Health, Labour and Welfare. According to the estimated dose–response curve reported by Gallagher *et al.*, by taking 2000 IU as a daily dose, 25OHD levels after the plateau phase would be 37 ng mL^{-1}.[13] Therefore, we chose 2000 IU as a daily dose in this study. Sesame oil, gelatin derived from swine and glycerin were used as the formulation for placebo as well as the active supplement. Blinding of the study was achieved by bottle numbering. Staff at the data monitoring center had no contact with participants. Thus, participants (high school students), care providers (parents), and medical doctors who assessed outcomes were blinded to the supplements.

3.2. Follow-up procedures and ascertainment of outcomes

3.2.1. Primary outcome. The students were asked to visit a doctor's clinic if they developed a fever (defined as body temperature higher than 37.0 °C) during the pandemic phase. As a school rule in Japan, students or parents are required to inform homeroom teachers of a doctor's diagnosis. Then, the homeroom teacher was asked to send a fax to the data monitoring center providing a detailed description from the students/parents as told to them and/or a certificate provided by the doctor regarding the diagnosis of or recovery from influenza A. Diagnosis was made by means of a RIDT and not polymerase chain reaction at the primary care setting in Japan. Participants were also asked to complete a daily log during the study period to: (1) reconfirm the diagnosis of influenza by a medical doctor, (2) assess adherence with the study supplement, and (3) assess other subjective symptoms, such as fever, runny nose, cough, sore throat, and arthralgia. For case identification, the study number was used and private information such as names and addresses of participants was not disclosed to the data monitoring center.

Because it takes several days for serum levels of 25OHD to start increasing, and a few days to allow for the incubation period of influenza, outcomes were only included in the analysis if they occurred after October 25 (7 days after starting study supplements). The primary outcome was the occurrence of influenza A, diagnosed by medical doctors with RIDT using nasopharyngeal swabs (*i.e.*, RIDT-positive influenza A). The sensitivity of the RIDT used in Japan for 2009 pandemic influenza A (H1N1) virus infection confirmed by polymerase chain reaction is approximately 77%.[14]

3.2.2. Secondary outcome. Secondary outcomes were: (1) doctor-diagnosed influenza-like illness, including not only RIDT-positive but also RIDT-negative influenza cases suspected by doctors due to clinical signs (*e.g.*, fever, headache, arthralgia, runny nose, and coughing) and close contact with patients with influenza; and (2) school absence and the reason for absence. Homeroom teachers were asked to send a fax to the data monitoring center to report any case of adverse events, including urinary tract stones or other serious signs/symptoms, as described to them by the students/parents.

3.3. Statistical analysis

3.3.1. Sample size calculation. We estimated that the primary outcome (RIDT-positive influenza A) would occur in 25% of students in the placebo group. A 3 : 2 divided sample of 260 was calculated as being sufficient for the detection of a 60% reduction in outcome, with a type I error (two-sided) of 5% and a power of 85%, on the assumption of no loss to follow-up. To detect the same 60% risk reduction with 85% power using a 1 : 1 ratio, the calculated sample size was 254. Because this number was almost the same if we used a 3 : 2 ratio, we chose the latter ratio because we assumed that the number of participants would increase in a study using a 3 : 2 ratio rather than a 1 : 1 ratio. Interim analyses were not used as the study period was only 2 months.

3.3.2. Efficacy analysis. Efficacy was assessed using an intention-to-treat analysis, which includes all students in the study, regardless of whether they were taking supplement after randomization. The incidence of both primary and secondary outcomes in the two groups was compared using a RR and 95% CI. All reported P values are two-sided and $P < 0.05$ was considered statistically significant. No adjustments were made for multiple comparisons. All analyses were performed using Stata 12.1 (StataCorp LP, College Station, TX).

4. Conclusions

We found that vitamin D3 supplementation did not decrease the overall incidence of RIDT-positive influenza A or influenza-like illness. However, the RCT also suggests that short-term (*i.e.*, 1 month) use of vitamin D3 dietary supplementation may temporarily decrease the incidence of influenza A during an influenza pandemic; the similarity of this finding with that of two other published RCTs[5,6] suggests that this finding is a result of more than chance. Future studies, with larger populations and serial 25OHD testing, are needed to further explore this novel finding and determine the optimal dose and duration of vitamin D supplementation to potentially decrease the risk of influenza and other acute respiratory infections.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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Bystander immunotherapy as a strategy to control allergen-driven airway inflammation

S Navarro[1,3], A Lazzari[1], A Kanda[2], S Fleury[2], D Dombrowicz[2], N Glaichenhaus[1] and V Julia[1]

Allergic asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness (AHR), lung infiltration of Th2 cells, and high levels of IgE. To date, allergen-specific immunotherapy (SIT) is the only treatment that effectively alleviates clinical symptoms and has a long-term effect after termination. Unfortunately, SIT is unsuitable for plurisensitized patients, and highly immunogenic allergens cannot be used. To overcome these hurdles, we sought to induce regulatory CD4$^+$ T cells (T_{reg}) specific to an exogenous antigen that could be later activated as needed *in vivo* to control allergic responses. We have established an experimental approach in which mice tolerized to ovalbumin (OVA) were sensitized to the *Leishmania* homolog of receptors for activated c kinase (LACK) antigen, and subsequently challenged with aerosols of LACK alone or LACK and OVA together. Upon OVA administration, AHR and allergic airway responses were strongly reduced. OVA-induced suppression was mediated by CD25$^+$ T_{reg}, required CTLA-4 and ICOS signaling and resulted in decreased numbers of migrating airway dendritic cells leading to a strong impairment in the proliferation of allergen-specific Th2 cells. Therefore, inducing T_{reg} specific to a therapeutic antigen that could be further activated *in vivo* may represent a safe and novel curative approach for allergic asthma.

INTRODUCTION

Allergic diseases affect up to 30% of the population and their prevalence has steadily increased in recent decades. Among allergic diseases, asthma is a chronic inflammation of the lungs caused by an inappropriate immune response to a single or multiple airborne allergens. This pathology has a substantial economic burden for which the only targeted method of treatment is allergen-specific immunotherapy (SIT). SIT involves the administration by either subcutaneous injection or mucosal application of increasing doses of the allergen to which the patient is allergic. Unfortunately, this approach is ineffective for treating multi-sensitized patients, and despite recent clinical studies, numerous allergens are too immunogenic to be used in desensitization protocols.[1] SIT induces a state of peripheral tolerance characterized mainly by the generation of allergen-specific regulatory T cells (T_{reg}), along with the suppression of effector cell proliferation and cytokine production against the targeted allergen.[2,3] Interleukin (IL)-10 and/or TGF-β producing T_{reg} are the key factors for specific immunotherapy in humans.[4–6] It has been shown that treating naive (non-sensitized) mice with ovalbumin (OVA) aerosols results in the development of OVA-specific T_{reg} and IgE unresponsiveness upon subsequent sensitization and challenge with OVA.[7,8] Akbari *et al.*[8] have shown that lung draining lymph node (LN) dendritic cells (DCs) are able to create a local pro-regulatory environment by transiently producing IL-10 following a first contact with an inhaled antigen (Ag). These phenotypically mature DC induce the development of T_{reg}, which in turn produces IL-10.[9] Respiratory tolerance can be achieved with only three intranasal (IN) administrations of Ag, which are sufficient to protect against airway hyperresponsiveness (AHR) and allergic inflammation upon further Ag sensitization and challenge.[10] TGF-β-expressing T_{reg} also have a role in tolerance induction to inhaled Ags.[6,11]

In fact, SIT not only has been shown to alter the development of new sensitizations, but it has also been suggested that tolerance generated by SIT is not solely limited to the targeted allergen and also confers extended protection against other allergens.[12,13] This non-specific and beneficial action of SIT could be explained by the activation of bystander T_{reg}.

[1]IPMC, CNRS UMR7275, INSERM U1080 Université de Nice-Sophia Antipolis, Valbonne, France and [2]Institut Pasteur de Lille, INSERM U1011, Université de Lille 2, Lille, France. Correspondence: S Navarro (severine.navarro@jcu.edu.au) or V Julia (vjulia@unice.fr)
[3]Present address: Australian Institute of Tropical Health and Medicine, CBMDT, James Cook University, E4 McGregor Road, Smithfield, QLD 4878, Australia

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Bystander immunosuppression was first described by Bullock *et al.*[14] as a process in which Ag-specific T_{reg} inhibit T effector cell responses directed to both the target Ag and to a colocalized third-party Ag. However, it was originally believed that in order for suppression to occur, the tolerogen and the Ag were required to be physically linked, i.e., presented by the same Ag-presenting cell (APC), at which point the terms linked suppression or linked recognition were coined. It was later shown that this state of induced tolerance was self-perpetuating and long lasting, and moreover, the tolerogen and the third-party Ag only needed to be in the vicinity of one another.[15–18] In the field of oral tolerance, the term bystander suppression was introduced to describe inhibition of a T-cell memory response as a result of a regulatory response generated to an unrelated but colocalized tolerogen.[16,19,20] In bystander suppression, the tolerogen and third-party Ag do not need to be presented by the same APC. Admittedly, while soluble mediators are critical to induce suppression of the response directed to the third-party Ag, contact-based cell interactions are not excluded[19,21–23] and have been recently demonstrated during graft tolerance.[24]

In this study, we have investigated whether T_{reg} specific for a third-party therapeutic Ag could control allergic immune responses and lung inflammation in mice. More specifically, we sought to demonstrate that therapeutic Ag-specific T_{reg} could be recruited into the airway mucosa as needed during acute inflammatory bursts, and alter the natural development of allergic disease by modifying the microenvironment. To this aim, the *Leishmania* homolog of receptors for activated c kinase (LACK) Ag was used as an allergen model as described previously.[25,26] Briefly, mice were sensitized to LACK, tolerized to OVA through three IN administrations as described previously,[8] and challenged with aerosols of LACK alone or with an additional OVA aerosol treatment to activate/recruit previously generated OVA-specific T_{reg}.

