Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

(see Fig E3, *A*). Similarly, mutant YFP-STIM1 (L74P) showed no response to thapsigargin but also appeared to form constitutive puncta, which was less distinct in appearance than that for the D76A mutant (see Fig E3).

We compared Ca^{2+} fluctuations in HEK293 cells transfected with ORAI-CFP and either wild-type YFP-STIM1, mutant YFP-STIM1 (D76A), or mutant YFP-STIM1 (L74P; see Fig E3, *B* and *C*). Both YFP-STIM1 (D76A) and YFP-STIM1 (L74P) transfected cells had increased basal Ca^{2+} concentrations compared with wild-type YFP-STIM1 and reduced peak and integral responses to CPA-induced SERCA inhibition (see Fig E3, *B* and *C*). However, in contrast to the EF-hand mutant YFP-STIM1 (D76A), YFP-STIM1 (L74P) did not demonstrate reduced SOCE after CPA washout and Ca^{2+} restoration, suggesting that the previously reported desensitization of SOCE observed with the YFP-STIM1 (D76A) mutant does not occur with the YFP-STIM1 (L74P) mutant form. Therefore the L74P mutation appears to result in a distinct molecular phenotype compared with the loss of function observed in immunodeficient patients and the constitutive activation observed in patients with myopathy.

This study is the first to report recessive *STIM1* mutations in patients presenting with AI and hypohidrosis without overt clinical immunodeficiency or myopathy. Clinical immunologic investigations were consistent with abnormal NK cell and T-lymphocyte function that might be expected to be associated with ongoing clinical immunodeficiency. However, despite severely abnormal SOCE, this was not the case in these patients. Missense mutations affecting the EF-hand can have very different clinical phenotypes with respect to the immune system, muscle, sweating, and enamel formation. This has important implications for clinical evaluation, as well as understanding the biological functions of STIM1.

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REFERENCES

1. Picard C, McCarl C-A, Papolos A, Khalil S, Lüthy K, Hivroz C, et al. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. N Engl J Med 2009;360:1971-80.
2. Fuchs S, Rensing-Ehl A, Speckmann C, Bengsch B, Schmitt-Graeff A, Bondzio I, et al. Antiviral and regulatory T cell immunity in a patient with stromal interaction molecule 1 deficiency. J Immunol 2012;188:1523-33.
3. Schaballie H, Rodriguez R, Martin E, Moens L, Frans G, Lenoir C, et al. A novel hypomorphic mutation in STIM1 results in a late-onset immunodeficiency. J Allergy Clin Immunol 2015;136:816-9.e4.
4. Zhang H, Clemens RA, Liu F, Hu Y, Baba Y, Theodore P, et al. STIM1 calcium sensor is required for activation of the phagocyte oxidase during inflammation and host defense. Blood 2014;123:2238-49.
5. Shaw PJ, Weidinger C, Vaeth M, Luethy K, Kaech SM, Feske S. $CD4^+$ and $CD8^+$ T cell-dependent antiviral immunity requires STIM1 and STIM2. J Clin Invest 2014;124:4549-63.
6. Böhm J, Chevessier F, Maues De Paula A, Koch C, Attarian S, Feger C, et al. Constitutive activation of the calcium sensor STIM1 causes tubular-aggregate myopathy. Am J Hum Genet 2013;92:271-8.
7. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE, et al. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. Curr Biol 2005; 15:1235-41.

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Antigen-presenting epithelial cells can play a pivotal role in airway allergy

To the Editor:

Professional antigen-presenting cells (APCs; ie, dendritic cells, macrophages, and B cells) react against exogenous antigens and initiate an adaptive immune response by presenting antigen peptides in the groove of the MHC class II molecules. During inflammation, ectopic expression of MHC class II has been reported on cells from multiple tissues, including the nasal mucosa, suggesting an antigen-presenting capacity of epithelial cells (ECs).[1-4] The present investigation was designed to examine the contribution of nasal epithelial cells (NECs) to the allergic inflammatory process. The abilities of NECs to take up antigen, express MHC class II and costimulatory molecules, and stimulate antigen-specific activation and proliferation of $CD4^+$ T cells were investigated by using a human mucosal specimen (see the Methods section in this article's Online Repository at www.jacionline.org).

First, the cell-surface expression of MHC class II and costimulatory molecules on human and mouse nasal epithelial cells (MNECs) was confirmed (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org). Then the ability of MNECs to present the antigen ovalbumin (OVA) to naive T cells was demonstrated. MNECs from sensitized mice displayed an enhanced MHC class II expression on coculture

