistochemical analyses

Immunohistochemical stainings were performed in a half-automatic stainer (Sequenza, Amsterdam, the Netherlands) as described previously [15]. Acetone-fixed slides were blocked in diluted normal goat serum (CLB, Amsterdam, the Netherlands) and stained with rat monoclonal antibodies against mouse MBP (J. J. Lee, Mayo Clinic, Scottsdale, AZ, USA), CD31 or CD54 [inter-cellular adhesion molecule-1 (ICAM-1)]. Primary antibodies were revealed by incubation with diluted appropriate secondary antibodies coupled to alkaline phosphatase for 30 min. Slides were subsequently incubated with New Fuchsin substrate for alkaline phosphatase conjugates. Finally, the sections were counterstained with Gills triple-strength haematoxylin and mounted in vecta mount (New Brunswick, Amsterdam, the Netherlands).

Light-microscopic evaluation

Cells were quantified (blinded) in two sections of each specimen, whereby epithelium and lamina propria were evaluated separately. Numbers of eosinophils were calculated per length basal membrane for the whole epithelium and for 100 μm depth in the lamina propria of the nasal mucosa. ICAM-1 was analysed based on the strength of

the signals in the lungs. Biopsies were ranked 1–28 (in case of $n = 28$) by continuously comparing the biopsies with each other, until all were ranked. A slide with section was taken at random, evaluated and put down. A next slide was randomly taken and graded for stronger or weaker signals, or signals in between the two previous sections. Finally, all sections were analysed and the weakest stained section received rank 1 and the strongest-stained section received rank 28. This method of analysis was previously shown to indicate clear differences in staining intensities [18].

Statistical analysis

For statistical evaluations, Kruskal–Wallis one-way ANOVA was used to calculate the overall P -value. A P -value of <0.05 was considered a significant difference between groups. The non-parametric Mann–Whitney U -test was performed to analyse each group with respect to each other.

Results

Lower airway allergen challenge induces the hallmarks of asthma in allergic rhinitis mice

We used our upper airway allergic inflammation mouse model (AR mice), in which a compartmentalized allergic Th2 response is exclusively induced in the nose [15], to study the effects of upper and lower airway interactions on a lower airway challenge and to identify the underlying mechanism.

First, we wanted to assess the validity of our model for the study of this interaction. We have previously reported that nasal allergen provocation of human AR subjects induces tissue eosinophilia and adhesion molecule expression in upper and lower airways [13, 14]. To induce upper airway allergic inflammation, mice were first i.p. immunized and boosted with OVA, and subsequently challenged by i.n. instillation either of OVA (resulting in AR mice) or PBS (resulting in sensitized control mice). In our system of i.n. application of OVA antigen in awake mice, there is no deposition of antigen in the lower airways. For this, we used the ultrasensitive method of administering mice a cohort of CFSE-labelled OVA-specific CD4^+ TCR transgenic T cells, to be able to trace the occurrence of antigen presentation. Using this system, which is very sensitive to the presence of minute amounts of antigen, we only found divisions of CFSE-labelled T cells in the lymphoid structures draining the upper airway, but not in the mediastinal LN draining the lower airway (Fig. 1).

Twenty-four hours after the last i.n. application of either OVA or PBS, AR or control mice were challenged with either OVA or PBS aerosol exposure, to induce lower airway inflammation (see Fig. 2a for a time

