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Artemisia pollen is the main vector for airborne endotoxin

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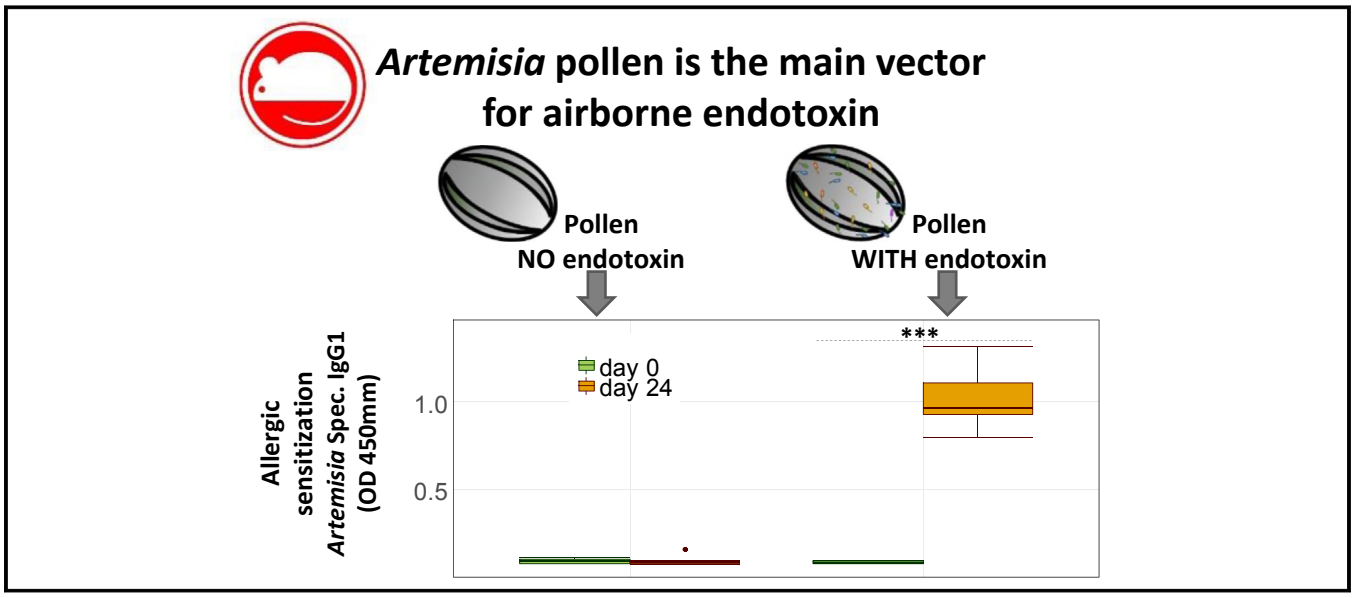
Munich, Germany, Davos, Switzerland, and Madrid, Spain

GRAPHICAL ABSTRACT



Background: Endotoxin (LPS) released from gram-negative bacteria causes strong immunologic and inflammatory effects and, when airborne, can contribute to respiratory conditions, such as allergic asthma.

Objectives: We sought to identify the source of airborne endotoxin and the effect of this endotoxin on allergic sensitization.

Methods: We determined LPS levels in outdoor air on a daily basis for 4 consecutive years in Munich (Germany) and Davos (Switzerland). Air was sampled as particulate matter (PM) greater than 10 μm (PM > 10) and PM between 2.5 and 10 μm. LPS levels were determined by using the recombinant Factor C assay.

Results: More than 60% of the annual endotoxin exposure was detected in the PM > 10 fraction, showing that bacteria do not aerosolize as independent units or aggregates but adhered to large particles. In Munich 70% of annual exposure was detected between June 12th and August 28th. Multivariate modeling showed that endotoxin levels could be explained by phenological parameters (ie, plant growth). Indeed, days with high airborne endotoxin levels correlated well with the amount of *Artemisia* pollen in the air. Pollen collected from plants across Europe (100 locations) showed that the highest levels of endotoxin were detected on *Artemisia vulgaris* (mugwort) pollen, with little on other pollen. Microbiome analysis showed that LPS concentrations on mugwort pollen were related to the presence of *Pseudomonas* species and *Pantoea* species communities. In a mouse model of allergic disease, the presence of LPS on mugwort pollen was needed for allergic sensitization.

Conclusions: The majority of airborne endotoxin stems from bacteria dispersed with pollen of only one plant: mugwort. This LPS was essential for inducing inflammation of the lung and allergic sensitization.

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Endotoxins (LPS) are macromolecules from the outer membrane of all gram-negative bacteria and are essential for their viability. These molecules consist of an O-polysaccharide, a core oligosaccharide, and lipid A. LPS is one of the most potent activators of the immune system, including in human subjects. There are many LPS types in nature, but all of them have a phosphorylated diglucosamine backbone substituted with several acyl chains and 1 or more 2-keto-3-desoxy-octonate residues.[1]

Endotoxin exposure can influence human health. Airborne endotoxin is a known immunotoxin causing inflammatory reactions of the respiratory system, with the main symptoms being fever, chest tightness, bronchospasm, pyrexia, and ultimately chronic neutrophilic airway inflammation. Excessive exposure to LPS results in a systemic inflammatory reaction, leading to multiple-organ failure, shock, and potentially death.[1] A positive association has been found between indoor LPS exposure and wheezing.[2] On long-term exposure, lung inflammation caused by LPS is a determinant for the progression of chronic respiratory diseases.[3] Inhaled endotoxin causes asthma intensification and adverse respiratory symptoms and is also a risk factor for increased asthma prevalence,[4] but not all studies show a consensus.[2] Although high LPS levels increase asthma symptoms, epidemiologic data show that low LPS exposure is related to less allergy and atopic sensitization.[5] The possibility of a protective effect of exposure to endotoxin in the development of allergy resulted in the hygiene hypothesis. Exposure to this proinflammatory agent can activate the immune system toward T_H1 responses. T_H1 responses suppress the development of IgE antibodies.

Endotoxin concentrations in the workplace and other indoor environments, where populations spend most of their time, have been extensively studied. However, there is a large gap in our knowledge about exposure to endotoxins in outdoor environments. The consequences of prolonged exposure to a constant outdoor LPS are still unknown. Although indoor LPS concentrations are affected by specific indoor factors (eg, pets in the house, the age of the building, building usage, number of occupants, and smoking),[6] they are also affected by conditions outdoors.[7] Furthermore, indoor bacterial communities show seasonality over the year,[8] which could be driven by outdoor variations.[9] Indoor LPS concentrations can be either greater or less than outside concentrations.[10,11]

The identity and source of bacteria producing airborne LPS outdoors is also not well known. Airborne bacteria are ubiquitous, but their communities vary depending on the surrounding environment and are much higher over terrestrial areas than over oceans.[12] Bacteria in outdoor air mostly originate from natural rather than anthropic sources.[13] Soil dust is thought to be one of the main natural sources of airborne bacteria, and plant leaf surfaces have been identified as one of the dominant sources of airborne bacteria during summer. Animal feces from pets could be a major contributor in urban areas during winter.[14] In addition to the natural sources of bacteria, agricultural areas, waste dumps, wastewater management installations, and other anthropic-related surfaces are also major sources of airborne endotoxin.[15-17]