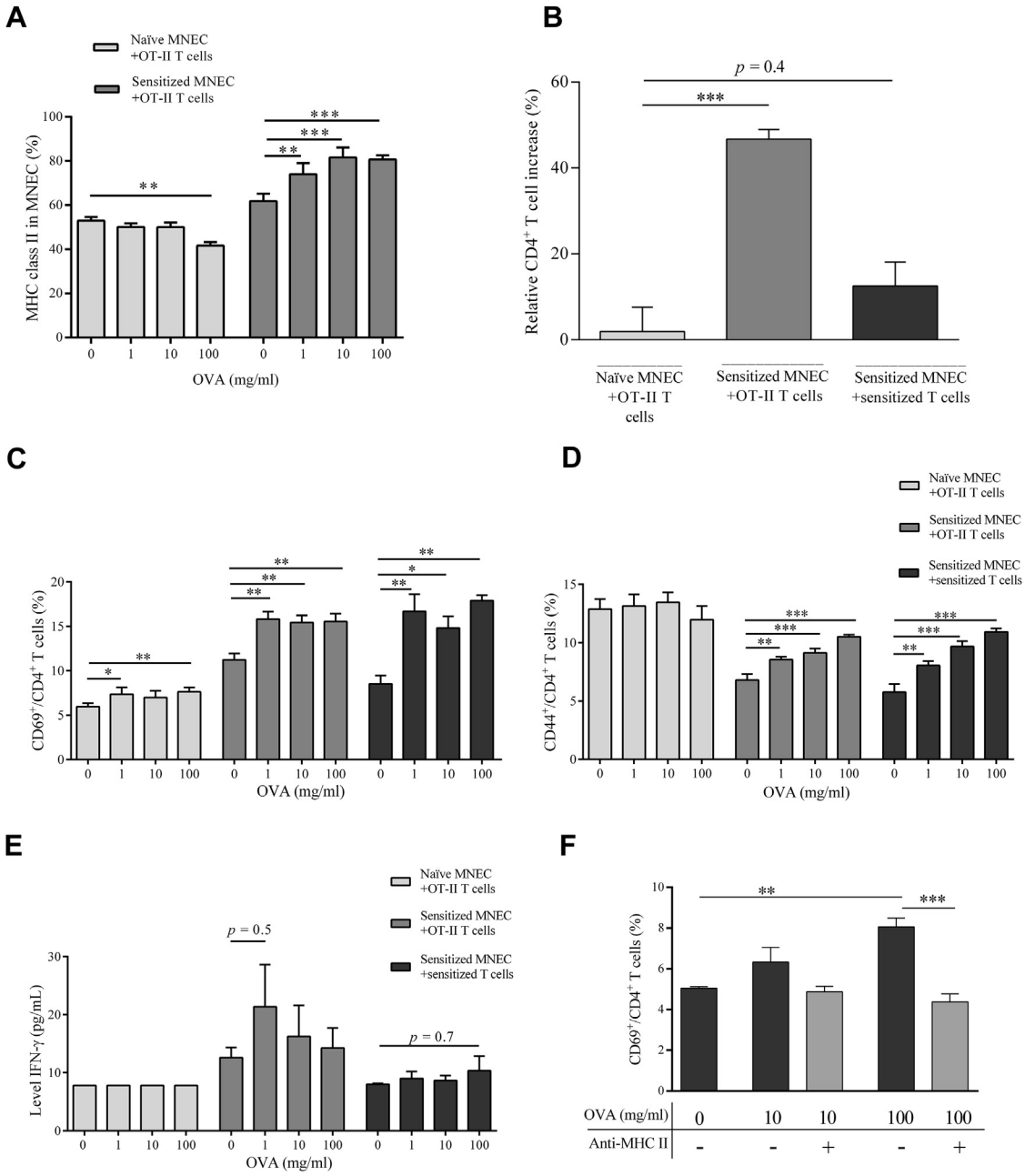

FIG 1. A, MHC class II expression on OVA-stimulated MNECs cocultured with T cells (4 hours). **B,** $CD4^+$ T-cell counts in cocultures with OVA-stimulated MNECs (24 hours). **C** and **D,** Fraction of $CD69^+/CD4^+$ (Fig 1, *C*) and $CD44^+/CD4^+$ (Fig 1, *D*) T cells after coculture with OVA-stimulated MNECs. **E,** INF-γ release in cocultures (24 hours). **F,** Cocultures with MNECs and T cells (both from sensitized mice) with anti–MHC class II antibodies *(anti-MHC II)*. $*P < .05$, $**P < .01$, and $***P < .001$.

with OT-II T cells compared with naive cells (Fig 1, *A*). The total number of OT-II $CD4^+$ T cells in the same cocultures was increased. A tendency toward an increase in $CD4^+$ T-cell counts was also seen when sensitized T cells were used as reporter cells (Fig 1, *B*). Analysis of T-cell activation revealed a pronounced increase in the total number (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org) and fraction (Fig 1, *C*) of activated $CD69^+$ OT-II cells, as well as sensitized T cells, when using sensitized MNECs as APCs. Notably, sensitized MNECs exhibited significantly increased activating capacity, even without added OVA, which was supposedly partially due to the remaining OVA in the MNECs from the sensitization process. In line with this, MNECs from sensitized mice augmented the absolute number (see Fig E3, *B*) and fraction (Fig 1, *D*) of $CD44^+$ OT-II cells, as well as sensitized T cells, in a dose-dependent manner. A tendency toward an increased IFN-γ release was simultaneously seen when sensitized MNECs were used as APCs (Fig 1, *E*). Finally, sensitized MNECs were unable to affect the