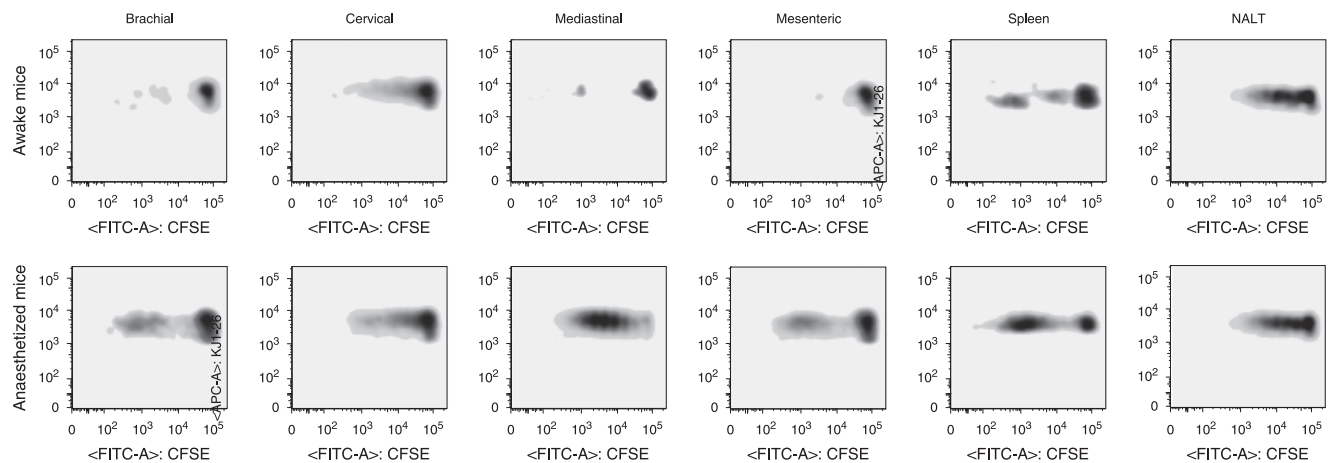


Fig. 1. No deposition of antigen in the lower airways in awake intranasally (i.n.) challenged mouse. CFSE-labelled DO.1110 cells (15e6 cells) were injected intravenously (i.v.). Subsequently, 24 and 30 h later, 10 μ L of ovalbumin (OVA) 1 mg/mL solution in phosphate-buffered saline (PBS) per nostril was applied i.n. in awake animals (upper row) and in anaesthetized animals (second row). In the NALT and Cervical lymph node (LN), clearly primary divisions of KJ1.26⁺ (transgenic OVA-specific CD4⁺ and CFSE⁺) cells could be observed in both awake and anaesthetized animals. In the Mediastinal LN, only primary divisions could be observed in anaesthetized animals. In the spleen, recirculating CFSE⁺ T cells could be observed in both groups. The Brachial and Mesenteric LN only show recirculating CFSE⁺ cells that were already undergoing a third or a fourth division cycle ($n = 4$ animals per group).

schedule). As demonstrated in Fig. 2b, significantly higher percentages and absolute numbers of eosinophils were present in the BAL fluid of the OVA aerosol-challenged AR group, when compared with the sensitized control group. No eosinophilia was detected in the PBS-challenged AR group. Percentages and absolute numbers of BAL fluid T cells were significantly higher in AR animals that were OVA-challenged, than in those that were PBS-challenged (Fig. 2b). However, upon OVA aerosol challenge, there was no difference between AR mice and sensitized control mice. Dendritic cell (DC) numbers were strongly induced in OVA aerosol-challenged AR mice, compared with control-sensitized mice or PBS-challenged AR mice (Fig. 2b).

When cervical LN samples were restimulated with OVA *in vitro* for 4 days and assayed for cytokine production, we observed the increased production of the Th2 cytokines IL-4, IL-5 and IL-13, as well as IL-10, in AR mice, compared with sensitized control mice, whether or not they had received an OVA aerosol challenge (Fig. 2c). IFN- γ was only elevated in PBS-challenged AR animals (Fig. 2c). In the mediastinal LN, significant amounts IL-4, IL-5, IL-10 and IL-13 were observed, but there were no significant differences between the groups (data not shown). Serum IgE data confirmed that all animals were sensitized for OVA (Fig. 2d), and there was a non-significant trend for OVA-specific IgE to be higher in OVA-challenged AR mice. Serum IL-5 levels was detected in all mice challenged with OVA aerosol (AR and sensitized) (Fig. 2d).

From these findings, we conclude that AR mice manifested increased numbers of eosinophils in BAL fluid when compared with OVA-sensitized controls.

In vivo T cell depletion before lung allergen challenge abrogated lower airway eosinophilic inflammation in allergic rhinitis mice

Remarkably, whereas BAL eosinophilia was stronger in OVA-challenged AR mice, when compared with OVA-challenged control mice (Fig. 2b), they had similar serum IL-5 concentrations (Fig. 2d). Although cellular sources of IL-5 include Th2 cells, eosinophils, mast cells and stromal cells, observations by Ogawa *et al.* [19, 20] indicate that T cells are the principal source of IL-5 in murine asthma. In murine asthma studies, depletion of CD4⁺ cells suppressed BAL fluid eosinophilia of wild-type and mast cell-deficient W/W^v mutant mice to a similar degree (77%–94% inhibition), consistent with the notion that the observed eosinophilia was dependent on T cells and IL-5 and independent of mast cells [19, 20].

To address the issue of T cell-dependency in nasobronchial cross-talk, AR and sensitized control mice were treated with anti-CD3 antibodies or isotype control antibodies before they were given i.t. injections of OVA or PBS, thereby bypassing the upper airways. The presence of anti-CD3 antibodies completely abrogated lower airway cellular inflammation, as is evident from the significantly reduced numbers of eosinophils, T cells and DC (Fig. 3a) and significantly lower concentrations of Th2 cytokines IL-4 and IL-13 (Fig. 3b), while for IL-5, only a trend is observed in cervical LN. The numbers of CLN cells and MLN cells were reduced by almost half after treatment with anti-CD3; this means that not only T cells from the circulation but also from local levels were reduced. Treatment with anti-CD3 had only a modest effect on total IgE or OVA-specific IgE in the serum, but completely abrogated the induction of serum IL-5 (Fig. 3c).

