Novel allergic asthma model demonstrates ST2-dependent dendritic cell targeting by cypress pollen

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Background: Cypress pollen causes respiratory syndromes with different grades of severity, including asthma. IL-33, its receptor ST2, and dendritic cells (DCs) have been implicated in human respiratory allergy.

Objective: We sought to define a new mouse model of allergy to cypress pollen that recapitulates clinical parameters in allergic patients and to evaluate the implications of DCs and the IL-33/ST2 pathway in this pathology.

Methods: BALB/c mice, either wild-type or ST2 deficient ($ST2^{-/-}$), were sensitized and challenged with the *Cupressus arizonica* major allergen nCup a 1. Local and systemic allergic responses were evaluated. Pulmonary cells were characterized by means of flow cytometry. DCs were stimulated with nCup a 1 and tested for their biological response to IL-33 in coculture assays.

Results: nCup a 1 causes a respiratory syndrome closely resembling human pollinosis in BALB/c mice. nCup a 1–treated mice exhibit the hallmarks of allergic pathology associated with pulmonary infiltration of eosinophils, T cells, and DCs and a dominant T_H2-type immune response. IL-33 levels were increased in lungs and sera of nCup a 1–treated mice and in subjects with cypress allergy. The allergen-specific reaction was markedly reduced in $ST2^{-/-}$ mice, which showed fewer infiltrating eosinophils, T cells, and DCs in the lungs. Finally, stimulation of DCs with nCup a 1 resulted in ST2 upregulation that endowed DCs with increased ability to respond to IL-33–mediated differentiation of IL-5– and IL-13–producing CD4 T cells.

Conclusions: Our findings define a novel preclinical model of allergy to cypress pollen and provide the first evidence of a functionally relevant linkage between pollen allergens and T_H2-polarizing activity by DCs through IL-33/ST2. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: *IL-33, dendritic cells, cypress pollen, allergic asthma, ST2, mouse model, eosinophils, T cells*

In the past 20 years, cypress pollen allergy has shown an upward trend, especially in Mediterranean countries, North America, Japan, and Australia, reaching approximately 30% of all cases of pollinosis in some areas.[1,2] Although pollens of the various cypress species show increased allergenic cross-reactivity, pollen extract of *Cupressus arizonica* exhibits a high allergenic potential. The major *C arizonica* allergen Cup a 1 is the predominant component for the frequency of recognition by IgE in allergic patients.[3] Treatments currently available for allergy include avoidance of exposure and pharmacotherapy, which only relieve symptoms. Conventional allergen-specific immunotherapy often results in an unsatisfactory outcome.[4] The major obstacles to the development of alternative and more effective therapies is the lack of a suitable *in vivo* experimental model that allows quantitative evaluation of the allergic inflammatory responses to inhaled allergen and dissection of the immunologic parameters related to the pathology.[5]

Pollinosis occurs as a consequence of dysregulated T_H1 and T_H2 immune responses. Most allergic subjects have a high proportion of IL-4–producing T_H2 cells and low numbers of IFN-γ–producing T_H1 cells.[6] The lung is equipped with an elaborate network of dendritic cells (DCs) with the special ability to capture inhaled allergens and migrate to the draining mediastinal lymph nodes (MLNs), where they interact with T cells to generate lung-homing, allergen-specific effector T_H2 cells.[7] Conditional depletion of airway DCs at the time of allergen challenge abolishes the feature characteristics of asthma, such as eosinophilic inflammation, goblet cell hyperplasia, and bronchial hyperreactivity, indicating a major role for DCs in mounting and maintaining immune responses to inhaled allergens.[8] In addition to DC–T-cell interaction, epithelial cell–DC cross-talk might also contribute to the T_H2-skewed immune response to inhaled allergens. Epithelial cell–derived factors, such as thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, might represent instructive signals to establish T_H2 cell development.[9] In particular, IL-33, a member of the IL-1 family, was shown to regulate eosinophilic airway inflammation and IL-4, IL-5, and IL-13 production.[10] Moreover, high IL-33 levels are observed in patients with allergic asthma[11] and allergic rhinitis.[12,13] IL-33 binds to its receptor, ST2, which exists in 2 isoforms: a soluble form, which acts as a decoy receptor, and a membrane-bound form, which is presented as a heterodimer together with IL-1 receptor–associated protein in the membrane of mast cells, T_H2 cells, DCs, basophils, and macrophages.[14] Although IL-33 has been implicated in airway

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Abbreviations used
BAL: Bronchoalveolar lavage
C_T: Cycle threshold
DC: Dendritic cell
FITC: Fluorescein isothiocyanate
HPRT: Hypoxanthine phosphoribosyltransferase
MLN: Mediastinal lymph node
OX40L: OX40 ligand
TSLP: Thymic stromal lymphopoietin
WT: Wild-type

inflammation in an ovalbumin-induced mouse model of allergic asthma[15] and has been shown to stimulate DCs to induce an atypical T_H2-type response,[16] a direct link between IL-33 levels, DCs, and T_H2 response in pollinosis *in vivo* is unknown.

Here we report a novel murine model of allergy to a natural allergen of *C arizonica* pollen, nCup a 1. The allergic disease induced closely resembles cypress pollinosis in human subjects. We demonstrate that exposure of BALB/c mice to nCup a 1 leads to recruitment of eosinophils and DCs in the lung, resulting in pulmonary allergic inflammation. Furthermore, this series of reactions is dependent on the IL-33/ST2 pathway through DCs and development of the T_H2 response.

METHODS

Supplemental information can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Eight-week-old female BALB/c and C57Bl/6 mice (Harlan, Bresso, Italy) were housed in individually ventilated and filtered cages under positive pressure in the Animal care Unit of the Istituto Superiore di Sanità. $ST2^{-/-}$ mice (deficient for membrane-bound ST2L and soluble ST2) on a BALB/c background were generated as previously described[17] and bred at the University of Glasgow. Mice were treated according to the local guidelines for animal care (D.L. 116/92, which has implemented in Italy the requirements of the European directive 86/609/EEC on laboratory animal welfare).

Statistical analysis

The Mann-Whitney test was used for nonparametric analysis of differences between groups. A P value of less than .05 was considered statistically significant.

RESULTS

nCup a 1 induces pathologic markers of allergic lung inflammation

We first established a mouse model of allergy to cypress pollen by using the native form of the major allergen nCup a 1 of *C arizonica*. BALB/c mice were sensitized with 2 intraperitoneal injections of nCup a 1 at weekly intervals, followed by 4 consecutive daily intranasal challenges of nCup a 1 (Fig 1, *A*). Because IgEs are crucial determinants of allergic reactions, we first assessed nCup a 1–specific IgE levels. nCup a 1–sensitized mice produced increasing levels of serum allergen-specific IgE antibodies after sensitization, peaking at day 26, which was 2 days after the last intranasal challenge (Fig 1, *B*). Next, we compared the reactivity pattern of mouse IgE induced in our model with Cup a 1–specific IgE present in the sera of patients with cypress allergy. Both sets of IgE specifically reacted with the major component Cup a 1 of *C arizonica* extract and purified nCup a 1, indicating the IgE-inducing ability of nCup a1 in both mice and human subjects (Fig 1, *C*). Histology revealed that the lungs of nCup a 1–treated mice exhibit the hallmarks of airway allergic pathology, with extensive peribronchial and perivascular inflammatory infiltrates associated with eosinophil accumulation, goblet cell hyperplasia, and mucus hypersecretion compared with control lungs (Fig 1, *D*). Moreover, lungs of nCup a 1–treated mice showed significant immune infiltrates by day 2 after challenge, peaking at day 4 and slightly decreasing thereafter (Fig 1, *E*). This inflammation is accompanied by increased expression of the mucus biomarkers hCLCA1/Gob5 and MUC5AC/Muc5ac in the lung, peaking at day 2 after challenge and gradually decreasing by day 9 (Fig 1, *F*). Notably, nCup a 1 induced similar patterns of allergic inflammation in C57Bl/6 mice (see Fig E1, *A-C*, in this article's Online Repository at www.jacionline.org). These data indicate that nCup a 1 sensitization and challenge result in the induction of an allergic disease in mice characterized by inflammatory airway changes resembling the human respiratory syndrome.

nCup a 1 sensitization and challenge induce a systemic T_H2 response

Because patients with pollinosis exhibit allergen-specific T_H1/T_H2 dysregulation,[2,18] we investigated whether nCup a 1–induced allergic disease in mice was characterized by the predominance of a T_H2 immune response. Numbers of $CD4^+$ T cells and, to a lesser extent, $CD8^+$ T cells were significantly increased both in spleens and MLNs over time after challenge compared with control mice (Fig 2, *A*). Splenic cells from nCup a 1–treated mice showed significantly enhanced proliferative response compared with control mice after *in vitro* recall with nCup a 1 (Fig 2, *B*). Supernatants from the same cultures of nCup a 1–treated mice also contained more T_H2 cytokines (IL-4, IL-5, IL-13, and IL-10) compared with supernatants from control animals (Fig 2, *C*). Similar results were obtained with the MLN cells, although with an earlier peak of response compared with that of the splenic cells (Fig 2, *D*). Furthermore, a similar allergen-specific T_H2 response was induced in C57Bl/6 mice (see Fig E1, *D-E*). These results clearly demonstrate the preferential induction of a T_H2 response in mice sensitized and challenged with nCup a 1.