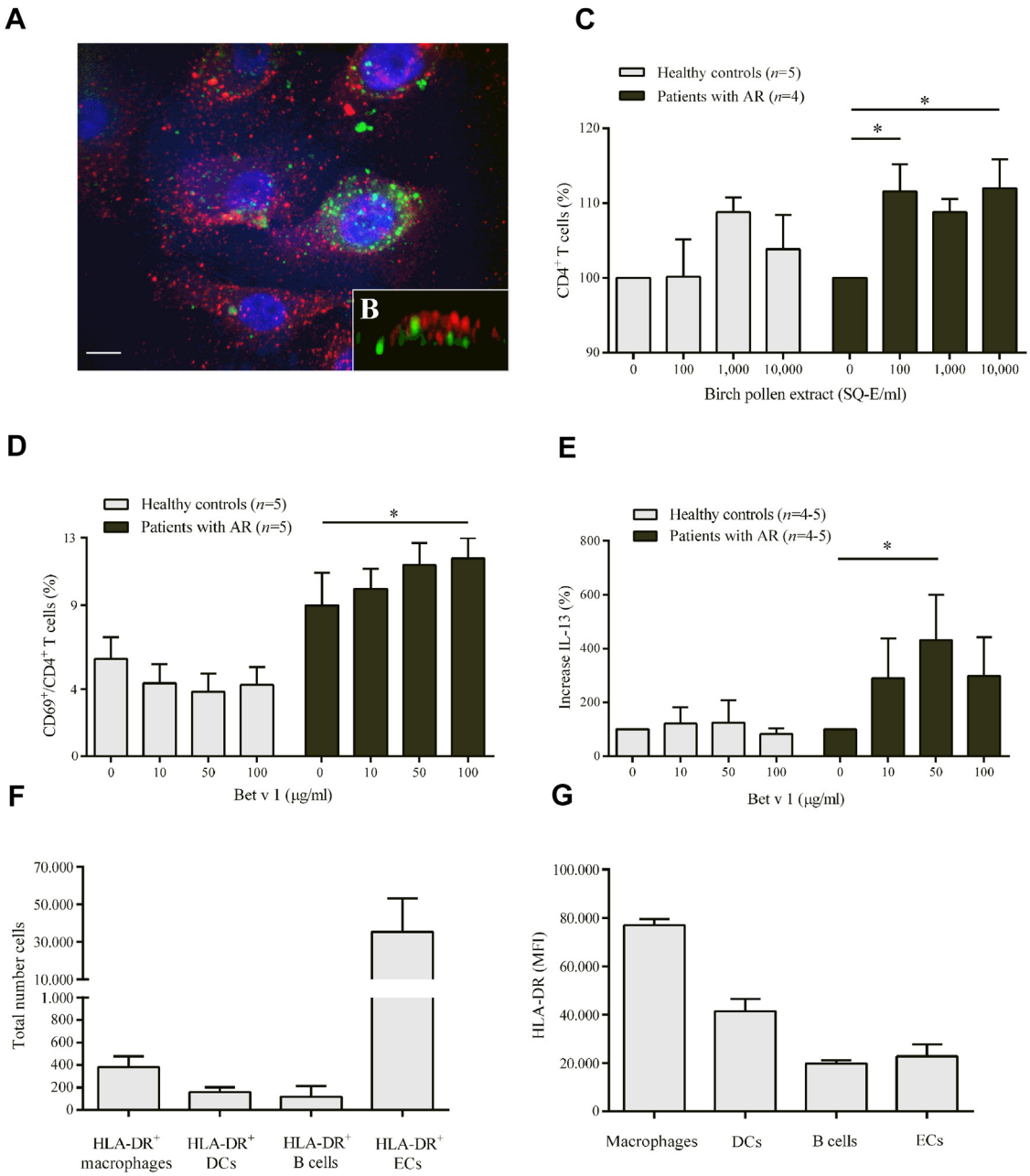

FIG 2. A, NECs from a patient with AR containing dextran *(green)* and MHC class II *(red)*. *Scale bar* = 10 μm. **B,** Three-dimensional composite image. **C,** Level of $CD4^+$ T cells after coculture with HNECs. **D,** Fraction of $CD69^+/CD4^+$ T cells after coculture with HNECs. **E,** Level IL-13 followed by 4 hours of coculture. **F** and **G,** Different cell types in nasal mucosa (Fig 2, *F*) and intensity of HLA-DR (Fig 2, *G*; n = 7). **P* < .05.

fraction of $CD69^+$ T cells in cocultures with neutralizing anti–MHC class II antibodies (Fig 1, *F*).

Human nasal epithelial cells (HNECs) were able to endocytose dextran, reaching the intracellular class II loading compartment (Fig 2, *A* and *B*, and see Video E1 in this article's Online Repository at www.jacionline.org). Autologous $CD4^+$ T-cell counts increased when cocultured with birch pollen–stimulated HNECs from patients with allergic rhinitis (AR; Fig 2, *C*), and the number of $CD69^+$ T cells increased in a dose-dependent manner when stimulating cocultures with Bet v 1, minimizing endotoxin contamination (Fig 2, *D*, and see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). IL-13 release was augmented in the same cocultures (Fig 2, *E*, and see Fig E4, *B*). Control experiments ruled out that the T-cell activation was mediated by activated MHC class II–expressing T cells (see Fig E4, *C*). Finally, a high number of HLA-DR–expressing HNECs were found, strengthening the importance of NECs in upper airway antigen presentation (Fig 2, *F* and *G*).

The OVA peptide amino acid 323-339 encompasses an allergic antigenic epitope of the OVA protein in mice. Thus activation of T-cell receptor (TCR) transgenic OT-II T cells by OVA requires antigen processing. The marked activation

and proliferation of OT-II $CD4^+$ T cells when cocultured with OVA-stimulated MNECs from sensitized animals demonstrates the antigen-processing and antigen-presenting capacity of MNECs. An experiment in which splenic cells from sensitized animals containing OVA-specific $CD4^+$ T cells were cocultured in the same manner was used to mimic a more natural process. This resulted in a T-cell response with IFN-γ release, demonstrating the T cell–activating capacity of NECs.

The use of a TCR transgenic system and murine ECs made it possible to use naive T cells for the responses, thus making the point that a T-cell response against allergen could be initiated by ECs. This could not be addressed in a human setting. HNECs from sensitized patients with AR demonstrated an allergen-specific increased capacity to activate $CD4^+$ autologous T cells, further corroborating the mouse data (Fig 2). This recall response, which was dependent on the presence of memory T cells, was likely to result in a T_H2 response reflected in the detection of IL-13 (Fig 2, *E*).

Professional APCs are believed to mainly present and activate T cells in the lymph nodes. NECs are local, and their numbers in the mucosa far outnumber those of local dendritic cells, making their ability to present allergen and activate T cells an important asset in the first line of defense (Fig 2, *F* and *G*). Increased expression of MHC class II in murine type II alveolar ECs has been demonstrated after *Mycobacterium tuberculosis* infection, further supporting the importance of NECs in the microbial response.[5] Several viruses, such as human coronavirus causing severe acute respiratory syndrome, have evolved mechanisms to inhibit MHC class II pathways in ECs.[6] Bet v 1 pollen allergen has further been demonstrated to bind and enter ECs within minutes after exposure in patients with AR, even during the nonsymptomatic winter season.[7]

In summary, for the first time, we have demonstrated the role of antigen-presenting NECs in patients with allergic airway disease. Previous information in the field is scarce, even though it has been shown that intestinal ECs can stimulate $CD4^+$ T cells *in vitro* to proliferate and secrete cytokines.[1] Furthermore, the importance of a local rapid response that enables antigen presentation and $CD4^+$ T-cell activation was recently demonstrated in an mouse inflammatory bowel disease model in which MHC class II–deficient mice were unable to induce and reinstate tolerance when fed with *Helicobacter hepaticus* and IL-10 receptor–blocking antibodies.[8] We demonstrated that MHC class II in NECs can be loaded with exogenous antigens (Fig 2, *A* and *B*, and see Video E1), that NECs stimulate allergen-specific $CD4^+$ T-cell responses in mice and human subjects (Figs 1 and 2), and that HNECs from patients with AR can amplify the allergic response contributing to the ongoing allergic $CD4^+$ T cell–mediated inflammation, with the latter likely contributing to the high number of activated $CD4^+$ T cells found in nasal mucosa of patients with AR.[9] Hence this is the first time that local immune activity within the nasal mucosa has been directly linked to a rapid and efficient allergen-induced T-cell activation.