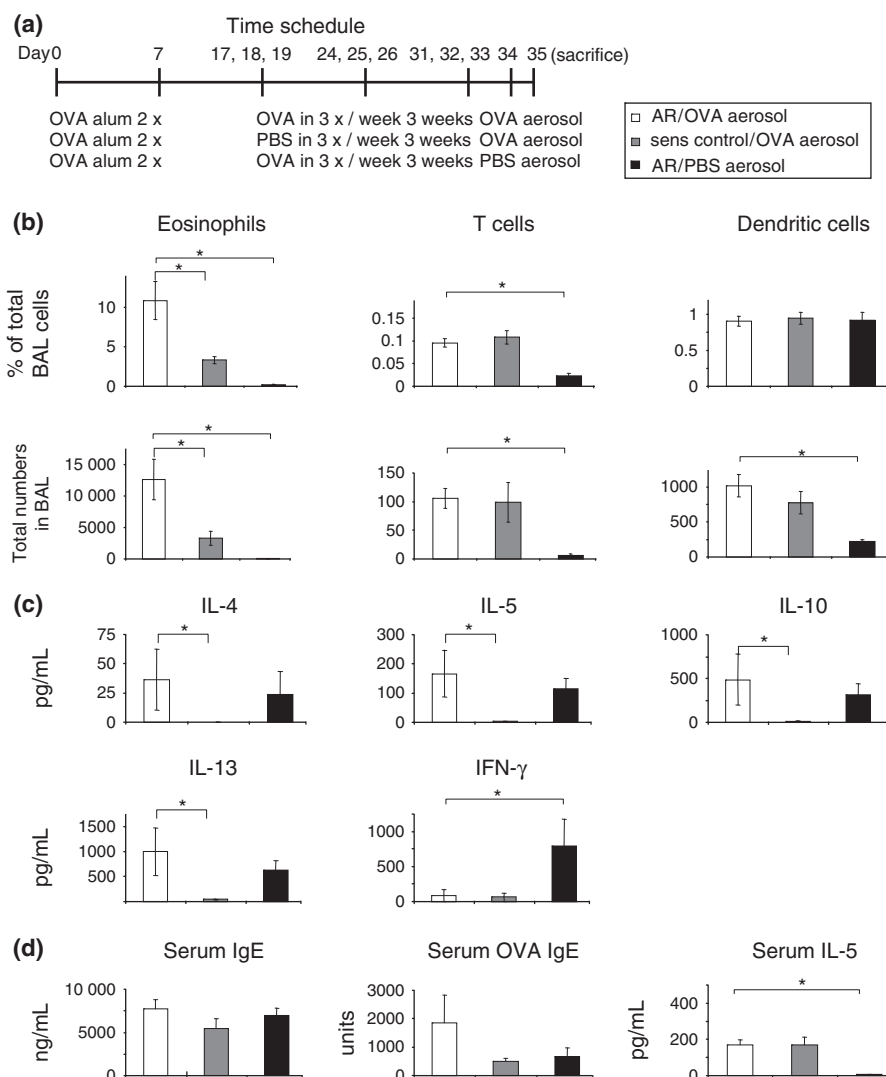


Fig. 2. Inflammation in lower airways appears after ovalbumin (OVA) aerosol challenge in allergic rhinitis (AR) mice. The experiment was set up in such a way that mice first developed AR or sensitized (control) only by first sensitizing them by giving OVA/alum on day 0 and day 7, followed by a challenge with OVA (AR) or phosphate-buffered saline (PBS) (sensitized control) intranasal (i.n.) applications on days 17–19; 24–26; and 31–33. At day 34, mice were divided into two AR groups; one that received an OVA aerosol challenge (OVA i.n./OVA aerosol) and one that received PBS aerosol (OVA i.n./PBS aerosol) and a third control group, which is only sensitized for OVA and received an OVA aerosol challenge (PBS i.n./OVA aerosol). On day 35, mice were killed and sampled (a) Animal treatment time schedule. Bronchoalveolar lavage (BAL) fluid was analysed (b) by flow cytometry for eosinophils, T cells and for dendritic cells (DCs). Cervical lymph node (LN) cell suspensions were restimulated *in vitro* for 4 days with OVA and assayed for cytokine production using an ELISA assay (c). Serum IgE and OVA-specific IgE and serum IL-5 using ELISA assay (d) Data are shown as mean $\pm$ SEM. * $P < 0.05$ ($n = 6$ animals per group).

Taken together, *in vivo* depletion of T cells before lung OVA allergen challenge showed that lower airway eosinophilic inflammation in AR mice, as evidenced by BAL eosinophilia and elevated levels of serum IL-5, is critically dependent on T cells.

Adoptive transfer of ovalbumin allergen-specific T helper type 2 cells is responsible for lower airway inflammation

We previously found a clear Th2 cytokine profile in the upper airway draining LN of AR mice [15]. To study the

role of Th2 effector cells, we cultured total LN and spleen cell fractions from DO11.10 transgenic (Balb/c) mice, in which all T cells express an MHC class II-restricted OVA-specific T cell receptor. Naïve DO11.10 CD4⁺ T cells were activated and expanded in a Th2-polarizing environment in the presence of IL-4 and IL-2 cytokines and antibodies to IFN- γ and IL-12 for 5 days, as described previously [21]. *In vitro* cultured Th2 effector cells or freshly isolated naïve DO11.10 T cells were injected into the tail vein of naïve (Balb/c) mice. Six hours later, animals were i.t. challenged with OVA. A significant

