Pollen metabolome analysis reveals adenosine as a major regulator of dendritic cell–primed T_H cell responses

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Background: Water-soluble components from pollen modulate dendritic cell (DC) functions, such as IL-12 secretion and 3′-5′-cyclic adenosine monophosphate (cAMP) signaling and migration, possibly contributing to the establishment of a T_H2-dominated immune response against pollen. Because these effects could not solely be attributed to the previously identified pollen-associated lipid mediators, the pollen metabolome was analyzed for candidate immunomodulatory substances.
Objective: We sought to perform an analysis of the effect of pollen-associated adenosine on DC function and T_H cell differentiation.
Methods: Fractions of aqueous pollen extracts (APEs) were generated by means of ultrafiltration and were subjected simultaneously to biological tests and metabolome analysis (ultra-high-resolution mass spectrometry) and ultraperformance liquid chromatography. Effects of pollen-derived adenosine on monocyte-derived DC cAMP signaling, cytokine response, and capacity to differentiate T_H cells were studied.
Results: The less than 3-kd fraction of APEs comprised thousands of substances, including adenosine in micromolar concentrations. Pollen-derived adenosine mediated A_2 receptor–dependent induction of cAMP and inhibition of IL-12p70 in DCs. APEs digested with adenosine deaminase failed to mediate IL-12 inhibition. DCs of nonatopic donors exposed to APEs showed an adenosine-dependent reduced capacity to differentiate T_H1 cells and an enhanced capacity to induce regulatory T cells and IL-10. DCs of atopic donors failed to induce IL-10 but instead induced IL-5 and IL-13.
Conclusion: This study identifies adenosine out of thousands of metabolites as a potent immunoregulatory substance in pollen. It acts on the level of the DC, with differential effects in atopic and nonatopic donors.

Key words: *Dendritic cells, immunomodulation, adenosine, pollen, allergy*

Pollen provoke strong T_H2-dominated immune responses in susceptible subjects. Allergic sensitization involves several factors, such as epithelial barrier defects, intrinsic features of allergens, and coexposure to "danger signals," which can break tolerance.[1] As endogenous, pollen-derived danger signals, nicotinamide adenine dinucleotide phosphate oxidases cause oxidant stress in lung epithelium and boost allergic lung inflammation.[2,3] Pollen also liberate bioactive lipids, the so-called pollen-associated lipid mediators.[4-7] The prostaglandin E–like E_1 phytoprostanes inhibit IL-12 production of dendritic cells (DCs) by blocking nuclear factor κB translocation and bias immune responses toward T_H2.[7,8] However, several findings suggest that the multiple immunomodulatory effects mediated by aqueous pollen extracts (APEs) cannot solely be attributed to the E_1 phytoprostanes.

First, APE is a potent inducer of 3′-5′-cyclic adenosine monophosphate (cAMP), whereas E_1 phytoprostanes are not. In fact, DCs exposed to APEs acquire a migratory phenotype resembling that of DCs exposed to classical cAMP-increasing agents, such as prostaglandin E_2.[9] Second, APEs, but not E_1 phytoprostanes, inhibit the LPS-induced upregulation of maturation markers in peripheral blood–derived 6-sulfo $LacNAc^+$ (slan) DCs.[10] Third, in an animal sensitization model to the allergen ovalbumin, animals exposed to intranasal APE/ovalbumin exhibit reduced T_H1 and enhanced T_H2 responses in draining lymph node $CD4^+$ T cells, whereas exposure to ovalbumin/E_1 phytoprostanes leads to a downregulation of both T_H1 and T_H2 responses.[11] Finally, APEs, but not PPE_1, induced Ca^{2+} transients and DC chemotaxis (unpublished data). These findings suggest that pollens release other potent immunomodulatory substances in addition to the known pollen-associated lipid mediators.

Adenosine is an important tissue hormone originally known for its role in inflammation, but it also plays a key role in immune suppression and regulation.[12,13] Extracellular adenosine acts through 4 G protein–coupled receptors: Gi/o-coupled A_1 and A_3 receptors that decrease intracellular cAMP levels and Gs-coupled A_{2a} and A_{2b} receptors that increase cAMP levels through the activation of adenylyl cyclase.[12]

In immature DCs adenosine is recognized mainly through A_1 and A_3 receptors, inducing calcium transients, phagocytosis, and chemotaxis.[14,15] During maturation, DCs alter the expression profile of adenosine receptors, switching from expression of A_1 and A_3 receptors to expression of A_2 receptors.[15,16] It has been proposed that many of the suppressive effects of extracellular ATP on DC function are due to its degradation product,

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Supported by the Faculty of Medicine of the Technische Universität München (S.G. and C.T.-H.), the Deutsche Forschungsgemeinschaft (C.T.-H.), and the Christine-Kühne-Stiftung (CK-Care).
Disclosure of potential conflict of interest: The authors have declared they have no conflict of interest.

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Abbreviations used

ADA: Adenosine deaminase
APE: Aqueous pollen extract
cAMP: 3′-5′-Cyclic adenosine monophosphate
CFSE: Carboxyfluorescein succinimidyl ester
DC: Dendritic cell
FITC: Fluorescein isothiocyanate
Foxp3: Forkhead box protein 3
PE: Phycoerythrin
SPE: Solid-phase extraction
Treg: Regulatory T
UPLC: Ultraperformance liquid chromatography
UPLC-PDA: UPLC coupled with photodiode array
UPLC–TOF-MS: UPLC coupled with time-of-flight mass spectrometry

adenosine.[17] Beyond its inhibitory effects on T_H1 differentiation, adenosine has been shown to induce certain tolerogenic features in DCs, such as expression of IL-10, TGF-β, and indoleamine 2,3-dioxygenase,[18,19] implicating its contribution to DC-mediated differentiation of regulatory T (Treg) cells. Beyond its effects on DCs, adenosine plays a pivotal role in Treg cell function. In particular, Treg cells express ectoenzymes, such as CD73 and CD39, converting extracellular ATP to adenosine, which inhibits effector T-cell functions through A_{2a} receptors.[20] Moreover, development of adaptive Treg cells and T-cell anergy are triggered by A_{2a} receptor signaling.[21]

In this study the pollen metabolome was screened through systematic chromatographic fractionation with subsequent biological testing. Within the active fraction, adenosine was found to be a major constituent. We demonstrate that pollen-derived adenosine transmits tolerogenic signals to DCs, which translate into inhibition of T_H1 and T_H2 responses and into the induction of Treg cells in allogeneic stimulation assays. These tolerogenic signals are less efficiently transmitted by DCs of patients with pollen allergy.

METHODS

Subjects for blood sampling

Healthy nonatopic volunteers (aged 22-37 years) were screened for total serum IgE levels and for specific IgE against common allergens, as previously described.[10] For details on donor characterization, see the Methods section of this article's Online Repository at www.jacionline.org.

Pollen and preparation of APEs

Pollen of *Betula verrucosa*, *Phleum pratense*, *Artemisia vulgaris*, and *Ambrosia artemisiifolia* were from Allergon (Angelholm, Sweden). Pollen from *Betula alba* were from Sigma-Aldrich (Taufkirchen, Germany). Birch pollen was also collected in the spring of 2009 during the birch-flowering season in southern Bavaria, as previously described.[22] For details on the preparation, ultrafiltration, and adenosine deaminase (ADA) digestion of APEs, see the Methods section in this article's Online Repository.

Solid-phase extraction and ultraperformance liquid chromatography of APEs

For more information, see the Methods section of this article's Online Repository.

High-precision mass spectrometry

Negative and positive Fourier transform ion cyclotron resonance mass spectometric spectra were acquired on a Bruker Daltonics (Bremen, Germany) Apex Qe 12T system equipped with an APOLLO II source and microspray infusion of 120 μL/h with a scan number of 256. Spectra were acquired in broadband mode and were calibrated externally on clusters of arginine and internally calibrated with fatty acids and phthalate diesters, allowing a maximum error of 100 ppb. Peaks exceeding a threshold signal/noise ratio of 3 were exported to peak lists and were (1) converted into corresponding C (carbon), H (hydrogen), N (nitrogen), O (oxygen) and S (sulfur) elementary compositions and (2) submitted to MassTRIX, a metabolite-annotation Web interface (www.masstrix.org), as described previously.[23,24] MassTRIX processes the submitted mass peak list by comparing the input of experimental masses against all compounds of the Kyoto Encyclopedia Genes and Genome chemical compound database.[25]

Solid-phase extraction and ultraperformance liquid chromatography of APEs

Solid-phase extraction and ultraperformance liquid chromatography of pollen extracts was performed for further characterization and identification of substances picked out of the metabolome analysis. For details on chromatographic separation of APEs, see the Methods section of this article's Online Repository.

Reagents, culture, stimulation of monocyte-derived DCs and allogeneic stimulation assay

Isolation, culture, and stimulation of human monocyte-derived DCs was performed as previously published.[7-10] For detailed information on cellular assays, see the Methods section of this article's Online Repository.

Statistical analysis

The Wilcoxon test for paired samples was used to demonstrate statistically significant differences between treatment groups. *P* values of .05 or less were considered significant.