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REFERENCES

1. Dotan I, Allez M, Nakazawa A, Brimnes J, Schulder-Katz M, Mayer L. Intestinal epithelial cells from inflammatory bowel disease patients preferentially stimulate CD4+ T cells to proliferate and secrete interferon-gamma. Am J Physiol Gastrointest Liver Physiol 2007;292:8.
2. Takizawa R, Pawankar R, Yamagishi S, Takenaka H, Yagi T. Increased expression of HLA-DR and CD86 in nasal epithelial cells in allergic rhinitics: antigen presentation to T cells and up-regulation by diesel exhaust particles. Clin Exp Allergy 2007;37:420-33.
3. Goldwich A, Burkard M, Olke M, Daniel C, Amann K, Hugo C, et al. Podocytes are nonhematopoietic professional antigen-presenting cells. J Am Soc Nephrol 2013;24:906-16.
4. McDonald GB, Jewell DP. Class II antigen (HLA-DR) expression by intestinal epithelial cells in inflammatory diseases of colon. J Clin Pathol 1987;40:312-7.
5. Debbabi H, Ghosh S, Kamath AB, Alt J, Demello DE, Dunsmore S, et al. Primary type II alveolar epithelial cells present microbial antigens to antigen-specific CD4+ T cells. Am J Physiol Lung Cell Mol Physiol 2005;289:15.
6. Josset L, Menachery VD, Gralinski LE, Agnihothram S, Sova P, Carter VS, et al. Cell host response to infection with novel human coronavirus EMC predicts potential antivirals and important differences with SARS coronavirus. MBio 2013;4:e00165-13.
7. Renkonen J, Mattila P, Lehti S, Makinen J, Sormunen R, Tervo T, et al. Birch pollen allergen Bet v 1 binds to and is transported through conjunctival epithelium in allergic patients. Allergy 2009;64:868-75.
8. Thelemann C, Eren RO, Coutaz M, Brasseit J, Bouzourene H, Rosa M, et al. Interferon-gamma induces expression of MHC class II on intestinal epithelial cells and protects mice from colitis. PLoS One 2014;9:e86844.
9. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326:298-304.

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RORγt inhibitors suppress T_H17 responses in inflammatory arthritis and inflammatory bowel disease

To the Editor:

Increased frequencies of T-helper cells producing the proinflammatory cytokine IL-17A (T_H17) have been reported in ankylosing spondylitis (AS), rheumatoid arthritis (RA), psoriatic arthritis (PsA), and inflammatory bowel disease (IBD).[1] Genome-wide association studies have linked multiple T_H17-associated genes, including *IL23R*, with AS, psoriasis, and IBD, further supporting a common pathogenic role for the IL-17/IL-23 axis in these diseases.[2] Neutralizing IL-17A with mAbs has shown efficacy in AS and PsA,[3] and some benefit in RA,[4] but has proved ineffective and possibly deleterious in patients with IBD.[5] There is therefore a need for new therapies, including small-molecule inhibitors, that target type 17 immune responses.

METHODS

Patients

Nine nonsmoking patients with AR to birch pollen (mean age, 32 years; 3 male and 6 female patients) were included in the study. They were given diagnoses based on clinical history and RAST results for allergen-specific IgE. In addition, 20 healthy nonsmoking control subjects were included (mean age, 29 years; 5 male and 15 female subjects). RAST results for allergen-specific IgE were negative for all healthy control subjects included in the study. The study was approved by the local ethics committee, and all patients provided written informed consent.

Human cell preparation and coculture

Primary HNECs were obtained by means of nasal brushing, as previously described.[E1] None of the patients had symptoms of allergy at the time of nasal brushing because samples were taken before the season, and all but 1 patient had been off antihistamine treatment for more than 1 week. All had been off topical steroids for at least 1 year. Cells were maintained in collagen-coated tissue-culture flasks in complete medium with Keratinocyte Serum-Free Medium (Gibco, Paisley, United Kingdom), 0.005 μg/mL epidermal growth factor, 0.05 mg/mL bovine pituitary extract, 20 U/mL penicillin, and 20 μg/mL streptomycin (Gibco, Grand Island, NY). In addition, 0.0125 μg/mL Fungizone Antimycotic (Gibco) was added to the cells stimulated with Bet v 1. All cells were cultured at 37°C in a humidified 5% CO_2 air atmosphere. Cells from passages 3 to 5 were used in the experiment, and they were all positive for epithelial cell adhesion molecule (EpCAM; >90%) and negative for CD11b (<1%) and CD11c (2% to 4%).

Isolation of HNECs and human T cells and coculture setup

Peripheral blood samples from all patients with AR and control subjects were obtained approximately 3 weeks after nasal brushing. Blood samples were taken in season, and all patients had nasal allergy symptoms. All patients had been off antihistamine treatment for more than 24 hours. T cells were isolated by using magnetic antibody cell-sorting separation (MACSxpress Pan T Cell Isolation Kit, human; Miltenyi Biotec, Cologne, Germany), according to the manufacturer's instructions. In brief, peripheral blood was added to the Pan T Isolation cocktail, put in the MACSmix Tube Rotator for 5 minutes, and then put in the magnetic field of the MACSxpress Separator for 15 minutes. The supernatant was then collected. Furthermore, erythrocyte depletion was performed on the T cells used for experiments with HNECs stimulated with Bet v 1, according to the manufacturer's instructions. In brief, the MACS xpress Erythrocyte Depletion Reagent was added to the supernatant containing T cells, put in the MACSmix Tube Rotator for 5 minutes, and then put in the magnetic field of the MACSxpress Separator for 10 minutes. The supernatant was then collected, washed, and centrifuged. The isolated cells were routinely screened for expression of CD3 by using flow cytometry, revealing highly purified T-cell preparations (>98%).

Coculture setup

A coculture of HNECs and T cells was set up to investigate T-cell activation. The cultured HNECs (250,000 cells/mL) were stimulated with 0, 100, 1,000, or 10,000 SQ-E/mL birch pollen extract (Aquagen SQ Birch; ALK-Abelló, Hørsholm, Denmark) or 0, 10, 50, or 100 μg/mL Bet v 1 (Biomay, Vienna, Austria) for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. T cells for control experiments (Fig E4, *C*) were stimulated with 10,000 SQ-E/mL birch pollen extract. HNECs were then washed thoroughly with PBS 4 times. Purified primary T cells were added to each well (0.5×10^6 to HNECs stimulated with birch pollen extract and 0.3×10^6 to HNECs stimulated with Bet v 1) and resuspended in RPMI medium (Gibco) containing 2 mmol/L L-glutamine (Gibco), 2% FBS (Gibco), 20 U/mL penicillin, 20 μg/mL streptomycin, and 0.0125 μg/mL Fungizone Antimycotic. On 4, 24, and 48 hours of coculture, T cells, HNECs, and supernatants were separated and collected. Cocultured control cells were kept on ice.

Human nasal biopsy specimens

Nasal biopsy specimens were taken from inferior turbinates of healthy nonsmoking control subjects after achievement of local anesthesia, as previously described.[E2] The control subjects had no history of nasal or sinus disease, and RAST results for allergen-specific IgE were negative. Tissue was put through a 100-μm cell strainer (BD Falcon, Franklin Lakes, NJ) into Dulbecco modified Eagle medium and Nutrient Mixture Ham F12 medium (DMEM/F-12, Gibco) containing 10% FBS and incubated in room temperature for 5 minutes. Cells were washed and centrifuged once, and the supernatant was subsequently aspirated and discarded. Cells were highly positive for EpCAM (approximately 50%).