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
CFU:	Colony-forming units
EU:	European units
MARS:	Multivariate adaptive regression splines
NGS:	Next-generation sequencing
PM:	Particulate matter
PM10:	Particulate matter larger than 10 μm
10>PM>2.5:	Particulate matter between 10 and 2.5 μm
rFC:	Recombinant Factor C

The main transport method of LPS is assumed to be dust particles,[18] and endotoxin was detected on the surfaces of combustion particles and other particulate matter (PM).[10,19] On the other hand, marine aerosols were identified as vectors in coastal areas.[20] We show here that the main vector of endotoxin in the studied environments (urban and rural) is none of the above but rather a biological particle: mugwort (*Artemisia vulgaris*) pollen. *Artemisia* species is the most relevant allergenic pollen in some countries, such as China, and is responsible for many asthma attacks.[21]

There is no comprehensive knowledge about endotoxin exposure outdoors. We show the results of continuous daily monitoring of LPS in 2 different environments for 4 years. Outdoor endotoxin is a critical part of the human exposome with relevant effects on health. The aim of this study was to investigate the dynamics of airborne endotoxin throughout the year in outdoor air and to identify its source. After identifying the main source of LPS, a second objective was to identify the bacterial communities responsible for environmental LPS. We then showed the health relevance of pollen with low and high LPS content in an animal model of allergic sensitization.

METHODS

Study area

Air was sampled daily on a noon-to-noon basis for 4 consecutive years by using a Chemvol high-volume cascade impactor run at 48 m^3/h and equipped with the stages for particulate matter larger than 10 μm (PM10) and particulate matter between 10 and 2.5 μm (10>PM>2.5 μm)[22] in Munich, Germany (520 m above sea level, 48.164478° latitude, and 11.593209° longitude), and Davos, Switzerland (1530 m above sea level, 46.829139° latitude, 9.856292° longitude; Fig 1, *C*). Samples from each air fraction (PM10 and 10>PM>2.5) were analyzed independently. Prewashed polyurethane filters served as impacting substrate. After collection, samples were stored at −80°C until analysis. In winter (November 1st to February 15th) sampling was weekly.

Munich has a continental climate influenced by the Alps, with an annual mean temperature of 10.7°C (between −1.8°C in January to 17.4°C in July). Total annual precipitation is 900 mm. Davos is the highest city in the Alps and has a continental subarctic climate, with an annual mean temperature of 4.6°C (between −4.3°C in January to 12.4°C in July). Total annual precipitation is 500 mm. There are also large differences in pollutant exposure between both locations (see Fig E1 in this article's Online Repository at www.jacionline.org).

Data assimilation

Endotoxin levels were measured by using the recombinant Factor C (rFC) method (Lonza, Basel, Switzerland), which is insensitive to cross-reacting 1,3-β-D-glucan from molds and pollen, according to the manufacturer's specifications.[23,24] Impacting substrates were head-over-head extracted with

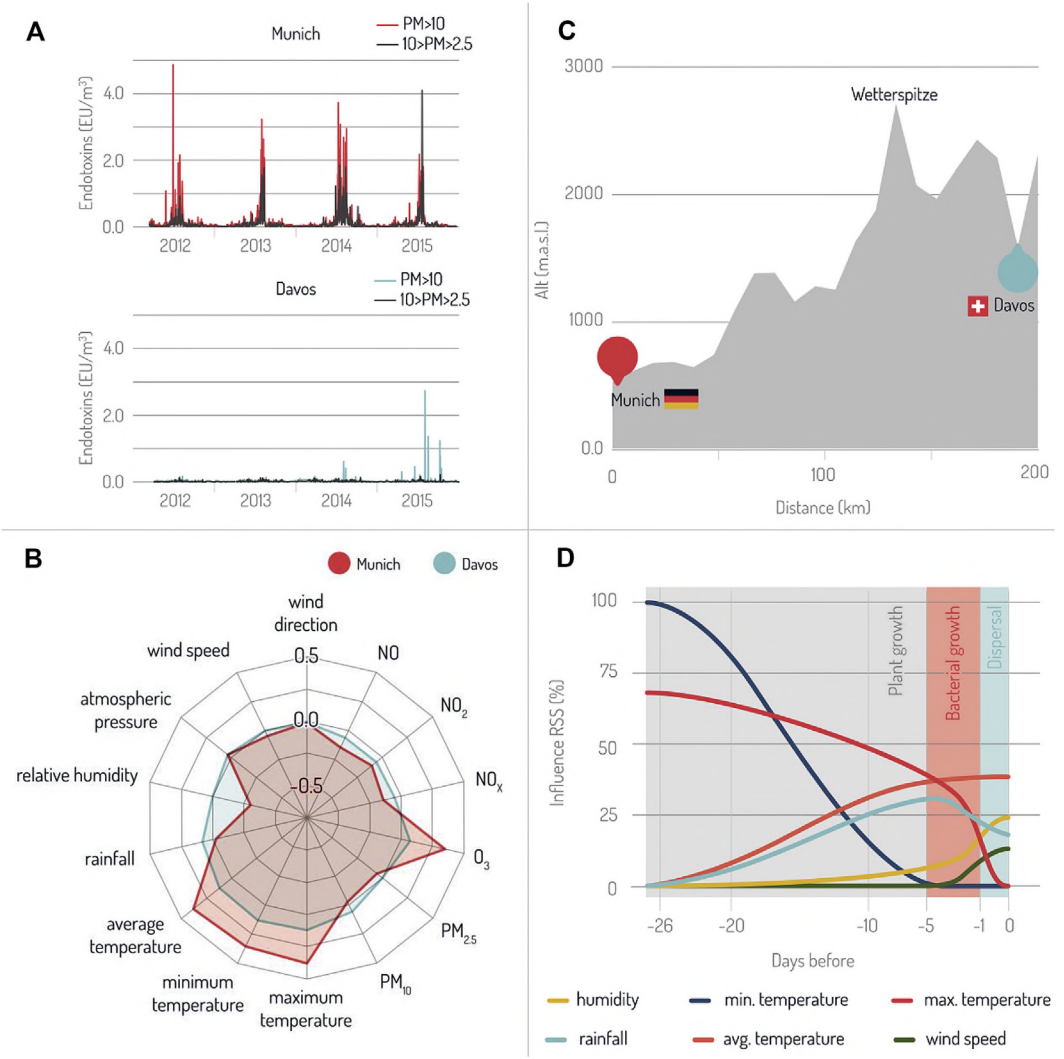

FIG 1. A, Daily endotoxin concentrations determined with the rFC method in Munich and Davos (2012-2015). Endotoxin concentrations were measured in 2 fractions of ambient air: PM>10 and 10>PM>2.5. **B,** Kiviat diagram with Pearson correlations between daily endotoxin concentrations and weather variables/aerosols in the atmosphere in Munich (Germany) and Davos (Switzerland). No correlation was at an *r* value of greater than 0.5, and the represented correlation range is between −0.5 and 0.5 (all $P > .05$). No correlation of greater than 0.5 existed with 30 different types of airborne pollen (data not shown). **C,** Altitude gradient between monitoring locations in central Europe: Davos (Switzerland) and Munich (Germany). **D,** Variables for forecasting daily endotoxin concentrations by using residual sum-of-square values at each of the 3 MARS models: model A (plant growth), model B (bacterial growth), and model C (pollen dispersal). Model A (explained 50% of variance) is based on weather conditions during day −26 to day −5 before the forecasted day. Model B (explained 59%) is based on model A plus weather conditions during the period from day −4 to day −1. Model C (explained 71%) is based on model B plus weather conditions on the forecasting day.

endotoxin-free water for 4 hours in borosilicate glass tubes. Replicates of each sample were spiked with an endotoxin standard to avoid possible enhancement or inhibition reactions of the assay. A standard calibration curve (0.005-5 European units [EU]/mL) was run with each assay. Pollen were sampled with a Hirst-type pollen trap at the endotoxin-monitoring site.[25,26] Concentrations were calculated as the amount of endotoxins in EU per cubic meter of air sampled during a day.