nCup a 1 induces pulmonary recruitment of immune cell populations sustaining T_H2 polarization

In allergic asthmatic patients pollens of diverse plant species trigger a chronic disease characterized by activation and recruitment of various inflammatory cells in the airways.[19,20] Therefore we analyzed immune cell recruitment in the bronchoalveolar lavage (BAL) fluid and lungs of nCup a 1–treated mice. BALB/c and C57Bl/6 treated mice exhibited a massive increase of cellularity in the BAL fluid characterized by the development of strong eosinophilia, peaking between days 2 and 4 after challenge (Fig 3, *A* and *B*, and see Fig E1, *F*). The predominance of eosinophils in the BAL fluid was confirmed by means of histochemistry (Fig 3, *C*). Macrophage numbers were initially decreased and then increased by day 9. No changes in neutrophil numbers were detected. In contrast, nCup a 1 treatment induced a 6-fold increase

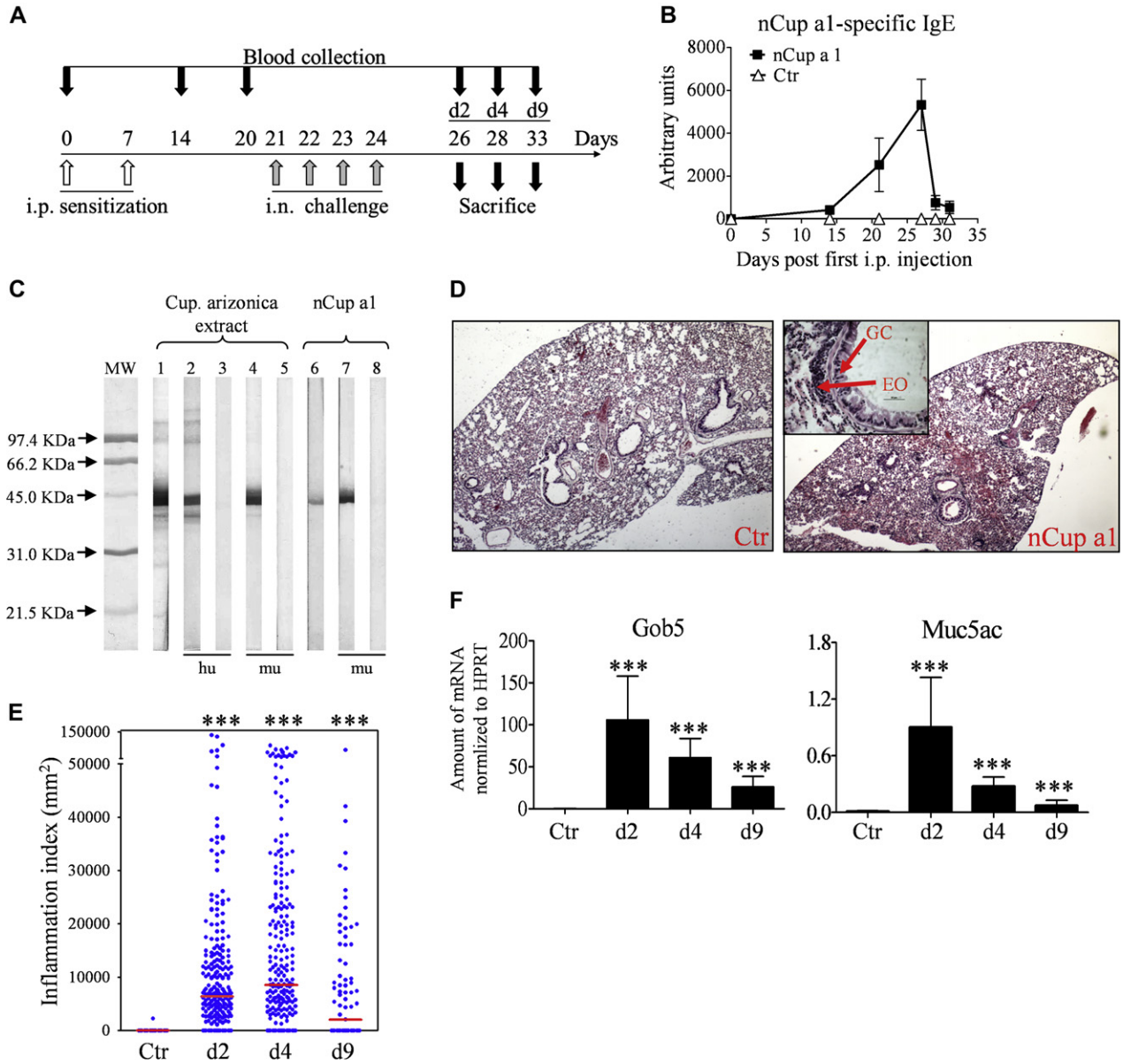

FIG 1. Induction of allergic reaction in mice by nCup a 1. **A,** nCup a 1 treatment schedule. **B,** nCup a 1–specific IgE in sera (n = 6 mice per group). **C,** IgE binding to *C arizonica* extract or nCup a 1. Amido black staining (*lanes 1* and *6*), serum from a subject with cypress allergy *(lane 2)* or a healthy subject (*lane 3*; *hu*), and serum from nCup a 1–sensitized (*lanes 4* and 7) or control (*lanes 5* and *8*) mice *(mu)* are shown. *MW*, Molecular weight. **D,** Lung sections on day 4 after challenge (×40 magnification). *Insert* = ×400 magnification (n = 6 mice per group). *EO*, Eosinophils; *GC*, goblet cells. **E,** Measure of lung infiltrated areas (n = 250-437 areas per group) in control and nCup a 1–treated mice at various days after challenge. *Dots* represent single areas. The *red line* indicates the mean. ***$P < .001$ versus control. **F,** Gob5 and Muc5ac expression in lungs (n = 6 mice per group). ***$P < .001$ versus control. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

in DC numbers by day 2 after challenge (Fig 3, *B*, and see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) and an augmented number of lymphocytes (Fig 3, *D*) in the BAL fluid. Consistent with a predominant T_H2 response, IL-4 and IL-5 levels in the BAL fluid of nCup a 1–treated mice were significantly increased (Fig 3, *E*).

Immune cell infiltration in the lungs of nCup a 1–treated mice was evident by day 2 and sustained to at least day 9 after challenge (Fig 4, *A* and *B*). Cellular distribution in the lungs was similar to that seen in BAL fluid, with a marked increase in the eosinophil, DC, and T-cell populations (Fig 4, *A* and *B*, and see Figs E1, *G*, and E2, *B*). Both $CD4^+$ and $CD8^+$ T-cell infiltrations were evident in the lungs of treated mice (Fig 4, *C*). A significant increase in IL-4, IL-5, and IL-13 message was observed in the lungs of nCup a 1–treated mice (Fig 4, *D*). We also found markedly increased expression of TSLP, IL-25, OX40/OX40 ligand (OX40L), and IL-33, molecules known to be important in the cross-talk between epithelial cells and DCs (Fig 4, *E*).[21] Together these results demonstrate that the nCup a 1–induced allergic reaction is associated with a T_H2 response characterized by marked