Mice

Eighty-seven C57BL/6 mice (female) aged 8 to 10 weeks were purchased from Charles River (Sulzfeld, Germany). The animals were allowed pelleted food and water *ad libitum* and housed in a climate-controlled environment with a 12-hour daylight cycle. The spleens of 4 OT-II TCR transgenic mice recognizing OVA peptide amino acid 323-339 in the context of $H2\text{-}IA^b$ were used with the permission of Dr F. Carbone.[E3] The study was approved by the Swedish Animal Experimental Review Board (N153-11 regarding C57BL/6 mice and N558/12 regarding OT-II mice). Fifty-nine C57BL/6 mice were sensitized by means of intraperitoneal injection of 10 μg of grade V OVA and 4 mg/mL $Al(OH)_3$ (Sigma-Aldrich, St Louis, Mo) suspended in 0.2 mL of PBS administered in the morning on days 0 and 7.

Isolation and culture of MNECs

All mice were killed by means of cervical dislocation. Septal mucosae were dissected from 79 mice 14 days after sensitization, as previously described.[E4] In brief, the skull was sectioned in the coronal plane, and the brain and posterior part of the skull were removed. The septum was separated from the nasal lateral walls. Nasal septums from 12 to 14 mice were pooled and put in DMEM/F-12 (Gibco) and then into dissociation medium for 1 hour containing minimal essential medium (Gibco), 100 IU/mL penicillin/100 μg/mL streptomycin, 1.4 mg/mL Pronase, and 0.1 μg/mL DNase (Sigma-Aldrich). DMEM/F12 containing 10% FBS was added to stop the reaction. Tissue was put through a cell strainer twice and thereafter in complete culture medium with DMEM/F12 (Gibco) containing 100 IU/mL penicillin–100 μg/mL streptomycin, 10% FBS, and 120 IU/mL ITS Premix (BD Biosciences, San Jose, Calif). The suspension was incubated at 37°C for 2 hours on 100-mm Primaria culture dishes (BD Bioscience). The cell suspension was then collected and cultured in collagen-coated tissue culture flasks containing complete culture medium at 37°C in a humidified 5% CO_2 atmosphere seeded at a density of 2.5×10^5 cells/mL.

Preparation of mouse splenic cells

Spleens were taken from 4 OT-II mice, 6 sensitized C57BL/6 mice, and 2 naive C57BL/6 mice. The tissue was put through a 100-μm cell strainer (BD Falcon). Cells were washed and centrifuged once, and the supernatant was subsequently aspirated and discarded. The cell populations contained approximately 15% (OT-II mice), approximately 25% (sensitized C57BL/6 mice), and approximately 12% (naive C57BL/6 mice) $CD3^+$ cells. T cells were defined as responders to stimulation of NECs.

Coculture setup

Cocultures of MNECs (pooled from 12-14 mice) and mouse splenic cells (from 1-2 mice) were set up to investigate T-cell activation. At epithelial confluence, they were put in DMEM/F-12 (Gibco) containing 2% FBS (Gibco) and 100 IU/mL penicillin–100 μg/mL streptomycin (Sigma-Aldrich). OVA was added at concentrations 1, 10, or 100 mg/mL, and cells were incubated for 24 hours at 37°C in a humidified 5% CO_2 atmosphere. They were then washed thoroughly with PBS 4 times. Splenic cells were cocultured with ECs by adding 1×10^6 splenic cells to each well. After 4 or 24 hours of coculture at 37°C in a humidified 5% CO_2 air atmosphere, splenic cells,

MNECs, and supernatants were collected. Cocultured control cells were put on ice. In the experiments with anti–MHC class II antibodies, 2.5 μg/mL anti-mouse I-A/I-E, or isotype control rat IgG2b, κ (both from BioLegend, San Diego, Calif) was added for 4 hours in addition to the splenic cells (4 hours).

Antibodies and flow cytometry

The following mAbs were purchased from BD Biosciences: anti-human CD3 (UCHT1, SK7), CD4 (RPA-T4), CD19 (SJ25C1), CD25 (2A3), CD45 (HI30), CD68 (Y1/82A), CD69 (FN50), CD11b (ICRF44), CD11c (B-ly6), EpCAM (EBA-1), HLA-DR (G46-6), CD86 (2331 [FUN-1]), CD80 (BB1), anti-mouse CD4 (RM4-5), CD8a (53-6.7), CD19 (ID3), CD44 (IM7), CD69 (H1.2F3), MHC class II (M5/114.15.2), and CD86 (GL1). Anti-human CD8 (DK25) was purchased from Dako (Glostrup, Denmark). Anti-human HLA-DR (L243) and anti-mouse EpCAM (G8.8), CD80 (16-10A1), and CD86 (GL-1) were purchased from BioLegend. All antibodies were titrated for optimum concentration before use, with dilutions ranging from 1:5 to 1:400. Cells were identified based on forward and side scatter properties. Cells were analyzed on an LRSFortessa analyzer (BD Biosciences), and data were processed with FlowJo software (Tree Star, Ashland, Ore).

Histology and fluorescence confocal microscopy

For optical imaging, HNECs from 1 patient with AR were cultured, as described above. For visualization of MHC class II expression, HNECs were preincubated with 10% normal donkey serum (Abcam, Cambridge, United Kingdom) for 30 minutes to block nonspecific binding. Subsequently, cells were incubated with an anti–MHC class II primary antibody (1:50, EPR11226, Abcam) for 1 hour, followed by an Alexa Fluor 488–labeled anti-rabbit secondary antibody (1:200, Abcam) for 1 hour. Imaging was performed with a Nikon Digital Sight DS-U1 camera coupled to a Nikon Eclipse TE2000-U microscope by using NIS Elements F. 2.20 software.

HNECs from another patient with AR were used to study dextran uptake. Fluorescein isothiocyanate–coupled 3000 molecular weight dextran (1 mg/mL D3306; Invitrogen, Carlsbad, Calif) was added to HNECs in complete medium for 2 hours in the dark at 37°C and thereafter washed with PBS to visualize endocytosis.[E5] Cells were fixed for 15 minutes in 4% formaldehyde, washed with PBS, and subsequently incubated with 10% normal donkey serum for 30 minutes to block nonspecific binding. MHC class II was visualized with an anti–MHC class II primary antibody (1:50, EPR11226, Abcam) and incubation for 1 hour, followed by an Alexa Fluor 555–labeled anti-rabbit secondary antibody (1:200, Abcam) and incubation for 1 hour. Stained cells were maintained in ProLong Gold antifade reagent with 4′-6-diamidino-2-phenylindole (DAPI; Invitrogen). For laser confocal microscopy, a Nikon Eclipse TE 300 microscope (PerkinElmer, Waltham, Mass) equipped with a solid-state laser (blue, 405 nm; green, 488 nm; and red, 568 nm) and connected to a Hamamatsu ORCA ER camera (Hammamatsu, Hammamatsu City, Japan) was used. Images were analyzed with UltraWIEW software. A 3-dimensional image was created with Image Processing and Analysis in Java (ImageJ; National Institutes of Health, Bethesda, Md) software.