RESULTS

Exposure to OVA aerosols inhibits LACK-induced allergic asthma in OVA-tolerized mice

To determine whether T_{reg} specific for a third-party Ag could control allergic airway inflammation, mice were sensitized to LACK, tolerized to OVA through IN administrations, a protocol reported to induce respiratory tolerance via the induction of OVA-specific Treg,[9,10] and tested for their ability to develop unresponsiveness to OVA upon further OVA immunization and challenge. When compared with phosphate-buffered saline (PBS)-treated mice, LACK-sensitized mice treated with OVA exhibited reduced AHR and airway allergic inflammation upon sensitization and challenge to OVA (**Supplementary Figure 1** online). In addition, OVA-treated mice also exhibited decreased OVA-specific IgE, further confirming that our treatment induced a state of tolerance to OVA (**Supplementary Figure 1**). To investigate whether tolerance to OVA could improve asthma-related symptoms in mice sensitized and challenged to LACK, we sensitized mice to LACK and tolerized them to OVA and further challenged them with LACK aerosols alone (control) or both LACK and OVA to activate and recruit OVA-specific T_{reg} to the airways (**Figure 1a**). When compared with the control group, mice treated with OVA aerosols showed decreased AHR (**Figure 1b**), reduced numbers of total cells, eosinophils, and lymphocytes in the bronchoalveolar lavage fluids (BALFs) (**Figure 1c**), and more specifically a reduced number of airway-infiltrating $CD4^+$ T cells expressing the Th2- marker T1/ST2 (IL-33Ra) (**Figure 1d**). Concentrations of IL-4, IL-5, and IL-13 in the BALF were also decreased upon exposure to OVA during LACK challenges, while interferon (IFN)-γ levels remained low and comparable in both groups (**Figure 1e**). Interestingly, intracellular staining showed that OVA aerosols significantly decrease both the frequency and the number of IL-4, and IL-5-secreting LACK-specific $CD4^+$ T cells in comparison with mice that received LACK only aerosols (**Figure 1f**). However, in our particular experimental settings, levels of LACK-specific IgE and IgG1 were not affected by OVA treatment (**Figure 1g**). Since therapeutic administration of OVA was given during the LACK challenge phase, it was possible that the decreased amplitude of LACK-specific responses could be due to an artifact related to a suboptimal availability of LACK-loaded APCs. For this reason, we sensitized mice to LACK, omitted the OVA IN administrations, and challenged them with either LACK or both LACK/OVA aerosols (**Supplementary Figure 2**). Both groups exhibited similar AHR and BALF cellular infiltration, providing further evidence that the decrease in inflammation observed in the LACK/OVA-treated group was not due to diminished LACK-derived Ag presentation but rather to the induction of suppressive mechanisms (**Supplementary Figure 2**).

To confirm our observations with a human relevant allergen, mice were tolerized to OVA, exposed to a series of ragweed (*Ambrosia artemisiifolia*) IN injections, and later administered an OVA IN analeptic (**Figure 2a**). Similar to our previous results, mice treated with OVA at the later phase of ragweed-induced inflammation showed decreased AHR (**Figure 2b**), reduced total numbers of cells, eosinophils, and lymphocytes in BALF (**Figure 2c**), and a significant reduction in both the number and frequency of $T1/ST2^+$ $CD4^+$ T cells when compared with the control group, suggesting that ragweed-specific Th2 responses have been controlled (**Figure 2d**). Likewise, BALF levels of IL-4 and IL-13 were also reduced upon later treatment with OVA, while IFN-γ levels remained low and comparable in both groups (**Figure 2e**). Altogether, our results suggested that OVA-induced tolerance could inhibit AHR, allergic airway inflammation and allergen-specific Th2 immune responses directed to another unrelated Ags.

Inhibition of LACK-induced airway inflammation in OVA-tolerized mice is mediated by OVA-specific $CD25^+$ T_{reg}

Previous reports have shown that, in the absence of reporter mice, regulatory cells could be purified from the spleen of tolerized mice.[6,26,27] To investigate whether OVA-specific T_{reg} were responsible for the suppression of LACK-induced airway inflammation, and whether this protection was Ag specific,

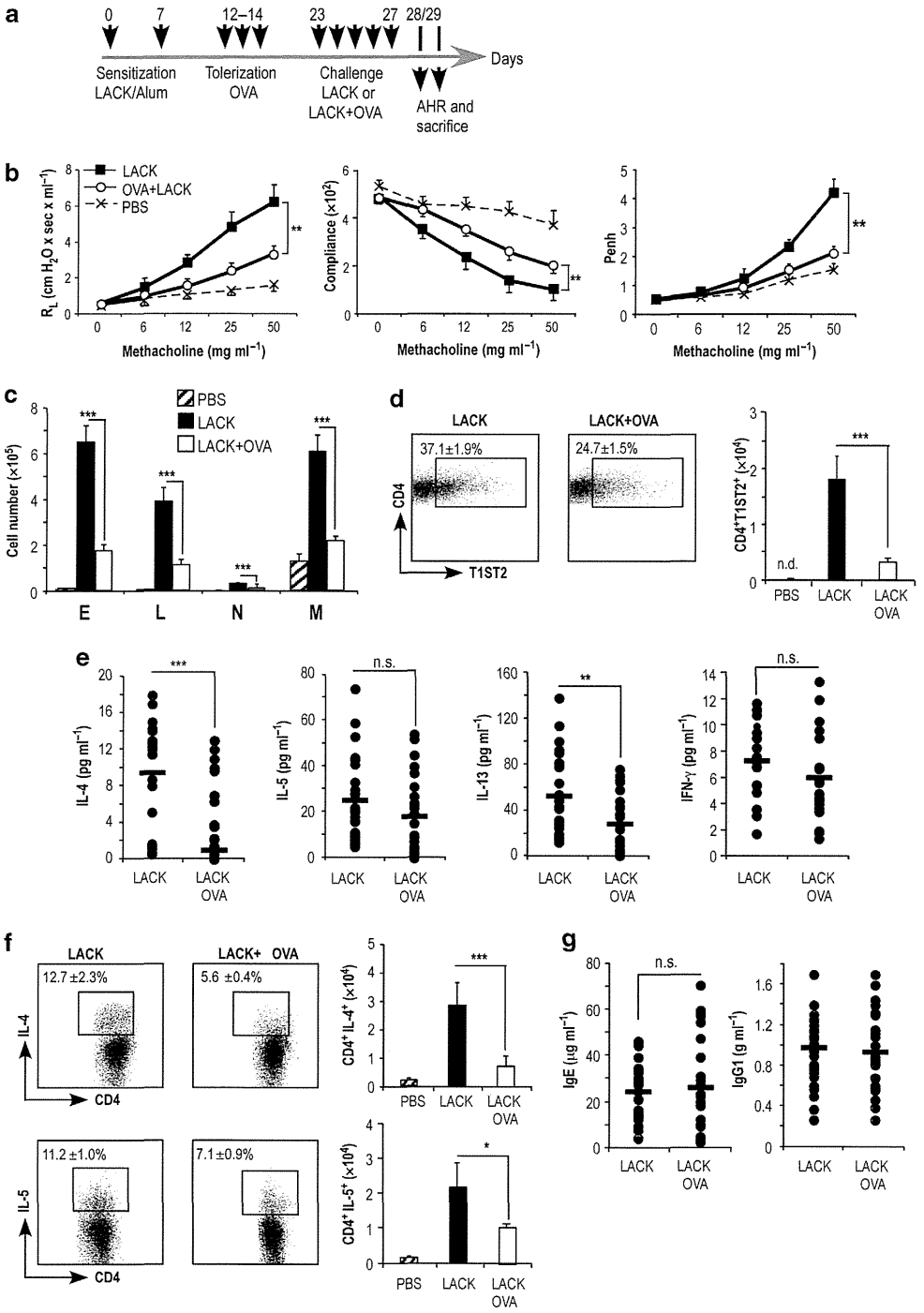

Figure 1 Airway hyperresponsiveness (AHR), airway inflammation, and cytokine levels in ovalbumin (OVA)-tolerized mice exposed to both OVA and *Leishmania* homolog of receptors for activated c kinase (LACK) aerosols. (**a**) Experimental protocol. Mice were sensitized with two intraperitoneal injections of LACK in Alum, treated with three intranasal (IN) injections of OVA, and challenged daily for 5 days with LACK aerosols or LACK/OVA aerosols. Mice were analyzed 1 and 2 days after the last aerosol. (**b**) AHR. Whole body plethysmography (right), and dynamic lung resistance and compliance (left) were monitored in mice exposed to LACK aerosols (filled squares), LACK/OVA aerosols (empty circles), or phosphate-buffered saline (PBS) (crosses, dashed line). (**c**) Number and phenotype of bronchoalveolar lavage fluid (BALF) cells. BALF cells were counted and analyzed by fluorescence *in situ* hybridization (FACS) in mice exposed to PBS (dashed bars), LACK (black bars), or LACK/OVA aerosols (empty bars). Data show the number of eosinophils (E), neutrophils (N), lymphocytes (L), and macrophages (M). (**d**) Frequency and number of Th2 cells in the airways. BALF cells were stained with anti-CD4, CD3, and T1/ST2 mAbs and analyzed by FACS. Data show representative FACS profiles, numbers indicate the mean frequency ± s.e.m. and histograms show the absolute numbers of T1ST2$^+$ CD4$^+$ T cells for the indicated groups. (**e**) Cytokine levels in BALF. Mice were analyzed for interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)-γ by cytometric bead array (CBA). (**f**) Cytokine secretion by lung CD4$^+$ T cells. IL-4 and IL-5-secreting CD4$^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1 mg ml^{-1}), anti-CD28 (1 μg ml^{-1}), and brefeldin A (5 μg ml^{-1}). Data show representative FACS profiles, numbers indicate the mean frequency ± s.e.m. and histograms show the absolute numbers for the indicated groups. (**g**) Immunoglobulins. Levels of serum LACK-specific IgE and IgG1 were assessed in mice upon challenge with LACK or LACK/OVA aerosols. All data show either individual mice with bar indicating the mean, with $n=6$ mice per group pooled from four different experiments. n.s., non-significant, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$.