Meteorological parameters for Munich were obtained from the German Weather Service, and PM10, nitric oxide, nitrogen dioxide, nitrogen oxide, and ozone concentrations were from the State Office for the Environment, Station Lothstrasse, which is 2 km from the endotoxin-monitoring site. For Davos, meteorological parameters were obtained from Davos-Seewaldhorn at 1 km from the monitoring site, and pollution data were from Davos-Promenade; both are operated by Grisons Agency for Nature and Environment.

Statistical modeling

We created a multivariate adaptive regression splines (MARS) model to explain daily endotoxin concentrations at both locations based on meteorological parameters.[27,28] MARS does not consider underlying relationships between parameters and can explain linear and nonlinear relationships. Independent variables in the MARS model were as follows: relative humidity, atmospheric pressure, rainfall, wind speed, and air temperature. We did not use simple daily meteorological variables for predicting endotoxin levels, but we used the average of each meteorological variable during an optimized

agglomeration period before the prediction date. All days inside the agglomeration period are consecutive. We optimized 2 features of the agglomeration period: (1) the number of days included (we tested from 1-30) and (2) the number of days between the agglomeration period and the prediction date (we tested from 0-15, where 0 is the same day as predicted). We developed an automatic algorithm for screening the optimal agglomeration lag period. To quantify the effect of each variable on the amount of endotoxin, we used the variable importance parameter, which was calculated by applying the residual sum-of-squares criterion. The model indicates which past environmental conditions determine the endotoxin level of a certain day.

Endotoxin source identification

We did not observe a simple correlation between endotoxin and weather parameters, pollutants, or individual pollens. However, the MARS model indicated that endotoxin was closely related to phenological parameters, such as accumulated temperature and humidity. We compared daily endotoxin concentrations between 2 groups (for each pollen type): (1) during days with the presence of specific pollen and (2) during days with the absence of this pollen. A statistical test (robust *t* test) was performed with the function yuen of the R package WRS2.[29]

Endotoxin on pollen

We measured endotoxin concentrations of 40 different pollen types by analyzing 100 samples of sifted pollen collected in Germany, Sweden, Poland, and the Czech Republic between 2000 and 2016 (each sample is a mixture of plants from a specific location). After collection and sieving, pollen was stored at 4°C until endotoxin analysis. The endotoxin determination followed the same protocol as for airborne endotoxin and also used the rFC assay. Results were expressed as EU per milligram of pollen.

Bacteria cultured from pollen

Water extracts from different batches of pollen were cultured with MacConkey agar, a medium used for screening gram-negative bacteria. We quantified the amount of colony-forming units (CFU) per pollen batch, isolated all phenotypical different colonies (minimum of 5 per pollen batch), and identified them by using biochemical methods. This biochemical method consisted in the application of the 2 specific diagnostic test from Biomérieux: API 20E (for Enterobacteriaceae and other nonfastidious gram-negative bacteria) and API 20NE (for gram-negative non-Enterobacteriaceae). From each isolated bacterial colony, we also quantified endotoxin release using the rFC assay and the amount of CFU/single colony after 24 hours growing in MacConkey agar at 35°C. Results were expressed as mEU/single CFU. A sample of *Sphingomonas* species was also tested for endotoxin production.

Microbiome sequencing

High-throughput sequencing analyses were performed with purified DNA from 7 samples of pure pollen collected directly from the plants. The samples included 3 pollen types*: Betula* species, Poaceae, and *Artemisia* species, with variable endotoxin concentrations. Universal primers attached to adaptors and multiplex identifier sequences were used to amplify specific regions from 16S rRNA for bacteria. Purified amplicon libraries were sequenced in the Illumina MiSeq platform (2 × 300 reads).

DNA was extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, Calif), according to the manufacturer's instructions. Purified DNA was eluted in a final volume of 60 μL and quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Molecular Probes, Eugene, Ore) by using a QuantiFluor Fluorometer (Promega, Madison, Wis). Aliquots from extracted DNA were used for next-generation DNA sequencing analyses. The sequencing analysis always includes an empty tube with the lysis buffer as a negative control. Primers used for sequencing were as follows: Bakt_341 (forward), 5′-CCTACGGGNGGCWGCAG-3′; Bakt_805 (reverse), 5′-GACTACHVGGGTATCTAATCC-3′.

Data from next-generation sequencing (NGS) were first submitted to general checking with FastQC software (Babraham Bioinformatics Group, Babraham Institute, Cambridge, United Kingdom). Paired-ends sequences were assembled with PANDAseq,[30] removing primer sequences and filtering by quality. Global processing was carried out in the Qiime suite environment.[31] Taxonomic assignment was performed with the Greengenes database.[32] Supplementary filtering was carried out in all analyses to remove operational taxonomic units with less than 5 counts ($n \geq 5$) in any sample. Operational taxonomic units were defined at 97% sequence similarity.

Mouse sensitization protocol

Extracts of 10 mg/mL *A vulgaris* pollen with low (10 EU/mg) and high (260 EU/mg) LPS content were made in PBS and frozen as aliquots. BALB/c mice housed under specific pathogen-free conditions were sensitized intranasally with 10 μL of pollen extract in each nostril for 11 days, followed by a 9-day pause and a subsequent 3-day boost using an extended protocol described in Wimmer et al.[33] Cell counts were reported for total bronchoalveolar lavage fluid (BALF). Three control groups of animals were used: (1) those receiving the same amount of PBS; (2) those receiving the same amount of LPS as the pollen with low LPS; and (3) those receiving the same amount of LPS as the pollen with high LPS.

Twenty-four hours after the last intranasal exposure, lung function analysis was performed in intubated and mechanically ventilated animals, BALF was collected and analyzed for inflammatory cell infiltration, and specific IgG_1 levels were measured in serum samples by means of ELISA, as in Wimmer et al.[33] The ANOVA *post hoc* Tukey test was used to test differences between treated groups and the control group. The paired *t* test was used to test differences within each group at different times of the experiment.

The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (approval no. 55.2-1-54-2532-156-12).