RESULTS

Low-molecular-weight fractions of APEs contain micromolar concentrations of adenosine

Mass spectrometric analysis of a low-molecular-weight fraction of APEs (<3 kd) revealed high amounts of adenosine, a known immune modulator (see Fig E1 in this article's Online Repository at www.jacionline.org). We therefore analyzed whether pollen-derived adenosine mediates modulation of DC cytokine production. First, the ultrafiltrated extract was separated by means of solid-phase extraction (SPE), and SPE fractions were analyzed for their capacity to inhibit the LPS-induced production of IL-12p70 (data not shown). The active fractions were further analyzed by means of ultraperformance liquid chromatography (UPLC) coupled with photodiode array (UPLC-PDA) analysis, and the presence of adenosine was confirmed (Fig 1). Consistent with the mass spectrometric data, APEs also contained other purine and pyrimidine nucleosides (Fig 1 and see Figs E2-E4 in this article's Online Repository at www.jacionline.org).

UPLC-PDA was performed with adenosine standard and APEs to quantify levels of adenosine in APEs. Adenosine concentrations in the extracts were estimated by comparison of peak areas with known standard concentrations. Adenosine levels in extracts of self-collected birch pollen were 3.9 ± 1.6 μmol/L (corresponding to aqueous extracts of 10 mg of pollen per

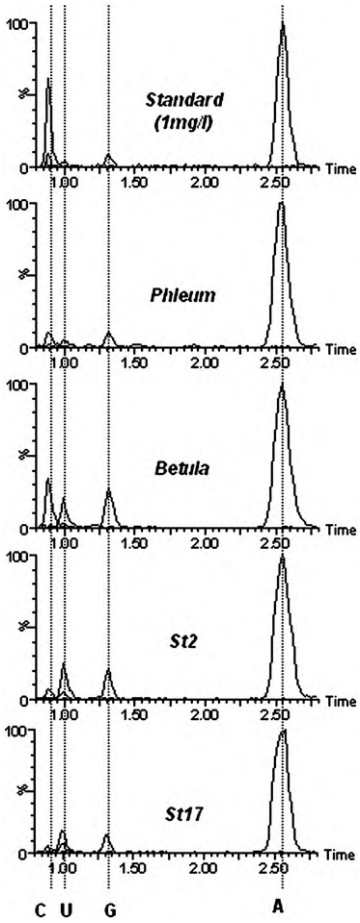

FIG 1. UPLC reveals the presence of adenosine in aqueous birch pollen extracts. Nucleoside standards and birch APEs were subjected to UPLC with subsequent detection by a photodiode array. The ion chromatograms were extracted at specific m/z per second values. Adenosine was detected as its protonated form (m/z of 268.103), and the other nucleosides were detected as their sodium adducts (m/z of 266.074 for cytosine, 267.060 for uridine, and 306.084 as guanosine). The retention time of cytosine, uridine, guanosine, and adenosine was 0.87, 0.98, 1.30, and 2.55 minutes, respectively.

milliliter, n = 40). Adenosine was also quantified in aqueous extracts of birch, grass, ragweed, and pine pollen. There was no adenosine detectable in mugwort pollen (Table I).

Pollen-mediated inhibition of LPS-induced IL-12 partly depends on pollen-derived adenosine

The control substance adenosine (Fig 2, *A*), as well as APEs (Fig 2, *B*), were digested with ADA, an enzyme catalyzing the degradation of adenosine to inosine to assess the contribution of adenosine to the pollen-mediated inhibition of IL-12 production by maturing DCs. Extracts were filtered after enzymatic digestion to remove the enzyme. Undigested APEs and adenosine, as well as the respective ADA-digested stimulants, were then added in different concentrations to DCs in the presence of LPS. Both ADA-digested APEs and ADA-digested adenosine were less potent in inhibiting the IL-12 response than the respective undigested stimulants (Fig 2). Inosine, the metabolite resulting from enzymatic digestion of adenosine, did not inhibit secretion of IL-12 (Fig 2, *A*).

cAMP induction and inhibition of IL-12 by pollen-derived adenosine occurs through A_2 receptors

Induction of cAMP and inhibition of IL-12 by adenosine have been reported to depend on A_{2a} or A_{2b} receptors in DCs.[14,17] Therefore pharmacologic inhibition assays with antagonists highly selective for the receptors A_{2a} or A_{2b} were performed. In DCs stimulated with adenosine (Fig 3, *A*) or APEs (Fig 3, *B*), an increase in intracellular cAMP levels was observed. This cAMP induction was blocked when DCs were pretreated with antagonists of A_{2a} and A_{2b} (SCH-442416 and MRS-1706; Fig 3, *A* and *B*).

Next, the contribution of A_2 receptors to the pollen-mediated inhibition of IL-12 production was assessed. In the presence of the A_{2a} antagonist SCH-442416, the adenosine-mediated inhibition of IL-12 was attenuated compared with that seen in DCs exposed to adenosine in the absence of antagonist (Fig 3, *C*). The A_{2a} antagonist, however, had no significant effect on APE-mediated IL-12 inhibition (Fig 3, *D*). In the presence of the A_{2b} antagonist MRS-1706, both adenosine (Fig 3, *E*) and APEs (Fig 3, *F*) were impaired in inhibiting the DC IL-12 response.

Pollen-derived adenosine contributes to inhibition of IFN-γ and induction of IL-10 in allogeneic T-cell stimulation assays

DCs stimulated with LPS plus adenosine, APEs, ADA-digested adenosine, or ADA-digested APEs were coincubated with allogeneic naive $CD4^+$ T cells to functionally verify the DC cytokine data. At day 7, cells and supernatants were subjected to intracellular cytokine staining and ELISA, respectively. Neither adenosine nor APEs mediated any significant modulation of the T_H17/T_H22-associated cytokines IL-17 (Fig 4, *A*) and IL-22 (Fig 4, *B*). However, the ability of DCs to differentiate IFN-γ–producing $CD4^+$ T cells was significantly reduced after exposure to adenosine or APEs. In contrast, DCs differentiated in the presence of ADA-digested adenosine or ADA-digested APEs were less efficient in inhibiting the T-cell IFN-γ response (Fig 4, *C*). Because adenosine is known to induce a tolerogenic DC phenotype,[18] we analyzed the capacity of pollen- or adenosine-exposed DCs to induce IL-10 in allogeneic naive $CD4^+$ T cells. Compared with LPS-matured DCs, DCs matured in the presence of LPS/adenosine or LPS/APEs induced an enhanced secretion of IL-10 in the T cells (Fig 4, *D*, and see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast, DCs exposed to ADA-digested adenosine or ADA-digested APEs failed to induce an enhanced IL-10 response in T cells (Fig 4, *D*). APE-induced IL-10 induction depended on A_2 receptors on DCs because DCs stimulated in the presence of A_2 antagonists failed to induce enhanced IL-10 levels in naive T cells (see Figs E5 and E6 in this article's Online Repository at www.jacionline.org).

Adenosine-exposed DCs derived from atopic donors induce less IL-10 and more T_H2 cytokines in allogeneic stimulation assays than nonatopic donor–derived DCs

The same nonatopic donor–derived naive $CD4^+$ T cells were coincubated with allogeneic DCs of a nonatopic donor and a

TABLE I. Adenosine concentrations in APEs

Concentration of adenosine (μmol/L)*							
***Betula* species (Allergon)**	***Betula* species (Sigma)**	***Betula* species #2**	***Betula* species #17**	***Phleum* species**	***Pinus* species**	***Artemisia* species**	***Ambrosia* species**
99.77	226.83	54.35	25.33	122.07	33.08	ND	32.86

Birch APEs enriched for compounds of less than 3 kd were analyzed by means of UPLC for the presence of adenosine. Adenosine concentrations in the extracts were estimated by comparison of peak areas with known standard concentrations. Pollen used to generate the extracts were from Allergon (*Betula verrucosa*, *Phleum pratense*, *Artemisia vulgaris*, *Ambrosia artemisiifolia*, and *Pinus sylvestris*) and Sigma-Aldrich (*Betula alba* species) or were isolated from birch catkins, as described in the Methods section.
ND, Not detectable.
*Corresponding to APEs generated from 50 mg of pollen per milliliter.

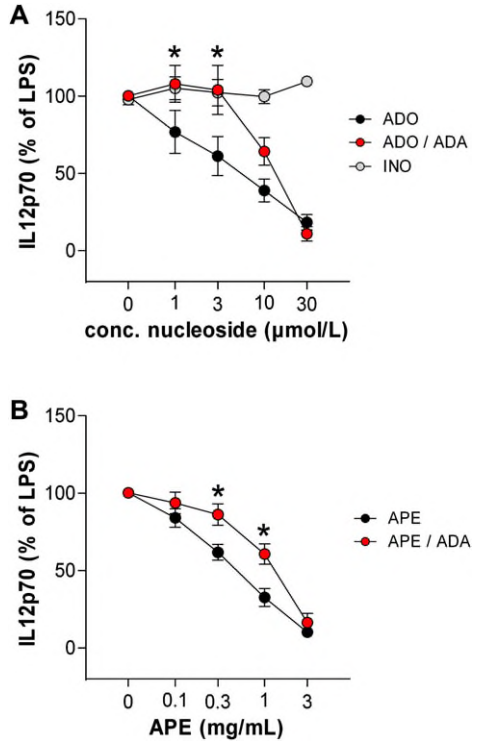

FIG 2. Pollen-associated adenosine contributes to inhibition of IL-12 from LPS-matured DCs. **A**, DCs were stimulated with LPS (100 ng/mL) plus inosine *(INO)*, mock-digested adenosine *(ADO)*, or ADA-digested adenosine *(ADO/ADA)*. Shown are the results of 8 (adenosine) or 3 (inosine) independent experiments. **B**, DCs were stimulated with LPS plus mock-digested or ADA-digested APEs *(APE/ADA)*. Shown are the results of 11 independent experiments, each performed in triplicate. Data are shown as percentages of mean ± SEM LPS-induced IL-12 (8.6 ± 1.8 ng/mL). *$P < .05$, one-tailed Wilcoxon signed-rank test for paired samples.