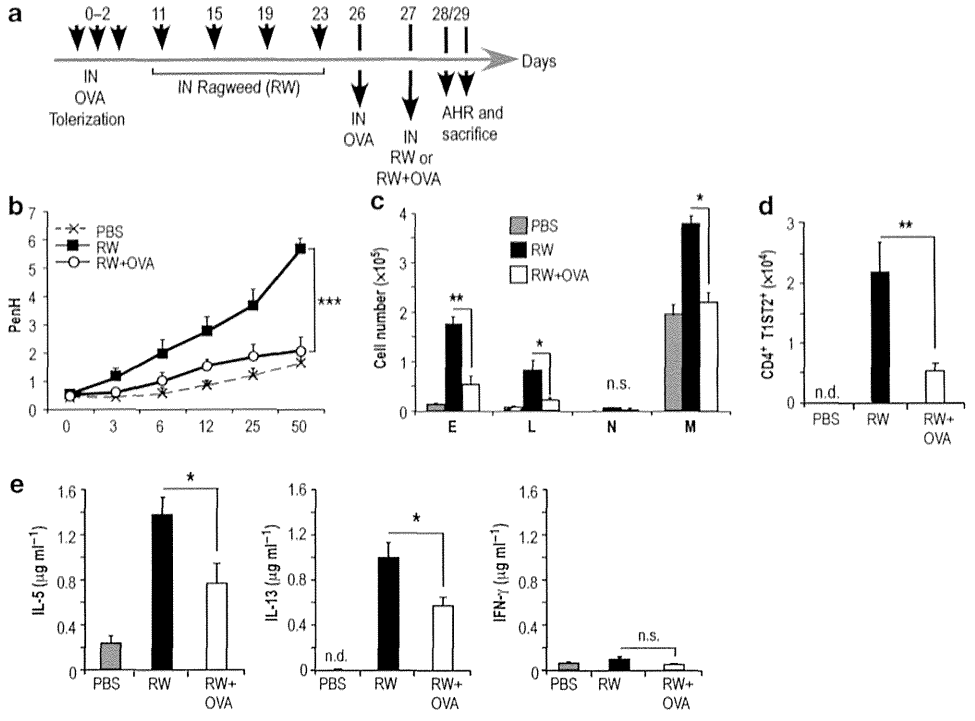

Figure 2 Airway hyperresponsiveness (AHR), airway inflammation, and cytokine levels in ragweed-sensitized, ovalbumin (OVA)-tolerized mice upon ragweed challenge. (**a**) Experimental protocol. Mice were treated with three intranasal (IN) injections of OVA, and four IN injections of ragweed. Mice were then challenged with either ragweed or ragweed/OVA, and analyzed 1 and 2 days after the last IN injection. (**b**) AHR. Whole body plethysmography in mice exposed to ragweed (filled squares), ragweed/OVA (empty circles), or phosphate-buffered saline (PBS) (crosses). (**c**) Number and phenotype of bronchoalveolar lavage fluid (BALF) cells. BALF cells were analyzed by fluorescence *in situ* hybridization (FACS) in mice exposed to ragweed only (black bars), to both ragweed and OVA aerosols (empty bars) or to PBS (gray bars). Eosinophils, E; neutrophils, N; lymphocytes, L; and macrophages, M. (**d**) Frequency and number of Th2 cells in the airways. BALF cells were stained with CD4 and T1/ST2 mAbs and analyzed by FACS. (**e**) Cytokine levels in lung cells. Interleukin (IL)-4, IL-5, and interferon (IFN)-γ levels were assessed by ELISA after *in vitro* stimulation with *Leishmania* homolog of receptors for activated c kinase (LACK) protein (0.1 mg ml^{-1}), anti-CD28 (1 μg ml^{-1}) and brefeldin A (5 μg ml^{-1}). Data are expressed as mean ± s.e.m. of two experiments with $n=8$ mice per group. n.s., non-significant; $^{*}P<0.05$; $^{**}P<0.01$.

total CD4$^+$ T cells were purified from the spleen of mice previously tolerized to OVA, or bovine serum albumin (BSA), or mock injection (PBS). Cells were injected into LACK-sensitized recipient mice that were further treated with OVA aerosols alongside LACK challenges (**Figure 3a**). In contrast to the mice injected with CD4$^+$ T cells purified from BSA-tolerized or PBS-treated donors, mice injected with CD4$^+$ T cells from OVA-tolerized mice exhibited decreased AHR (**Figure 3b**), reduced numbers of total cells, eosinophils, and lymphocytes (**Figure 3c**) as well as T1/ST2$^+$ CD4$^+$ T cells in BALF (**Figure 3d**), and reduced numbers of lung IL-4- and IL-5-producing LACK-specific CD4$^+$ T cells (**Figure 3e**).

Interestingly, CD4$^+$ T cells from OVA-tolerized mice were unable to transfer protection when depleted from the CD25-expressing cell subpopulation (**Figure 3f–i**). However, adoptive transfer of purified CD25$^+$ CD4$^+$ T cells from OVA-tolerized mice provided similar levels of protection to that of the whole CD4 population (**Figure 3f–i**). Taken together, these results suggest that not only does the inhibition of LACK-induced airway inflammation rely on the activation of CD25-expressing T_{reg}, but also that in order for these cells to suppress LACK-induced inflammatory processes, they need to be reactivated and/or recruited with their Ag of specificity, i.e., OVA.

OVA-T_{reg} depend on CTLA-4 and ICOS to control LACK-induced asthma

It is well documented that T_{reg} mediate suppression through various mechanisms including the secretion of inhibitory cytokines, the induction of cytolysis, metabolic disruption, the inhibition of Ag presentation by DCs via CTLA-4-dependent mechanisms[28] and ICOS–ICOS-ligand interactions.[29] Consequently, we first sought to determine the role of CTLA-4 and ICOS in the inhibition of LACK-induced inflammation in our model. To this aim, LACK-sensitized OVA-tolerized mice were treated with CTLA-4, ICOS, blocking mAbs, or isotype control, during LACK/OVA challenge (**Figure 4a**). Interestingly, inhibition of the T_{reg} activation marker CTLA-4 restored AHR, airway cellular infiltration of eosinophils, lymphocytes, and T1/ST2$^+$ Th2 cells, along with the number of lung IL-4- and IL-5-producing LACK-specific CD4$^+$ T cells (**Figure 4b–d**). Similar results were obtained by blocking ICOS/ICOSL signaling as shown by the restoration of AHR and comparable airway infiltration of eosinophils and lymphocytes to the LACK challenged group (**Figure 4f, g**). Taken together, our results demonstrate that OVA-specific T_{reg} control LACK-induced AHR and allergic airway inflammation in a CTLA-4 and ICOS-dependent mechanism.

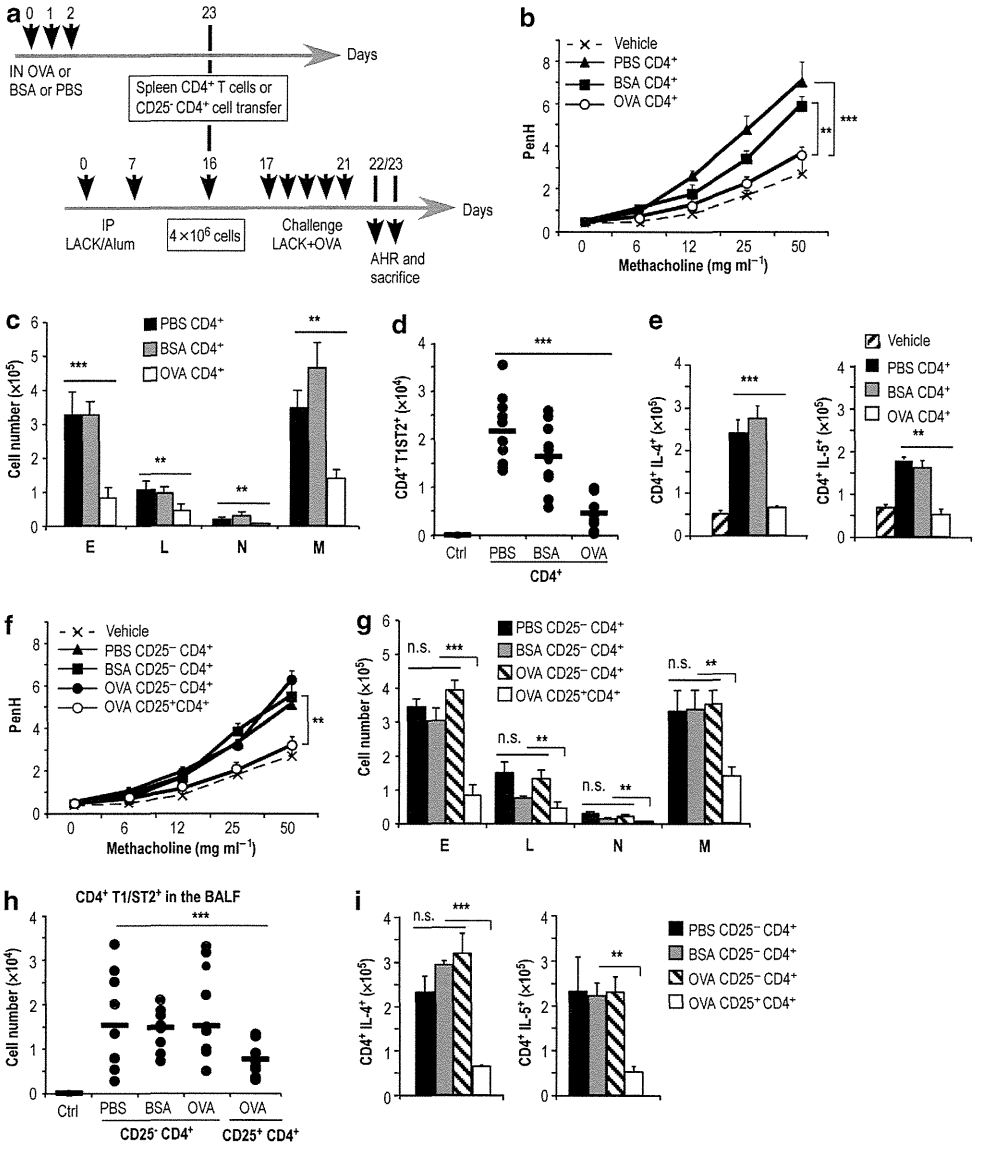

Figure 3 Airway hyperresponsiveness (AHR), airway inflammation, and cytokine levels in mice injected with CD4$^+$ T cells from ovalbumin (OVA)-tolerized mice. (**a**) Experimental protocol. Mice were sensitized with two intraperitoneal (IP) injections of *Leishmania* homolog of receptors for activated c kinase (LACK) in Alum, and injected 9 days later with 4×10^6 CD4$^+$ T cells (**a–e**), or 4×10^6 CD25$^-$CD4$^+$ T cells (**f–i**) or 1.5×10^6 CD25$^+$CD4$^+$ T cells (**f–i**) prepared from the spleen of mice exposed to OVA, BSA, or phosphate-buffered saline (PBS). Sensitized mice were then challenged with LACK/OVA aerosols for 5 days and analyzed 1 and 2 days after the last aerosol. (**b**, **f**) AHR. Whole body plethysmography was monitored in the indicated mice challenged to LACK/OVA aerosols in response to increased doses of inhaled methacholine. Control mice (vehicle) were sensitized with LACK, non-transferred and challenged with PBS. (**c**, **g**) Number and phenotype of bronchoalveolar lavage fluid (BALF) cells. BALF cells were analyzed by fluorescence *in situ* hybridization (FACS) for the number of eosinophils (E), neutrophils (N), lymphocytes (L), and macrophages (M). (**d**, **h**) Frequency and number of Th2 cells in the airways. BALF cells were stained with anti-CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. (**e**, **i**) Cytokine secretion by lung CD4$^+$ T cells. Interleukin (IL)-4 and IL-5-secreting CD4$^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1 mg ml^{-1}), anti-CD28 (1 μg ml^{-1}), and brefeldin A (5 μg ml^{-1}). Data show numbers of IL-4 and IL-5-secreting CD4$^+$ T cells in lungs. All data show either individual mice or are expressed as mean ± s.e.m., with $n = 5$ mice per group pooled from two experiments. *P*-values have been calculated by comparing OVA-tolerized mice with BSA-tolerized mice, n.s., non-significant; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$.