RESULTS

Endotoxin in outdoor air

Daily airborne endotoxin concentrations in Munich and Davos for 4 consecutive years are shown in Fig 1, *A*. In preliminary experiments 0.8% ± 0.7% of daily total endotoxin was detected in the fraction of ambient air containing fine particles (2.5>PM>0.12, data not shown), and this fraction was not sampled further.

Over the 4 years, the average yearly endotoxin load (summation of the 365/366 daily endotoxin concentrations per year) in Munich in PM>10 was 49.4 ± 11.2 $\sum EU/m^3/y$ and 29.1 ± 9.2 $\sum EU/m^3/y$ in 10>PM>2.5, which was about 5 times greater than that in Davos with 10.9 ± 4.7 $\sum EU/m^3/y$ (>PM10) and 6.4 ± 2.0 $\sum EU/m^3/y$ (10>PM>2.5, both $P < .01$). Fig 1, *A*, shows a low background level of endotoxin during the year, but concentrations increase dramatically during summer to resemble an "endotoxin season," which occurs during similar periods in both locations. This endotoxin season is particularly noticeable in Munich from about June 12th to August 28th (70% ± 3% of the total endotoxin).

Endotoxin and environmental parameters

We calculated the correlation between airborne endotoxin and different weather parameters (Fig 1, *B*). No linear correlation between atmospheric endotoxin levels and any of the weather parameters examined was detected because all correlation coefficients *(r)* were less than 0.5 (all with $P > .05$). As with weather, we observed no correlations (all $r < 0.5$, $P > .05$) with other airborne components. The correlations that we observed (eg, only endotoxin in a specific range of

PM_{10} or nitrogen dioxide) could be due to the overlapping of their seasons because environmental parameters show seasonality as well.

Modeling endotoxin drivers

No correlations between "same-day conditions" and LPS in air were detected, but endotoxin could be related to conditions from preceding periods. We developed an automatic screening algorithm to look for a period in time in which weather conditions would correlate with airborne LPS by using a MARS model. MARS regression is used for relating phenomena that do not maintain a fixed linear relationship, as is the case of most natural events. For our results, we also obtained 3 critical periods for explaining airborne endotoxin, with each period affected by different parameters (Fig 1, *D*).

During the first period (which we termed the plant growth model), covering the time frame from day −26 to day −5, the R^2 value of this model was 0.50. The second model uses prediction performed by the plant growth model as a copredictor, assuming that this variable cumulates all the variability explained by the previous model. This model, which we termed the bacterial growth model, increased the explained variability from 50% to 59%. Finally, the last model increased the R^2 value from 0.59 to 0.71. We termed this the pollen dispersal model.

We interpret the complete model for endotoxin in outdoor air as coming from a natural source with a temperature-dependent phenological development, as with the plant growth model. Once the conditions for this source are reached during the week before pollination, specific conditions for microorganism growth are involved in the bacterial growth model. Finally, conditions for the dispersion of LPS during the day of detection, like wind and rain, were also significant in the dispersal model.

Airborne pollen of *Artemisia* species is the vector for LPS

Based on Fig 1, *D*, we focused on a wind-dependent plant source for endotoxin (ie, pollen). Fig 2, *A*, shows the relationship between each pollen type and endotoxin in Munich (2012-2015): daily endotoxin in the presence of a specific pollen type versus endotoxin in the absence of this pollen. The presence of 5 pollen types was related to significantly greater amounts of endotoxin in the air ($P < .05$). However, from these 5 pollen types, only one is systematically related to the highest concentrations of endotoxin (ie, *Artemisia* species; Fig 2, *B*).

Fig E2 in this article's Online Repository at www.jacionline.org shows the time series of airborne endotoxin and *Artemisia* species at both study locations simultaneously. The figure suggests a correlation between both aerosols, although not a linear correlation. For each peak of LPS, we observed at least 1 pollen/m^3 of *Artemisia* species.

Endotoxin on pollen and pollen cultures

We analyzed the amount of endotoxin from 100 pure pollen samples on 40 different pollen types that were harvested directly from different plants from different European countries. We included anemophilous and entomophiles pollen, from herbaceous plants and woody perennials and from angiosperms and gymnosperms (Fig 2, *C*). All pollen showed an endotoxin concentration of less than 20 EU/mg, except pollen for *A vulgaris*, *Lolium* species, and *Chrysanthemum leucanthemus*.

A vulgaris was the pollen type with the highest endotoxin concentrations (on average, 88.31 EU/mg) and released concentrations of LPS of up to 778 EU/mg. Interestingly, we also observed *A vulgaris* pollen with no endotoxin. *Lolium* species had a median endotoxin concentration of 12 EU/mg. In addition to *Lolium* species, we analyzed another 25 samples from the Poaceae family, which were all less than 10 EU/mg. The other pollen type with endotoxin concentrations of greater than 20 EU/mg was *C leucanthemus*. Both *A vulgaris* and *C leucanthemus* belong to the Asteraceae family, but only *A vulgaris* is anemophilous. Plants of the genus *Chrysanthemum* are entomophilous, and their pollen is unlikely to be the source of endotoxin in the air.

The amount of endotoxin and CFUs from different batches of *A vulgaris* pollen grown on gram-negative specific MacConkey agar are positively correlated (see Table E1 in this article's Online Repository at www.jacionline.org). The most frequent bacteria were *Pseudomonas luteola*, which is present in more than 95% of samples, followed by *Pantoea* species (see Fig E3 in this article's Online Repository at www.jacionline.org). *Sphingomonas* species could not be cultured in the screening medium but was identified by using NGS.

NGS

An NGS analysis corroborated the observed relationship between bacterial content and the amount of endotoxin (Fig 2, *D*). We observed a negative correlation of −1 ($P < .001$) between the amount of endotoxin and the proportion of DNA coming from plants (pollen DNA from chloroplast and mitochondria). A positive correlation (ie, $r = 1$; $P < .001$) was observed between the amount of endotoxin and the proportion of DNA coming from Proteobacteria (the group including the LPS-forming bacteria). From this group, only 3 genera showed a significant correlation ($P \leq .01$) with the amount of endotoxin: *Sphingomonas* species ($r = 0.96$), *Pantoea* species ($r = 0.96$), and *Pseudomonas* species ($r = 0.89$). This coincided with *Pseudomonas* and *Pantoea* species as the most frequent bacteria isolated from cultures; *Sphingomonas* species could not be isolated because they did not grow on the screening media.

We analyzed LPS concentrations of a pure *Sphingomonas* species sample but obtained an LPS concentration of less than the detection limit. Although endotoxin could stem from many gram-negative bacteria, our results with *Artemisia* species pollen show that most endotoxin in outdoor ambient air probably stems from *Pseudomonas* and *Pantoea* species.