Measurement of cytokines in human supernatants by using the Luminex assay

Supernatants collected after coculture of stimulated HNECs and autologous T cells (4, 24, and 48 hours) were analyzed with Luminex (Luminex, Austin, Tex) regarding levels of IL-13 and IL-5. A Human Cytokine Standard 17-plex (Bio-Rad Laboratories, Hercules, Calif) was used and run in a Luminex200 system. The analyses were carried out according to the manufacturer's instructions. In brief, specific antibodies labeled with spectrally encoded beads were applied to the samples and standards. After incubation, the beads were washed and mixed with specific biotinylated detector antibodies. Subsequently, excess biotinylated antibodies were removed by using a washing step, followed by addition of streptavidin-R-phycoerythrin to conjugate and label the detector antibodies. By monitoring the spectral properties of the beads and the amount of associated streptavidin-R-phycoerythrin fluorescence with the Bio-Plex System (Bio-Rad Laboratories), cytokine concentrations could be determined. The minimum detectable concentration was 0.12 pg/mL (IL-13) and 0.31 pg/mL (IL-5).

Measurement of IFN-γ levels in mouse supernatants by means of ELISA

IFN-γ levels in supernatants of cocultures of OVA-stimulated MNECs and splenic cells were measured with a mouse IFN-γ–specific ELISA kit (BioLegend), according to the manufacturer's instructions. The minimum detectable concentration of IFN-γ for the kit was 8 pg/mL.

Statistics

Statistical differences were determined by using 1-way repeated-measures ANOVA with the Bonferroni posttest (for more than 2 sets of paired data). For unrepeated measurements, 1-way ANOVA was used together with the Bonferroni posttest. Statistical differences between MNECs and HNECs, respectively, without stimulation were performed by using unpaired *t* tests. Statistical analyses were performed with GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, Calif). All data are shown as means ± SEMs. For human data, *n* equals the number of patients.

RESULTS

Our initial experiments were carried out to confirm cell-surface expression of MHC class II on isolated NECs from healthy control subjects (Fig E1, *A*, left panel) and patients with birch pollen–induced AR sampled before the start of the season (Fig E1, *B*, left panel). The HLA-DR expression on isolated HNECs was verified by means of immunofluorescence staining (Fig E1, *C*). ECs must express costimulatory molecules, such as CD86 or CD80, to act as APCs. Isolated HNECs from healthy control subjects (Fig E1, *A*, right panel) and patients with AR (Fig E1, *B*, right panel) displayed cell-surface expression of the costimulatory molecule CD86. No expression of CD80 was found (data not shown). There were no significant differences in the fraction of HLA-DR or CD86 between healthy control subjects and patients with AR before the season (data not shown). There was no significant difference in the fraction of HLA-DR or CD86 expression between HNECs derived from nasal biopsy specimens or HNECs obtained from nasal brushing, validating the nasal brush method for use in our forthcoming experiments (Fig E1, *D*).

In mice we found a marked H2-IA^b expression on MNECs, as well as cell-surface expression of the costimulatory molecules CD86 and CD80 (Fig E2, *A*). To mimic allergic patients, we sensitized C57BL/6 mice with OVA in conjunction with aluminum hydroxide. NECs from OVA-sensitized mice expressed marked levels of H2-IA^b (Fig E2, *B*, left panel). Expression levels of CD86 were similar to those in naive mice (Fig E2, *B*, middle panel), whereas we found increased expression levels of the costimulatory molecule CD80 in OVA-sensitized mice (Fig E2, *B*, right panel). The fraction of MNECs expressing H2-IA^b and CD80 was significantly increased on OVA-sensitized epithelium (based on 28 animals) compared with that on naive epithelium (based on 25 animals), whereas no difference was seen in CD86 expression (Fig E2, *C*).

Notably, sensitized MNECs exhibited significantly increased activating capacity demonstrated by the increased number and fraction of CD69 expression on OT-II T cells seen even without OVA stimulation compared with that seen on naive MNECs

without OVA stimulation ($P < .001$ for both number and fraction of CD69; Figs 1, *C*, and E3, *A*).

Splenic $CD4^+$ T cells from naive mice were added to stimulated naive and sensitized MNECs in all experiments to rule out nonspecific T-cell activation. No signs of T-cell proliferation were seen in these experiments (data not shown), demonstrating the necessity of antigen presentation and engagement of antigen-specific T cells.

A neutralizing anti–MCH class II antibody (anti-mouse A-I/E-I) was added to the cocultures to test whether the activation was class II dependent, resulting in no increase of $CD69^+/CD4^+$ T-cell counts after 4 hours. When adding an isotype antibody instead, the increase in cell counts persisted. This indicated that activation of T cells was class II dependent.

Control experiments were carried out for both the mouse and human coculture experiments in which samples were kept on ice. These experiments revealed no T-cell proliferation or activation (data not shown), supporting the hypothesis that the enhanced T-cell activation is dependent on epithelial antigen processing and presentation.

T cells from Bet v 1–sensitized patients harvested in the season demonstrated increased cell-surface expression of CD69, even without the addition of Bet v 1 allergen (Figs 2, *D*, and E4, *A*), suggesting that sensitization might activate the ECs and augment their antigen-presenting capacities by, for example, increasing expression of HLA-DR and costimulatory molecules.

In the supernatants of the human cocultures, no increase in IL-13 levels was seen on 24 hours of coculture in either of the groups (data not shown). No increase in IL-5 levels was detected in either of the groups on 4, 24, or 48 hours of coculture (data not shown).

The frequencies of different cell types in nasal mucosa and the magnitude of HLA-DR were analyzed in the final experiments. These experiments revealed a high number of HLA-DR–expressing NECs (total NECs, 67.313 ± 1.566; Fig 2, *F*).

REFERENCES

E1. Rydberg C, Mansson A, Uddman R, Riesbeck K, Cardell LO. Toll-like receptor agonists induce inflammation and cell death in a model of head and neck squamous cell carcinomas. Immunology 2009;128(Suppl):e600-11.

E2. Fransson M, Benson M, Erjefalt JS, Jansson L, Uddman R, Bjornsson S, et al. Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. Respir Res 2007;8:17.