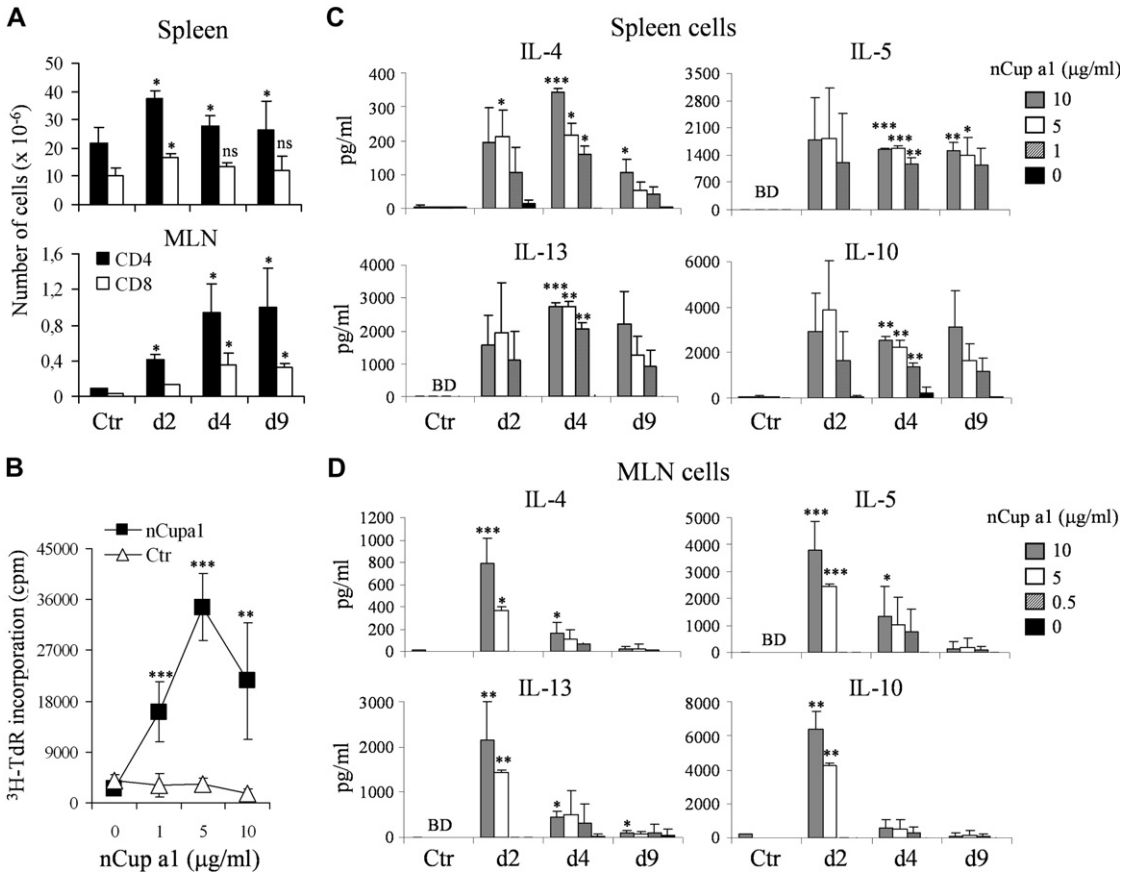

FIG 2. nCup a 1 induces a systemic T_H2 response. **A,** $CD4^+$ and $CD8^+$ T-cell numbers in spleens and MLNs of control and nCup a 1–treated mice at various days after challenge (n = 6 mice per group). $*P < .05$ versus control. *ns*, Not significant. **B,** nCup a 1–specific proliferation of splenic cells from control or nCup a 1–treated mice on day 4 after challenge (n = 6 mice per group). **C** and **D,** Cytokine production by splenic (Fig 2, *C*) and MLN (Fig 2, *D*) cells. $*P < .05$, $**P < .01$, and $***P < .001$ versus control.

airway infiltration of eosinophils, DCs, and T cells associated with increased T_H2 cytokine production and expression of mediators essential for the interaction of the various cell subsets present in the airway.

Activation of the IL-33/ST2 pathway during Cup a 1–induced pulmonary allergic reaction

IL-33 has emerged as an important cytokine in T_H2-mediated diseases, including allergic asthma, that involve interactions between different cell types leading to inflammation in the lung.[22] Therefore we investigated the role of IL-33 and its receptor ST2 in nCup a 1–induced allergy. We found that IL-33 levels were significantly increased in the lungs of nCup a 1–treated mice (Figs 5, *A*, and see Fig E1, *H*). IL-33 levels were also increased in the sera of treated mice (Fig 5, *B*). Importantly, IL-33 concentrations were significantly higher in the sera of monosensitive patients (allergic exclusively to Cupressaceae pollen) compared with those seen in nonallergic subjects or polysensitive patients (allergic to substances other than Cupressaceae pollen; Fig 5, *C*).

To directly determine the contribution of IL-33, we sensitized and challenged wild-type (WT) and $ST2^{-/-}$ mice with nCup a 1. Both the $ST2^{-/-}$ and WT mice had similar concentrations of serum nCup a 1–specific IgE 4 days after challenge (770 ± 143 vs 752 ± 123 aribitrary units). However, histology of the lung showed markedly reduced airway inflammation, eosinophil accumulation, and goblet cell hyperplasia in nCup a 1–treated $ST2^{-/-}$ mice compared with their WT counterparts (Fig 6, *A* and *B*). Flow cytometric analysis of lung immune cell populations confirmed a less evident increase in eosinophil numbers in $ST2^{-/-}$ mice compared with WT mice after nCup a 1 exposure (Fig 6, *C*). Remarkably, after nCup a 1 treatment, although DC numbers were increased in WT mice, no increase in DC numbers was found in $ST2^{-/-}$ mice (Fig 6, *C*). Moreover, no accumulation of T cells was observed in the lungs of treated $ST2^{-/-}$ mice with respect to WT mice in response to nCup a 1 challenge (Fig 6, *D*). IL-13 levels in the lungs of nCup a 1–treated $ST2^{-/-}$ mice were substantially lower compared with those in WT mice (Fig 6, *E*).

To determine the induction of an allergen-specific T_H2 response, we restimulated the splenic cells from nCup a 1–treated $ST2^{-/-}$ and WT mice with graded doses of allergen *in vitro*. Splenic cells of $ST2^{-/-}$ mice showed a significantly reduced proliferative response compared with that seen in WT mice (Fig 6, *F*). Moreover, culture supernatants of splenic cells from nCup a 1–treated $ST2^{-/-}$ mice contained significantly less IL-4, IL-5, and IL-13 compared with their WT counterparts (Fig 6, *G*). These results demonstrate an important role of ST2 in mediating lung inflammation and recruitment of eosinophils, DCs, and T cells in nCup a 1–induced lung allergy and associated T_H2 responses.

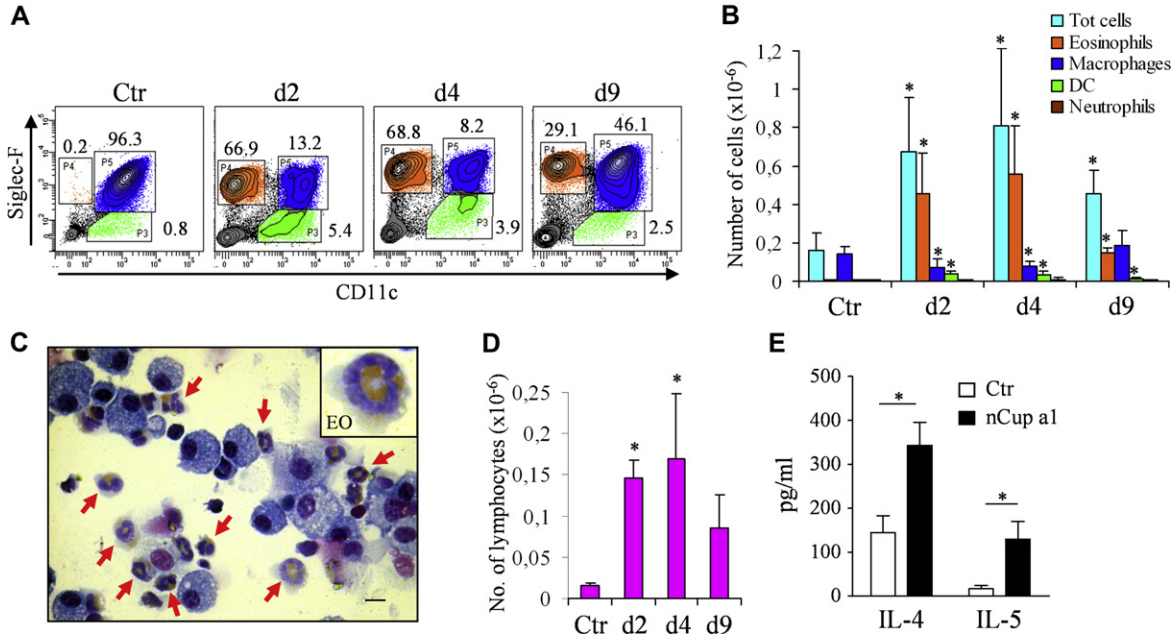

FIG 3. nCup a 1 induces allergic inflammation in airways. **A,** Representative fluorescence-activated cell sorting analysis of BAL cells in nCup a 1–treated and control mice. *Orange*, Eosinophils; *blue*, macrophages; *green*, DCs. **B,** Mean (± SD) cell numbers (n = 6 mice per group). **C,** Histochemistry of BAL fluid cells on day 4 after challenge (×200 magnification). The *arrows* and *insert* depict eosinophils *(EO)*. **D,** Number of BAL fluid lymphocytes (mean ± SD; n = 3 mice per group). **E,** Cytokines in BAL fluid on day 4 after challenge (n = 6 mice per group). *$P < .05$ versus control.