donor with pollen allergy to investigate whether DCs derived from nonatopic and atopic donors differ functionally in their capacity to induce regulatory versus T_H2-associated cytokines. After 7 days, IL-10, IL-13, and IL-5 levels were measured in the supernatants (Fig 5). Absolute IL-10 concentrations in cultures set up with LPS-DCs were as follows (mean ± SEM): 106 ± 13 pg/mL for nonatopic subjects and 155 ± 30 pg/mL for atopic subjects. Mean absolute IL-10 concentrations in Treg controls were 244 ± 28 pg/mL for nonatopic subjects and 183 ± 3 pg/mL for atopic subjects. Although nonatopic donor–derived DCs stimulated with LPS/APE or LPS/adenosine induced an enhanced secretion of IL-10 compared with that seen in LPS-DCs (Fig 5, *A*), DCs derived from donors with birch allergy and exposed to the same stimulants did not induce an enhanced IL-10 response in the same T cells (Fig 5, *A*). Concurrently, LPS/APE-stimulated DCs and LPS/adenosine-stimulated DCs of atopic donors induce an enhanced secretion of IL-13 (Fig 5, *B*) and IL-5 (Fig 5, *C*) compared with that seen in LPS-stimulated DCs, and this induction is not observed with DCs of nonatopic donors (Fig 5, *B* and *C*). Absolute LPS-induced cytokine concentrations in supernatants were as follows (mean ± SEM): IL-13 nonatopic, 713 ± 231 pg/mL; IL-13 atopic, 326 ± 74 pg/mL; IL-5 nonatopic, 112 ± 36 pg/mL; and IL-5 atopic, 164 ± 48 pg/mL. Absolute cytokine concentrations in the Treg controls were as follows: IL-13 nonatopic, 1,840 ± 242 pg/mL; IL-13 atopic, 1,949 ± 697 pg/mL; IL-5 nonatopic, 961 ± 150 pg/mL; and IL-5 atopic, 847 ± 220 pg/mL.

Exposure to pollen-derived adenosine triggers DCs to induce functional Treg cells in allogeneic T-cell stimulation assays

The induction of IL-10 in naive $CD4^+$ T cells by adenosine- and APE-exposed DCs prompted us to investigate the induction of Treg cells in the experimental setup. LPS/adenosine- and LPS/APE-stimulated DCs induced significantly higher levels of $CD4^+CD25^{high}$ forkhead box protein 3 (Foxp3)–positive cells in allogeneic stimulation assays than LPS-matured DCs (Fig 6, *A*). Moreover, LPS/APE- and LPS/adenosine-stimulated DCs derived from patients with birch allergy did not induce higher numbers of *bona fide* Treg cells compared with LPS-DCs (Fig 6, *B*). Intracellular cytokine staining revealed $CD3^+$ T cells as the only source of IL-10 in these cocultures (see Fig E5). Furthermore, IL-10 induction was abrogated by blocking A_2 receptors on the DCs (see Figs E5 and E6). Likewise, the induction of $CD4^+CD25^{high}Foxp3^+$ cells by nonatopic DCs depended on A_2 receptors (see Fig E7 in this article's Online Repository at www.jacionline.org).

The capacity of the *bona fide* Treg cells to suppress T-cell proliferation was tested by adding day 7 coculture supernatants or cells to autologous, carboxyfluorescein succinimidyl ester (CFSE)–labeled responder $CD4^+$ T cells. At day 5, Treg control, as well as LPS/adenosine and LPS/APE supernatants significantly inhibited the proliferation of responder T cells (Fig 7). Responder suppression likewise occurred when coculture cells were used at a coculture/responder cell ratio of 1:1 (data not shown). Furthermore, the suppressive capacity of coculture supernatants was lower when DCs for allogeneic stimulation were derived from patients with pollen allergy (Fig 7, *B*).

DISCUSSION

Analyzing the pollen metabolome for low-molecular-weight substances with immunomodulatory potential, we identified

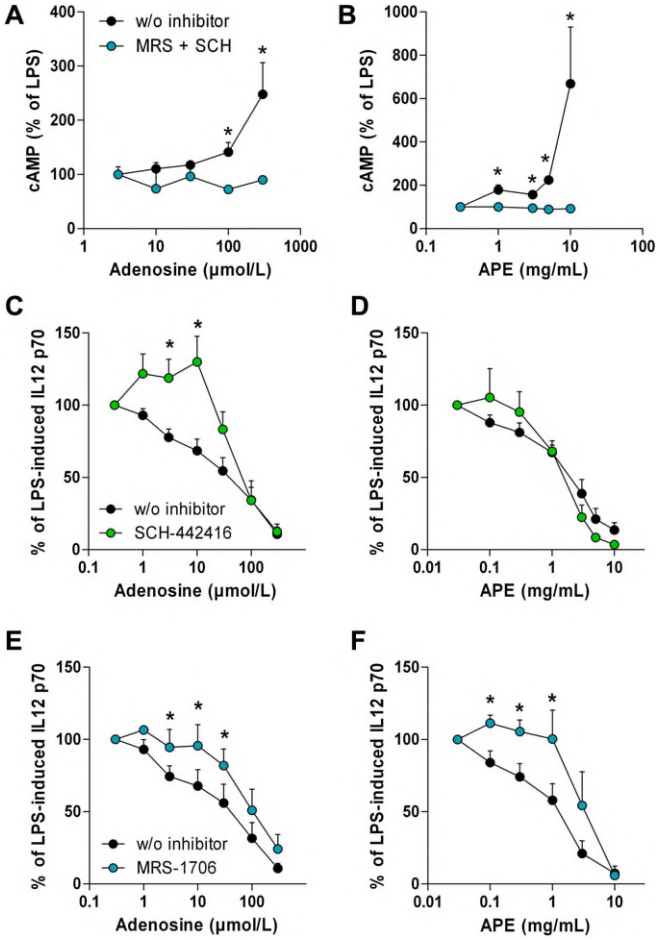

FIG 3. Pollen-derived adenosine induces cAMP and inhibits IL-12 production in DCs through A_2 receptors. **A** and **B**, DCs were preincubated for 1.5 hours with LPS (100 ng/mL) plus A_2 receptor antagonists (0.1 μmol/L MRS-1706 and 1 μmol/L SCH-442416) and subsequently treated for 40 minutes with adenosine (Fig 3, *A*) or APEs (Fig 3, *B*). Lysates were analyzed for cAMP by means of ELISA. Data are expressed as means ± SEMs of LPS-induced cAMP (7.8 ± 0.9 nmol/L). Shown are the results of 3 experiments. **P* < .05, Wilcoxon signed-rank test. **C** and **D**, DCs were preincubated for 1.5 hours with vehicle or A_{2a} antagonist (1 μmol/L SCH-442416). LPS (100 ng/mL) plus adenosine (Fig 3, *C*) or APEs (Fig 3, *D*) was added. After 24 hours, IL-12p70 levels were measured in supernatants. **E** and **F**, DCs were preincubated with vehicle or A_{2b} antagonist (0.1 μmol/L MRS-1706). LPS plus adenosine (Fig 3, *E*) or APEs (Fig 3, *F*) was added. Data are expressed as mean LPS-induced IL-12 production (+ SEM) of 6 independent experiments. **P* < .05 (with vs without inhibitor), Wilcoxon signed-rank test.

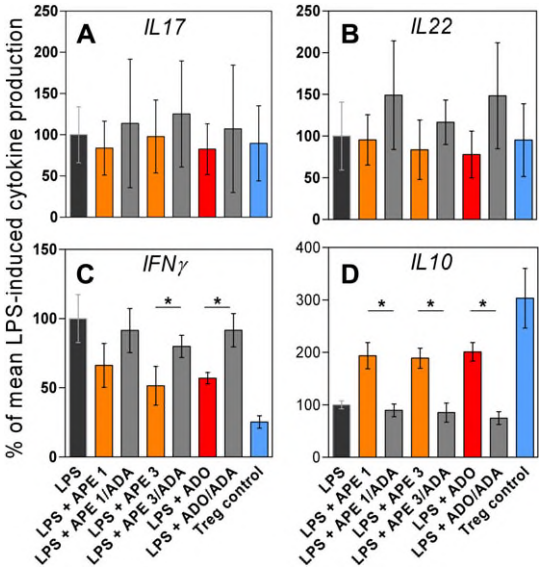

FIG 4. Pollen- and adenosine *(ADO)*–exposed DCs inhibit T_H1 differentiation and induce IL-10 in allogeneic stimulation assays. DCs were matured in the presence of 100 ng/mL LPS plus the indicated stimulants (APE1: 1 mg/mL; APE3: 3 mg/mL; adenosine: 10 μmol/L). Stimulants were either mock digested or digested with ADA. Allogeneic naive $CD4^+$ T cells were coincubated at a DC/T-cell ratio of 1:10. At day 7, IL-17 **(A)**, IL-22 **(B)**, IFN-γ **(C)**, and IL-10 **(D)** were analyzed. Shown are means ± SEMs of 4 independent experiments. **P* < .05, Wilcoxon signed-rank test.

nucleosides as major bioactive constituents of APEs. The presence of nucleosides in pollen might result from active, carrier-dependent import, which is a prerequisite for RNA synthesis during early pollen germination.[26,27] APEs generated from freshly collected birch pollen contain adenosine in micromolar concentrations, whereas concentrations of other nucleosides are low. The unique accumulation of adenosine in fresh birch pollen grains might be a consequence of ATP breakdown. Recently, extracellular ATP has been discovered as an important signaling molecule in *Arabidopsis* species growth, pollen germination, and pollen-tube elongation.[28-31] Whether adenosine or other nucleosides serve as signaling molecules in plants is not known.