E3. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol 1998;76:34-40.

E4. Antunes MB, Woodworth BA, Bhargave G, Xiong G, Aguilar JL, Ratner AJ, et al. Murine nasal septa for respiratory epithelial air-liquid interface cultures. Biotechniques 2007;43:195-6.

E5. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 1995;182:389-400.

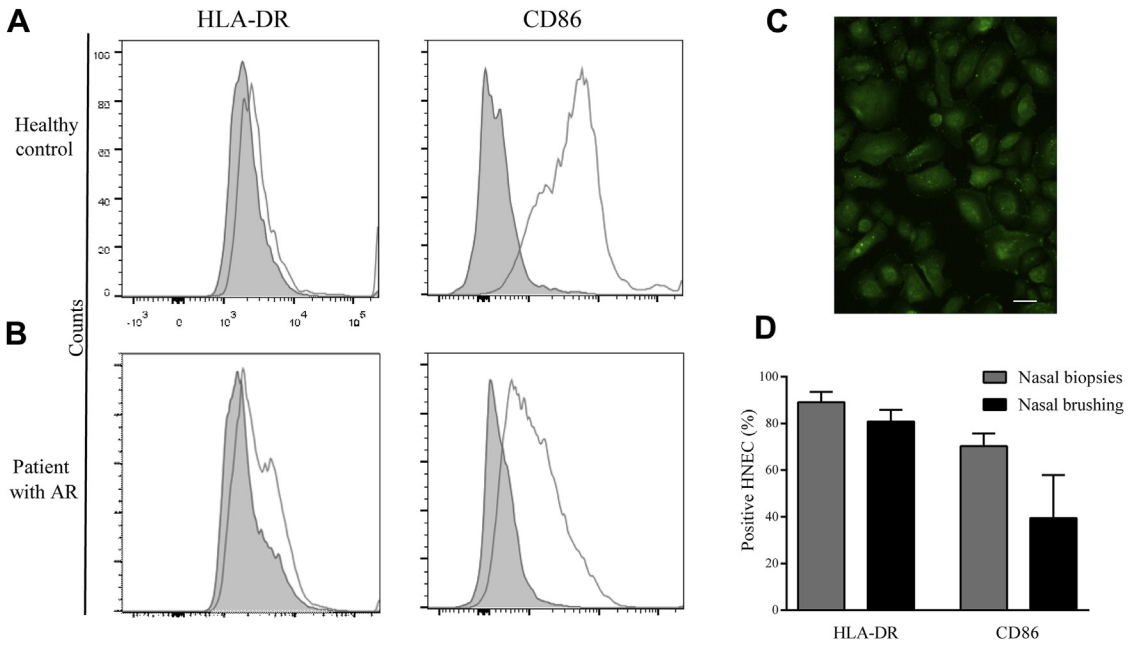

FIG E1. A and **B,** Expression of HLA-DR and CD86 *(open histogram)* in cultured HNECs from a healthy control subject (Fig E1, *A*) and a patient with AR (Fig E1, *B*; representative data from 9 different experiments, respectively). The isotype control is a *filled histogram*. **C,** HNECs stained for MHC class II *(green)*. *Scale bar* = 50 μm. **D,** Expression of HLA-DR and CD86 on freshly isolated HNECs from healthy control subjects in nasal biopsy specimens (n = 4) and nasal brushings (n = 6).

