

Arabinogalactan isolated from cowshed dust extract protects mice from allergic airway inflammation and sensitization

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Background: Extract from cowshed dust (CDE) is a source of immunomodulating substances. We have previously shown that such substances protect from experimental allergic disorders in a mouse model of asthma.

Objective: The objective of this study was to identify immunomodulatory molecules in extracts of dust from an allergy protective farming environment.

Methods: Polysaccharides were isolated from CDE and plants by chromatography and precipitation with specific reagents. Polysaccharides were then characterized by nuclear magnetic resonance spectroscopy. Subsequently, the allergy-protective potential of isolated polysaccharides was tested in a mouse model of asthma.

Results: The authors demonstrate that plant arabinogalactans are contained in CDE in high concentrations. The source of this arabinogalactan is fodder, in particular a prevalent grass species known as *Alopecurus pratensis*. Treatment of murine dendritic cells with grass arabinogalactan resulted in autocrine IL-10 production. Interestingly, these dendritic cells were not able to induce an allergic immune response. Furthermore, intranasal application of grass arabinogalactan protected mice from developing atopic sensitization, allergic airway inflammation and airway hyperreactivity in a mouse model of allergic asthma. This allergy-protective effect is specific for grass arabinogalactan because control experiments with arabinogalactan from gum arabic and larch revealed that these molecules do not show allergy-protective properties. This is likely because of structural differences because we were able to

show by nuclear magnetic resonance spectroscopy that although they are predominantly composed of arabinose and galactose, the molecules differ in structure.

Conclusions: The authors conclude that grass arabinogalactans are important immunomodulatory substances that contribute to the protection from allergic airway inflammation, airway hyperresponsiveness, and atopic sensitization in a mouse model of asthma. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Allergic airway inflammation, sensitization, immunomodulation, arabinogalactan

Cowshed dust from animal farms is a source of immune-modulating substances. Numerous epidemiologic studies have shown that frequent contact to cowsheds protects children from developing allergic diseases. Environmental exposure to microbes and their nonviable compounds has been suggested to underlie this protective effect. In fact, levels of LPS (endotoxin) and muramic acid have been found to be associated with a reduced risk of childhood asthma and allergic disease.¹⁻⁴ Unmethylated bacterial nucleic acids have also been detected in farm barn dusts.⁵ When applied at sufficiently high concentrations, all these substances resulted in protection from allergic disease in murine asthma models.⁶⁻¹⁰ Not only microbial compounds but also distinct bacterial species were isolated from cowshed dust and have been shown to protect from allergic disease in a mouse model of allergic asthma.¹¹

We and others showed previously that a low LPS concentration as found in extracts of cowshed dust (CDE) do not account for the allergy protective effect.^{12,13} Thus, substances other than those of microbial origin may be of importance.

The most abundant substances detectable in cowshed dusts are derived from plant material originating from fodder such as hay and grass. Only recently, high counts of grass pollen were found in cowsheds of traditional farms, and inhalation of these high concentrations may be associated with allergy protection.¹⁴ One class of substances derived from plants detectable in abundance in various parts of the plant organism are arabinogalactans. On the basis of structural characteristics, these polysaccharides are divided into 2 groups: type I and type II arabinogalactans. Type I arabinogalactan consists of long β -(1 $\rightarrow$ 4)-linked D-galactopyranose (D-Galp) backbones substituted with short α -linked and β -linked L-arabinofuranose (L-Araf) side chains. In contrast, type II arabinogalactan consists of a backbone of β -(1 $\rightarrow$ 3)-linked D-Galp substituted with β -(1 $\rightarrow$ 6)-linked D-Galp side chains with terminal L-Araf residues.^{15,16} Interestingly, some authors demonstrated that arabinogalactans might have immune modulating

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Abbreviations used

AG/BMDC:	Arabinogalactan treated bone marrow–derived dendritic cells
BAL:	Bronchoalveolar lavage
BMDC:	Bone marrowderived dendritic cell
CDE:	Cowshed dust extract
DC:	Dendritic cell
D-Galp:	D-galactopyranose
L-Araf:	L-arabinofuranose
NMR:	Nuclear magnetic resonance
OVA:	Ovalbumin
P1:	High-molecular-mass fraction

properties.¹⁷⁻²⁰ Moreover, it was recently reported that pollen contains large amounts of type II arabinogalactan and that these polysaccharides might induce immunomodulation.²¹

In search of relevant immunomodulating factors in CDE, we can now show that grass arabinogalactan is an essential factor contributing to cowshed dust–induced allergy protection.

METHODS**Extraction of cowshed dust, hay, and fresh grass**

Cowshed dust was collected and extracted with isotonic sodium chloride solution as previously described.¹³ Hay was collected from barns, and sorted grass species (*Alopecurus pratensis*, *Holcus lanatus*) were freshly harvested from meadows that were used to generate hay for feeding cattle. Hay and grass were frozen in liquid nitrogen and ground in a mortar under sterile conditions. Pulverized material was extracted with isotonic sodium chloride solution and subsequently dialyzed against water. All extracts were stored lyophilized. After resolubilization in isotonic sodium chloride solution, extracts were sterile-filtered (0.22 μ m) before use.