Effect of pollen carrying LPS on lung allergic inflammation

In an animal model for allergic sensitization and inflammation of the lung, repetitive intranasal instillation of *A vulgaris* pollen with either low or high LPS resulted in an enhanced inflammatory cell infiltration in BALF, as characterized by eosinophils, neutrophils, lymphocytes, and macrophages, compared with PBS (Fig 3, *A*). *A vulgaris* pollen with high LPS evoked the strongest influx of eosinophils, neutrophils, and lymphocytes in BALF (Fig 3, *A*), increased lung hyperresponsiveness on methacholine challenge

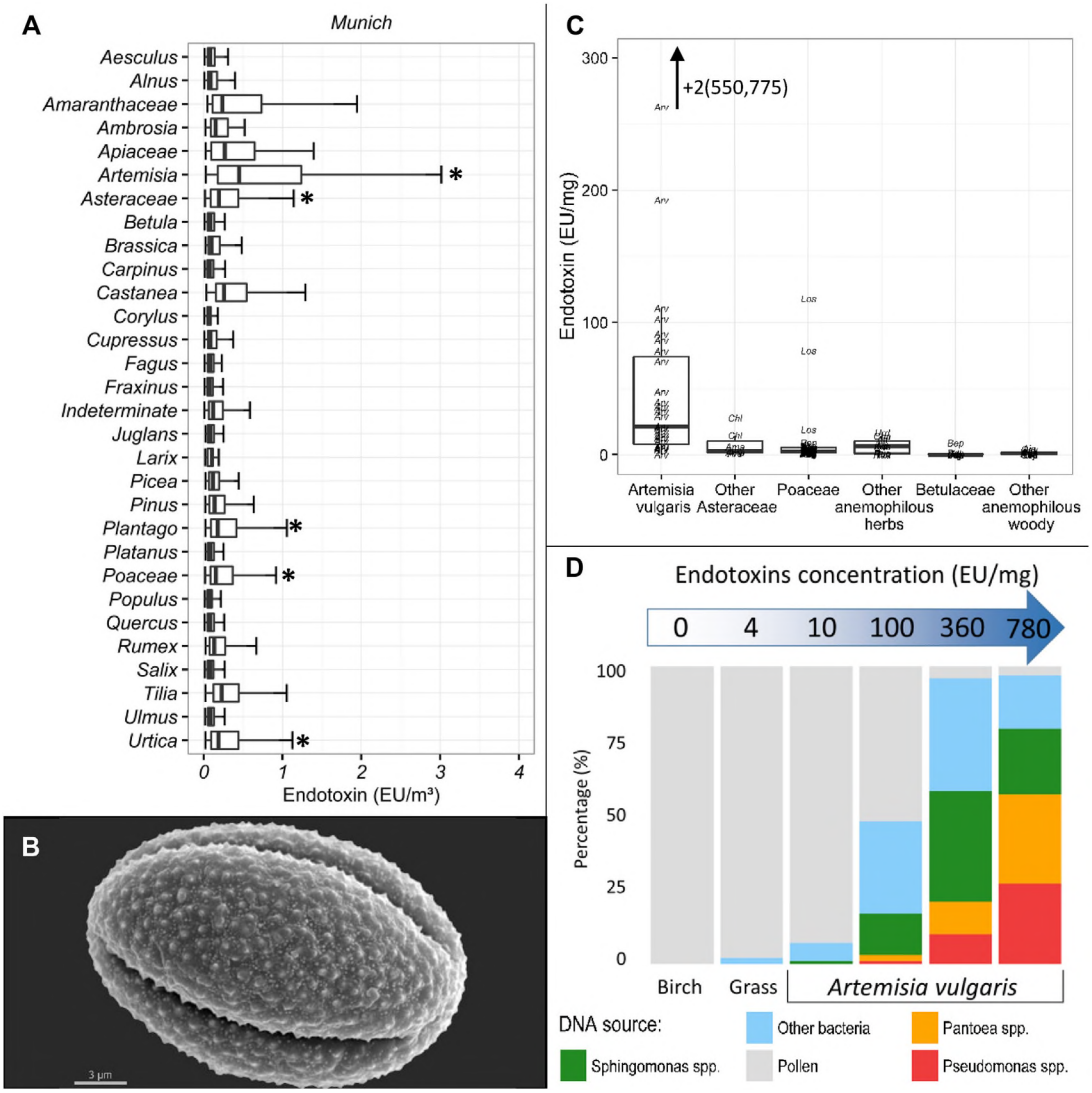

FIG 2. **A,** Box plots showing correlation between pollen type and endotoxin concentration in Munich. *Asterisks* show significant differences ($P < .05$) based on *t* tests in endotoxin levels between 2 groups: days with one pollen type and days without that pollen type (data not shown). **B,** *Artemisia* species pollen determined by using electronic microscopy. **C,** Endotoxin concentration. Group 1: *(A vulgaris [Arv]).* Group 2 (other Asteraceae): *Ambrosia artemisiifolia (Ama), Chrysanthemum leucanthemus (Chl), Iva xanthiifolia (Ivx),* and *Artemisia absinthium (Ara).* Group 3 (Poaceae): *Agrostis capillaris (Agc), Alopecurus pratensis (Alo), Anthoxanthum odoratum (Ano), Arrhenatherum elatius (Are), Bromus erectus (Bre), Cynodon dactylon (Cyd), Cynosurus cristatus (Cyc), Dactylis glomerata (Dag), Festuca pratensis (Fep), Festuca rubra (Fer), Holcus lanatus (Hol), Lolium* species *(Los), Phleum pratense (Php),* and *Poa pratensis (Pop).* Group 4 (Other anemophilous herbs)*: Atriplex littoralis (Atl), Atriplex patula (Atp), Chenopodium album (Cha), Kochia scoparia (Kos), Plantago lanceolata (Pll), Rumex acetosella (Rua), Rumex crispus (Ruc),* and *Urtica dioica (Urd).* Group 5 (Betulaceae): *Alnus glutinosa (Alg), Alnus incana (Ali), Betula pendula (Bep), Carpinus betulus (Cab),* and *Corylus avellana (Coa).* Group 6 (Other anemophilous Woody): *Acer negundo (Acn), Aesculus hippocastanum (Aeh), Ailanthus altissima (Aia), Calluna vulgaris (Cav), Cryptomeria japonica (Crj), Cupressus arizonica (Cua),* and *Cupressus sempervirens (Cus).* **D,** Results of microbiome NGS analysis from several pollen samples.

(Fig 3, *B*), and increased *Artemisia* species−specific IgG_1 levels (Fig 3, *C*). The latter 2 effects were absent in *A vulgaris* pollen with low LPS or in the control groups (see Fig E4 in this article's Online Repository at www.jacionline.org for specific IgG_1). LPS alone at concentrations similar to "high LPS-containing pollen" induced only an increase in BALF neutrophil counts, although to a smaller extent when compared with *A vulgaris* pollen with high LPS; LPS alone at both concentrations had no effect on total BALF eosinophil counts (see Fig E4).