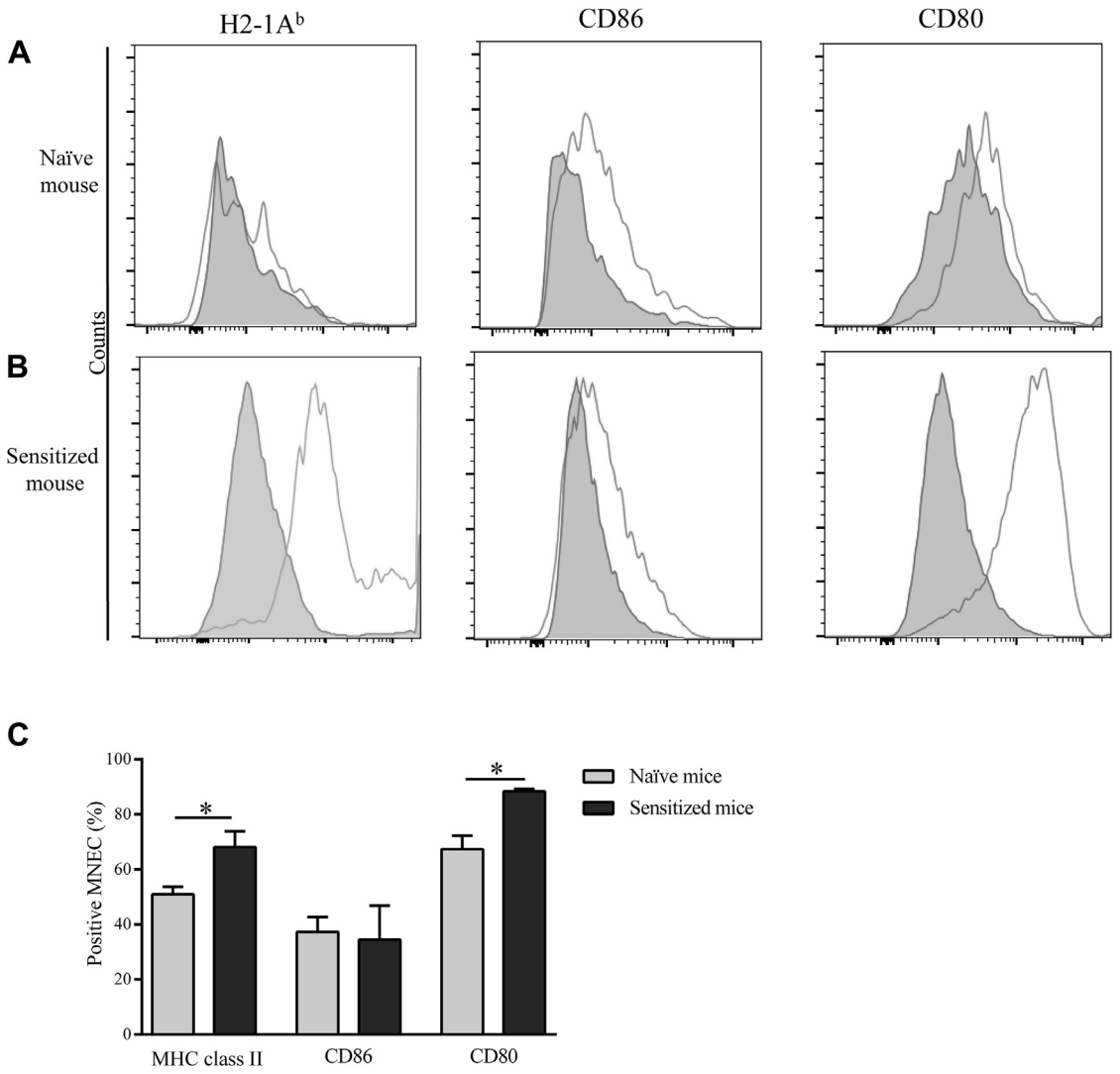

FIG E2. **A** and **B,** Expression of H2-IA^b, CD86, and CD80 on freshly isolated MNECs from a naive mouse (Fig E2, *A*) and from a mouse sensitized with OVA (Fig E2, *B*; representative data from 25-28 animals). **C,** Expression of H2-IA^b, CD86, and CD80 on cultured MNECs from naive and OVA-sensitized mice. *$P < .05$.

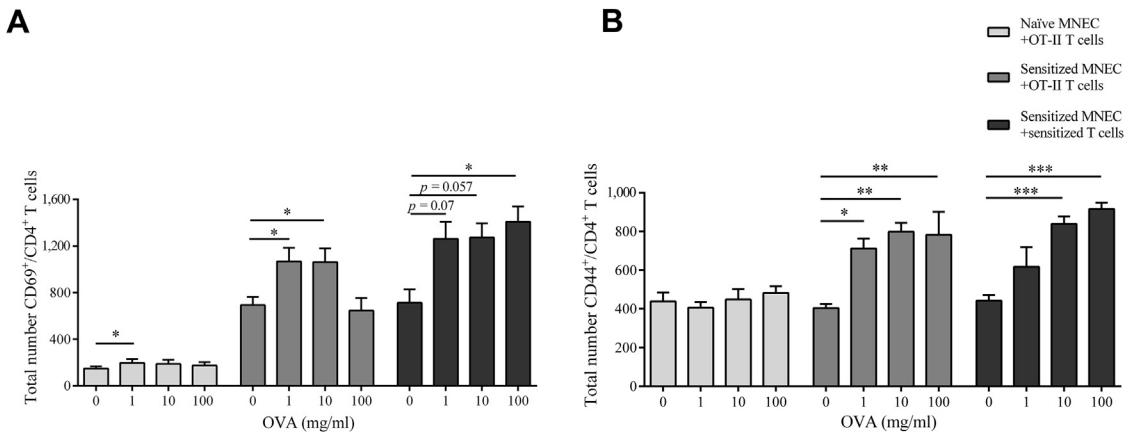

FIG E3. Total number of $CD69^+/CD4^+$ **(A)** and $CD44^+/CD4^+$ **(B)** T cells after coculture with OVA-stimulated MNECs. $*P < .05$, $**P < .01$, and $***P < .001$.

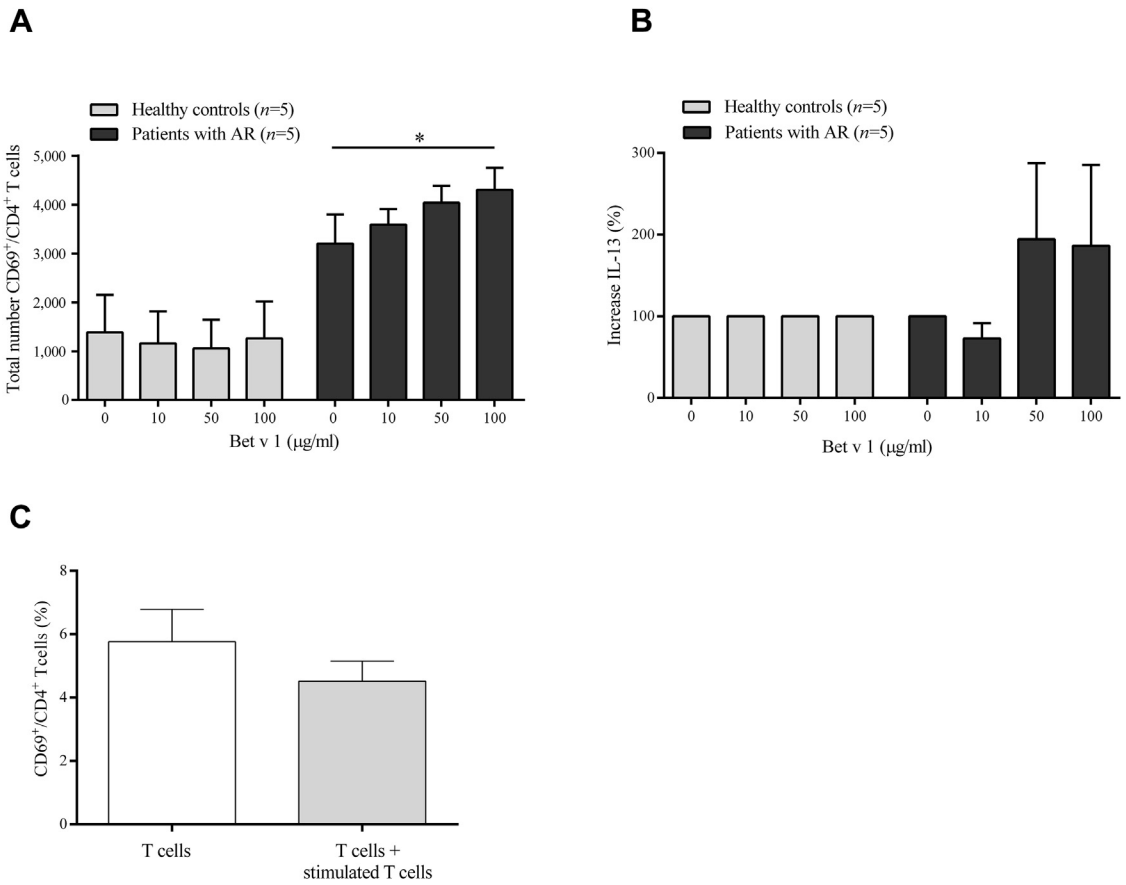

FIG E4. **A,** Total number of $CD69^+/CD4^+$ T cells after coculture with HNECs. **B,** IL-13 level after 48 hours of coculture. **C,** Fraction of $CD69^+/CD4^+$ T cells in coculture without and with birch pollen–stimulated T cells, ruling out that the T-cell activation was mediated by MHC class II–expressing T cells. *$P < .05$.