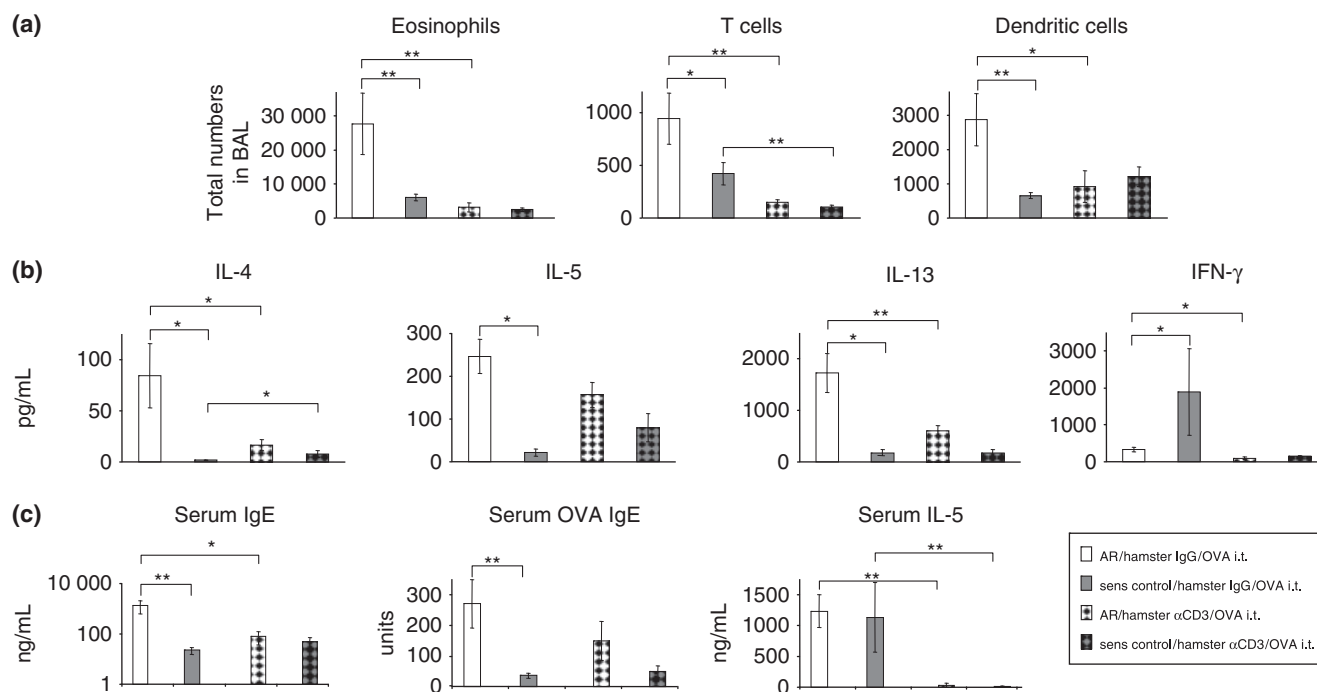


Fig. 3. Anti-CD3 treatment in allergic rhinitis (AR) mice completely abrogated lower airway inflammation after an ovalbumin (OVA) lower airway challenge. The experiment was set up in such a way that mice first developed AR or sensitized (control) only by first sensitizing them by giving OVA/alum on day 0 and day 7, followed by a challenge with OVA or phosphate-buffered saline (PBS) (sensitized) intranasal (i.n.) applications on days 17–19; 24–26; and 31–33. On days 32 and 33, animals were treated with hamster Ig or hamster anti-CD3 antibodies 100 µg/animal. Animals were challenged with 80 µg lipopolysaccharide (LPS)-free OVA intratracheal (i.t.), AR OVA i.t. and sensitized OVA i.t. On day 35, mice were killed and sampled. White bars, AR hamster IgG OVA i.t.; grey bars, sensitized hamster IgG OVA i.t.; black-white bars, AR hamster αCD3 OVA i.t.; black-grey bars, sensitized hamster αCD3 OVA i.t. BAL fluid was analysed by flow cytometry (a) for eosinophils, T cells and for dendritic cells (DCs) and Cervical lymph node (LN) by ELISA (b) for IL-4, IL-5, IL-13 and IFN-γ. Mediastinal LN showed no differences between the groups (data not shown). Serum levels (c) for total IgE, OVA-specific IgE and IL-5. Data are shown as mean ± SEM. * $P < 0.05$ ($n = 5$ animals per group).

increase in eosinophilic cells, both in proportions and in absolute cell numbers, was observed in the BAL fluid of OVA-challenged mice that received Th2 effector cells, when compared with OVA-challenged mice that received naïve DO11.10T cells (Fig. 4a). In the control groups, including mice that received cultured Th2 effector cells but were challenged with PBS or mice that were OVA-challenged but did not receive T cells, we observed only limited eosinophilia. When compared with the control groups, the OVA-challenged mice that received Th2 effector cells manifested significantly increased concentrations of IL-5 in serum and eotaxin levels in BAL fluid, consistent with the observed enhanced attraction of eosinophils in lower airway inflammation in these mice (Fig. 4b). In these experiments, the levels of IL-5 in BAL fluids were below the detection threshold in all four groups and the serum levels of eotaxin showed no differences between the groups (data not shown).

In summary, adoptive transfer of *in vitro* cultured Th2 effector cells, but not of naïve T cells, induced significant eosinophilic inflammation of lower airways after challenge with OVA in naïve mice. Thus, the presence of circulating OVA-specific Th2 effector cells is sufficient to

induce strong eosinophilic lower airway inflammation after OVA allergen challenge in naïve mice.

Blocking T cell recirculation in allergic rhinitis animals abrogates lung inflammation upon lower airway challenge

Next, we investigated the importance of circulation of effector T cells in the induction of asthma in AR mice. To this end, we used the sphingosine-1 analogue FTY720 (Fig. 5a time schedule for animal treatment), which has the capacity to prevent recirculation of lymphocytes by blocking their egress from LN medullary sinuses [22]. Intraperitoneal treatment with FTY720 during the development of upper airway allergic inflammation (Fig. 5b) did not prevent the onset of AR: by immunohistochemical MBP staining for eosinophils, we identified low numbers of eosinophils in sensitized control mice and yet substantial eosinophilia in the epithelium and in the lamina propria of FTY-treated AR mice (Fig. 5b), comparable with what we normally see in this AR model [15]. As FTY720 treatment can also block the development of lower airway inflammation directly [23], we allowed for an FTY720 washout period of 13 days before OVA lung challenge was