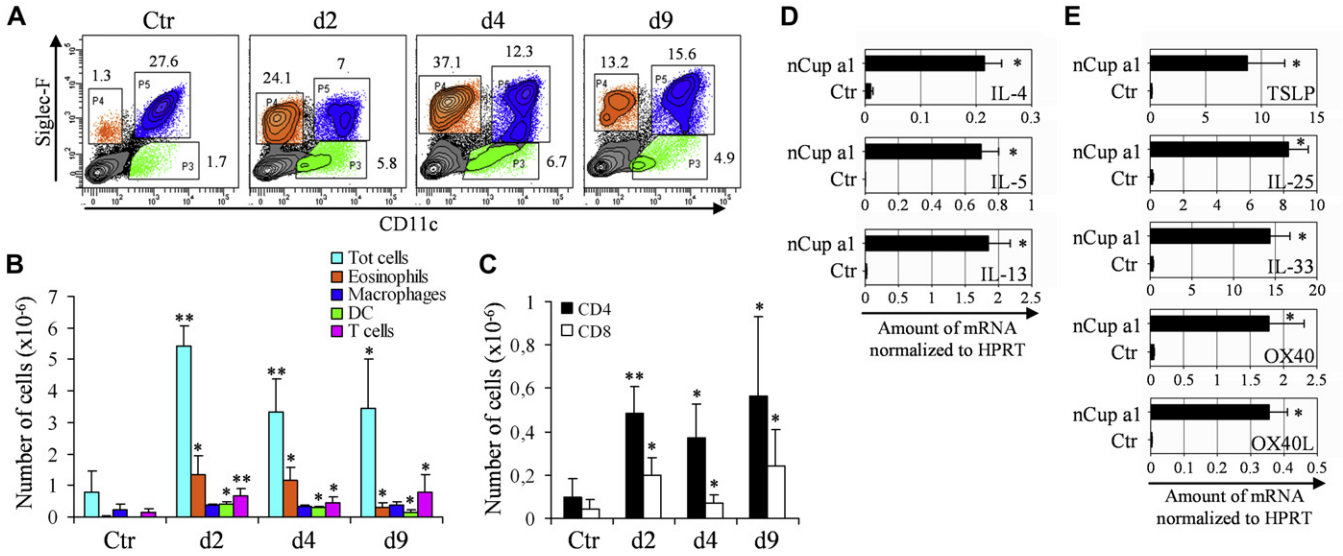

FIG 4. nCup a 1 induces allergic inflammation in lung tissue. **A,** Representative fluorescence-activated cell sorting analysis of lung cells in nCup a 1–treated and control mice. *Orange*, Eosinophils; *blue*, macrophages; *green*, DCs. **B** and **C,** Mean (± SD) numbers of cell subpopulations (n = 6 mice per group). *$P < .05$ and **$P < .01$ versus control. **D** and **E,** Expression of T_H2 cytokines (Fig 4, *D*) and epithelial cell–derived factors (Fig 4, *E*) in lungs 4 days after challenge (n = 6 mice per group). *$P < .05$ versus control mice.

DC-driven T_H2 polarization is ST2 dependent

Although DC-targeted modulatory activity by allergenic extracts has been reported, the functional relevance of this effect is unclear, particularly at the level of the single allergenic molecule.[23,24] We then investigated whether nCup a 1 could affect the expression of ST2 by treating naive DCs with nCup a 1 *in vitro*. ST2 expression in DCs was increased up to 5-fold after 24 hours of exposure to nCup a 1 compared with that seen in DCs treated with LPS or left untreated (Fig 7, *A*). nCup a 1 did not affect IL-33 expression in DCs, excluding possible autocrine pathways (data not shown). The expression of TSLP receptor and IL-25 receptor was not affected. However, nCup a 1 significantly enhanced the expression of OX40L, as did LPS. nCup a 1 did not induce IL-12 expression, which was instead enhanced by LPS

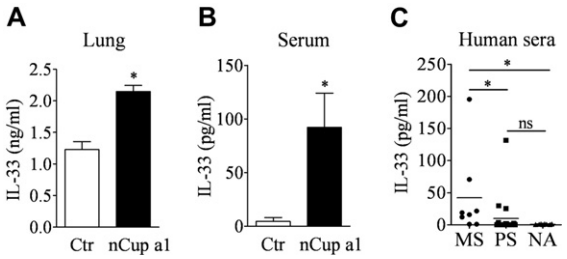

FIG 5. Increased IL-33 levels in nCup a 1–treated mice and patients with cypress allergy. **A** and **B,** IL-33 protein in lungs (Fig 5, *A*) and serum (Fig 5, *B*) from control or nCup a 1–treated mice on day 4 after challenge (n = 6 mice per group). *$P < .05$ versus control. **C,** IL-33 in sera of monosensitive (*MS*; n = 8) and polysensitive (*PS*; n = 20) patients with cypress allergy and nonallergic (*NA*; n = 8) subjects. *$P < .05$. *ns*, Not significant.

(Fig 7, *A*). The ability of nCup a 1 to induce ST2 expression on DCs was confirmed *in vivo*. DCs isolated from the spleens of WT mice 24 hours after intravenous injection of nCup a 1 expressed significantly higher levels of ST2 message in a dose-dependent manner (Fig 7, *B*).

Because IL-33 can polarize DCs to induce the differentiation of IL-5– and IL-13–producing naive $CD4^+$ T cells,[15,16,25] we tested whether nCup a 1–induced ST2 expression could lead to enhanced biological response of DCs to IL-33. To this end, we cultured bone marrow–derived DCs from $ST2^{-/-}$ mice and DCs from WT mice with nCup a 1 or medium for 24 hours. DCs were then harvested and cocultured with naive $CD4^+$ T cells with or without IL-33. To exclude any direct action of IL-33 on ST2-expressing CD4 T cells,[26] we used $ST2^{-/-}$ CD4 T cells as responders. DCs of $ST2^{-/-}$ and WT mice showed comparable levels of costimulatory (CD40, CD80, and CD86) and MHC class I and MHC class II molecules (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, treatment of DCs with nCup a 1 also did not alter the expression of these markers (see Fig E4 in this article's Online Repository at www.jacionline.org), indicating that the allergen had no direct effects on DC maturation. DCs from WT mice cultured with IL-33 were able to induce the production of IL-5 and IL-13 by $ST2^{-/-}$ $CD4^+$ T cells (Fig 7, *C*). Remarkably, concentrations of these cytokines were significantly increased when the DCs were precultured with nCup a 1. No IL-5 and IL-13 was detected in any of the cultures with DCs from $ST2^{-/-}$ mice (Fig 7, *C*). No IL-4 release was observed in either coculture setting (data not shown), confirming previous reports.[16] Together, these data demonstrate that nCup a 1 can directly target DCs and enhance ST2 expression and that the subsequent exposure of the DCs to IL-33 promotes the polarization of T_H2 cells.

DISCUSSION

Although many mouse models reproduce a collection of features that characterize most common forms of asthma and yield a basic core of phenotypic consequences, none mimics the full range of clinical manifestations of asthma.[27] Most mouse models of asthma use ovalbumin and not a natural allergen, thus raising concerns when attempting to interpret experimental findings in the context of human pollinosis.[28] Despite cypress allergy accounting for 30% of all cases of pollinosis, the development of effective treatments is hampered by the lack of an appropriate preclinical model. Here we describe a novel mouse model of allergic inflammatory reaction to the purified *C arizonica* major allergen nCup a 1 that recapitulates the major features of clinical pollinosis. Sensitization of BALB/c mice with Cup a 1 induced high levels of IgE antibodies that recognized nCup a 1 as a component of the crude cypress pollen extract, which was recognized also by the IgE of patients with cypress allergy. This observation might have relevant implications in view of a possible use of the nCup a 1 molecule in vaccination protocols.

nCup a 1 intranasal challenge of sensitized mice induced an asthma-like reaction in the pulmonary district, as evidenced by eosinophilic inflammation, goblet cell metaplasia, and expression of hCLCA1/Gob5 and MUC5AC/Muc5ac,[29] 2 biomarkers correlating with airway hyperreactivity in mice.[30] The analysis of the immune response associated with the allergic reaction to nCup a 1 reveals multiple components resembling the clinical trait of allergic responses to pollens in human subjects.[31]

First, a marked recruitment of eosinophils occurred in the lungs and airways of nCup a 1–treated mice soon after challenge. In this regard eosinophilia is most commonly seen in classic atopic asthma with allergen-mediated inflammation and plays a role in the recruitment of effector T cells into the lung.[32,33]

Second, the significant infiltration of DCs in the lungs and airways of sensitized mice after nCup a 1 challenge supports a role for this population in induced allergic pulmonary inflammation.[7]

Third, the pulmonary cytokine milieu was characterized by the presence of increased IL-4 and IL-5 levels and strongly correlated with a substantial recruitment of T cells, especially CD4 T cells, in both the lungs and airways of challenged mice. The induction of an allergen-specific T_H2 response in our model was confirmed at the systemic level based on a high rate of proliferation and IL-4, IL-5, and IL-13 production by splenic and MLN cells from treated mice. These data demonstrate that in sensitized mice nCup a 1 challenge generates a strong allergic inflammatory reaction supported by a specific T_H2 immune response.

The major factors dictating the interplay between the epithelium and T_H2 immune response[9] were significantly upregulated in the lungs of nCup a 1–treated mice, as evidenced by the simultaneous upregulation of TSLP, IL-25, OX40, OX40L, and IL-33. These observations suggest an involvement of epithelium-derived factors in the nCup a 1–induced T_H2 response. In this regard IL-33, an alarmin cytokine released by epithelial cells and implicated in airway inflammation during allergic asthma, has been shown to promote a DC-induced atypical T_H2-type response.[16,20] Several large-scale genome-wide association studies have shown that the genes for IL-33 and its specific receptor ST2 are crucial for asthma onset.[34] Moreover, serum IL-33 level and the IL-33 genetic variant were found to be associated with pollinosis to Japanese cedar, a highly cypress pollen cross-reactive species.[35] Our data showing a significant increase in IL-33 concentrations in the sera of Cupressaceae pollen–monosensitive allergic patients compared with nonallergic subjects are consistent with the observations reported by Sakashita et al[35] and suggest the potential involvement of IL-33 in clinical pollinosis. In the present study we show that the IL-33/ST2 pathway plays a significant role in mediating pulmonary and systemic allergic reactions to nCup a 1 pollen. First, we found a substantial increase in IL-33 levels both in the lung and in the sera of nCup a 1–treated mice. Second, when we sensitized and challenged $ST2^{-/-}$ mice, which lack a functional receptor for IL-33, with nCup a 1, we found markedly decreased allergen-specific T_H2 immune responses and allergic reactions in the airways. The observation that IgE levels were similar in the sera of allergen-treated WT and