OVA-T_{reg} inhibit LACK-specific Th2 cell proliferation and subsequent airway inflammation

To further understand the molecular mechanisms underpinning OVA-induced protection in this system, and more particularly the effects of OVA-T_{reg} on LACK-specific responses, we sought to investigate the fate of LACK-specific Th2 effector cells. To this end, we modified our disease model and generated *in vitro* LACK-specific Th2 cells from Thy1.1 expressing LACK WT15 TCR transgenic RAG$^{-/-}$ mice. As described previously, CD4$^+$ T cells from the spleen of naïve WT15 mice were differentiated under Th2 polarizing conditions (**Supplementary Figure 3**),[25,30] subsequently labeled with carboxyfluorescein succinimidyl ester (CFSE), and injected into OVA- or BSA-tolerized recipients before receiving a single

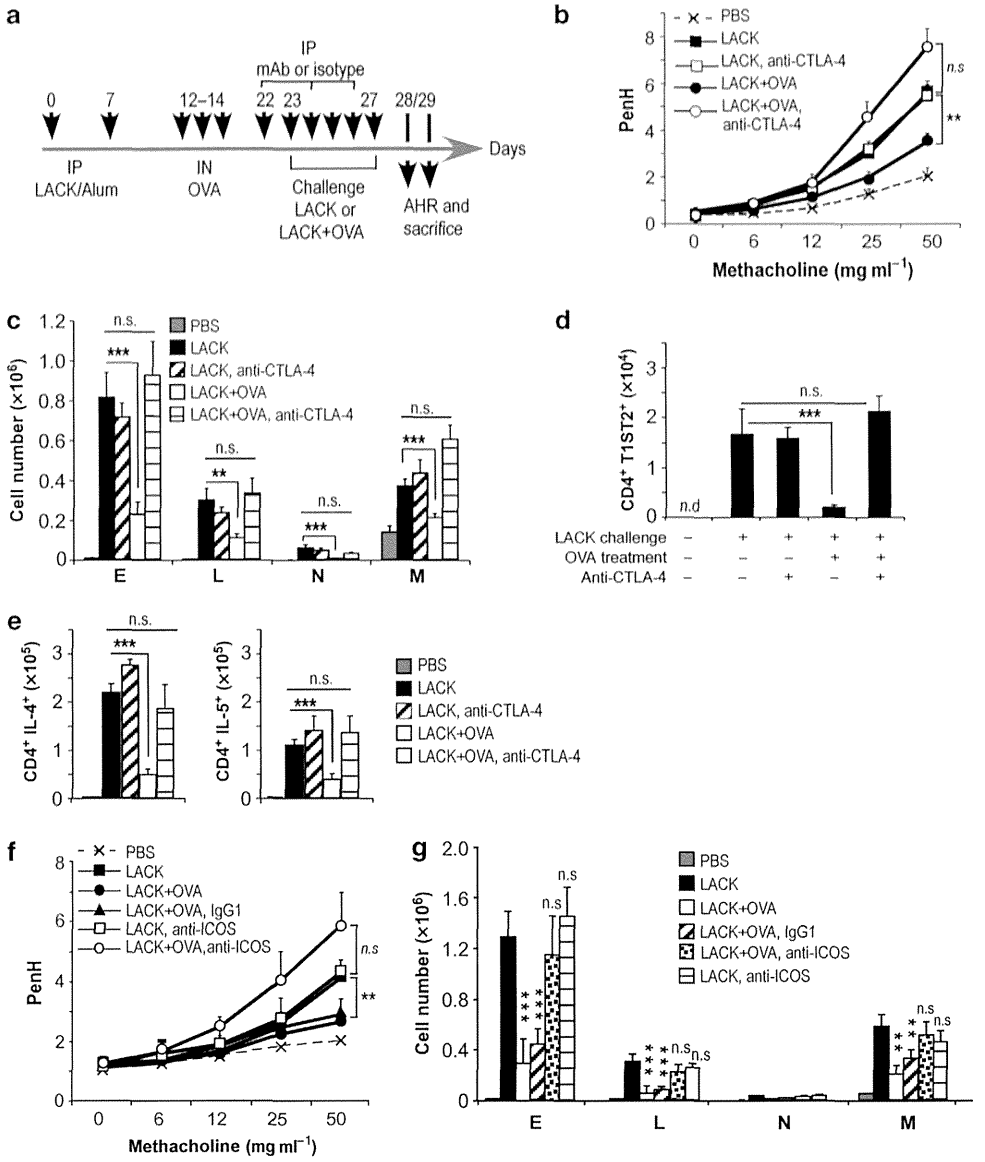

Figure 4 Airway hyperresponsiveness (AHR) and airway inflammation in mice treated with anti-CTLA-4 or anti-ICOS. (**a**) Experimental protocol. Mice were sensitized with two intraperitoneal (IP) injections of *Leishmania* homolog of receptors for activated c kinase (LACK) in Alum, treated with three intranasal (IN) injections of ovalbumin (OVA), treated or not with anti- CTLA-4 mAb, -ICOS, or IgG1 isotype mAb at the indicated time, and challenged daily for 5 days with LACK or LACK/OVA aerosols. Mice were analyzed 1 and 2 days after the last aerosol. (**b**, **f**) AHR. Whole body plethysmography in response to increasing doses of inhaled methacholine in the indicated groups of mice. (**c**, **g**) Number and phenotype of bronchoalveolar lavage fluid (BALF) cells. BALF cells were analyzed by fluorescence *in situ* hybridization (FACS) in the indicated groups of mice. Eosinophils, E; neutrophils, N; lymphocytes, L; and macrophages, M. (**d**) Number of Th2 cells in the airways. BALF cells were stained with anti-CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. (**e**) Interleukin (IL)-4 and IL-5-secreting $CD4^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1 mg ml^{-1}), anti-CD28 (1 μg ml^{-1}), and brefeldin A (5 μg ml^{-1}). Data are expressed as mean ± s.e.m. of three experiments with $n = 6$ mice per group. Experimental groups were compared with untreated LACK-challenged mice. n.s., non-significant; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$.

LACK + OVA IN challenge (**Figure 5a**). In this system, mice injected with LACK-specific Th2 cells that received an IN challenge displayed comparable inflammatory responses to the mice sensitized with LACK/Alum and challenged with aerosols used previously (**Figure 5b–d**). When compared with the BSA-tolerized group, mice treated with OVA IN and injected with LACK-specific Th2 cells exhibited decreased AHR (**Figure 5b**), and reduced numbers of eosinophils and lymphocytes in the BALF (**Figure 5c**). Interestingly, while no difference was observed in the blood, both the frequency and the number of donor LACK-specific Th2 cells were significantly reduced in the BALF, lung and mediastinal LN (MedLN) of OVA-tolerized mice when compared with BSA-tolerized mice (**Figure 5d**). CFSE dilution profiles revealed that LACK-specific Th2 cells had undergone more divisions in BSA-tolerized mice than in OVA-tolerized mice (**Figure 5e**), further suggesting that OVA-T_{reg} inhibited LACK-specific Th2 cell proliferation. To address the ability of APCs to present LACK at the time of the challenge,