Extracellular adenosine concentrations in normal and inflamed tissues range from 300 to 1200 nmol/L, respectively.[32] Adenosine concentrations in APEs of all examined pollen species ranged from 25 to 250 μmol/L. Taking into account pollen count during the season, tidal volume, breaths per minutes, exposure time, and weight of the pollen grains, the estimated pollen exposure of a subject is on the order of 0.1 to 5 mg/d. Assuming a mean nasal fluid volume of 20 mL/d and a mean adenosine concentration of 100 μmol/L in APEs obtained after eluting 50 mg of pollen per milliliter of buffer, one can estimate that at a peak pollen exposure of 5 mg/d, the concentration of pollen-derived adenosine in the nasal fluid would be approximately 0.5 μmol/L. However, this approximation does not allow for potential additive and cumulative effects during prolonged exposure time. Also, adenosine concentrations might peak at the site where single pollen grains are situated. It is thus conceivable that hydration of pollen on the mucosa of the respiratory epithelia leads to the local accumulation of physiologically relevant concentrations of adenosine, affecting resident antigen-presenting cells.

Previously, we showed that pollen strongly influences effector functions of DCs by inhibiting LPS-induced IL-12 production and modulating the chemokine profile, resulting in the generation of a T_H2-dominated immune response.[7,9] We showed that the prostaglandin E_2 receptors EP_2 and EP_4 account in part for the cAMP signaling in APE-exposed immature DCs.[8] Herein we expand these results, showing that monocyte-derived DCs respond to pollen-derived adenosine by inducing intracellular cAMP through adenosine receptors A_{2a} and A_{2b}. Our earlier finding is in agreement with the present results because immature DCs showed only a partial reduction of the cAMP response in the presence of A_2 receptor antagonists (data not shown). The APEs used in this study, although strongly enriched for molecules of less than

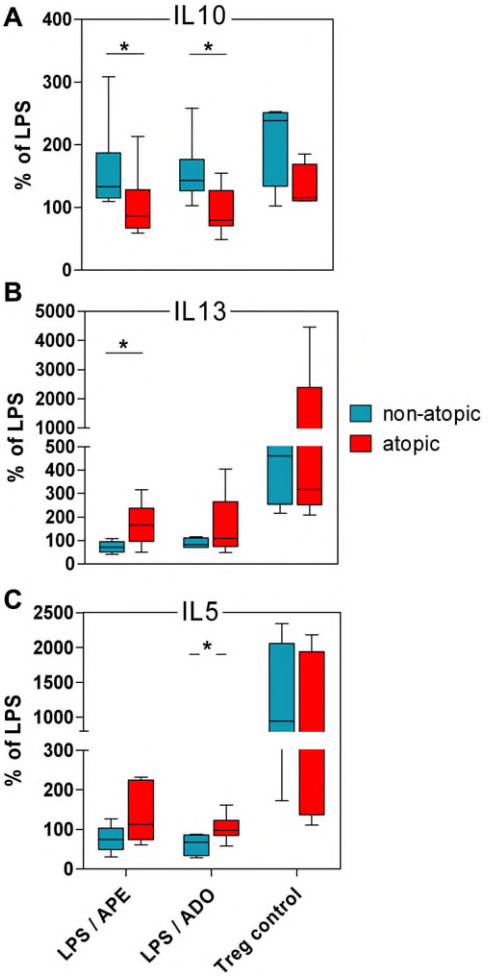

FIG 5. Adenosine *(ADO)*–stimulated DCs of atopic donors induce less IL-10 and more T_H2 cytokines than DCs of nonatopic donors. Pairs of DCs derived from monocytes of nonatopic and atopic donors were treated with 100 ng/mL LPS, LPS plus 1 mg/mL APEs *(LPS/APE)*, or LPS plus 10 μmol/L adenosine *(LPS/ADO)*; washed; and coincubated with the same allogeneic naive $CD4^+$ T cells (nonatopic donors) at a DC/T-cell ratio of 1:10. After 7 days, supernatants were analyzed for IL-10 **(A)**, IL-13 **(B)**, and IL-5 **(C)**. Results are expressed as percentages of mean LPS-induced cytokine production. Shown are the results of 6 independent experiments. *$P < .05$, Wilcoxon signed-rank test.

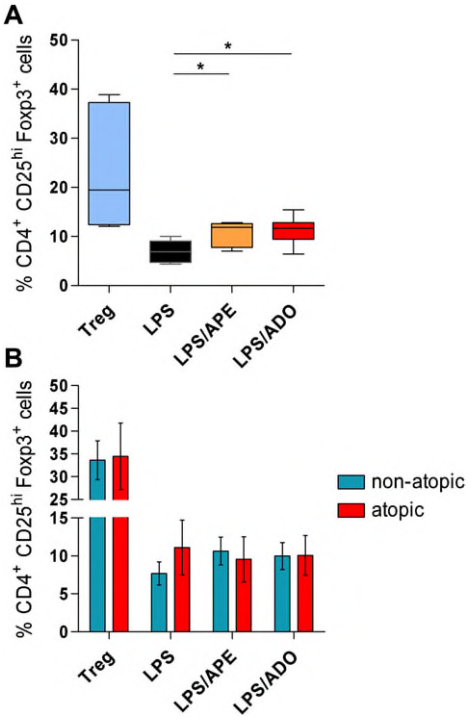

FIG 6. Adenosine *(ADO)*– and pollen-stimulated DCs induce $CD4^+CD25^+Foxp3^+$ T cells. **A**, DCs of nonatopic donors were coincubated with allogeneic naive $CD4^+$ T cells. At day 7, cells were stained for CD4, CD25, and intracellular Foxp3 and analyzed by means of flow cytometry. Data are expressed as percentages of $CD4^+CD25^{high}Foxp3^+$ cells (percentage of total coculture cells). Data from 7 independent experiments are shown. *$P < .05$, Wilcoxon signed-rank test. **B**, DCs in cocultures were either derived from nonatopic donors (blue) or patients with pollen allergy (red). Shown are means ± SEMs of 3 independent experiments (APE: 1 mg/mL; adenosine: 10 μmol/L).

3 kd, are complex mixtures of more than a thousand compounds. Thus it is conceivable that lipid mediators displaying a prostaglandin E_2–like spectrum of activity coexist with other cAMP-increasing compounds, such as adenosine.

In line with cAMP induction, pollen-derived adenosine contributed substantially to the inhibition of IL-12 secretion of DCs through A_{2a} and A_{2b} receptors. This is in agreement with previous work emphasizing the role of A_{2a} and A_{2b} receptors in inhibiting the IL-12 response of adenosine-exposed maturing DCs.[14,17,33]

The modulation of DC function by pollen-derived adenosine translated into an altered capacity to drive T_H cell differentiation from naive precursors. Differentiation of IFN-γ–producing T_H1 cells was inhibited in the presence of pollen in an adenosine-dependent manner because the effect was abrogated in the presence of APEs and ADA. The differentiation of T_H17/T_H22 cells was not modulated significantly. This might be explained by the fact that the secretion of IL-23 in contrast to IL-12 was only moderately inhibited in DCs by pollen-derived adenosine (data not shown).

Furthermore, pollen-associated adenosine confers the capacity to induce IL-10 and $CD4^+CD25^{high}Foxp3^+$ Treg cells on DCs. Upregulation of IL-10 coincided with the inhibition of T_H2 cytokines and did not occur when DCs of atopic donors were used for the allogeneic T-cell stimulation. Recently, it was found that the induction of $Foxp3^{hi}$ Treg cells in PMBCs stimulated with rye grass extract was reduced in allergic patients compared with that seen in healthy control subjects.[34] Furthermore, during the birch pollen season, allergen-specific immunosuppressive functions of $CD4^+CD25^+$ natural Treg cells from peripheral blood of patients with birch allergy are impaired compared with those seen in nonallergic control subjects.[35,36] In our experimental setup the induction of $CD4^+CD25^{high}Foxp3^+$ cells was mediated by DCs that had not been exposed to allergen but only to a low-molecular-weight fraction of APEs. Furthermore, it depended on A_2 receptors on the DC. These results implicate that defective Treg cell induction or function in patients with birch allergy might in fact be due to defective transmission of tolerogenic signals by DCs. One such signal might be pollen-derived adenosine. Although mRNA levels of A_2 receptors did not differ significantly between DCs derived from atopic and nonatopic donors (data not shown), DCs of atopic donors failed to induce an IL-10 response in naive $CD4^+$ T cells of nonatopic subjects on pollen