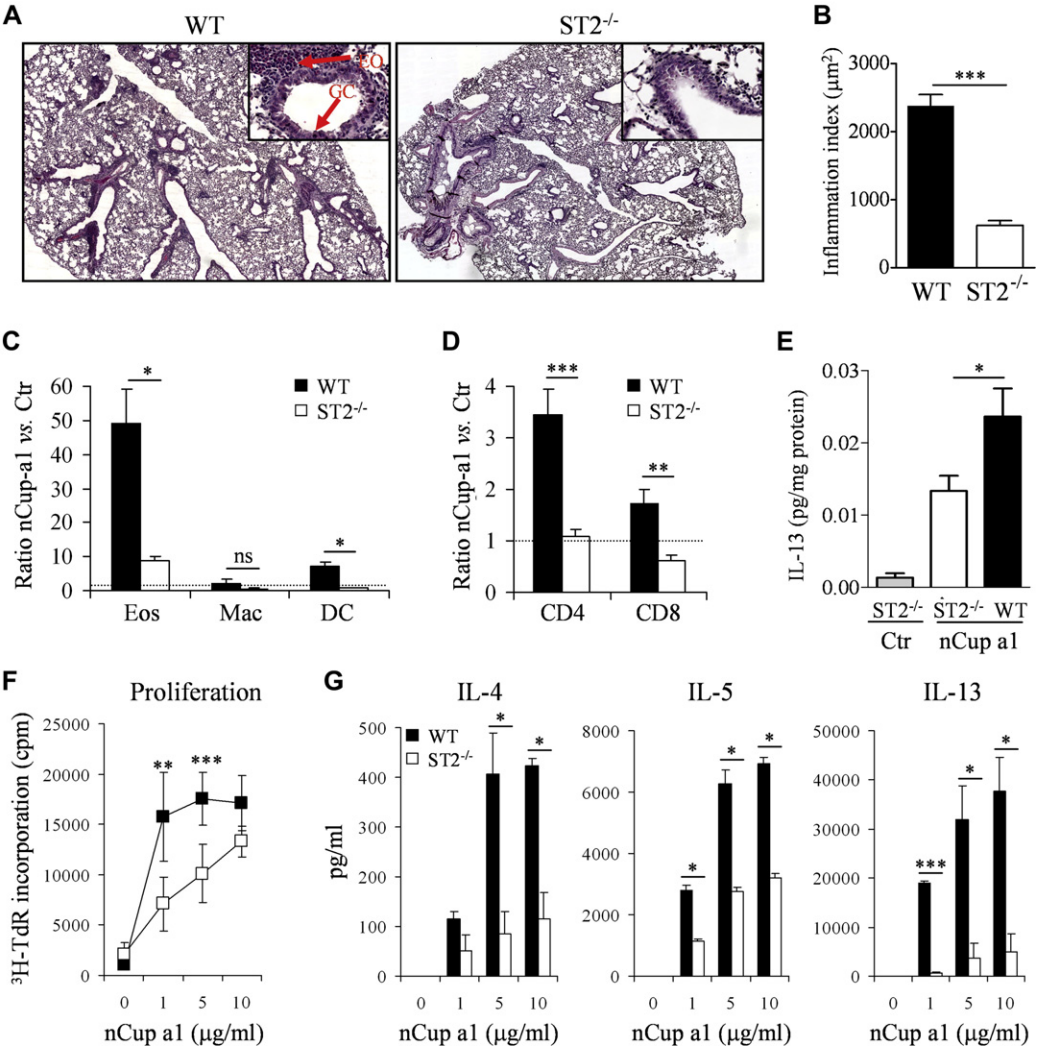

FIG 6. Activation of the IL-33/ST2 pathway in nCup a 1–treated mice. **A,** Lung sections from nCup a 1–treated WT and ST2$^{-/-}$ mice on day 4 after challenge (×40 magnification). *Insert* = ×400 magnification. *EO*, Eosinophils; *GC*, goblet cells. **B,** Measure of lung infiltrated areas (mean ± SD; n = 700-750 areas per group). **C** and **D,** Cell population increase in lungs of treated versus control mice (mean ± SD; n = 6 mice per group). *Dotted lines*, Ratio = 1. **E,** IL-13 in lungs. **F** and **G,** nCup a 1–specific proliferation (Fig 6, *F*) and cytokine production (Fig 6, *G*) by splenocytes (n = 6 mice per group). *$P < .05$, **$P < .01$, and ***$P < .001$ WT versus ST2$^{-/-}$ mice.

ST2$^{-/-}$ mice suggests that different mechanisms regulate IgE-secreting plasma cells and lung inflammation.[36,37] Consistent with this notion, the systemic nCup a 1–specific T-cell response was not completely abolished in ST2$^{-/-}$ mice, indicating the existence of additional IL-33–independent pathways in this model. Remarkably, after nCup a 1 challenge, ST2$^{-/-}$ mice exhibited reduced lung recruitment of eosinophils in accordance with recent observations with house dust mite and peanut allergens[38,39] and no accumulation of T cells and DCs with respect to control mice. The impaired infiltration of T cells in the lungs of ST2$^{-/-}$ mice after nCup a 1 exposure is in agreement with previous findings showing that IL-33 can act as a chemoattractant for T_H2 cells.[40] Interestingly, the lack of DC recruitment in ST2$^{-/-}$ lungs suggests that IL-33 can regulate DC trafficking in the airways. Evidence for a regulatory role of IL-33 on DCs *in vivo* comes from studies showing that IL-33 promotes DC development from bone marrow by triggering GM-CSF production.[41]

Another important finding reported in this study is that nCup a 1 can directly modulate DC activity by upregulating ST2 expression both *in vitro* and *in vivo*. Direct effect of allergens on DCs has been suggested as one DC-modulatory event occurring at the interface between the external environment and the innate immune system.[7] In this respect the possibility to manipulate nCup a 1 at the molecular level to reduce or eliminate such modulatory effects might be exploited in strategies of hypoallergenic vaccine design.[42] Of note, nCup a 1 treatment did not affect TSLP receptor or IL-25 receptor mRNA levels, indicating a direct and specifically IL-33–mediated action of nCup a 1 on DCs. To our knowledge, this is the first report showing a direct effect of a pollen compound on ST2 expression by DCs. The upregulation of IL-33 receptor on DCs by nCup a 1 resulted in an increased biological response of the DCs to IL-33–induced T_H2 skewing. In fact, pre-exposure of ST2-sufficient DCs (WT-DCs) to nCup a 1 significantly enhanced their capacity to induce IL-13 and

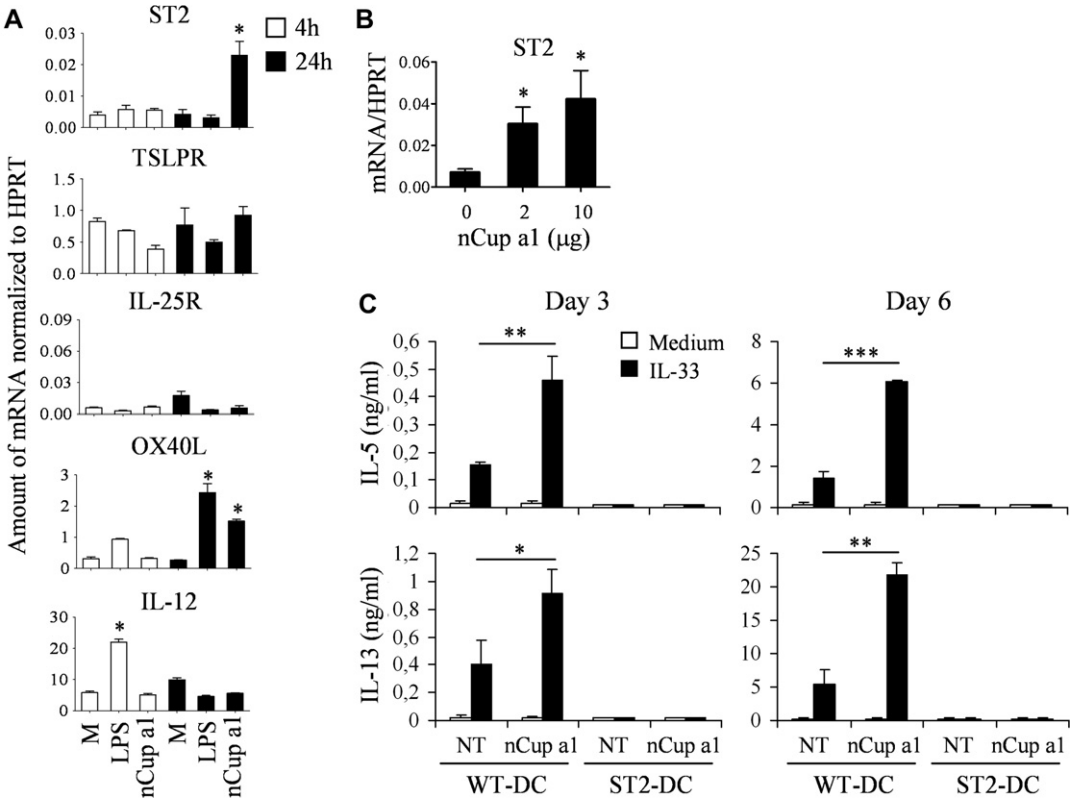

FIG 7. nCup a 1 upregulates ST2 on DCs and enhances DC responsiveness to IL-33. **A,** Expression of indicated genes in naive DCs cultured with nCup a 1, LPS, or medium *(M)*. $*P < .05$ versus medium. **B,** ST2 expression in DCs after nCup a 1 treatment *in vivo* (n = 7 mice per group). $*P < .05$ versus 0 μg. **C,** Cytokine release by $ST2^{-/-}$ $CD4^{+}$ T cells after 3 and 6 days of coculture with nCup a 1–treated or untreated *(Medium)* $ST2^{-/-}$ and WT DCs ± IL-33. $*P < .05$, $**P < .01$, and $***P < .001$ versus medium. *NT,* Not treated.