DISCUSSION

The source of outdoor airborne LPS is not well understood but must stem from airborne gram-negative bacteria or their

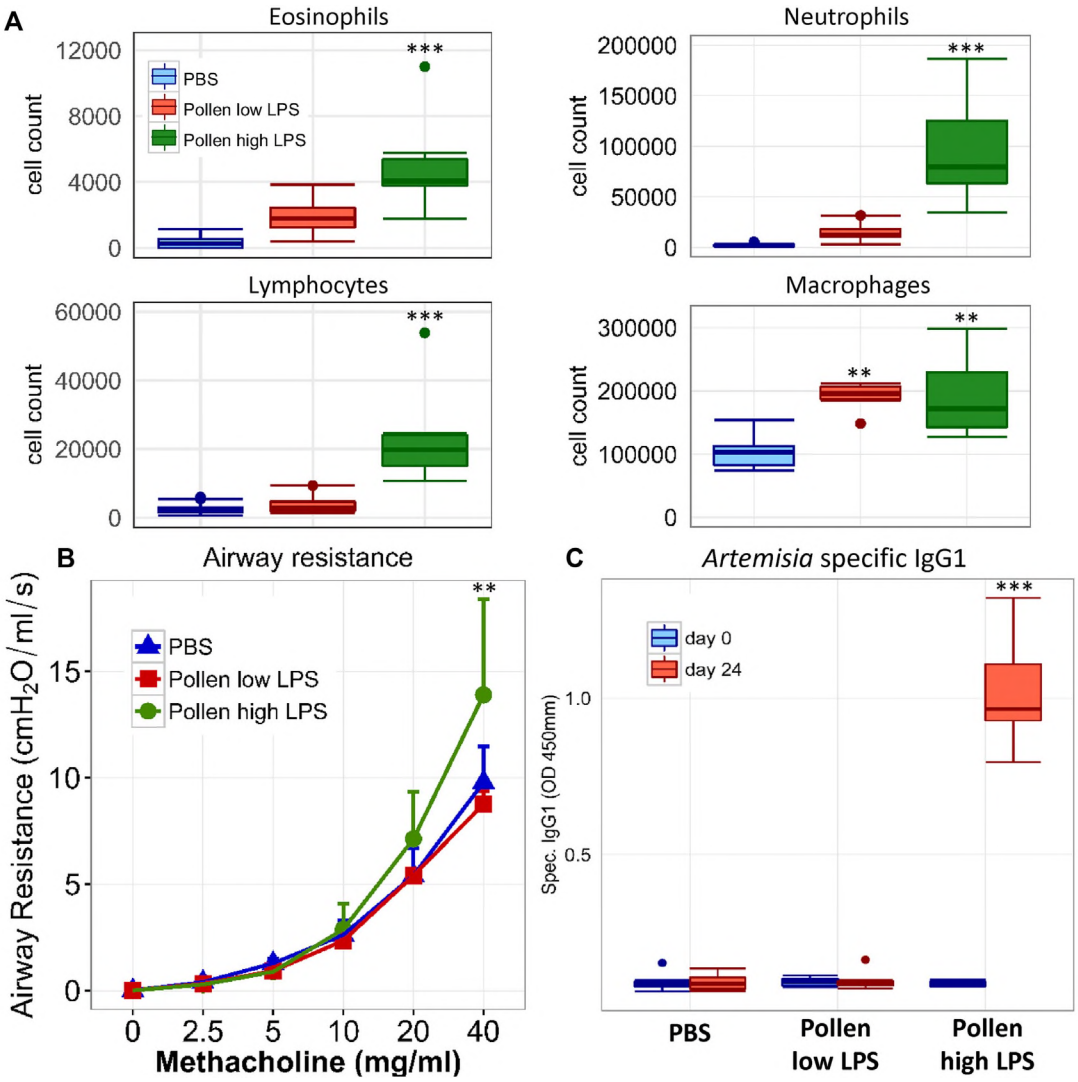

FIG 3. **A,** BALF total cell counts (see the Methods section) analyzed 24 hours after the last intranasal instillation: eosinophils, neutrophils, lymphocytes, and macrophages. Data are presented as box plots (n = 6-11): $^{**}P \leq .05$ and $^{***}P \leq .01$ versus PBS, ANOVA Tukey *post hoc* test. **B,** Lung function analysis performed 24 hours after the last intranasal instillation (n = 9 mice in the PBS group, n = 4 mice in the *Artemisia* species pollen with low LPS group, and n = 5 mice in the *Artemisia* species pollen with high LPS group). $^{**}P \leq .05$ and $^{***}P \leq .01$ versus PBS, ANOVA Tukey *post hoc* test. **C,** *Artemisia* species–specific IgG_1 levels were measured in mouse serum before (day 0, *blue plots*) and after (day 24, *red plots*) sensitization and challenge protocol (n = 11 mice in the PBS group, n = 6 mice in the *Artemisia* species pollen with low LPS group, and n = 6 mice in the *Artemisia* species pollen with high LPS group). $^{***}P \leq .01$ versus day 0, paired *t* test).

components.[34] However, conditions required for bacterial growth, such as temperature or humidity, did not correlate well with our data. Similarly, there were no significant correlations between airborne LPS and other environmental factors (ie, wind speed, wind direction, maximum or minimum temperature, rainfall, or humidity) or particle emissions or pollutants (ie, atmospheric PM10, nitrogen dioxide, nitric oxide, nitrogen oxide, ozone, or pollen), results that agree with those of other studies.[10,19]

We could predict daily airborne LPS by using environmental conditions from the preceding 26 days. For the MARS model, we developed an algorithm that tested all possible models with every weather parameter over this period. The model that produced the best prediction of daily LPS combined 3 periods, each dominated by different weather factors: plant growth factors, bacterial growth factors, and dispersion factors.

According to our model, plant growth conditions affected airborne LPS. The model coincides with other authors who also pointed out rainfall and temperature in the days before prediction[10] and in the preceding weeks.[35] Spatial modeling of endotoxin showed no correlation with any land use,[15,19] but agricultural areas, waste dumps, wastewater management installations, and other anthropic-related surfaces were postulated as the main sources for outdoor endotoxin.[15-17,36]

We then focused on pollen as a plant growth marker. Days with high LPS concentrations coincided with high concentrations of

airborne *Artemisia* species pollen. Bacteria are abundant on insect-pollinated pollen. Entomophilous pollen is sticky for better transport by insects because of sugars and lipids on their outer wall (termed pollenkitt), which can serve as nutrients for bacteria. Wind-pollinated species have a less nutrient-rich coating and less bacteria and endotoxin.[37,38] Unlike other plants belonging to the Asteraceae family, which are normally insect pollinated, *Artemisia* species (and a few other exceptions, such as *Ambrosia* species) are wind pollinated, but the pollen could, like in most of the Asteraceae, contain nutrients for bacteria.

The most frequent bacteria on pollen were *Pseudomonas* and *Pantoea* species, and *Sphingomonas*. *Pseudomonas*, and *Pantoea* species bacteria released abundant amounts of LPS per bacterium compared with other gram-negative bacteria from pollen. *Sphingomonas* species was not identified by using culture media because MacConkey agar is not a suitable culture medium for the isolation of this particular bacterium. Nevertheless, *Sphingomonas* species grown on other media did not release LPS.

Artemisia species pollens do not produce LPS *per se* but can host bacteria that release LPS. The reason why specific bacteria are particularly prone to grow on *Artemisia* species pollen is unclear. Plants have a high degree of species specificity in their microbiome.[39] Growing bacteria could limit infection by molds, and therefore the presence of bacteria could be a protection mechanism of pollen against fungal pathogens or bacteria could use pollen as a vector for dispersal.