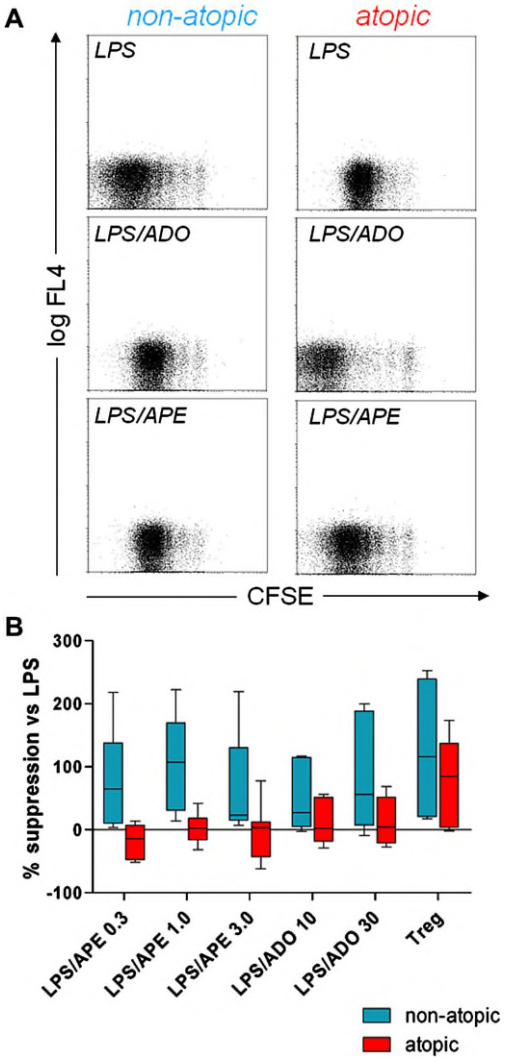

FIG 7. Adenosine *(ADO)*– and pollen-stimulated DCs induce functional Treg cells. **A**, Representative CFSE staining of $CD4^+$ responder T cells stimulated with anti-CD3 and anti-CD28 and mixed 1:1 with day 7 supernatants of allogeneic stimulation assays. DCs for the allogeneic stimulation assay were derived from a nonatopic and an atopic donor. Concentrations or DC stimulants were 100 ng/mL (LPS), 1 mg/mL (APE), and 10 μg/mL (adenosine). **B**, Quantitation of suppressive capacity of coculture supernatants. Pairs of nonatopic (blue) or atopic (red) donor-derived DCs were used for the allogeneic stimulation assays. Suppressive capacity of supernatants from LPS samples were defined as zero percent. Shown are results of 7 independent nonatopic/atopic pairs.

and adenosine exposure. This implies a defect in adenosine responsiveness in DCs of patients with birch allergy, resulting in impairment of the differentiation, function, or both of inducible Treg cells. Beyond that, DCs of atopic patients seem to display a more general defect in Treg cell induction because DCs used for Treg controls and generated in the absence of adenosine tended to induce lower IL-10 levels than DCs derived from nonatopic subjects. From the data presented herein, we hypothesize that pollen induces a regulatory response that could be in part due to pollen-derived adenosine. This pathway seems to be altered in susceptible atopic subjects.

We thank Julia Kolek, Heidi Neumeir, and Brigitte Look for excellent technical assistance.

Key messages

- **Pollen from birch and other species release high amounts of nucleosides, foremost adenosine.**
- **Pollen-associated adenosine contributes substantially to the modulation of DC function, such as inhibition of T_H1 responses and induction of Treg cells.**
- **Atopic donors exhibit a deficiency to induce Treg cells when primed by APEs or adenosine-stimulated DCs.**

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METHODS

Reagents, antibodies for flow cytometry, and ELISA kits

Adenosine was purchased from Sigma-Aldrich and ADA from Calbiochem. Receptor antagonists (SCH442416, A_{2a}; MRS1706, A_{2b}) were from Tocris Bioscience (Bristol, United Kingdom). Ultrapure *Escherichia coli* LPS was obtained from Invivogen (Toulouse, France). Antibodies for flow cytometry were fluorescein isothiocyanate (FITC)–labeled anti-human IFN-γ, phycoerythrin (PE)–labeled anti-human IL-4 (BD PharMingen, Heidelberg, Germany), PE-labeled anti-human IL-17 (eBioscience, Heidelberg, Germany), allophycocyanin-labeled anti-human IL-22 (R&D Systems, Minneapolis, Minn), FITC-labeled anti-human CD4 (BD PharMingen), PE-labeled anti-human CD25 (BD PharMingen), allophycocyanin-labeled anti-human Foxp3 (Miltenyi Biotech, Bergisch Gladbach, Germany), and FITC-labeled anti-human CD3 and PE-labeled anti-human IL10 (eBioscience, Heidelberg, Germany). Fixation and permeabilization of cells for intracellular cytokine staining was performed with 2% paraformaldehyde (Sigma-Aldrich) in PBS and 0.5% Saponin from Quillaja bark (Sigma-Aldrich) in FACS buffer. For intracellular staining of Foxp3, the Foxp3 Staining Buffer Set (Miltenyi Biotech) was used. For determination of cytokines in cell-culture supernatants, the following ELISA reagents were used: IL-12, human IL-12p70 matched pair (BD PharMingen); IL-10, BD OptEIA Human IL-10 ELISA Set (BD PharMingen); IL-13, DuoSet Human IL-13 (R&D Systems); and IL-5, Mouse/Human IL-5 ELISA Capture and Human IL-5 ELISA Detection (eBioscience).

Subjects for blood sampling

Healthy nonatopic volunteers (aged 22-37 years) were screened for total serum IgE and for specific IgE against common allergens, as previously described before.[E1] All blood donors were without medication for at least 15 days before blood sampling. Atopic donors were characterized by increased total serum IgE levels (554 ± 136 IU/mL); sensitization against birch (specific IgE, 26 ± 6; 554 ± 136 IU/mL), hazel (20 ± 7 IU/mL), grass (46 ± 12 IU/mL), and mugwort pollen (4 ± 2 IU/mL); and a positive history and current symptoms of seasonal allergic rhinitis. The ethics board of the Technische Universität München approved the study, and volunteers were enrolled after obtaining written informed consent.

Pollen

Pollen of *Betula verrucosa*, *Phleum pratense*, *Artemisia vulgaris*, and *Ambrosia artemisiifolia* were from Allergon. Pollen from *Betula alba* species were from Sigma-Aldrich. Birch pollen was also collected in the spring of 2009 during the birch-flowering season in southern Bavaria. Catkins were sampled on sunny days (temperature, 20°C ± 3°C) from 40 trees at 2 m above the ground. Pollen was extracted by sieving catkins with a 100-μm sieve followed by a 70-μm sieve and stored at −80°C until further processing.

Preparation of APEs

Pollen grains were incubated at a concentration of 50 mg of pollen per milliliter of PBS for 30 minutes at 37°C with vortexing every 10 minutes. The suspension was centrifuged (10 minutes at 3,000*g*), and the aqueous supernatant was passed through a 0.2-μm pore sterile filter (Millipore, Schwalbach, Germany) to avoid transfer of pollen grains. The extracts were incubated overnight at 4°C with Detoxigel matrix (Pierce, Rockford, Ill) under constant agitation to remove endotoxin. The extracts were ultrafiltered with 3-kd and 10-kd cutoff filters (Amicon ultra YM3 and YM10; Millipore, Schwalbach, Germany) to fractionate APEs based on molecular size. The absence of Bet v 1 in the fraction less than 3 kd was verified by means of ELISA. For dose-response studies, *Betula* species APEs were serially diluted in complete DC medium. The concentrations of APEs given in the text and figures correspond to the amount of pollen used to generate the extract in a given volume (eg, 1 mg/mL = 50 mg of pollen extracted in 1 mL of PBS; pollen-free supernatant of the extraction diluted 1:50 into the culture medium). All cellular experiments shown in the present study were conducted with the less than 3-kd fraction of APEs.

ADA digestion of APEs

APEs and the reference substance, adenosine, were digested with 100 U/mL ADA for 1 hour at 25°C. The enzyme was removed by means of ultrafiltration with 3-kd cutoff filters (Amicon ultra YM3, Millipore).

SPE and UPLC of APEs

The APEs were fractionated by means of SPE with a MegaBond Elute cartridge filled with 1 g of octadecyl functionalized silica gel (Varian, Darmstadt, Germany). After conditioning with 2 mL of methanol and water, the 0.5-mL sample extract was introduced onto the solid phase. The sample constituents were fractioned with increasing amounts of methanol in water from 0% to 100%, increasing in 10% steps. The eluents were then dried in a self-constructed Thermo Scientific Savant SPD121P SpeedVac Concentrator.

Nucleotides in the pollen extracts and fractions thereof were analyzed with the Acquity UPLC System equipped with a 2996 PDA detector (Waters, Eschborn, Germany). The reversed-phase separation was achieved on BEH C18 packing with a 1.7-μm particle diameter and column dimensions of 100 × 2.1 mm (Waters). The thermostat of the column was set to 40°C, and the autosampler was set to 27°C. The flow rate was 0.5 mL/min, and the injection volume was 5 μL through a partial loop. Isocratic elution with 10 mmol/L ammonium formate in water containing 10% (vol/vol) acetonitrile was applied. The detection wavelength was set to 260 nm (1.2 nm in width), with a scan rate of 20 Hz. The peak areas were integrated with Empower 2 (Waters). Before measurement of the samples, the method was characterized for repeatability, linearity, and accuracy.

Confirming the determination, UPLC coupled to a quadrupole time-of-flight mass spectrometer (UPLC–TOF-MS) was applied (Synapt; Waters, Manchester, United Kingdom). The UPLC method was as above, although the flow rate had to be decreased to 0.35 mL/min for achieving efficient ionization. Capillary voltage and cone voltage were 3,000 V and 30 V, respectively. Nitrogen was used as nebulization (600 L/h, 300°C) and cone gas (50 L/h). Source temperature was set to 120°C. The mass spectrometer was operated in the positive electrospray ionization mode, and data between m/z 50 and 1,000 were recorded. The data acquisition rate was set to 0.3 seconds, with a 0.1-second interscan delay. Lock mass of Leucine-Emphakiline for mass calibration was measured every 15 seconds.