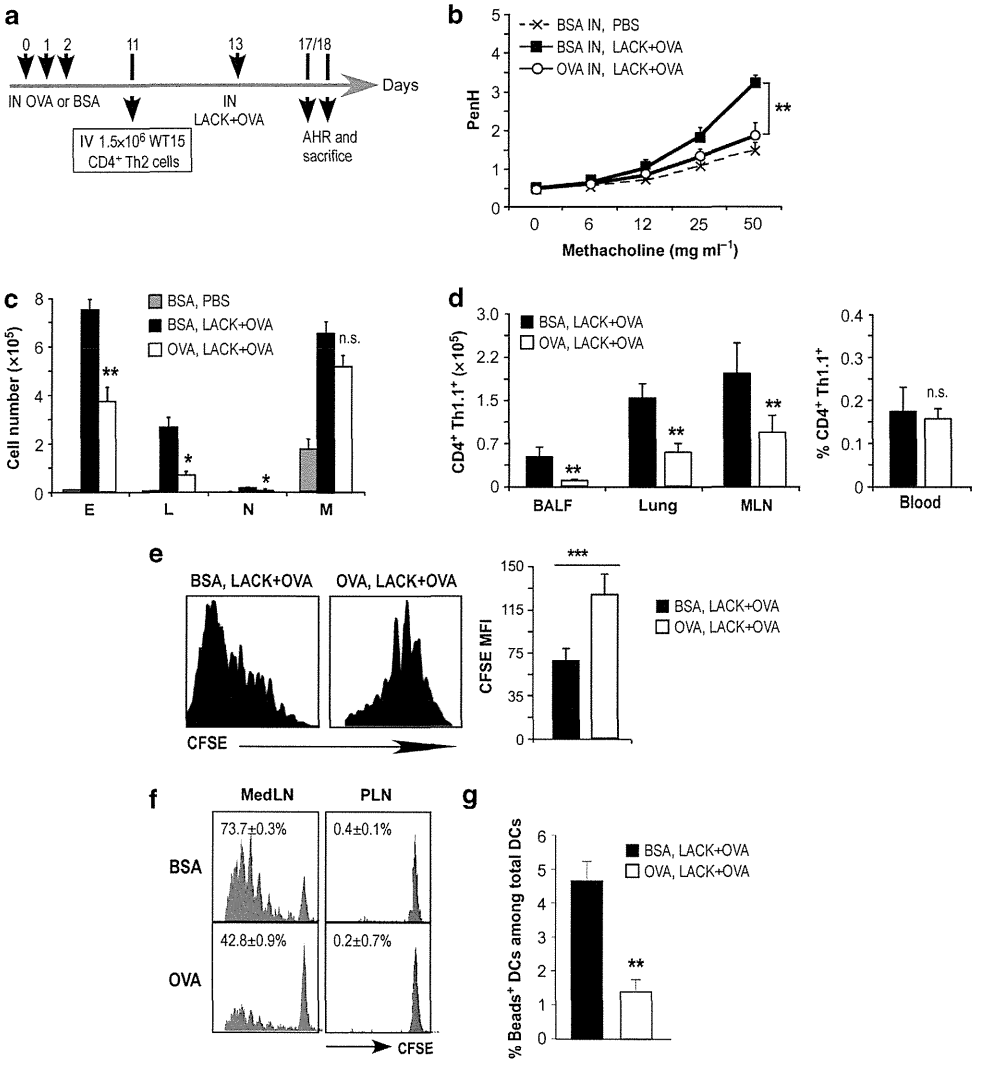

Figure 5 Airway hyperresponsiveness (AHR), airway inflammation, cytokine levels, and T-cell proliferation in ovalbumin (OVA)-tolerized mice injected with *Leishmania* homolog of receptors for activated c kinase (LACK)-specific Th2 cells and exposed to LACK/OVA. (**a**) Experimental protocol. Mice were treated with three intranasal (IN) injections of OVA or BSA, injected with 1.5×10^6 Carboxyfluorescein succinimidyl ester (CFSE)-labeled Thy1.1$^+$ LACK-specific Th2 cells. Mice were injected intranasally 2 days later with LACK/OVA and analyzed 4 and 5 days later. (**b**) AHR. Whole body plethysmography was monitored in mice tolerized to OVA (empty circles) or BSA (filled squares, and crosses) and exposed to aerosols of LACK/OVA (full lines) or to phosphate-buffered saline (PBS) (dashed lines). Data are expressed as mean ± s.e.m. (**c**) Number and phenotype of bronchoalveolar lavage fluid (BALF) cells. BALF cells were analyzed by fluorescence *in situ* hybridization (FACS) in the indicated groups of mice. Eosinophils, E; neutrophils, N; lymphocytes, L; and macrophages, M. Data are expressed as mean ± s.e.m. of three experiments with $n = 6$–8 mice per group. (**d**) Numbers of Thy1.1$^+$ LACK-specific Th2 cells in BALF, lung, and medLN and frequency in the blood of BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA aerosols. (**e**) Representative plots of CFSE (left panels) and mean fluorescence intensity of CFSE (right panels) of the indicated mice. (**f**) *In vitro* antigen presentation assay. Whole-cell suspensions prepared from the MedLN and PLN of OVA- or BSA-exposed mice were incubated for 3 days with CFSE-labeled LACK-specific Th2 cells. Data show representative CFSE plots for the indicated mice with the frequency of divided cells as mean ± s.e.m. of two experiments with $n = 6$ mice per group. (**g**) Mice underwent the same protocol as shown in (**a**), but also received fluorescent latex beads during LACK/OVA challenge. Beads$^+$CD11c$^+$ migratory DCs were analyzed by FACS in the MedLN of BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA aerosols. n.s., non-significant; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.0001$.

cells from the MedLN and distal popliteal LN were purified 16 h after the LACK + OVA IN challenge and incubated *in vitro* with naïve CFSE-labeled LACK-specific WT15 CD4$^+$ T cells to assess their ability to induce proliferation. As expected, popliteal LN cells did not promote cell division (**Figure 5f**). However, LACK-specific T cells proliferated more vigorously when incubated with MedLN cells from BSA-tolerized mice than MedLN cells from OVA-tolerized mice (**Figure 5f**). Furthermore, to understand whether this phenomenon resulted from a quantitative (i.e., different number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice) or qualitative defect (i.e., similar number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice but different ability to induce T-cell proliferation), we administered PE-conjugated latex beads to OVA- and BSA-tolerized mice at the time of the challenge with LACK + OVA, and the frequency of MedLN bead$^+$ DCs was

assessed 16 h later. While bead$^+$ DCs were readily detected in the MedLN of both OVA- and BSA-tolerized mice and expressed similar levels of CD80, CD86, OX40L, and MHC class II molecules (not shown), the frequency of bead$^+$ DCs was decreased from $4.7 \pm 0.7\%$ in BSA-tolerized mice to $1.4 \pm 0.3\%$ in OVA-tolerized animals (**Figure 5g**). Altogether, our data suggest that OVA-specific T_{reg} inhibited the proliferation of LACK-specific Th2 cells by preventing the migration of airway DCs to MedLN.

DISCUSSION

Tolerance induction protocols, in the absence of adjuvant, accompanied with the generation of T_{reg} cells have been reported using multiple distinct approaches.[6,10,31–34] In this report, we have chosen to administer three IN injections with OVA to induce Ag-specific T_{reg}. In contrast to chronic exposure to Ag aerosols,[7] this treatment does not confer a complete IgE unresponsiveness to further OVA sensitization and challenge (**Supplementary Figure 1**), suggesting that the underlying mechanisms of suppression are qualitatively and/or quantitatively different. However, the suppression induced by these series of IN injections was previously described as an effective method to prevent graft rejection[32] but also as a way to suppress airway allergic inflammation.[10] In the present study, we have demonstrated that OVA-T_{reg} generated following this approach are able to alleviate allergic symptoms induced by a different Ag upon their reactivation. We believe that these results represent an interesting therapeutic strategy; as such suppression would only be active upon local administration of the therapeutic Ag.

OVA-induced protection was not a result of Ag competition for the presentation of OVA or LACK, as non-tolerized mice challenged with both OVA and LACK displayed similar inflammatory responses as the LACK only challenged group. More importantly, experiments in which CD4$^+$ T cells were purified from the spleen of PBS-treated, BSA- or OVA-tolerized mice and injected into LACK-sensitized recipients revealed that the suppression of inflammation was mediated by T_{reg} that require their cognate Ag to be reactivated.

Inhibition studies showed that CTLA-4 was absolutely required for OVA-induced suppression of LACK-mediated allergic asthma. While we did not determine the role of CTLA-4 in our model, it has been shown to be critical for the suppressive ability of T_{reg} cells both *in vivo* and *in vitro*.[35] CTLA-4 could prevent CD28 signaling in effector T cells (T_{eff}) by competing with CD80 and CD86, and/or to induce the synthesis of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase by APCs, leading to T-cell suppression by both local depletion of tryptophan and induction of apoptosis via tryptophan catabolites.[35] While we were unable to detect increased apoptosis of LACK-specific T cells in OVA-tolerized mice upon challenge (not shown), we cannot rule out the possibility that this phenomenon was partially responsible for OVA-induced protection.

Blocking ICOS–ICOSL interactions induced the same effect as CTLA-4 inhibition. This result supports a previous study showing that ICOS$^{-/-}$ T_{reg} did not confer protection upon transfer to asthmatic mice, demonstrating a crucial role of ICOS in their suppressive function.[29] Taken together, these data highlight the multiple and complex role of CD25$^+$ T_{reg}. While these cells have been proposed to act via cell contact-dependent mechanisms *in vitro*, many more may also take part such as inhibitory cytokine production as well as non-cytokine-dependent mechanisms *in vivo* depending on the experimental conditions.[36]

Adoptive transfer of CFSE-labeled LACK-specific Th2 cells into OVA-tolerized recipient mice demonstrated that OVA-specific T_{reg} significantly reduced the number of LACK-specific Th2 cells in the BALF, lung, and MedLN by affecting their proliferation. Additional experiments using of fluorescently labeled latex beads revealed that OVA treatment affected the migration of airway DCs into the MedLN, suggesting that one possible suppressive mechanism of OVA-T_{reg} is their interaction with APCs to decrease the total load of Ag to reach the draining LNs, dampening therefore the amplitude of T_{eff} response. These results are in agreement with the previous findings, which reported that airway T_{reg} could decrease the expression of CD86 on DCs altering therefore their ability to migrate to the lung draining LNs.[37] While we did not characterize the state of activation of LACK-loaded DCs in our model, it is possible that OVA-T_{reg} are using a similar approach, supporting further the crucial role of CTLA-4 in the protection against LACK-induced inflammation. However, it is important to notice that, while the effect on DCs could explain the decrease in proliferation of LACK-specific Th2 cells, it is also possible that OVA-T_{reg} perform a second level of suppression in the MedLN by interacting directly with the T effector responses. Indeed, imaging data in mice have shown that T_{reg} interact with DCs, altering the latter and diminishing subsequent DC-T_{eff} cell conjugate formation *in vivo*.[38,39] In addition, Derks *et al.*[40] have envisioned two hypotheses of APC function in bystander suppression: a passive APC model, in which the APCs would present MHC peptide to the T_{reg}, stimulating them to produce immunosuppressive cytokines that would further binds their cognate receptors on the third-party T_{eff}, or an active APC model, in which the APCs would propagate regulatory effects from the T_{reg} to the T_{eff} through various APC products. These two hypotheses remain to be tested in our model.

To eliminate the possibility of this protection being restricted to OVA protein, BSA-tolerized mice treated with BSA during the LACK challenge phase were equally protected from LACK-induced inflammation (data not shown). In conjunction with our findings with the human relevant allergen, ragweed, our data suggest that neither this protective phenomenon is restricted to the specific therapeutic Ag OVA, nor it is restricted to a specific allergen LACK.

Taken together, our results demonstrate that T_{reg} specific for a third-party therapeutic Ag could control allergic immune responses and lung inflammation when re-stimulated *in vivo*. Our results may explain the protective effects of SIT against allergens that are different from those used during the desensitization treatment, also primary referred to as "infectious tolerance", as reported in several epidemiological studies.[12,13,41]

In the same line of evidence, Campbell and his collaborators demonstrated the phenomenon of "linked epitope suppression" in which treatment with selected epitopes from a single allergen resulted in suppression of responses to other epitopes within the same molecule. They found that peptide treatment led to decreased proliferation and cytokine production to both treatment and non-treatment peptides. Using an experimental mouse model, they further showed that the IN administration of a single dose of peptide derived from the cat allergen Feld1 induced Ag-specific tolerance and resolution of allergic airway inflammation driven by exposure to whole cat allergen extract. While some questions remain to further understand the precise molecular and cellular mechanisms of such suppression, our study together with previous epidemiological and experimental evidence pave the way for the development of a novel curative approach that could control allergen-specific Th2 responses in patients with allergic asthma, and more specifically in patients sensitized to multiple allergens.

METHODS

Mice. Six-week-old BALB/c mice were purchased from The Centre d'Elevage Janvier (France) and housed under standard pathogen free conditions. LACK TCR transgenic mice (WT15 RAG-1 KO) on the BALB/c background as previously described[42] were bred in our animal facility at the Institut de Pharmacologie Moleculaire et Cellulaire (Valbonne, France). In this study, WT15 transgenic mice were further crossed onto RAG-$1^{-/-}$ Thy$1.1^{+/+}$ BALB/c mice. All experimental protocols were approved by the local animal ethic committee.