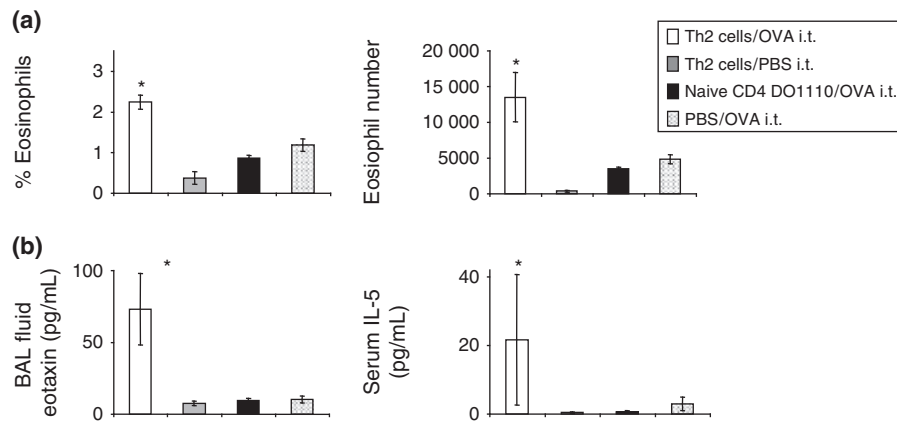


Fig. 4. To study the role of circulating Th2 effector cells, total lymph nodes (LNs) and spleen cells were grown from naïve DO11.10 cells in a proTh2 cell environment with IL-4 and IL-2 as growth factors and anti-IFN- γ and anti-IL-12 for 5 days as described previously [21]. Th2 features were confirmed with RT-PCR for IL-4. Adoptive transfer of 2×10^6 Th2 DO.11.10 cells or 5×10^6 naïve DO11.10 cells were given intravenously. Six hours later, 80 μ g ovalbumin (OVA) or phosphate-buffered saline (PBS) was given intratracheally (i.t.) Four different groups were used; Th2-cells+OVA (open bars); Th2-cells+PBS (grey bars); DO11.10-cells+OVA (black bars); and Naïve animals receiving PBS+OVA (dotted bars). The next day, animals were killed and bronchoalveolar lavage (BAL) fluids and blood were taken for analysis. Percentage and numbers of eosinophils (a) and eotaxin in BAL fluid and serum IL-5 (b) ($n = 4$ animals per group).

given. An almost complete lack of eosinophilic inflammation in BAL fluid was observed in OVA-challenged FTY720-treated AR mice (Fig. 5c). Although in the presence of FTY720, we noticed some eosinophilia above the level seen in OVA-challenged FTY-treated control sensitized mice, this was only a fraction of the exacerbation observed in the OVA-challenged AR mice given a saline treatment instead of an FTY720 treatment. Furthermore, in FTY720-treated AR mice, the numbers of T cells and DC in the BAL fluid were strongly reduced, when compared with the saline controls (Fig. 5c). In BAL fluids, significantly elevated levels of IL-5 were observed when animals were i.t. challenged with OVA compared with PBS, irrespective of their treatment with FTY720 or saline (Fig. 5d). A slightly elevated level of eotaxin could be observed in BAL fluid after an i.t. OVA challenge compared with a PBS i.t. challenge, although this does not reach significance (Fig. 5d). The production of cytokines in cervical and mediastinal LN cell suspensions was similar in FTY720- or saline-treated mice, indicating that the presence of FTY720 did not affect T cell function in the LN (Fig. 5e).

Collectively, these findings show that blocking T cell circulation during AR development by the migration inhibitor FTY720 effectively prevents lower airway inflammation in AR mice upon a single lower airway challenge, without affecting the signs of AR.

Adhesion molecule up-regulation and lung priming in allergic rhinitis mice

To investigate the mechanism by which recirculating T cells might affect the extravasation of eosinophils in OVA-challenged AR mice, we analysed the role of ICAM-1 expression. ICAM-1 is an important adhesion molecule

for the extravasation of eosinophils in lung tissue. Immunohistochemical staining of lung tissue for ICAM-1 showed significant up-regulation of ICAM-1 on endothelial cells in OVA-challenged AR mice, when compared with OVA-challenged control mice (both given saline treatments as a control for FTY720 treatment). Figures 6a and b shows representative photographs. Ranking of ICAM-1 staining intensities indicated the strongest ICAM-1 up-regulation in OVA-challenged AR mice, followed by OVA-challenged control mice. (In Fig. 6c, the ranking order of the strongest staining to the weakest staining is shown.) PBS-challenged AR mice showed only slightly but significantly ($P = 0.02$) lower ICAM-1 expression compared with OVA-challenged AR mice (Fig. 5c). By comparing the lungs of OVA i.n./FTY/OVA i.t. mice with OVA i.n./saline/OVA i.t. mice, we found that ICAM-1 expression was significantly lower when lymphocyte circulation was blocked during the development of upper airway inflammation ($P = 0.02$).

Thus, we conclude that upper airway allergic inflammation (like AR) is associated with T cell priming of lower airway endothelial cells, which results in a stronger expression of ICAM-1 on lung endothelial cells after a lower airway allergen challenge.