Culture of monocyte-derived DCs

PBMCs of nonatopic and atopic donors were isolated from peripheral blood by means of density gradient centrifugation. $CD14^+$ monocytes were purified by means of MACS (Miltenyi Biotech) and cultured in DC medium (RPMI-1640, 10% FCS, 2 mmol/L L-glutamine, 20 μg/mL gentamicin, and 500 μmol/L 2-mecaptoehanol) in the presence of 50 U/mL recombinant human (rh) GM-CSF and 50 U/mL rhIL-4 (PromoCell, Heidelberg, Germany). Immature DCs harvested on day 5 were greater than 98% pure, as assessed by means of FACS analysis ($CD14^-CD1a^+HLA\text{-}DR^+CD80^{low}CD83^-CD86^{low}CD40^{low}$, data not shown).

cAMP assays

Monocyte-derived DCs of nonatopic donors were preincubated with LPS in the absence or presence of MRS1706 (1 μmol/L) and SCH442416 (1 μmol/L) for 1.5 hours and subsequently stimulated for 40 minutes with different concentrations of APEs (1-10 mg/mL) or adenosine (10^{-5} to 3×10^{-4} mol/L). Lysates were analyzed for cAMP by means of ELISA, according to the manufacturer's instructions (cAMP Screen; Applied Biosystems, Foster City, Calif).

Stimulation of DCs for cytokine measurements

Immature DCs derived from monocytes of nonatopic donors were resuspended at 1×10^6 cells/mL in DC medium, and LPS was added to 100 ng/mL before addition of different concentrations of APEs (*Betula* species), adenosine, inosine, ADA-digested APEs, or ADA-digested adenosine. For pharmacologic inhibition assays, cells were preincubated for 1.5 hours with adenosine receptor antagonists or vehicle before adding the stimulants. After 24 hours, culture supernatants were analyzed for IL-12 by means of ELISA.

Cells were recovered and subjected to propidium iodide staining and FACS analysis to determine survival rates in response to respective stimulants (data not shown).

DC-driven T_H cell differentiation

Monocyte-derived DCs of nonatopic and atopic donors were stimulated with LPS or LPS plus indicated stimulants, as described above. After 24 hours, DCs were washed twice with Ca^{2+}-free PBS. Allogeneic $CD4^+CD45RA^+$ T cells were isolated from PBMCs of nonatopic donors by using a $CD4^+CD45RA^+$ Naïve T Cell Isolation Kit (Miltenyi Biotech). T cells were added to DCs at a T-cell/DC ratio of 10:1. Treg controls were run by using a standard protocol. Briefly, DCs are left unstimulated for 24 hours, washed twice, and cocultured with allogeneic naive $CD4^+$ T cells after addition of 5 ng/mL TGF-β, 1 μg/mL anti–IFN-γ, 25 ng/mL anti–IL-12, and 100 U/mL rhIL-2. After 7 days, cells were stained for CD4 and CD25, fixed, permeabilized, and stained for intracellular Foxp3. Supernatants were analyzed for IL-10 by means of ELISA (eBioscience). For intracellular cytokine staining, cells were restimulated for 6 hours with phorbol 12-myristate 13-acetate/ionomycin in the presence of monensin and brefeldin A, fixed and permeabilized, and stained for IFN-γ, IL-17, IL-22, and IL-10, followed by flow cytometry.

Suppression assays

After depletion of $CD14^+$ cells from PBMCs, total $CD4^+$ cells were isolated with $CD4^+$ Micro Beads (Miltenyi Biotech). $CD4^+$ cells were labeled with CFSE (Sigma-Aldrich) and seeded onto plates coated with anti-CD3 antibody (eBioscience). Soluble anti-CD28 antibody (BD PharMingen) and 10 U/mL rhIL-2 were added. From day 7 allogeneic stimulation assays, cells or supernatants were transferred to the labeled responder cells (ratio 1:1). Naive $CD4^+$ T cells from the allogeneic stimulation assays and responder $CD4^+$ T cells were derived from the same donor. At day 5 after anti-CD3/anti-CD28 stimulation, CFSE-positive cells were acquired on a FACScalibur (Becton Dickinson, Heidelberg, Germany). Suppressive capacity was quantitated by setting a gate on the proliferated cells and comparing mean fluorescent intensities within this gate. The suppressive capacity of LPS-DC cocultures was set to zero percent.

RESULTS

Pollen metabolome analysis reveals more than 1,000 low-molecular-weight compounds

To find candidate low-molecular-weight immunomodulatory substances from pollen, ultrafiltered APEs were subjected to infusion electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry for exact mass analysis. The less than 3-kd fractions of APEs showed distinct 12,305 exact mass signals that could be converted into 1,611 CHNOS elementary compositions, and the annotation of the exact masses with MassTRIX in the Kyoto Encyclopedia Genes and Genome metabolite data base yielded more than 900 annotated compounds (Fig E1). Among them were several metabolites of purine and pyrimidine metabolism (ie, adenosine, guanosine, deoxyguanosine, uridine, pseudouridine, and dihydroorotate; data not shown).

UPLC–TOF-MS confirms the presence of adenosine in APEs

To confirm the identification and the purity of the UPLC-PDA, the UV-Vis spectra, TOF-MS spectra, and MS^E spectra of the peak were studied. The UPLC-PDA separation provided clean separation of adenosine from the matrix constituents. Additionally, identical molecule peaks (m/z of 268.103 ± 0.001) and fragmentation peaks (m/z of 136.062 ± 0.001 and 119.035 ± 0.001), reflecting the loss of ribose in the standard solution and in the extracts, were observed in UPLC–TOF-MS (Fig E2).

Determination of guanosine, cytosine, and uridine in APEs

UPLC-PDA could not apply for the determination of guanosine, cytosine, and uridine at a high confidence level because of the partial coelution of the sample constituents and lower intensity of the analyte peak (Fig E3). Therefore UPLC–TOF-MS was applied as more selective detection instead of PDA where the chromatograms were extracted at the molecular peak of the nucleosides, as shown in Fig 1 (more details in Fig E4).

Induction of IL-10 and *bona fide* Treg cells in allogeneic T-cell stimulation assays depends on A_2 receptors on the DCs

Day 7 cocultures were stained for IL-10 and counterstained for the T-cell marker CD3 to identify the source of IL-10. Low numbers of $CD3^+$ IL-10–producing cells (0.25% to 2.3%) were detectable, none of which were $CD3^-$ (Fig E5). LPS/APE- and LPS/ADO-stimulated DCs induced higher percentages of $CD3^+IL\text{-}10^+$ cells than LPS-DCs. This induction of $IL\text{-}10^+$ T cells was reduced when DCs were preincubated with the A_2 receptor antagonists MRS-1706 and SCH-442416 (Fig E5) and was less pronounced when atopic donor-derived DCs were used to differentiate the T cells (Fig E5). Concordantly, in cell-culture supernatants the induction of IL-10 by LPS/APE- and LPS/adenosine-stimulated DCs was blocked by A_2 antagonists (Fig E6).

Additionally, percentages of $CD4^+CD25^{high}Foxp3^+$ T cells were compared in cocultures of naive T cells with DCs either untreated or pretreated with A_2 antagonists. LPS/APE- and LPS/adenosine-stimulated DCs failed to induce increased numbers of $CD4^+CD25^{high}Foxp3^+$ T cells when preincubated with A_2 antagonists (Fig E7).

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E1. Gilles S, Jacoby D, Blume C, Mueller MJ, Jakob T, Behrendt H, et al. Pollen-derived low-molecular weight factors inhibit 6-sulfo LacNAc+ dendritic cells' capacity to induce T-helper type 1 responses. Clin Exp Allergy 2010;40:269-78.