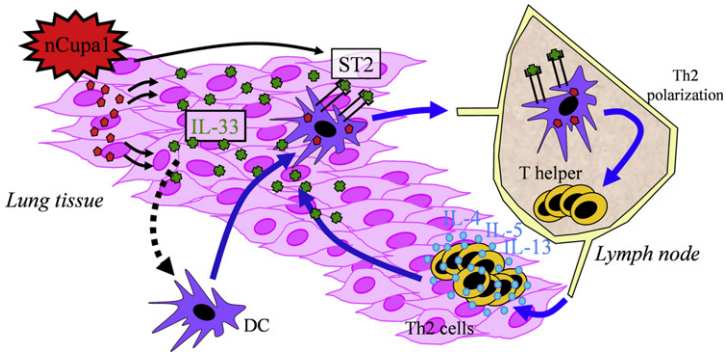

FIG 8. Hypothetical model for role of the IL-33/ST2 pathway and DCs in nCup a 1–induced allergic reactions. Inhaled nCup a 1 induces intrapulmonary secretion of IL-33 by epithelial cells. nCup a 1 also increases ST2 expression on DCs migrating to the lung. DCs then respond to IL-33 through ST2 and polarize CD4 T_H cells toward T_H2 cells that mediate and perpetuate airway inflammation.

IL-5 production by cocultured $ST2^{-/-}$ CD4 T cells in response to IL-33. However, when using DCs unresponsive to IL-33 (ST2-DCs), the capability of $ST2^{-/-}$ CD4 T cells to produce cytokines was completely lost, indicating the specificity of the IL-33/ST2 pathway through DCs. Although a role for DCs on T_H2 polarization by the ST2/IL-33 pathway has been reported, there was no direct demonstration of the action of IL-33 on DCs in mediating this effect.[15,16] Because in our cultures we used $ST2^{-/-}$ CD4 T cells, our results conclusively demonstrate that IL-33 can operate through DCs in stimulating the production of high IL-5 and IL-13 levels by naive CD4 T cells. The finding that nCup a 1 treatment also increased OX40L expression by DCs, together with the observation of OX40/OX40L upregulation in the lungs of mice with nCup a 1 allergy, might suggest the existence of a mechanism linking IL-33, DCs, and T_H2 polarization in conditions of absence of IL-12, as observed for TSLP.[43] This possibility is currently under investigation.

A previous study showed that Cupressaceae pollen grains activate murine bone marrow–derived DCs *in vitro* and induce serum IgE levels and airway eosinophil infiltration *in vivo* when

pollen grains were coadministered with ovalbumin.[44] Our results extend these findings and provide the first direct evidence that a natural allergen can modulate the biological activity of DCs by directly enhancing ST2 expression and responsiveness to IL-33–induced T_H2 polarization. In view of recent reports addressing the importance of T cell–polarizing activity of allergenic molecules, the evidence of a functionally relevant DC-modulatory role by a pollen allergen inform the design of effective vaccines against allergy.[39,45-47] On these bases, we propose a model by which nCup a 1 inhalation could lead to the induction and release of IL-33, most likely by epithelial cells. nCup a 1 could then induce increased ST2 levels on lung DCs. IL-33–activated DCs would then selectively activate T_H2 cells, producing IL-5 and IL-13, which mediate airway inflammation, including the infiltration of eosinophils and T cells (Fig 8). We propose that the cross-talk between epithelial cells and DCs through activation of the IL-33/ST2 pathway might represent an important component for development of the T_H2 immune response and for initiation/perpetuation of pulmonary allergic inflammation in pollinosis.

In conclusion, we established a novel experimental model of *C arizonica* pollinosis suitable for the development of preclinical studies. Furthermore, our results highlight the therapeutic potential of targeting DCs and the IL-33/ST2 pathway for improving the treatment of clinical pollinosis and other allergic disorders.

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Clinical implications: This study provides an appropriate preclinical model of cypress pollinosis and identifies a modulatory activity of the cypress major allergen on DCs through the IL-33/ST2 pathway exploitable for therapeutic strategies.

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METHODS

nCup a 1 sensitization and challenge

Groups of 6 BALB/c mice, either WT or $ST2^{-/-}$, were treated according to the protocol shown in Fig 1. Mice were injected intraperitoneally on days 1 and 7 with 0.5 μg of purified nCup a 1 (Bial, Bilbao, Spain)[E1] adsorbed in 2.5 mg aluminum hydroxide (Sigma-Aldrich, St Louis, Mo) in sterile PBS. On days 21, 22, 23, and 24, mice were anesthetized with Rompum (Bayer, Leverkusen, Germany) and Zoletil (Virbac, Carros Cedex, France) intraperitoneally and then challenged by intranasal instillations of 10 μg of nCup a 1. Age-matched naive mice injected intraperitoneally with aluminum hydroxide alone and challenged with PBS were used as controls.

Measurement of allergen-specific serum IgE levels

At defined time points after the first allergen intraperitoneal injection, mice were bled from the retro-orbital venous plexus, and individual sera were stored at −20°C until analyzed. nCup a 1–specific IgE was monitored by using direct ELISA for individual mice. Plates (Greiner Bio-One, Monroe, NC) were coated with nCup a 1 (5 μg/mL) in carbonate/bicarbonate buffer, pH 9.6, for 3 hours at 37°C plus overnight at 4°C. Serum samples were added in duplicates and incubated o.n. at 4°C. Preimmune sera from each mouse were used in parallel as controls. Bound antibodies were detected by adding peroxidase-labeled rat monoclonal anti-mouse IgE (SouthernBiotech, Birmingham, Ala) for 5 hours at room temperature. The peroxidase substrate orthophenylenediamine chloride (Sigma-Aldrich) was then added, and absorbance was determined with an ELISA reader (Bio-Rad Laboratories, Hercules, Calif) at 492 nm. Pooled sera from hyperimmune mice were used for calculation of standard curves. Results are presented as arbitrary units per milliliter.

Human sera

Human sera from 8 monosensitized and 20 polysensitized Italian patients with cypress allergy and 8 nonatopic subjects were purchased from PlasmaLab International (Everett, Wash).

SDS-PAGE and immunoblotting

SDS-PAGE was performed with 12% (wt/vol) polyacrylamide gels under reducing conditions with a Mini-protean apparatus (Bio-Rad Laboratories). SDS-PAGE and immunoblotting experiments were carried out, as previously described,[E2,E3] with minor modifications. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Shuell, Dassel, Germany). After blocking with 3% (wt/vol) gelatin in PBS, the membranes were incubated o.n. at room temperature with sera pooled from mice allergic to nCup a 1 or with preimmune sera as controls diluted in 0.05% Tween 20 in PBS (PBS-T). Bound mouse IgE was detected by a peroxidase-labeled rat monoclonal anti-mouse IgE (SouthernBiotech). Pooled sera from 5 patients with Cupressaceae allergy were diluted in PBS-T, and bound IgE was detected by using peroxidase-labeled goat anti-human IgE (KPL, Gaithersburg, Md). The 3, 3′-diaminobenzidine peroxidase substrate (Sigma-Aldrich) was then added to develop the colorimetric reaction.

Histopathology

At defined times after challenge, lungs were collected and fixed with 10% buffered formalin and then embedded parasagitally in paraffin. Sections (5 μm) of paraffin-embedded, formalin-fixed tissues were deparaffinized through xylene and hydrated through washes of ethanol and stained with hematoxylin and eosin. Morphometric analysis was performed with a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany). For each mouse, 5 lung sections were assessed. In each lung section all of the cross-sections of vessel and bronchus ramifications were counted, and the area of inflammatory infiltrate around these cross-sections was quantified with IAS2000 software. Lung inflammation was determined as the surface of infiltrated areas and expressed as the inflammation index. For each cross-section, areas were examined for the presence of infiltrates. The surface of each infiltrated area was measured and expressed in square micrometers.