Reagents. LACK recombinant protein was produced in *E. coli*, purified as described previously,[43] and detoxified using an Endotrap column (Profos, Bernried am Starnberger See, Germany). Lipopolysaccharide contents in LACK protein were below 5 ng mg^{-1} as determined using Limulus Amoebocyte Lysate (LAL) assay (Pierce, Illkirch, France). $LACK_{156\text{-}173}$ peptide was purchased from Mimotopes (Clayton, Victoria, Australia). T1/ST2 mAbs were purchased from MD Biosciences (St Paul, MN). Monoclonal antibodies to CD3, CD4, CD25, Thy1.1, CD11c, IA/IE, CD80, CD86, IL-4, and IL-5 were purchased from BD Biosciences (Le Pont de Claix, France).

Induction of allergic asthma and tolerization to OVA. Sensitization was performed by two intraperitoneal injections of 10 μg of LACK in 2 mg of Aluminium hydroxide (Alum) (Pierce) at days 0 and 7. On days 12, 13, and 14, mice were tolerized to OVA by injecting IN 100 μg of LPS-free OVA (Profos) as described previously.[8] From day 23 to day 27, mice were exposed either to LACK (0.15%) or to LACK plus OVA (0.2%) aerosols (administered 8 h apart) for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia, Le Tholonet, France). Mice were analyzed on days 28 and 29 for AHR and airway inflammation, respectively. When indicated, mice were injected with either 0.5 mg of anti-CTLA-4 mAb (9H10) anti-ICOS (17G9) or isotype control (IgG1, GL113) every other day over the challenge period starting 1 day before the first aerosol. For ragweed-induced asthma, mice were first tolerized to OVA by receiving IN injections of OVA on days 0, 1, and 2 and further sensitized to ragweed via IN administrations of 25 μg ragweed (Greer Laboratories, Lenoir, NC) on days 11, 15, 19, and 23. Mice received a last challenge of ragweed on day 27 or ragweed and OVA on days 26 and 27. Mice were analyzed on day 28 for AHR and on day 29 for airway inflammation.

Th2 cell transfers. In some experiments, mice were first tolerized to OVA, BSA, or PBS and injected i.v on day 11 with LACK-specific $CD4^+$ Th2 cells. Mice were challenged 24 h later with a single IN injection of LACK (30 μg) and OVA (100 μg), and assessed for AHR, and immunological parameters 3 and 4 days later, respectively.

Airway hyperresponsiveness. For non-invasive measurements, mice were analyzed 1 day after the last aerosol challenge using whole body plethysmography as described previously.[44] Invasive measurements of dynamic lung resistance and compliance were performed 1 day after the last aerosol challenge using a Flexivent apparatus (SCIREQ, Emka Technologies, Paris, France) as previously described.[25] Briefly, mice were anesthetized (5 ml kg^{-1} Dormitor 10% (Medetomidine, Pfizer, Paris, France)—Imalgene 10% (Ketamine, Merial, Lyon, France) tracheotomized, paralyzed (5 ml kg^{-1} Pavulon 1% (Pancuronium bromide, Organon, Fresnes, France) and immediately intubated with an 18-G catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths per minute with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of methacholine (0–24 mg ml^{-1}) were administered at the rate of 20 puffs per 10 s, with each puff of aerosol delivery lasting 10 ms, via a nebulizer aerosol system with a 2.5–4 μm aerosol particle size generated by a nebulizer head (Aeroneb, Aerogen, Galway, France). Baseline resistance was restored before administering the subsequent doses of methacholine.

Analysis of BALF cells. Mice were bled and a canula was inserted into the trachea. Lungs were washed three times with 1 ml of warmed PBS. For differential BALF cell counts, cells were stained with mAb anti-CCR3 (R&D, Lille, France), anti-Gr1, anti-CD3, and anti-CD19 mAbs (Becton Dickinson, Le Pont de Claix, France) and analyzed by fluorescence *in situ* hybridization (FACS) using a FACScalibur flow cytometer (BD Bioscience) and Cellquest software (BD Bioscience). Eosinophils were defined as $CCR3^+$ $CD3^-CD19^-$, neutrophils as $Gr\text{-}1^{high}$ $CD3^-CD19^-$, lymphocytes as $CD3^+CD19^+$, and alveolar macrophages as large autofluorescent cells.

Serum antibody measurements. Serum LACK-specific IgG1 and IgE were measured by ELISA. For IgG1 quantification, Ag-coated Maxisorp plates (Nunc, Illkirch, France) were incubated with serial dilution of sera and biotinylated anti-IgG1 mAb (BD). For Ag-specific IgE, plates were first coated with the respective capture mAb (BD), and incubated with serum dilutions. Biotinylated-LACK Ag was then added. HRP-conjugated streptavidin (BD) and TMB (KPL, Gaithersburg, MD) were used for detection.

Tissue processing. Lungs, LN, or spleens were cut into small pieces in HBSS containing 400 U type I collagenase and 1 mg ml^{-1} DNAse I and digested for 30 min at 37 °C. Cells were strained through a 70-μm cell strainer. Erythrocytes were lysed with ACK lysis buffer.

Cytokine assays. Lung samples were homogenized in C^{2+}- and Mg^{2+}-free HBSS. BAL and lung supernatants were used. Multiplex IL-4, IL-5, IL-13, and IFN-γ analysis was performed with CBA using FACS array (BD Biosciences). For intracellular staining, cells were incubated with 100 μg ml^{-1} LACK and 1 μg ml^{-1} of anti-CD28 (BD) for 6 h. Brefeldin A (5 μg ml^{-1}, Sigma, Saint Quentin Fallavier, France) was added during the last 4 h. Cells were then stained with anti-CD4 mAb, fixed, permeabilized using cytofix/cytoperm reagent (BD), stained with anti-IL-4, or IL-5 (BD) and analyzed by FACS.

$CD4^+$ T-cell transfer. Donor mice were tolerized to OVA, BSA, or PBS as described above. Cells were prepared from spleens 21 days later, and $CD4^+$ T cells were enriched by negative depletion using CD4 isolation kit (Dynal, Illkirch, France) and further sorted using a high-speed sorter VANTAGE SETLO$^+$ flow cytometer (BD) after staining with anti-CD3 and anti-CD4 mAbs. CD4 purity was >95%. In some experiments, enriched $CD4^+$ T cells were stained with antibodies to CD25, CD4, and CD3, and $CD25^-$ and $CD25^+$ $CD4^+$ T-cell populations were sorted by FACS. Sorted cells were then injected i.v. into sensitized mice (4×10^6 or 1.5×10^6 cells per mouse, respectively) as described previously.[26,27]

Statistic analysis. ANOVA for repeated measures was used to determine the levels of difference between groups of mice for plethysmography measurements. Comparisons for all pairs were performed by Mann–Whitney *U* test. Significance levels were set at a *P*-value of 0.05.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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Case Report

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A novel therapeutic use of HFA-BDP metered-dose inhaler in asthmatic patients with rhinosinusitis: Case series

Yoshiki Kobayashi[1,2], Mikiya Asako[2], Akira Kanda[2], Koich Tomoda[2], and Hirotaka Yasuba[1]

[1]*Department of Airway Medicine, Mitsubishi Kyoto Hospital, Kyoto, and*
[2]*Department of Otolaryngology, Kansai Medical University, Osaka, Japan*

Key words
airway medicine– asthma – exhalation through the nose – rhinosinusitis – small airway obstruction

Abstract. Objective: Most asthmatics have been found to have rhinosinusitis (RS). Patients with ethmoid sinusitis, in particular, often suffer from an impaired sense of smell; this is clinically important and necessitates concurrent treatment for both asthma and RS. As a rational therapeutic strategy, we focused on a fine particle HFA-134a-beclomethasone dipropionate (HFA-BDP) metered-dose inhaler. Because of its small size, the medication is still present in the exhaled breath after inhalation. Methods: Five mild-to-moderate asthmatics with ethomoid-predominant sinusitis characterized by an impaired sense of smell and mild peripheral blood eosinophilia received a single-agent treatment with orally-inhaled HFA-BDP which was then exhaled through the nose. In addition, the stained small particles were created by an ultrasonic nebulizer and flow image of them during oral inhalation and nasal exhalation was evaluated by using nasal endoscopy. Results: After treatment, the sense of smell was restored in all cases with a concomitant improvement in sinusitis as confirmed by computerized tomography. In addition, amelioration of peripheral blood eosinophilia as well as small airway obstruction as indicated by pulmonary function tests was observed. Macroscopical imaging revealed that small particles flow toward olfactory cleft during both the inhalation and exhalation phases. Conclusion: We have presented 5 cases of asthmatic patients with RS treated with a concurrent single therapy, HFA-BDP exhaled through the nose (ETN). A clinical trial must be considered to establish this new therapeutic strategy based on the concept of "one airway, one disease."

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Correspondence to
Yoshiki Kobayashi, MD, PhD
Department of Otolaryngology, Kansai Medical University, 2-5-1 Shinmachi, Hirakata, Osaka 573-1191, Japan
kobayosh@hirakata.kmu.ac.jp

Introduction

Bronchial asthma, a chronic inflammatory airway disease, can be well controlled by the regular use of inhaled corticosteroids (ICS) with or without long-acting β_2-agonists (LABAs) [1]. Despite effective treatment strategies, its prevalence is still increasing. Further, many asthmatics have rhinosinusitis (RS) as demonstrated by computed tomography (CT) scans [2] and they suffer from nasal obstruction or impaired sense of smell. Pfister et al. [3] observed mucosal thickening of sinuses on CT scans in 74% of asthmatic patients. Along with the abnormalities of sinus on CT scans, patients with severe asthma also have eosinophilia in peripheral blood and induced sputum, impaired pulmonary function, and elevated levels of exhaled NO [4]. Furthermore, patients with severe asthma tend to have more severe RS [5, 6]. Therefore, recognizing the close relationship between asthma and RS is crucial for asthma control.