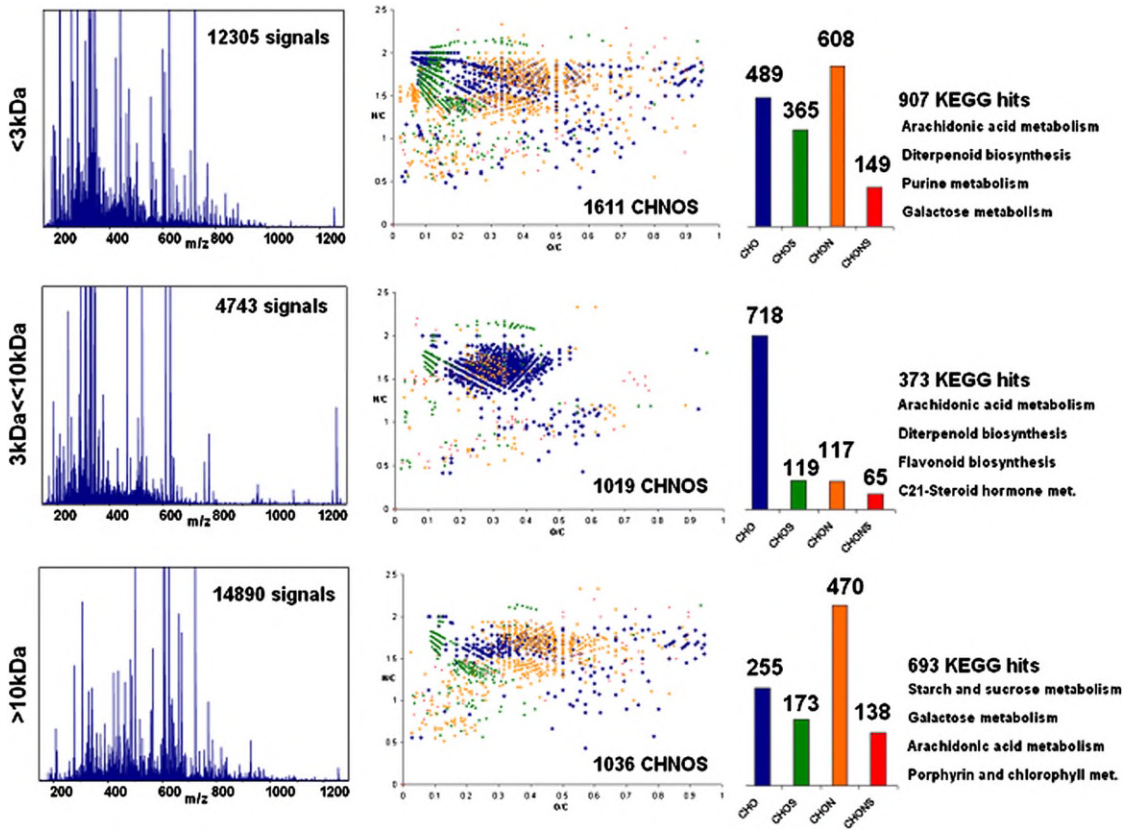

FIG E1. The pollen metabolome comprises more than 1,000 low-molecular-weight substances. Negative-mode electrospray ionization Fourier transform ion cyclotron resonance mass spectra of the 3 APE ultrafiltration fractions show the number of m/z signals, the number of corresponding calculated CHNOS elementary compositions, and the number of hits when annotated with MassTRIX in the Kyoto Encyclopedia Genes and Genome *(KEGG)* database in addition to the 4 pathways with the most of hits. The CHO, CHNO, CHOS, and CHNOS compositions are visualized with van Krevelen diagrams (H/C vs O/C), enabling us to differentiate structural information.

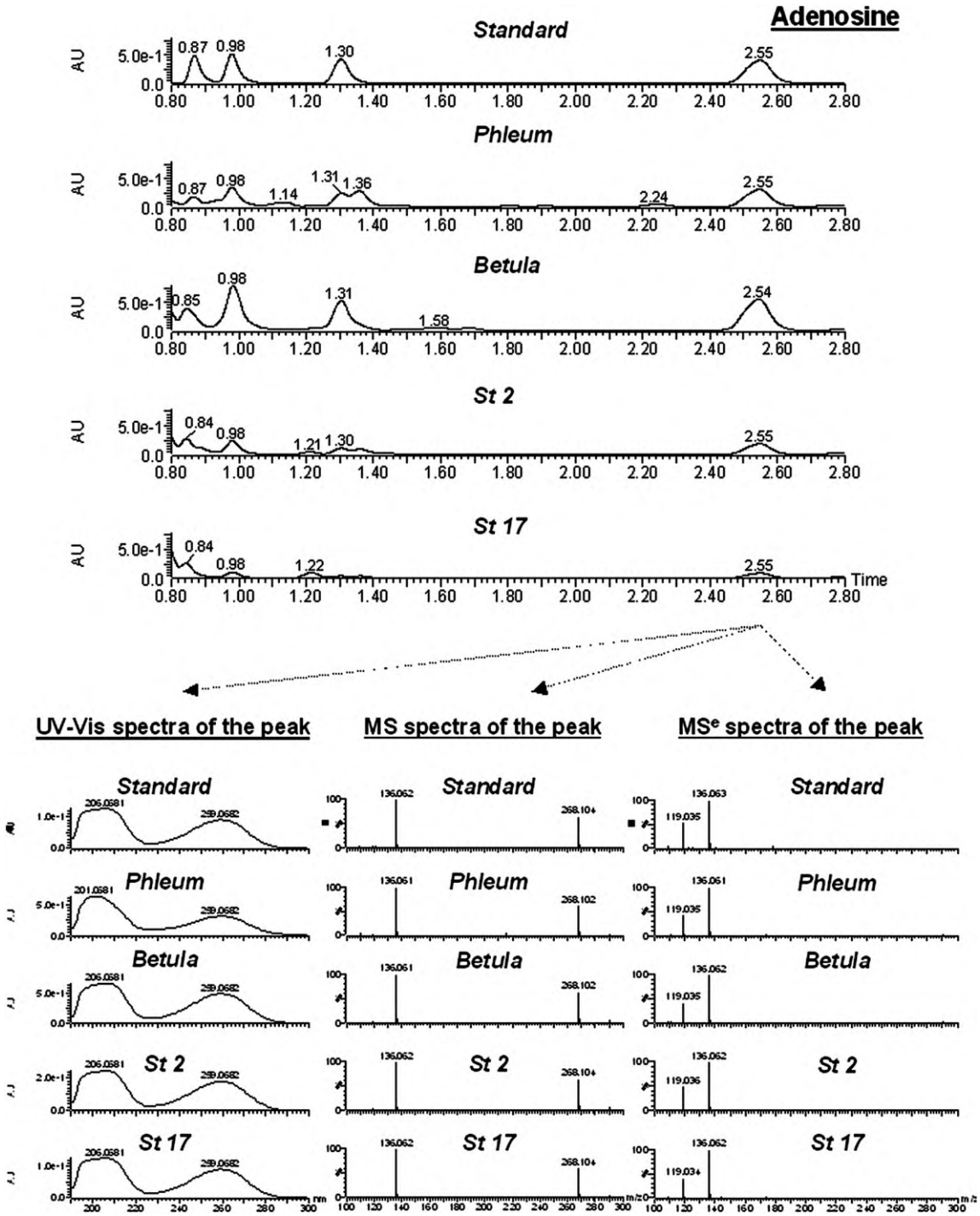

FIG E2. Application of 3 different detection techniques after liquid chromatographic separation for the reliable determination of adenosine in the pollen extracts. The nucleoside standards and the constituents of pollen extracts were separated by means of UPLC. The retention time of adenosine was 2.55 ± 0.01 minutes in the standard solution and also in different pollen extracts. The separated peaks were detected with 3 different detection techniques, UV spectrophotometry, mass spectrometry of the molecule ion *(MS)*, and mass spectrometry after fragmentation of the molecule ion *(MS^E)*, to investigate the possible interference of the sample constituents. The UV, MS, and MS^E spectra at 2.55 ± 0.01 minutes (retention time of adenosine) were identical in the samples as in the standard solutions, confirming the unique presence of adenosine in the separated peak.

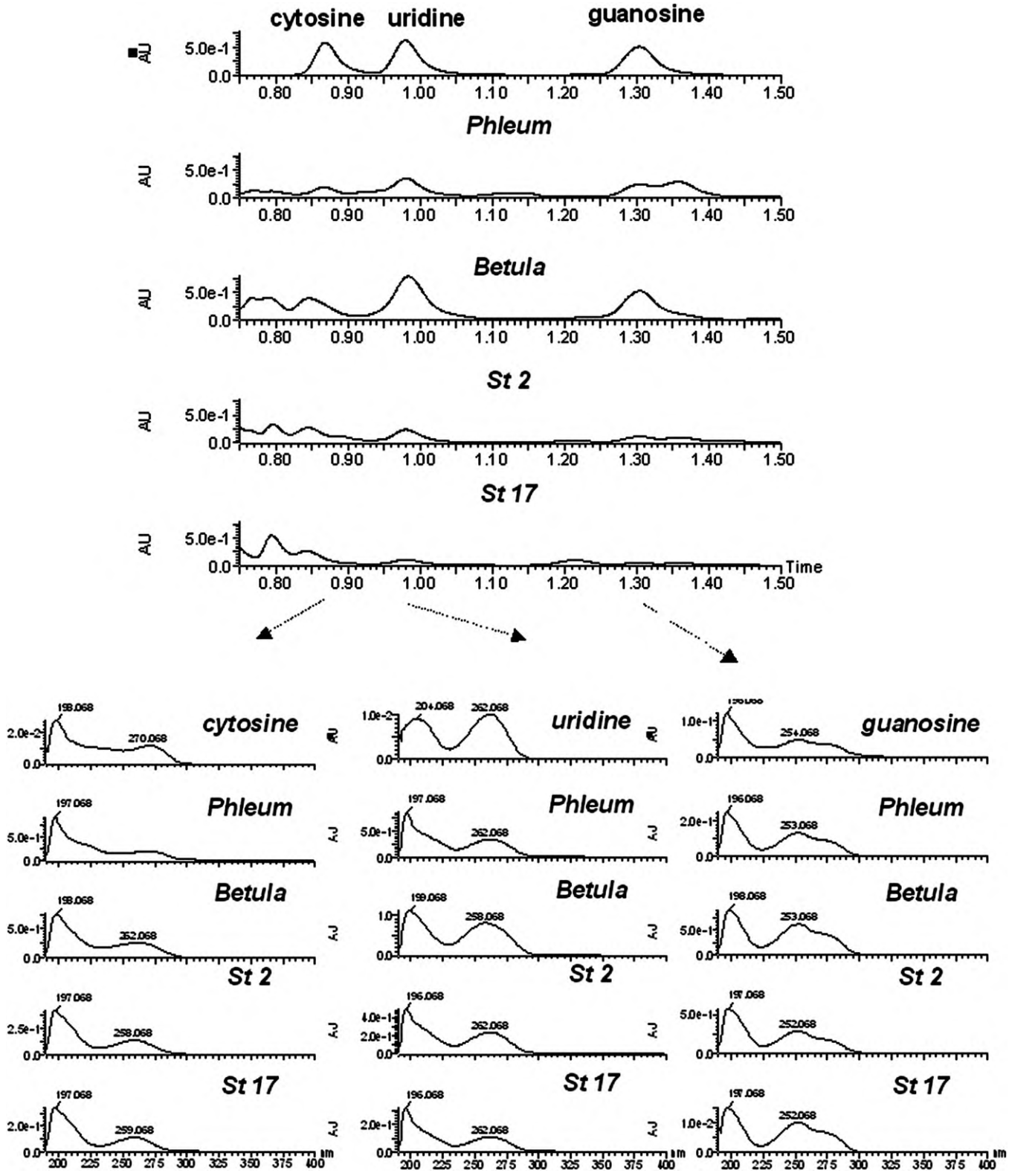

FIG E3. UPLC-UV determination applying the recent condition of cytosine, uridine, and guanosine was not sufficient for their reliable determination in pollen extract. Simultaneous determination of cytosine, uridine, and guanosine was attempted by applying the method developed for the adenosine analysis. Small or no peaks were observed at the retention time of cytosine in the pollen samples. Although peaks at the retention time of uridine were detectable in the extracts, the UV-Vis spectra of the sample peaks were only similar to the standard solution. The peak area of guanosine differed in the different pollen extracts, and sometimes coelution of constituents was observed, showing difference in the composition of pollen types. Thus more selective detection, TOF-MS, was also applied that increases the reliability of cytosine, uridine, and guanosine analysis.