Isolation of BAL fluid, lungs, MLNs, and spleens

Mice were killed, and BAL was performed with 3× 1 mL of PBS containing 0.1 mmol/L EDTA. Fluid recovered from the first instillation was centrifuged, and the supernatant was stored at −20°C for cytokine analysis. Cells collected from 3 instillations were pooled and centrifuged, and the residual red blood cells were lysed with ammonium chloride. Lungs were perfused with 20 mL of PBS through the right ventricle and harvested. To obtain single-cell suspensions, lungs, spleen, and MLNs were cut into small fragments and digested in 1 mg/mL type III collagenase (Worthington Biochemicals, Lakewood, NJ) and 325 Kunitz units/mL DNase I (Sigma-Aldrich) for 25 minutes, followed by EDTA (0.1 mol/L, pH 7.2, Sigma) for 5 minutes. Tissues were passed through a cell mesh, and the resulting cell suspension was subjected to red cell lysis.

mAbs and flow cytometry

The following mAbs were used to label BAL fluid, lung, MLN, and splenic cells: anti-CD45, peridinin-chlorophyll-protein complex–Cy5.5; anti-Siglec-F, phycoerythrin; anti-CD11c, fluorescein isothiocyanate (FITC); anti-CD4, FITC; anti-Gr1/Ly6G, phycoerythrin; anti-CD8a, QE; anti-CD3, allophycocyanin (all from BD PharMingen, San Jose, Calif). Stained cells were analyzed on a FACSaria flow cytometer (Becton Dickinson). Viable cells were selected for analysis based on forward- and side-scatter properties and propidium iodide negativity. For qualitative and quantitative analysis, cell populations ($CD45^+$ gated) were determined as follows: eosinophils, $CD11c^-SiglecF^+Gr\text{-}1^{low}$; neutrophils, $CD11c^-SiglecF^-Gr\text{-}1^{hi}$; DCs, $CD11c^+SiglecF^-Gr\text{-}1^-$; macrophages, $CD11c^+SiglecF^+Gr\text{-}1^{low/-}$; CD4 T cells, $CD3^+CD4^+CD8^-$; and CD8 T cells, $CD3^+CD4^-CD8^+$. Absolute numbers of cell populations were obtained by relating the percentage values to total cell counts. For characterization of bone marrow–derived DC phenotype, the following mAbs were used: FITC anti-CD11c coupled with biotin-labeled CD40, CD80, CD86, H-2D, or I-A, followed by streptavidin, peridinin-chlorophyll-protein complex–Cy5.5 (BD PharMingen).

Cytochemistry of BAL fluid

A single-cell suspension (150 μL) of BAL fluid (2×10^5/mL) was placed on a microscope slide and cytospun at 350 rpm for 5 minutes at room temperature and then stained with May Grunwald-Giemsa. Cells with eosinophilic cytoplasmic granules and ring-shaped nuclei were scored as eosinophils. Total lymphocytes, excluding plasma cells, were identified by using morphologic criteria, and the percentages of a total of 200 cells per slide were computed.

In vitro restimulation of splenic and MLN cells and cytokine analysis

For proliferation assays, 4×10^5 splenic cells from immunized or control mice were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL Polymyxin B sulphate (all from Lonza, Basel, Switzerland) in the presence of graded doses of nCup a 1 for 3 days at 37°C in 5% CO_2. Cultures were performed in triplicates by using 96-well U-bottomed plates. On the fourth day, cultures were pulsed with tritiated thymidine (1 μCi per well) for 16 hours. Incorporation of tritiated thymidine was analyzed by means of liquid scintillation counting. For cytokine detection, cell suspensions from spleens (2×10^6/mL) and MLNs (0.5×10^6/mL) were cultured in 24-well flat-bottom plates in the presence or absence of graded amounts of nCup a 1 in Polymixin B–containing medium. Supernatants were harvested at day 5 of culture. IL-4, IL-5, IL-10, and IL-13 levels in culture supernatants and BAL fluid were determined by using ELISA (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

Stimulation of DCs with nCup a 1 and coculture with T cells

DCs were isolated from spleens of naive mice by means of magnetic cell sorting with CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD11c^+$ DCs (>92% pure) were cultured in Polymixin

B–containing medium in the presence or absence of nCup a 1 (1 μg/mL) or with LPS (0.5 μg/mL) without Polymixin B. After 4 and 24 hours, cells were harvested, and total RNA was extracted. Alternatively, naive BALB/c mice were injected intravenously with graded amounts of nCup a 1 in PBS or PBS alone. One day later, splenic DCs were harvested and assayed by means of quantitative PCR. In some experiments DCs were generated from naive BALB/c or $ST2^{-/-}$ mice by culturing the bone marrow cells in medium containing 10 ng/mL recombinant murine GM-CSF (Miltenyi Biotech). Cytokines and fresh medium were added every other day. At day 6, DCs were harvested and either surface labeled for flow cytometric analysis or replated in the presence or absence of 1 μg/mL nCup a 1. One day later, DCs were harvested, washed, and either assayed by means of quantitative PCR or used for coculture assays. Briefly, $CD4^+$ T cells were isolated from spleens and lymph nodes of naive $ST2^{-/-}$ mice by means of magnetic cell sorting with a $CD4^+$ T cell negative selection kit (Miltenyi Biotec). In total, 10^4 WT or $ST2^{-/-}$ DCs were seeded in round-bottom 96-well plates and cultured with 10^5 purified $ST2^{-/-}$ CD4 T cells in the presence of 1 ng/mL IL-33 (R&D Systems) or medium alone in triplicates. Supernatants were collected at days 3 and 6 of culture and assayed for IL-4, IL-5, and IL-13 by using ELISA.

Quantitative PCR

Total RNA was extracted from 0.5 to 2×10^6 DCs or lung tissue by using Trizol (Invitrogen, Carlsbad, Calif). mRNA was reverse transcribed with the Verso cDNA kit (Thermo Scientific, Uppsala, Sweden). Briefly, 100 ng of total RNA was incubated at 54°C for 45 minutes with oligo-p$(dT)_{15}$ in the presence of RNase inhibitors and reverse transcriptase in a final volume of 20 μL (10 mmol/L Tris, 50 mmol/L KCl, 5 mmol/L $MgCl_2$, and 1 mmol/L dNTPs, pH 8.3). Quantitative PCR was performed with Sensimix Plus SYBR kit containing the fluorescent dye Sybr Green (Quantace, London, United Kingdom). Forward and reverse primers (Table E1) at a final concentration of 20 μmol/L and 2 μL of each cDNA sample were also used (Primm, Milan, Italy). Primers for IL-25, IL-33, and TSLP were purchased from SA Biosciences (Qiagen, Hilden, Germany). The conditions of PCR reaction were as follows: 15 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C (45 cycles). PCR products were continuously measured with an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, Calif). The quality and specificity of amplicons in each sample were determined by using dissociation curve analysis and agarose gel. Triplicates were performed for each experimental point. For quantitation, cycle threshold (C_T) values were determined with the Sequence Detection System software (Applied Biosystems), and ΔC_T values were obtained by subtracting the C_T value of the reference gene hypoxanthine phosphoribosyltransferase (HPRT) from the C_T value of the target gene. The amount of mRNA, normalized to HPRT, was calculated as $2^{-\Delta Ct}$, as described previously.[E4]

Statistical analysis

The Mann-Whitney test was used for nonparametric analysis of differences between groups. A P value of less than .05 was considered statistically significant.

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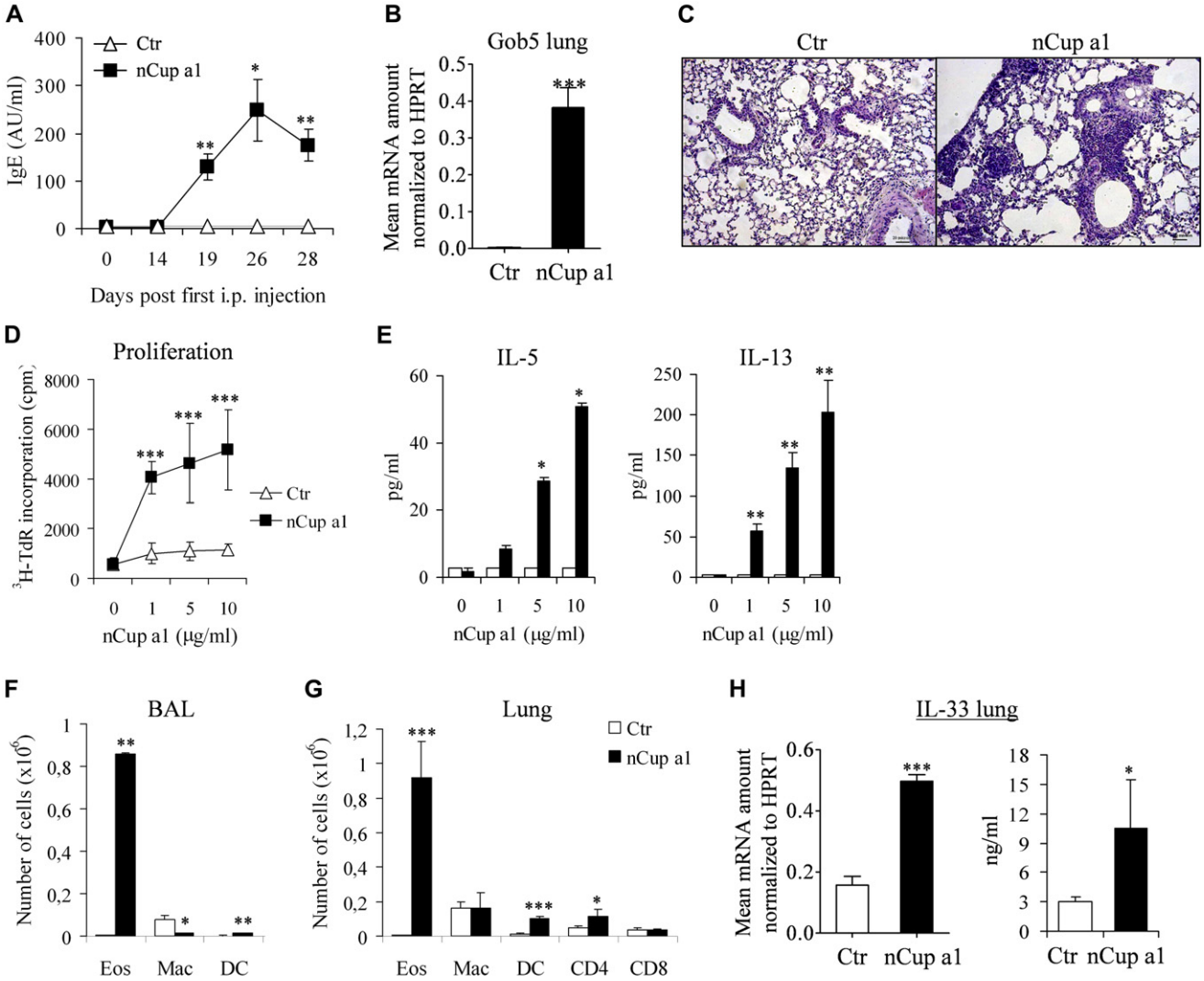