For simultaneous treatment of both asthma and RS based on the concept of "one airway, one disease", we previously evaluated the hydrofluoroalkane-134a-beclomethasone dipropionate (HFA-BDP) metered-dose inhaler. This inhaler contains an extra-fine particle, which remains in the expired breath due to its small size (average diameter: 1.1 μm) [7, 8]. To optimally use HFA-BDP, we examined its beneficial effect when it was orally-inhaled and then exhaled through the nose (ETN) on a trial basis. In our preliminary study, HFA-BDP ETN converted from the conventional ICS therapy (equivalent doses of fluticasone propionate or HFA-BDP) improved both small airway obstruction and nasal symptoms (such as nasal obstruction and impaired sense of smell) along with the improvement in sinus CT findings [9]. However, in that study we could not exclude the possibility of synergistic effects with other medications such as LABAs or leukotriene receptor antagonists. Here, we report a series of 5 cases of bronchial asthma with RS treated with the single-agent therapy using HFA-BDP ETN.

Table 1. Clinical characteristic of 5 cases.

Case no.	1	2	3	4	5	Mean ± SD
Age/sex	69/F	60/F	71/F	69/F	60/F	65.8 ± 5.4
Severity of asthma	mild	moderate	mild	moderate	moderate	
Smoking status	never	never	never	never	never	
Total IgE (IU/mL)	122	99	237	325	218	200 ± 92
Eosinophil (/μL)	595	596	310	312	760	515 ± 198
FEV_1 (L)	1.71	1.36	1.64	1.60	1.53	1.57 ± 0.13
$\%FEV_1$ (%)	89.6	69.6	84.9	86.7	70.4	80.2 ± 9.5
Reversibility						
ΔFEV1 (mL)	280	260	320	240	230	266 ± 36
ΔFEV1 (%)	16.4	19.4	21.6	17.6	15.0	18.0 ± 2.6
FVC (L)	2.42	2.00	2.21	2.34	2.07	2.21 ± 0.18
$FEV_1\%$ (%)	70.7	68.0	74.2	68.4	73.9	71.0 ± 2.9
$\%FEF_{25-75}$ (%)	46.0	30.6	53.1	40.3	39.2	41.8 ± 8.4
ACT	24	18	24	25	22	22.6 ± 2.8
LMS						
Total	9	10	8	12	9	9.6 ± 1.5
Ethmoid	5	4	4	5	4	4.4 ± 0.5
Pretreatment						
ICS (μg)/LABA	0/–	0/–	200/+	400/+	0/–	
LTRA	–	+	–	–	–	

FEV_1 = forced expiratory volume in 1 s; FVC = forced vital capacity; FEF_{25-75} = forced expiratory flow between 25% and 75% of vital capacity; ACT = asthma control test; LMS = Lund-Mackay scale; ICS = inhaled corticosteroid (equivalent doses of fluticasone propionate); LABA = long-acting β_2-agonist; LTRA = leukotriene receptor antagonist.

Methods

Subjects

Five patients with mild-to-moderate asthma, determined according to the Global Initiative for Asthma (GINA) guidelines [10], were evaluated. They all were never-smokers with positive reversibility and had mild eosinophilia, evidence of small airway obstruction, and an impaired sense of smell due to RS, caused predominantly by CT-confirmed ethmoid sinusitis (evaluated using the Lund-Mackay scale). Two patients were receiving no medication, another patient used only a leukotriene receptor antagonist, and the other two had been treated with conventional ICS/LABA treatment which was orally-inhaled and exhaled through the mouth. No patients had received any treatments, such as topical corticosteroids or endoscopic sinus surgery, for RS. All patients were converted to a single-agent therapy using HFA-BDP ETN without a wash out period for the pre-treatment and continued on only HFA-BDP ETN without any concomitant treatment. Briefly, HFA-BDP (QVAR, 3M Pharmaceuticals, St. Paul, MN, USA; 400 μg twice a day) was inhaled orally using a spacer (VORTEX®, PARI-Japan, Osaka, Japan), followed by breath-holding for a few seconds and exhalation through the nose. We confirmed the procedures at the beginning of HFA-BDP ETN treatment and on each visit to make sure that patients were able to perform them properly. Two patients had diabetes or hypercholesterolemia, and the other three did not have any comorbidity requiring treatment. The patient characteristics are summarized in Table 1. All patients gave their written informed consent for inclusion in this report.

Macroscopic imaging

Normal saline solution was stained with natural dye (Kiriya's brilliant green SP, KIRIYA Chemical Co., Ltd. Osaka, Japan). Three healthy volunteers orally inhaled the small particles (a single-digit μm range) created by an ultrasonic nebulizer, followed by breath-holding for a few seconds and exhalation through the nose. The particle flow during oral inhalation and nasal exhalation was observed by using nasal endoscopy. All healthy volunteers gave their written informed consent for inclusion in this study.

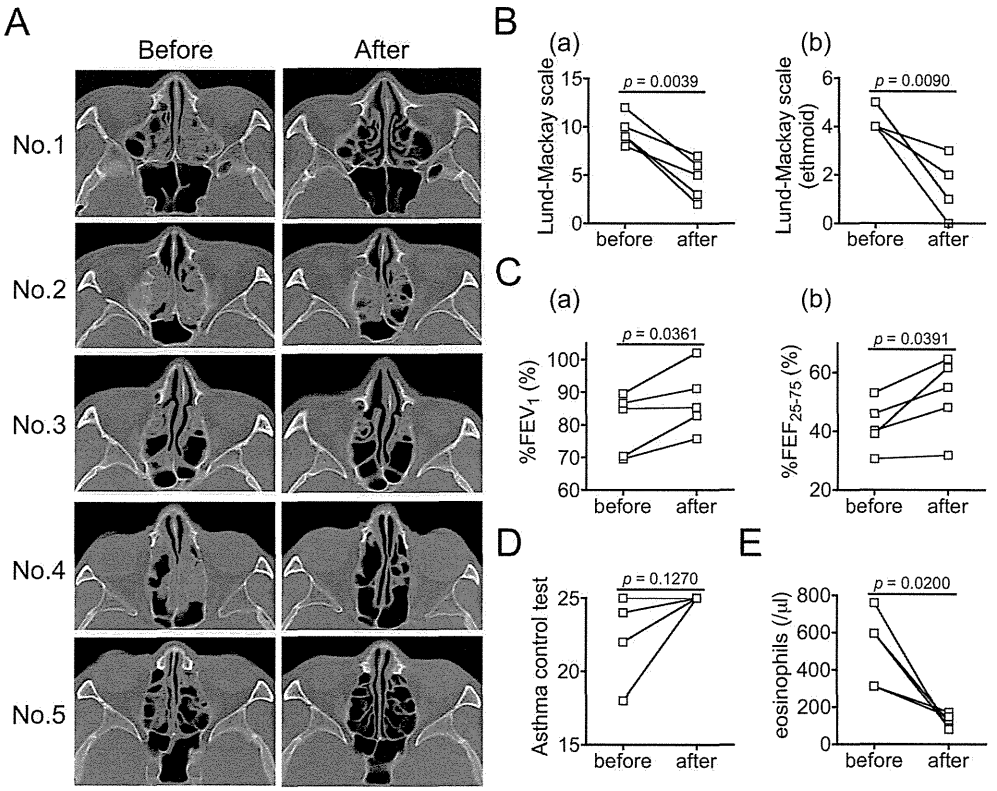

Figure 1. Effects of HFA-BDP ETN treatment. All 5 patients inhaled HFA-BDP using a tube-type spacer, followed by exhalation through the nose. Each parameter was evaluated before and after treatment with HFA-BDP ETN (Case no. 1, 3, and 5: 2 months after treatment; Case no. 2: 4 months after treatment; Case no. 4: 1 month after treatment). The indicated periods depend on the first visit after converting to HFA-BDP ETN. A: CT findings in the sinus; B: Lund-Mackay scale (panel a: total score; panel b; ethmoid sinus score). Comparisons of data before and after treatment were performed using a paired t-test; C: Lung function test (panel a: $\%FEV_1$; panel b: $\%FEF_{25-75}$); D: Asthma control test; E: Peripheral blood eosinophil counts.

Results

After the commencement of HFA-BDP ETN therapy, the restoration of their sense of smell with concomitant improvement in the Lund-Mackay scale was observed within 2 months in 4 patients (Figure 1A, B). Furthermore, HFA-BDP ETN treatment also provided good asthma control, as evaluated by the Asthma Control Test with decreased airway obstruction including small airways, as shown by improvements in forced expiratory volume in 1 second ($\%FEV_1$) and forced expiratory flow between 25% and 75% of vital capacity ($\%FEF_{25-75}$) (Figure 1C, D). These results were supported by the amelioration of mild eosinophilia linked to asthma as well as RS with ethmoid-predominant sinusitis (Figure 1E). Four patients had no recurrence of symptoms with continued HFA-BDP ETN treatment. The dose of HFA-BDP was successfully tapered for one of these patients (case no. 4). Another patient stopped the HFA-BDP treatment because of mild treatment-induced stomatitis; the discontinuation resulted in the recurrence of RS and asthma symptoms after 4 months (case no. 2).

In macroscopic examination, we found that small particles flow toward the olfactory cleft during both the inhalation and exhalation phases (Figure 2).

Discussion

As shown in our case series, a rational and simultaneous therapy for asthma and RS is required based on the concept of "one airway, one disease". Nonaka et al. [11] recently reported a case of refractory RS with comorbid asthma successfully treated with orally-inhaled fluticasone propionate (FP). They speculated that inhaled FP (100 μg twice a