Discussion

AR and allergic asthma are two features of allergy of the respiratory tract. Here, we prove for the first time that AR has a significant impact on lower airway inflammation in an animal model of AR. In particular, we show that circulating Th2 effector cells are necessary and sufficient to connect upper and lower airway eosinophilic inflammation. Intranasal OVA application in awake animals only

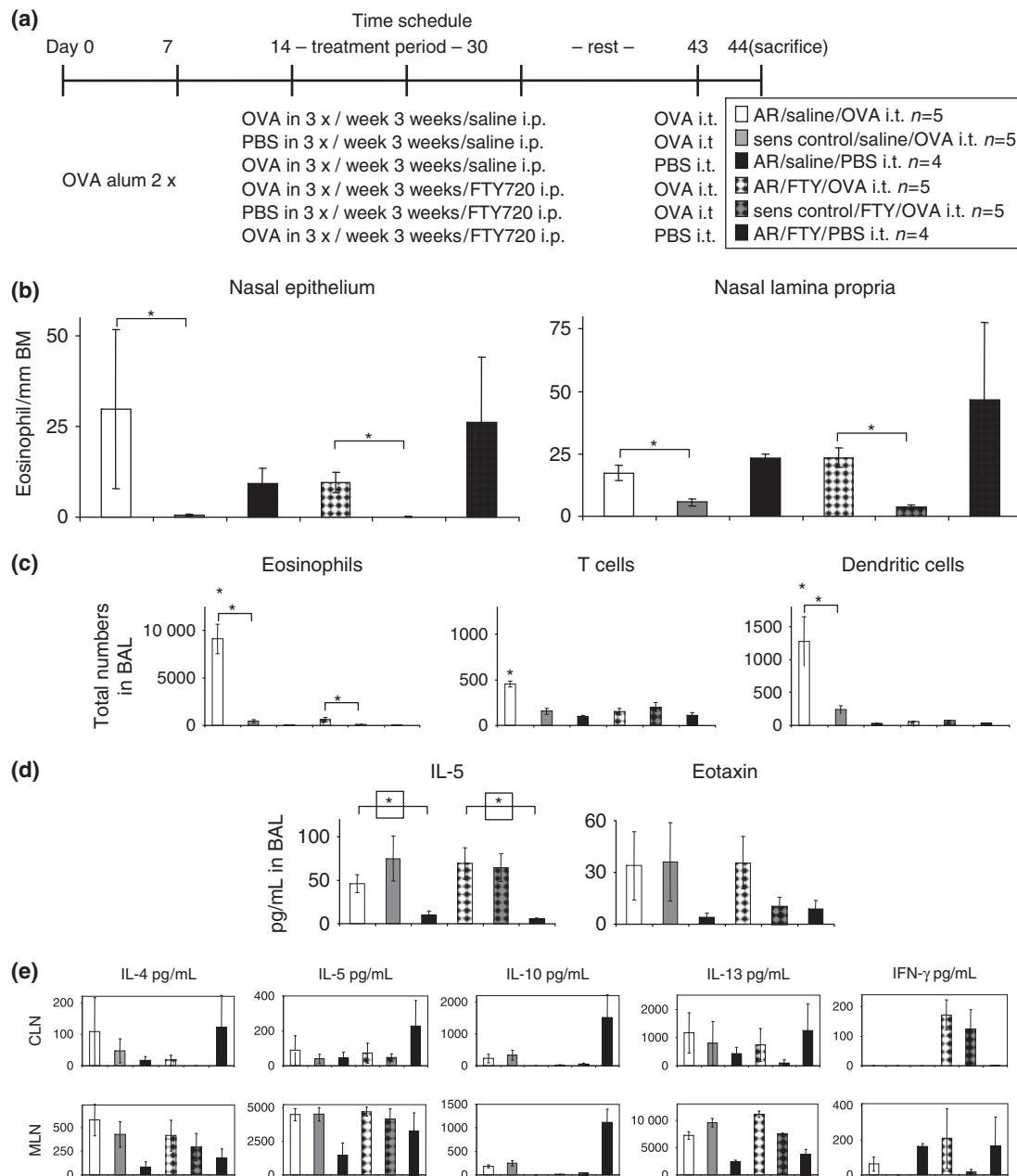


Fig. 5. Systemic treatment with FTY720 does not affect upper airway allergic inflammation development. FTY720 6 μ g or placebo was given intraperitoneally (i.p.) 3 days per week during the period that mice were intranasally (i.n.) challenged with ovalbumin (OVA) or phosphate-buffered saline (PBS) to induce allergic rhinitis (AR) to block T cell circulation. Two weeks after the last challenge and treatment, mice were intratracheally (i.t.) challenged with 80 μ g lipopolysaccharide (LPS)-free OVA or PBS i.t. Six experimental groups were used, OVA sensitized with AR; OVA sensitized without AR during the period of i.n. application of OVA FTY treatment or saline were given. After the treatment, a washout period of 12 days was used to recover the lymphopenia. On day 13 after the last i.n. challenge, animals were i.t. challenged with 80 μ g LPS-free OVA or PBS, followed by harvesting nasal mucosa tissue, lungs, cervical lymph nodes (LN), mediastinal LN, blood and BAL 24 h after the i.t. challenge (a) Animal treatment time schedule. Nasal mucosal sections were stained for murine Major Basic Protein (mMBP), a specific marker for eosinophils (b). Murine eosinophils have a ring-shaped or a bilobular nucleus ($*P < 0.05$) BAL fluid was analysed by flow cytometry for (c) eosinophils, T cells and for dendritic cells (DCs) and by ELISA (d) for eotaxin, and IL-5. Data are shown as mean $\pm$ SEM. $*P < 0.05$. Cervical LN and Mediastinal LN cell suspensions were restimulated *in vitro* for 4 days with OVA and assayed for cytokine production using ELISA assay (e). Data are shown as mean $\pm$ SEM. $*P < 0.05$ (i.t. challenge with OVA $n = 5$ animals per group and i.t. challenge with PBS $n = 4$ animals per group).

affects the upper airways as indicated by the ultrasensitive method of giving mice a cohort of CFSE-labelled OVA-specific CD4⁺ TCR transgenic T cells, to be able to trace the occurrence of antigen presentation. Using this system,

which is very sensitive to the presence of minute amounts of antigen, we only found divisions of CFSE-labelled T cells in the lymphoid structures draining the upper airway, but not in the mediastinal LN draining the lower airway.