FIG E1. Induction of nCup a 1–mediated allergic reaction and T_H2 response in C57Bl/6 mice. C57Bl/6 mice were immunized and challenged with nCup a 1, as in Fig 1, *A*. **A,** nCup a 1–specific IgE in sera (n = 6 mice per group). **P* < .05 and ***P* < .01 versus control. **B,** Gob5 expression in lungs on day 4 after challenge (n = 6 mice per group). ****P* < .001 versus control. **C,** Lung sections on day 4 after challenge (×40 magnification). **D** and **E,** nCup a 1–specific proliferation (Fig E1, *D*) and cytokine production (Fig E1, *E*) of splenic cells from control or nCup a 1–treated mice at day 4 after challenge (n = 6 mice per group). **P* < .05, ***P* < .01, and ****P* < .001 versus control. **F** and **G,** Numbers of indicated cell populations in BAL fluid (Fig E1, *F*) and lungs (Fig E1, *G*) of control and nCup a 1–treated mice at day 4 (mean ± SD; n = 6 mice per group). **P* < .05, ***P* < .01, and ****P* < .001 versus control. **H,** IL-33 mRNA and protein levels in lungs from control and nCup a 1–treated mice at day 4 (n = 6 mice per group). **P* < .05 and ****P* < .001 versus control.

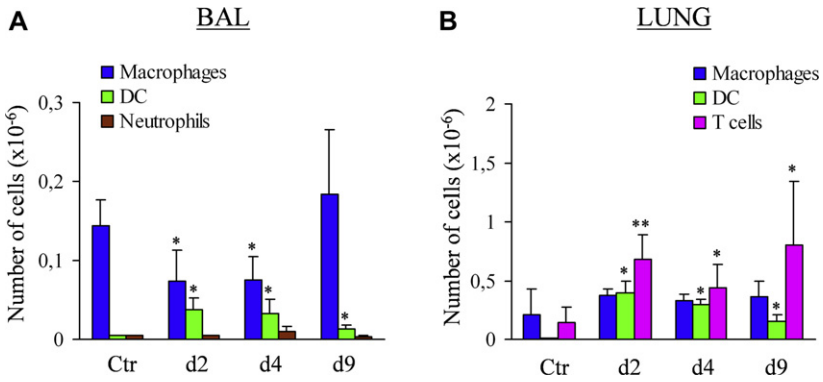

FIG E2. Cell populations in BAL fluid and lungs of nCup a 1–treated mice. BALB/c mice were treated with nCup a 1 as in Fig 1, *A*. **A,** Mean (± SD) numbers of macrophages, DCs, and neutrophils in BAL fluid of control and nCup a 1–treated mice at the indicated days after challenge (n = 6 mice per group). *$P < .05$ versus control. **B,** Mean (± SD) numbers of macrophages, DCs, and T cells in the lungs control and nCup a 1–treated mice at the indicated days after challenge (n = 6 mice per group). *$P < .05$ and **$P < .01$ versus control.

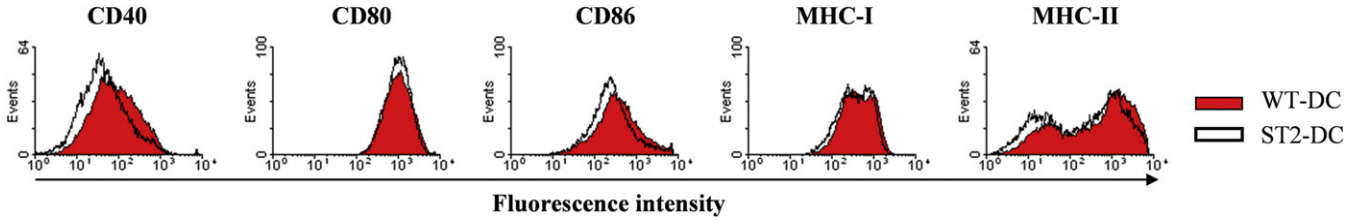

FIG E3. Phenotype of bone marrow–derived DCs from ST2$^{-/-}$ and BALB/c WT mice. Phenotype of DCs generated from the bone marrow of WT and ST2$^{-/-}$ BALB/c mice by culture in GM-CSF–containing medium for 6 days. One representative experiment of 2 is shown.

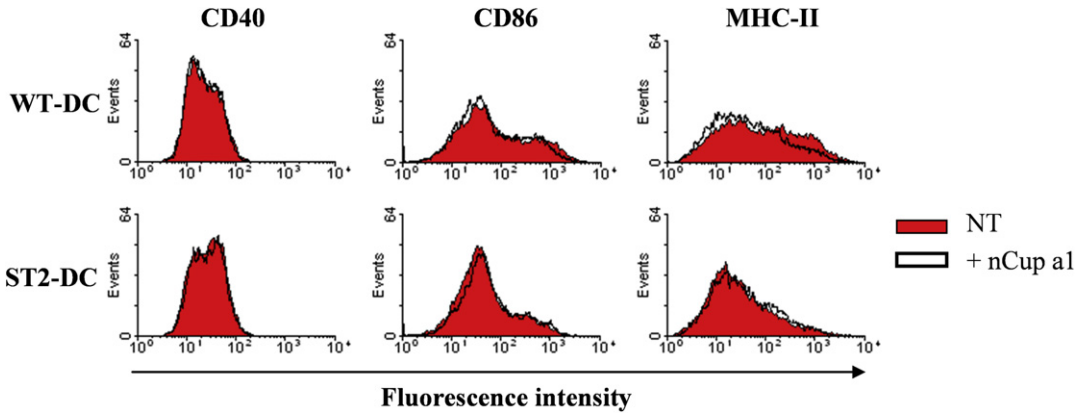

FIG E4. Phenotype of ST2$^{-/-}$ and BALB/c WT DCs after nCup a 1 treatment. Phenotype of DCs from WT and ST2$^{-/-}$ mice after overnight culture with 1 μg/mL nCup a 1 (day 7). One representative experiment out of 2 is shown. *NT,* Not treated.

TABLE E1. Primers used for quantitative PCR

Gene	Forward and reverse primers (5′ → 3′)	GenBank ID no.	Amplicon size (bp)
OX40	GTAGACCAGGCACCCAACC	NM_011659.2	268
	GGCCAGACTGTGGTGGATTGG		
OX40L	TCTCCGGCAAAGGACCCTCCA	NM_009452.2	145
	GCCCATCGCACTTGATGACAACC		
IL-4	ACAGGAGAAGGGACGCCAT	NM_021283.1	123
	TCCAGAACATGCCGCAGAG		
IL-5	AGCACAGTGGTGAAAGAGACCTT	NM_010558.1	150
	TCCAATGCATAGCTGGTGATTT		
IL-13	AGACCAGACTCCCCTGTGCA	NM_008355	116
	TGGGTCCTGTAGATGGCATTG		
	CGTGATGGCAGAGGGTGACGG		
ST2	GAATGGGACTTTGGGCTTTG	NM_001025602	76
	CAGGACGATTTACTGCCCTCC		
IL-25R	CGGGCGCCTGGATAAGAGGACCC	NM_019583.3	182
	CATCCACTCTGGAGATGGCCCTGT		
TSLPR	TGGAGTTCCGTTATGGCACTGGC	NM_001164735.1	83
	GATGCACCCGGAAGTGACACCAG		
Muc5ac	CAGGACTCTCTGAAATCGTACCATGAACAC	NM_010844	128
	AAGGCTCGTACCACAGGGA		
Gob5	CATCGCCATAGACCACGACG	NM_017474.1	150
	TTCCAGCTCTCGGGAATCAAA		
HPRT	CTGGTGAAAAGGACCTCTCG	NM_013556.2	109
	TGAAGTACTCATTATAGTCAAGGGCA		