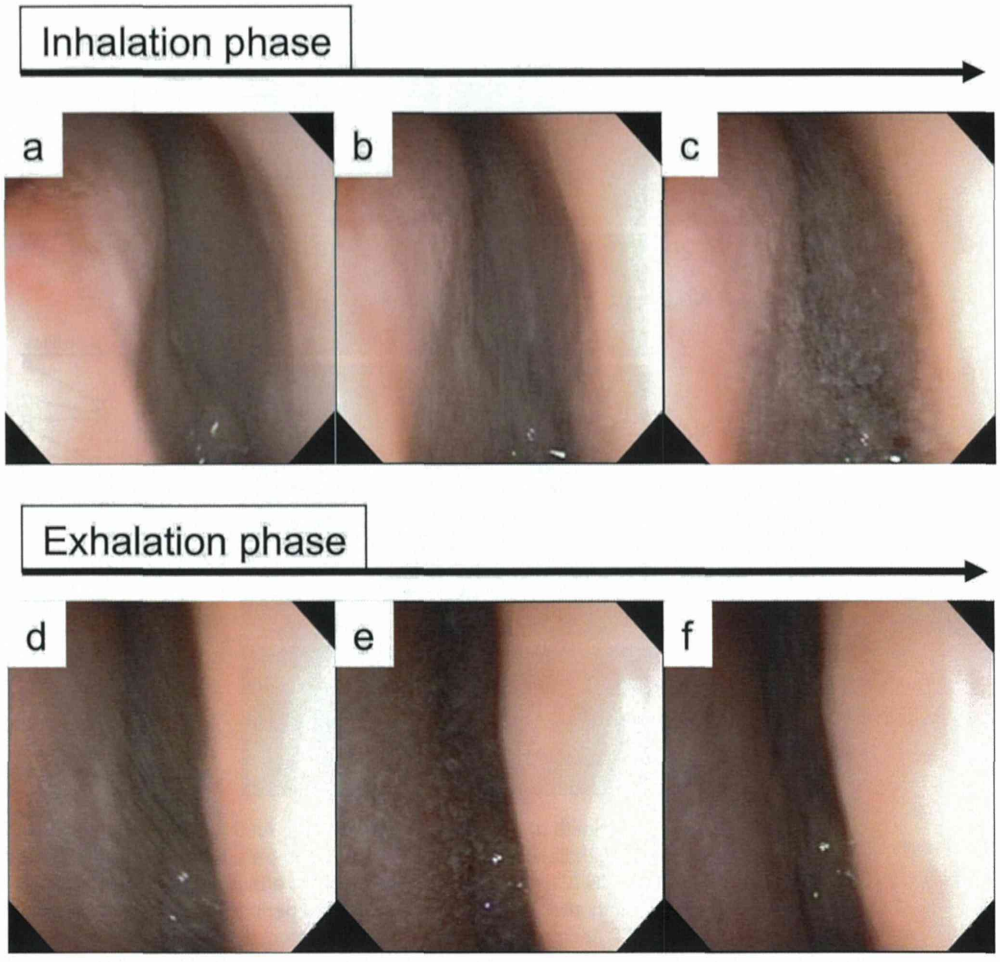

Figure 2. Time-lapse flow images of small particles from the inhalation phase to the exhalation phase. Small particles (a single-digit μm range) made by an ultrasonic nebulizer were inhaled orally and exhaled through the nose. Flow images towards the olfactory cleft were serially observed from inhalation phase (upper; a – c) to exhalation phase (lower; d – f) every second using nasal endoscopy. Panels a and f show the middle meatus at the beginning of the inhalation phase (a) and the end of the exhalation phase (f). Panels b and d show the flow lines of particles towards the olfactory cleft (from bottom to top). Panels c and e show the clusters of particles floating in the middle meatus for 1 s.

day) attenuated lower airway inflammation mainly associated with eosinophils, resulting in an amelioration of recurrent RS following endoscopic sinus surgery [11]. However, in our case (no. 4), RS could not be controlled even with the administration of higher doses of orally-inhaled FP (200 μg twice a day). In the case reported by Nonaka et al. [11], it is possible that the distribution of orally-inhaled FP to the ethmoid sinus mucosa may have been enhanced by endoscopic sinus surgery [12].

RS with ethmoid-predominant sinusitis characterized by activated eosinophil infiltration is associated with olfactory dysfunction [13]. This explains why the amelioration of ethmoid sinusitis as confirmed by CT led to a restoration of the sense of smell in the patients evaluated in this study. Scheibe et al. [14] indicated that drug delivery to the olfactory cleft provides a beneficial effect on the restoration of sinonasal smell disorders. To confirm whether the small particles (a single-digit μm range) flow toward the olfactory cleft during oral inhalation and nasal exhalation, we observed the particles by using nasal endoscopy. Macroscopical imaging revealed that small particles easily and effectively reach the olfactory cleft during both the inhalation and exhalation phases. This finding may explain not only the lasting improvement of olfactory impairment by HFA-BDP ETN but also the beneficial effects with the conventional orally-inhaled ICS in some cases, such as that reported by Nonaka et al. [11]. However, Hyo et al. [15] has shown that only

3% of the small particles (μm range) reach the paranasal sinuses, supporting our previous report that the conventional orally-inhaled and orally-exhaled HFA-BDP did not improve RS [9]. In the cases without endoscopic sinus surgery, continuous treatment with HFA-BDP ETN may increase the accumulation into paranasal sinuses, resulting in suppression of airway inflammation. Beneficial effects of topical nasal corticosteroids for patients with RS have been demonstrated [16, 17] whereas in our case series, all patients improved by single-agent HFA-BDP ETN therapy. Intranasal administration using a squirt system could efficiently deliver corticosteroids to the olfactory cleft, thereby exerting a beneficial effect on olfactory dysfunction; however, these effects are incomplete and transient [18]. It is notable that the conversion from conventional ICS to HFA-BDP ETN (equivalent doses of ICS) provided beneficial effects for patients with poorly controlled asthma with RS even under treatment with ICS and intranasal corticosteroid [9].

RS with ethmoid-predominant sinusitis is closely related to bronchial asthma and peripheral blood eosinophilia [19]. As peripheral blood eosinophil counts correlate with infiltrating eosinophil counts in nasal polyps [19], we confirmed decreased peripheral blood eosinophil counts with concomitant improvement in sinus CT findings after treatment. Even after converting from conventional ICS/LABA therapy to HFA-BDP ETN, asthma in these patients was well-controlled without exacerbation which reflected the decreased eosinophil counts in the peripheral blood. Furthermore, HFA-BDP inhibited eosinophilic inflammation in the small airways of asthmatics more potently compared with an equivalent dose of FP treatment [20]. In addition, latent small airway inflammation has a close relationship with RS [21], suggesting that it is important to regulate inflammation of both small airway and paranasal sinuses. We believe that HFA-BDP ETN therapy may be a reasonable option due to the small particle size, which is capable of reaching both the small airways and olfactory cleft (or paranasal sinuses) and control both upper and lower airway inflammation more effectively.

Since the sample size in this case report was too small to conclude beneficial effect of HFA-BDP ETN treatment, a crossover study or a blinded placebo-controlled study need to be done to confirm our preliminary findings.

Conclusion

We have reported 5 cases of asthmatic patients with RS (ethmoid-predominant sinusitis) treated with HFA-BDP ETN. A clinical trial must be conducted to confirm the utility of this novel strategy based on the concept of "one airway, one disease".

Consent

Written informed consent was obtained from the patients for publication of this case report and any accompanying images.

Funding

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Conflict of interest

The authors declare that they have no competing financial interests.

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The Laryngoscope

Subclassification of Chronic Rhinosinusitis With Nasal Polyp Based on Eosinophil and Neutrophil

Katsuhisa Ikeda, MD; Akihito Shiozawa, MD; Noritsugu Ono, MD; Takeshi Kusunoki, MD; Mikio Hirotsu, MD; Hirotomo Homma, MD; Tatsuya Saitoh, MD; Junko Murata, MD

Objectives/Hypothesis: Japanese patients with chronic rhinosinusitis with nasal polyps (CRSwNP), differing from European and U.S. patients, are suggested to show two distinct phenotypes: Th2-polarized and Th1-shifted immunity. The purpose of this study was to conduct clinical subgrouping of CRSwNP based on inflammatory cell infiltration, which was evaluated and supported by clinical backgrounds and immunological characteristics.

Study Design: A cross-sectional study.

Methods: One hundred thirty Japanese patients with CRSwNP were classified by the infiltration of eosinophils and neutrophils in nasal polyps. Immunohistochemical analysis was performed in 42 patients.

Results: The patients were classified into three groups: 1) 42 patients with eosinophilic type, 2) 27 patients with neutrophilic type, and 3) 61 patients with noneosinophilic nonneutrophilic type. Both the number of serum eosinophils and the recurrence rates were significantly higher in the eosinophilic group compared to the other two groups. The IgE value was significantly higher in the eosinophilic group, followed by the noneosinophilic nonneutrophilic and neutrophilic groups. Both the symptomatic and CT scores were significantly greater in the eosinophilic group than in the neutrophilic group. The expressions of eotaxin, IL-17A, MUC5AC, and CD68 were greater in the eosinophilic group than in the other two groups.

Conclusion: The eosinophilic CRSwNP phenotype is clinically characterized by serum eosinophilia, atopy, extensive disease, and poor prognosis compared to the neutrophilic and the noneosinophilic nonneutrophilic groups. We clearly demonstrated that all three subgroups of CRSwNP had characteristic differences in those inflammatory markers, which allows for pathophysiologically meaningful differentiations with likely therapeutic consequences.

Key Words: Chronic rhinosinusitis, cytokine, eosinophil, nasal polyp, neutrophil.

Level of Evidence: 3b.

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INTRODUCTION

Although chronic rhinosinusitis (CRS) is a multifactorial disease within a heterogenous group of diseases with different underlying etiologies and pathophysiologies, European and U.S. studies proposed the classification into four categories: 1) acute bacterial rhinosinusitis, 2) CRS without nasal polyps, 3) CRS with nasal polyps (CRSwNP), and 4) allergic fungal rhinosinusitis.[1,2] The histomorphological patterns of CRSwNP are characterized by Th2-driven immune responses, including the predominance of eosinophils and mixed mononuclear cells with a relative paucity of neutrophils.[3] Japanese patients with CRSwNP, differing than European and U.S. patients, are suggested to show two distinct phenotypes: 1) tissue eosinophilia characterized by Th2-polarization[4, 5] and marked expression of eotaxins,[6] as well as tissue remodeling and eosinophilia dependent upon IL-17A expression[7]; and 2) poorly expressed eosinophilia characterized by Th1-shifted immunity and prominent expression of IL-8.[5,8] The former type can be designated as eosinophilic CRSwNP. The latter type displays neutrophilic inflammation in which neutrophil recruitment into the sinus effusion is mediated by both the upregulation of adhesion molecules of the vascular endothelium induced by IL-1β and the enhanced secretion of IL-8 from epithelial cells and neutrophils[8,9]; thus, it can be identified as neutrophilic CRSwNP. Recently, polyps found in the Asian population are suggested to be biased toward neutrophilic inflammation, together with less eosinophilia, in contrast to an eosinophil bias in the Belgian population.[10] Furthermore, the former showed a Th1/Th17 cell pattern and were dominated by a non-IL5 proposed Treg pathway. Thus, CRSwNP shows heterogenous pathogeneses and various underlying immunologic and pathophysiologic aspects.

In the present study, we conducted a clinical subgrouping of CRSwNP based on inflammatory cell infiltration. The subclassified categories of CRSwNP were evaluated and supported by the clinical backgrounds such as disease severity, atopic status, recurrence, etc. Furthermore, the expression patterns of inflammatory parameters in each group were compared in order to

From the Department of Otorhinolaryngology, Juntendo University Faculty of Medicine, Tokyo, Japan

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The study was approved by the ethics committee of the Juntendo University Faculty of Medicine. The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Katsuhisa Ikeda, Department of Otorhinolaryngology, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113–8421, Japan. E-mail: ike@juntendo.ac.jp

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