Bacteria are known adjuvant-inducing inflammatory T-cell responses.[40] Some models show that LPS is critical for the development of allergic disease.[41] We investigated whether the presence of LPS on *Artemisia* species pollen could have a health effect, and we showed that the combination of *Artemisia* species pollen extract with high LPS is critical for the development of allergic inflammation of the lung. Furthermore, *A vulgaris* pollen with low LPS was unable to induce allergic sensitization in a mouse model. Only the combination of both factors produced allergic sensitization in animals and not each factor by itself. A limitation was that we were unable to detect sIgE against *A vulgaris*. Although LPS is thought to be protective against allergic sensitization, new findings indicate the existence of good and bad LPS,[42] which could explain our results. Although there is still a lack of knowledge about the different health effects of the different kinds of LPS, LPS from *Pantoea* species found in *Artemisia* species pollen is considered one of the strongest immune stimulants and is thought to be protective against lung cancer development.[43] Perhaps the LPS being carried by *Artemisia* species pollen could be an adjuvant for other concomitant allergens during the *Artemisia* species pollen season, such as *Ambrosia* species pollen or *Alternaria* species fungal spores.

We also observed that *Artemisia* species pollen with high LPS increased lung resistance in mice. This could be the reason why *Artemisia* species pollen is highly related to asthma attacks and why *Artemisia* species is the number one airborne allergen in countries like China.[21] Davos is well known for the healing climate, which alleviates asthma symptoms.[44] We observed a difference in exposomes between Davos and Munich, including for LPS, and think that the absence of LPS in Davos could be one of the explanations for this beneficial effect.

In conclusion, we identified pollen to be a natural vector that carries specific bacteria. Anemophilous pollen is an optimal vector for bacteria because these pollens evolved to be airborne, sometimes over long distances. This could be an essential phenomenon in many ecosystem dynamics, promoting the exchange of microbiome between plants or the dispersal of certain bacteria. Here we describe a phenomenon in which few airborne pollen of *Artemisia* species (particularly *A vulgaris*) carry the majority of yearly airborne LPS. Furthermore, in an animal model we observed that the combination of pollen and LPS represents a necessary factor for inducing lung hyperreactivity and allergic disease. Our data represent Davos and Munich, and for other geographic regions, the situation might be different.

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Key messages

- **Seventy percent of airborne endotoxin was dispersed with only 1 specific pollen type: *Artemisia* species pollen.**
- **In an animal model the endotoxin was essential for inducing allergic sensitization and lung inflammation.**
- **The microbial load of pollen could enhance its allergenic effect.**

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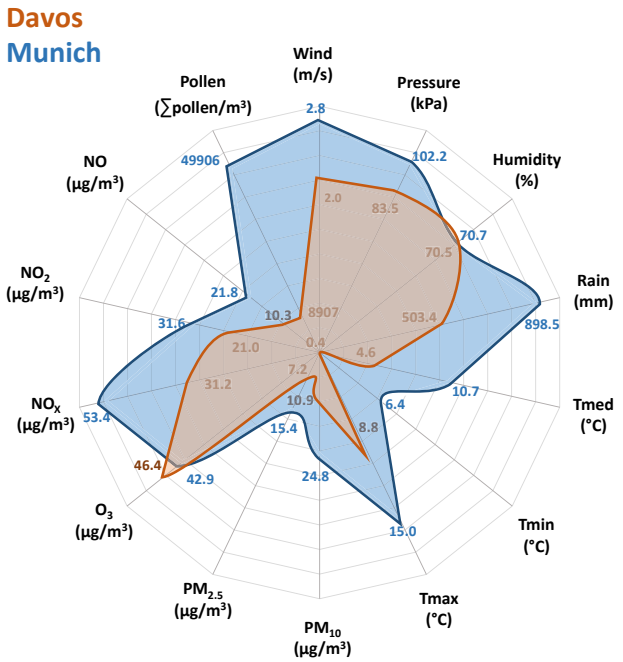

FIG E1. Kiviat diagram of the average of yearly averages of weather/aerosol daily values during the study period (2012-2015) in the atmosphere of Munich (Germany) and Davos (Switzerland).

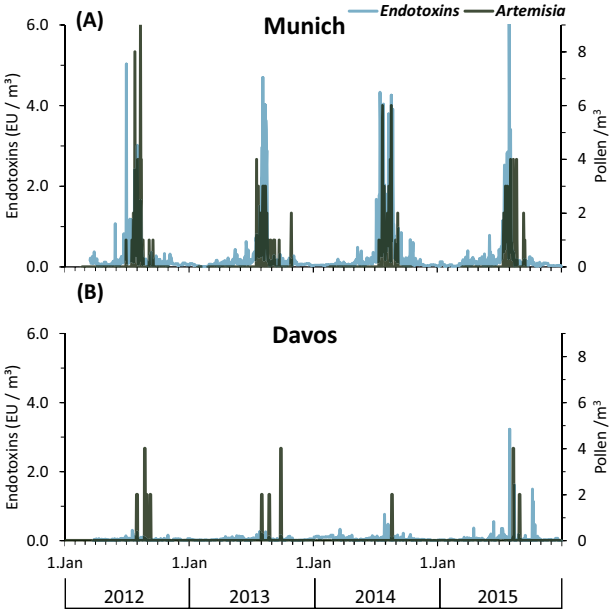

FIG E2. Time series of daily endotoxin (*blue*) or *Artemisia* species pollen (*green*) concentrations measured in Munich **(A)** and Davos **(B)** during the 4 study years.

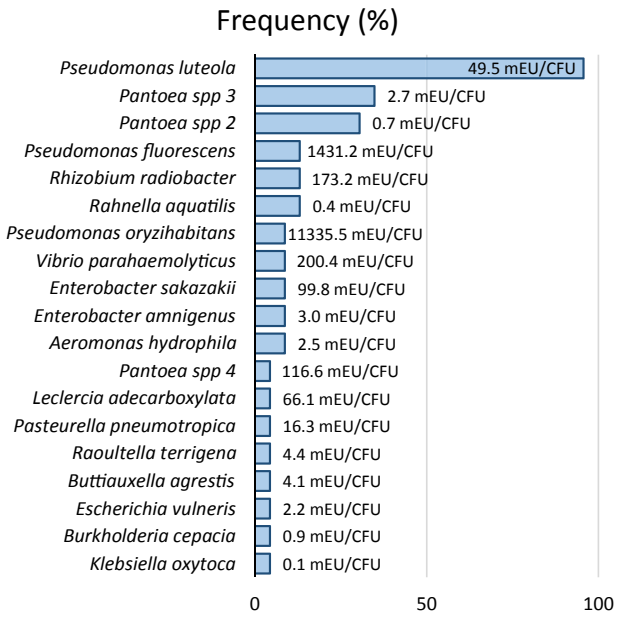

FIG E3. Frequency of bacterial taxa identified from the 23 different *A vulgaris* samples, excluding 6 pollen samples without bacterial growth. Endotoxin production capacity of each bacterium species is beside the labels (in EU/CFU).