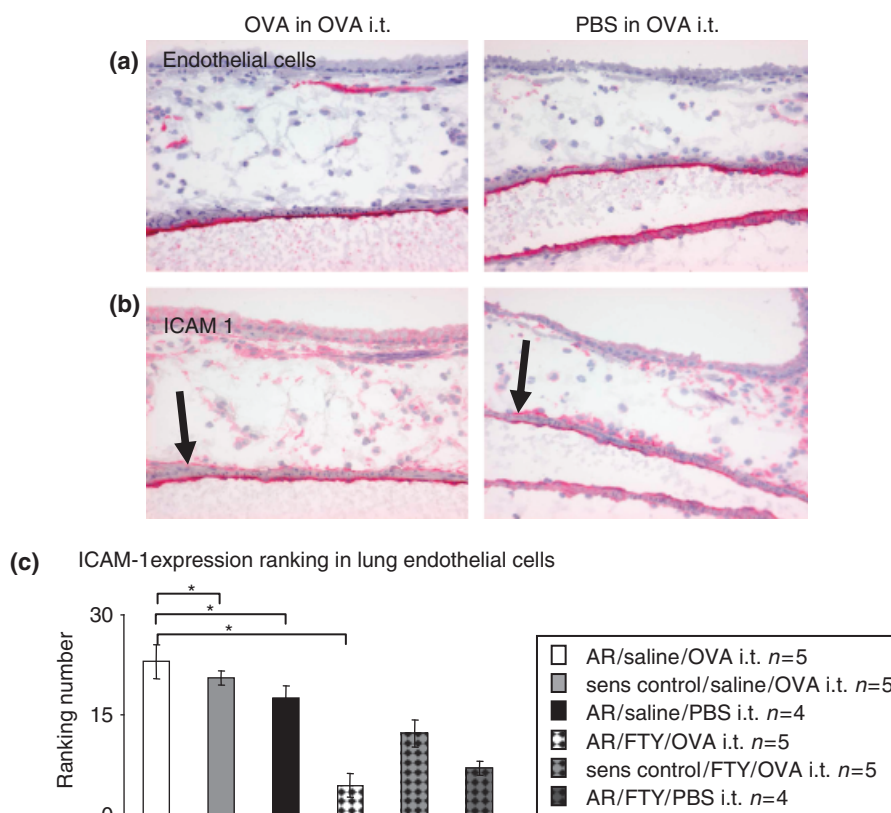


Fig. 6. Lung endothelial inter-cellular adhesion molecule 1 (ICAM-1) expression is elevated in allergic rhinitis (AR) mice upon ovalbumin (OVA) allergen lower airway challenge. Representative photographs of bronchial mucosal sections were stained for murine CD31, a marker for endothelial cells (a, upper panel) and for murine ICAM-1 (b, lower panel). Sensitized AR mice challenged with OVA showed strong ICAM-1 expression on endothelial cells. Sensitized phosphate-buffered saline (PBS) mice challenged with OVA showed some ICAM-1 expression on endothelial cells. Sensitized AR PBS-challenged mice showed almost no ICAM-1 expression on endothelial cells. Ranking the ICAM-1 staining intensity of lung endothelial cells showed the strongest ICAM-1 staining in AR mice upon OVA lower airway challenge (c).

The presence of Th2 cells in AR mice and the fact that these Th2 cells can recirculate strongly suggested that the induction of recirculating effector Th2 cells is the most obvious explanation for the link between AR and asthma. To demonstrate that T cells are a crucial factor in nasobronchial interaction, we made use of T cell depletion by anti-CD3 antibodies just before bronchoalveolar challenge. It might be argued that due to the depletion of T cells, all features of allergy disappear, including the interaction between upper and lower airways. Our finding that T cell depletion abrogates lower airway eosinophilic inflammation excludes an important role for other pathways that may have a significant impact on nose-lung interaction, such as neurological cross-talk and mediators. Next, we took advantage of FTY720, a compound that blocks lymphocyte recirculation by blocking their egress from LN medullary sinuses [22]. When we treated mice systemically with FTY720, we found that AR as characterized by nasal tissue eosinophilia was unaffected. Nevertheless, these animals showed far less lower airway inflammation than saline-treated animals, substantiating the importance of recirculating Th2 cells from the upper to the lower airways

as a link between upper and lower airway allergy. To show that Th2 cells are not only necessary but also sufficient to induce lower airway eosinophilic inflammation, we performed adoptive transfer experiments of Th2 effector cells demonstrating that circulating Th2 cells are indeed the key factor for lower airway allergy in AR. This experiment of adoptive transfer of OVA-specific Th2 cells has been performed by others as well, with similar results [24]. In this context, our data also indicate that recirculating Th2 cells are essential for priming of endothelial cells, which results in a rapid and higher expression of ICAM-1 than in control animals. This adhesion molecule enables eosinophils to extravasate and appear in the lungs and alveolar space [25].