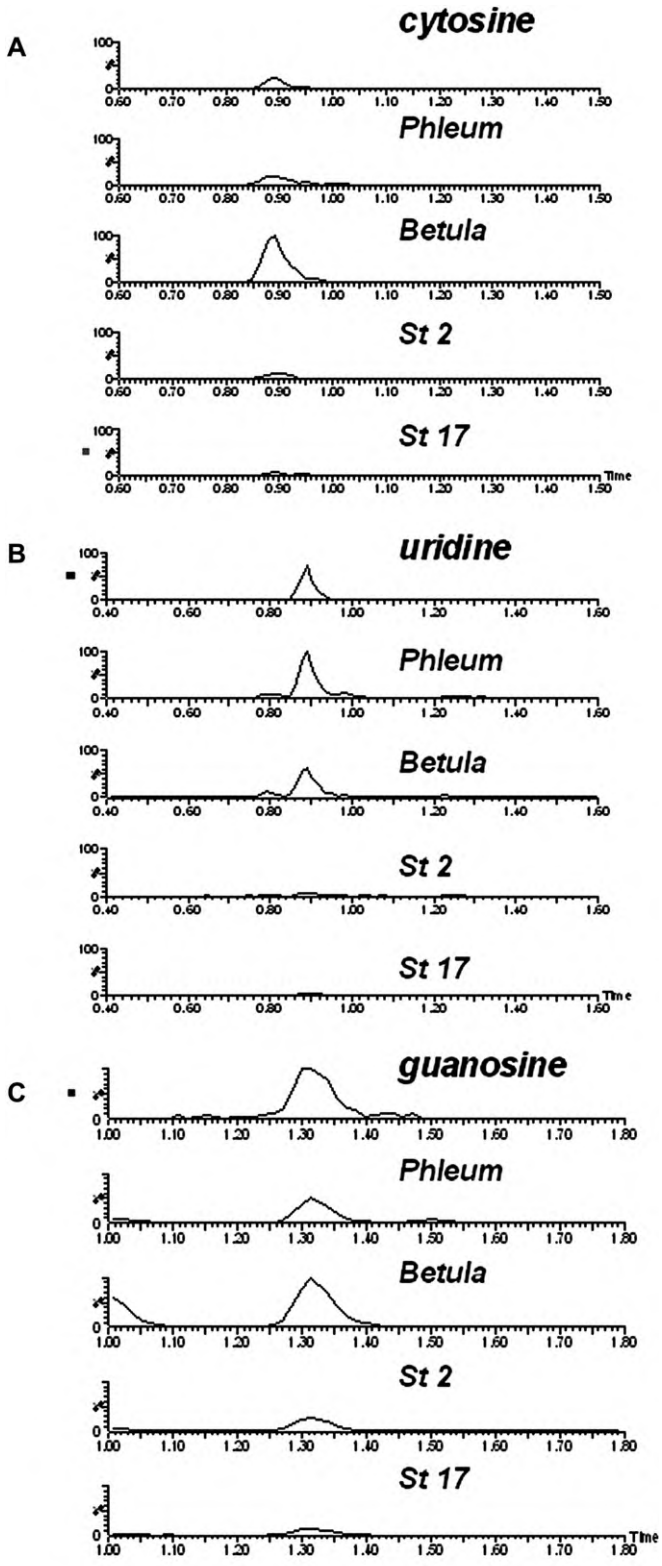

FIG E4. Detected peaks of cytosine, uridine, and guanosine with UPLC–TOF-MS. More selective detection after the liquid chromatographic separation of standard solutions and pollen extracts for the determination of cytosine, uridine, and guanosine were applied because only the ionized molecular mass of the analytes was monitored. The peak areas of cytosine, uridine, and guanosine differed, showing their concentration dependence on the pollen type.

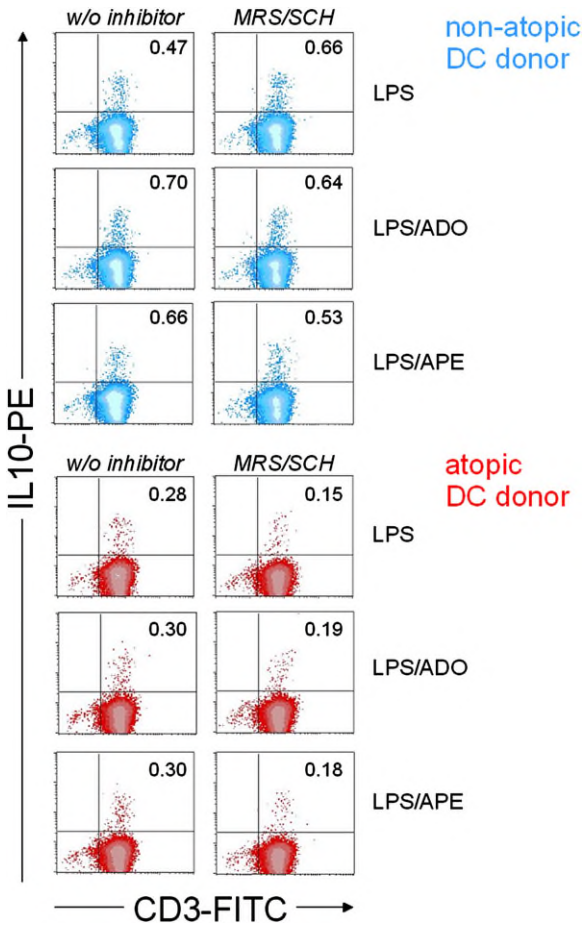

FIG E5. DCs exposed to pollen-associated adenosine *(ADO)* induce IL-10–producing T cells in allogeneic stimulation assays. DCs from a nonatopic (blue) and an atopic (red) donor were treated with indicated stimulants for 24 hours, washed, and coincubated with allogeneic naive $CD4^+$ T cells at a ratio of 1:10. At day 7, cocultures were restimulated with phorbol 12-myristate 13-acetate/ionomycin and then stained with FITC-labeled anti-human CD3, fixed and permeabilized, and stained with PE-labeled anti-human IL-10. Shown is one of 3 representative stainings (LPS, 100 ng/mL; APE, 1 mg/mL; adenosine, 10 μmol/L).

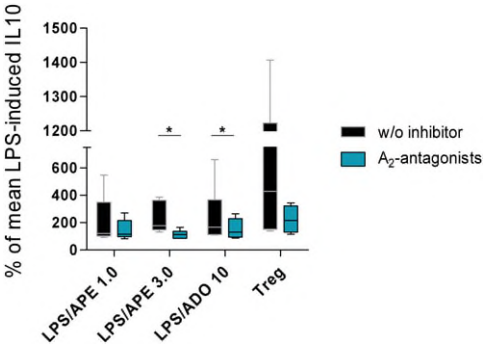

FIG E6. Induction of IL-10 in allogeneic stimulation assays depends on A_2 receptors on the DC. IL-10 levels were measured in day 7 supernatants of allogeneic T-cell stimulation assays. DCs for allogeneic T-cell stimulation were derived from nonatopic donors. They were pretreated with either vehicle *(w/o inhibitor)* or with the A_2 antagonists MRS-1706 (0.1 μmol/L) plus SCH-442416 (1 μmol/L) and then incubated with the indicated stimulants (APE1, 1 mg/mL; APE3, 3 mg/mL; ADO 10, 10 μmol/L adenosine). Data are displayed as a percentage of mean LPS-induced IL-10 production. Results from 7 independent experiments are shown. *$P < .05$, Wilcoxon signed-rank test.

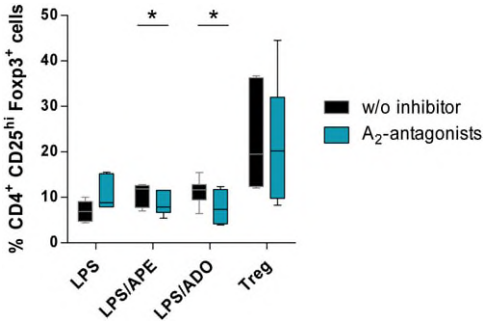

FIG E7. Induction of $CD4^+CD25^+Foxp3^+$ Treg cells depends on A_2 receptors on the DC. Day 7 cocultures were stained for extracellular CD4 and CD25, fixed and permeabilized, and stained for intracellular Foxp3. Shown are data (percentage of total) from 7 independent experiments. *$P < .05$, Wilcoxon signed-rank test.