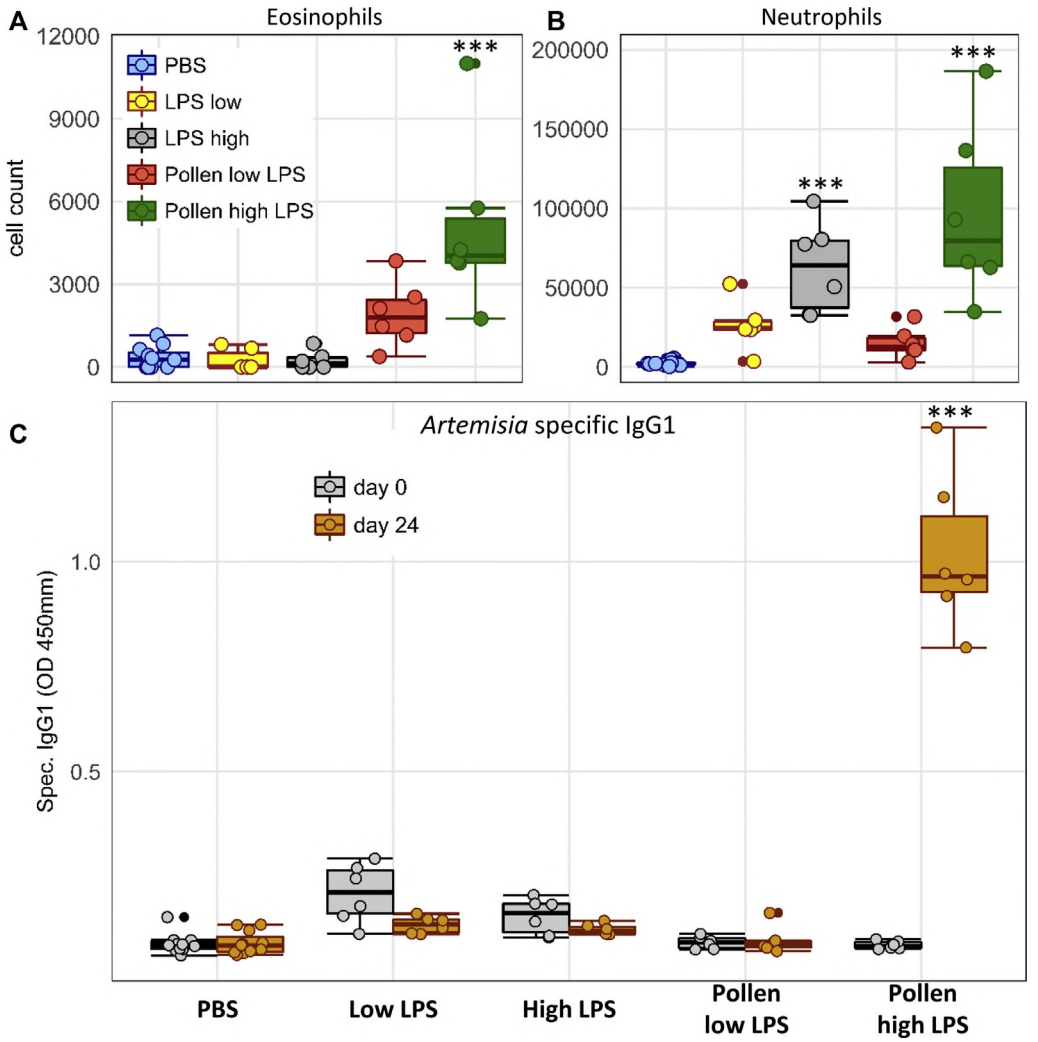

FIG E4. **A** and **B,** BALF total eosinophil counts (Fig E4, *A*) and total neutrophil counts (Fig E4, *B*) analyzed 24 hours after last intranasal instillation. Data are presented as box plots (n = 5-11): $^{**}P \leq .05$ and $^{***}P \leq .01$ versus PBS, ANOVA Tukey *post hoc* test. **C,** *Artemisia* species–specific IgG_1 levels measured in mouse serum before (day 0) and after (day 24) sensitization and challenge protocols (n = 5-11). $^{**}P \leq .05$ and $^{***}P \leq .01$ versus day 0, paired *t* test.

TABLE E1. Endotoxin concentrations and CFU from different pure pollen samples of *A vulgaris* cultivated on MacConkey agar at 35°C during 24 hours

Sample	Country	Year	EU/mg	CFU/mg	Gram-negative bacteria
1	Germany	2016	778.21	1.30E+06	B1, B5, B6
2	Germany	2016	462.77	1.28E+06	B1, B11
3	Germany	2011	262.81	4.05E+05	B1, B15, B17, B18, B19, B20
4	Sweden	2000	192.46	4.75E+03	B1, B6, B10, B13, B16
5	Poland	2016	110.74	4.16E+05	B1, B4, B5, B7
6	Germany	2016	102.32	7.67E+03	B1, B6
7	Germany	2016	91.08	1.94E+04	B1, B6, B9, B11, B17
8	Poland	2016	86.48	7.95E+05	B1, B2, B6
9	Poland	2016	78.04	1.23E+06	B1, B2, B3, B5
10	Germany	2016	70.53	6.00E+04	B1, B16, B20
11	Germany	2016	47.46	5.23E+03	B1
12	Germany	2016	39.63	3.14E+03	B1, B9
13	Poland	2015	36.12	6.93E+05	B1, B5
14	Czech Republic	2011	32.70	3.71E+03	B1
15	Poland	2015	29.04	6.13E+03	B5, B6, B15
16	Germany	2016	21.28	0.00E+00	No bacterial growth
17	Germany	2016	20.02	7.27E+02	B1, B2, B5
18	Poland	2015	17.48	3.64E+02	B1, B8, B9
19	Germany	2016	15.04	0.00E+00	No bacterial growth
20	Poland	2015	13.05	2.65E+04	B1, B6
21	Germany	2016	12.51	1.54E+03	B1, B5
22	Poland	2015	10.04	1.18E+03	B1, B11
23	Poland	2015	6.10	1.63E+04	B1, B5
24	Germany	2016	6.10	2.82E+03	B1
25	Germany	2016	5.86	0.00E+00	No bacterial growth
26	Poland	2014	5.34	6.40E+03	B1, B3, B5, B6, B7
27	Germany	2016	4.17	0.00E+00	No bacterial growth
28	Germany	2016	3.66	0.00E+00	No bacterial growth
29	Czech Republic	2015	0.00	0.00E+00	No bacterial growth

Year indicates year of harvest. Gram-negative bacteria were as follows: *B1*, *Pseudomonas luteola*; *B2*, *Pseudomonas fluorescens*; *B3*, *Pseudomonas oryzihabitans*; *B4*, *Burkholderia cepacia*; *B5*, *Pantoea* species 2; *B6*, *Pantoea* species 3; *B7*, *Enterobacter amnigenus*; *B8*, *Escherichia vulneris*; *B9*, *Rahnella aquatilis*; *B10*, *Leclercia adecarboxylata*; *B11*, *Rhizobium radiobacter*; *B13*, *Pantoea* species 4; *B15*, *Vibrio parahaemolyticus*; *B16*, *Aeromonas hydrophila*; *B17*, *Enterobacter sakazakii*; *B18*, *Pasteurella pneumotropica*; *B19*, *Raoultella terrigena*; *B20*, *Klebsiella oxytoca*; and *B21*, *Buttiauxella agrestis*.