Although our experiments show that circulating Th2 effector cells are necessary and sufficient to connect upper and lower airway allergy, they also allow a role for non-T cells in controlling nasobronchial interaction. In the experiments in which T cell recirculation was blocked with FTY720, we still noticed a slight induction of eosinophilia in OVA-challenged AR animals, when compared with sensitized control animals (Fig. 5c). This small induction of eosinophilia could be due to mast cells'

contribution to the lung inflammation by their production of Th2 cytokines [19, 20] or the effect of abolishing the lymphocyte recirculation blockade. A single lower airway PBS challenge in OVA-immunized AR animals was not controlled by a PBS challenge in OVA-immunized PBS control animals. In these experiments, we omitted the control group of a single lower airway PBS challenge in OVA-immunized PBS control mice. The reason was that even AR mice with nine OVA upper airway challenges and OVA-alum immunization did not show any signs of lower airway inflammation upon i.t. PBS challenge [15].

We also found that eosinophilic inflammation did not show a strict correlation with IL-5 levels: IL-5 levels in the serum of AR and sensitized control mice were similar, but eosinophilic inflammation was significantly enhanced in AR mice (Fig. 2b). IL-5 levels were not detectable in AR mice 24 h after the last i.n. challenge (and PBS aerosol, Fig. 2d), suggesting that IL-5 was produced locally by cells in the lower airways. In this context, it is suggested that CD34⁺ cells are involved in the formation of eosinophilic progenitors from haematopoietic stem cells following allergen exposure. The CD34⁺ cells have the capacity to release and contribute to the production of IL-3, IL-5 and GM-CSF [26–29]. IL-5 is, however, not the only Th2 cytokine important for the recruitment of eosinophils [30, 31]. Another important eosinophil chemokine is eotaxin, which was clearly detectable in BAL fluids and was induced upon challenge with OVA aerosols, but failed to discriminate between AR mice and sensitized controls, thus offering no explanation for the nasobronchial interactions. Serum levels for eotaxin were not significant changed in serum of PBS- and OVA-challenged animals.

Before an eosinophil reaches the alveolar space, a whole cascade of events is necessary including the chemoattraction by cytokines or chemokines and the endothelial up-regulation of adhesion molecules like ICAM-1 and VCAM-1 [32]. The expression of these adhesion molecules is controlled by the Th2 cytokines IL-4 and IL-13 and TNF- α [30, 31], and therefore one possible explanation for the effect of circulating Th2 cells might be the induction of these adhesion molecules on lung structural cells. We indeed found evidence for this mechanism by showing the increased expression of ICAM-1 in AR mice, dependent on recirculating Th2 cells (as inhibited by FTY720). How exactly ICAM-1 is induced by Th2 cells is less clear from our studies, but could certainly involve the secretion of IL-4 by Th2 cells [33, 34]. TNF- α in serum and in BAL fluid did not turn out to be an important factor in induction of ICAM-1 because no significant difference in levels could be observed between the groups with or without AR (data not shown). A recent report, however, suggested that adoptive transfer of OVA-immune Th2 cells led to up-regulation of ICAM-1 on lung cells in a process requiring TRAF-1 (TNF receptor-associated factor-1) [35]. Although other pro-inflammatory cytokines like IL-1 and

IL-6 also have the potential to induce the up-regulation of ICAM-1, we currently do not know whether these would be involved.

The induction of ICAM-1 expression of lung endothelial cells could explain not only the increase in eosinophils but also the early lung accumulation of DCs and T cells seen in allergen-challenged AR mice, as this might lead to increased retention of these cells in the lungs [35]. We have previously reported that monocyte-derived CD11c⁺ DCs are essential for mounting allergic inflammation in the nose and lung [15, 21] and enhanced recruitment of this cell type might therefore explain the increased sensitivity to allergen challenge in these mice. The benefit of blocking antibodies against ICAM-1 was also shown to reduce hapten-induced colon eosinophilic inflammation as well as isocyanate-induced asthma [36], showing that this might be a generic mechanism for promoting Th2-dependent inflammation [37].

Although we have reached these conclusions in a mouse model of AR, comparable results were observed in previous studies in human AR subjects without asthma. Here, we also noticed the up-regulation of ICAM-1 on vessels in the lower airways as well as eosinophilic inflammation in the lower airways after nasal and bronchial allergen challenge [13, 14]. The current observations enable us to interpret our human results as a priming effect of recirculating Th2 cells on lung mucosa. Our observations are in agreement with observations from allergy models in the skin and in the gut that having such an allergy in a shock organ outside the lungs can worsen antigen-induced airway inflammation [25, 38–40].

In conclusion, AR mice are significantly more susceptible to eosinophilic inflammation following a lower airway challenge and induce increased lower airway inflammation, thereby providing an animal model for early onset of asthmatic inflammation. We demonstrated that recirculating effector Th2 cells are necessary and sufficient for a nose–lung interaction to occur in the context of allergic eosinophilic inflammation. In this process, Th2 cell-dependent priming on lung endothelial cells results in enhanced sensitivity of the lung for allergen challenge. Therapies aimed at interfering with Th2-mediated increase of ICAM-1 should 1 day benefit patients with United Airways disease.

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