Isolation of arabinogalactan

Concentration of arabinogalactan in the high molecular mass fraction of the gel-permeation chromatography was estimated by radial diffusion in a 1% agarose gel with incorporated β -glycosyl Yariv reagent (Biosupplies Inc, Parkville, Australia). Precipitation rings of gum arabic arabinogalactan (Biosupplies Inc) at known concentrations (100–1000 μ g/mL) were used for comparison.

For isolation, 1 mg arabinogalactan from the high-molecular-mass fraction of gel permeation chromatography (see this article's Methods in the Online Repository at www.jacionline.org) was mixed with 1 mg β -glycosyl Yariv reagent.²² The resulting precipitate was washed 2 times with 0.9% NaCl solution and dissolved by adding up to 2% (wt/vol) sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) and heating to 50°C until the color changed from red to light yellow. Finally the solution was dialyzed extensively against water. Concentration of purified arabinogalactan was measured by radial diffusion and resorcin assay using gum arabic arabinogalactan as standard.

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) age 7 to 8 weeks were purchased and then adapted to the animal facility for 14 days before experiments. Animals had access to food and water *ad libitum*. All experimental procedures were approved by the animal ethics committee at Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany.

Sensitization and treatment of mice in a model of allergic airway inflammation by systemic sensitization with ovalbumin in aluminum hydroxide

Sensitization of mice was performed as described previously.¹³ Briefly, mice were sensitized by intraperitoneal injection of 20 μ g ovalbumin emulsified in 2.2 mg aluminum hydroxide (ImjectAlum; Pierce, Rockford, Ill) in a

volume of 200 μ L on days 1 and 14. On days 28 and 38, mice were challenged via the airways with 1% OVA aerosol for 20 minutes by using a PARI-Boy aerosol generator (see this article's Fig E1 in the Online Repository at www.jacionline.org). Mice were treated by intranasal application of the different substances (as specified in the result section) in 50 μ L volume. Mice were treated a total of 14 times starting on day 1 of sensitization with the last application 10 days before the final challenge. For this procedure, mice were anesthetized with a mixture of ketamine and xylazine.

Sensitization of mice in a model of allergic airway inflammation induced by bone marrow–derived dendritic cells

Bone marrow–derived dendritic cells (BMDCs) were generated as described previously.²³ On day 6, low-adherent dendritic cells (DCs) were collected and subsequently cultured with 10 μ g/mL arabinogalactan from *A pratensis* for an additional 4 days (from day 6 to 10). In some experiments, 10 μ g/mL IL-10 neutralizing antibody (clone JES52A5) or an appropriate isotype control (both from BD Bioscience, Heidelberg, Germany) was added to the cell culture medium during this period of stimulation with arabinogalactan. On day 8, medium was exchanged, and cells were pulsed with 100 μ g/mL chromatographically purified OVA for 2 days (Worthington Biochemical Corp, Lakewood, NJ). This OVA preparation contained 2 ng LPS/mg protein. On day 10, cells were used to sensitize mice intranasally. Ten days later, mice were challenged on 3 consecutive days by inhalation of OVA.

Statistical analysis

All data were analyzed by the 2-tailed Mann-Whitney test. Graph Pad Prism Software, La Jolla, Calif (Version 5) was used for analysis. Values of $P < .05$ were considered statistically significant (* $P < .05$; ** $P < .01$; *** $P < .001$). Results are presented as medians or means $\pm$ SDs as indicated.

RESULTS**Arabinogalactan isolated from CDE showed immune modulating activity *in vivo***

In search for arabinogalactans in CDE, we identified most of them in a high-molecular-mass fraction (P1) after size exclusion chromatography of CDE. Concentration of arabinogalactan in CDE as measured by sandwich ELISA was found to be 131 μ g arabinogalactan mg^{-1} CDE. This equals about 13 weight percent in CDE, indicating that the arabinogalactan concentration is significantly higher than the LPS concentration in CDE.¹³ To test the potential immune modulatory activity of arabinogalactan in the OVA mouse model, the high-molecular-mass polysaccharides were isolated by means of size exclusion chromatography. Arabinogalactan was then precipitated from the high-molecular-mass fraction by using the Yariv reagent. Subsequently mice were treated with purified arabinogalactan (1 μ g) in the established OVA mouse model, and the effect was compared with CDE (50 μ g), the high-molecular-mass fraction (P1), and the arabinogalactan-free supernatant of the high-molecular-mass fraction treated with Yariv reagent. Treatment of mice with 1 μ g arabinogalactan resulted in significant reduction of eosinophilic airway infiltration (Fig 1, A), IL-5 and IL-13 production by splenocytes (Fig 1, B), and IgE production in bronchoalveolar lavage (BAL) fluid (Fig 1, C). The reduction of these parameters was comparable to the reduction observed by treatment with CDE or the high-molecular-mass fraction P1. Although the supernatant of Yariv precipitation exhibited significantly less allergy protective activity than arabinogalactan, CDE, or P1–treated mice, it still resulted in reduced eosinophilic airway infiltration, IL-5 production of splenocytes, and lower IgE levels in BAL.

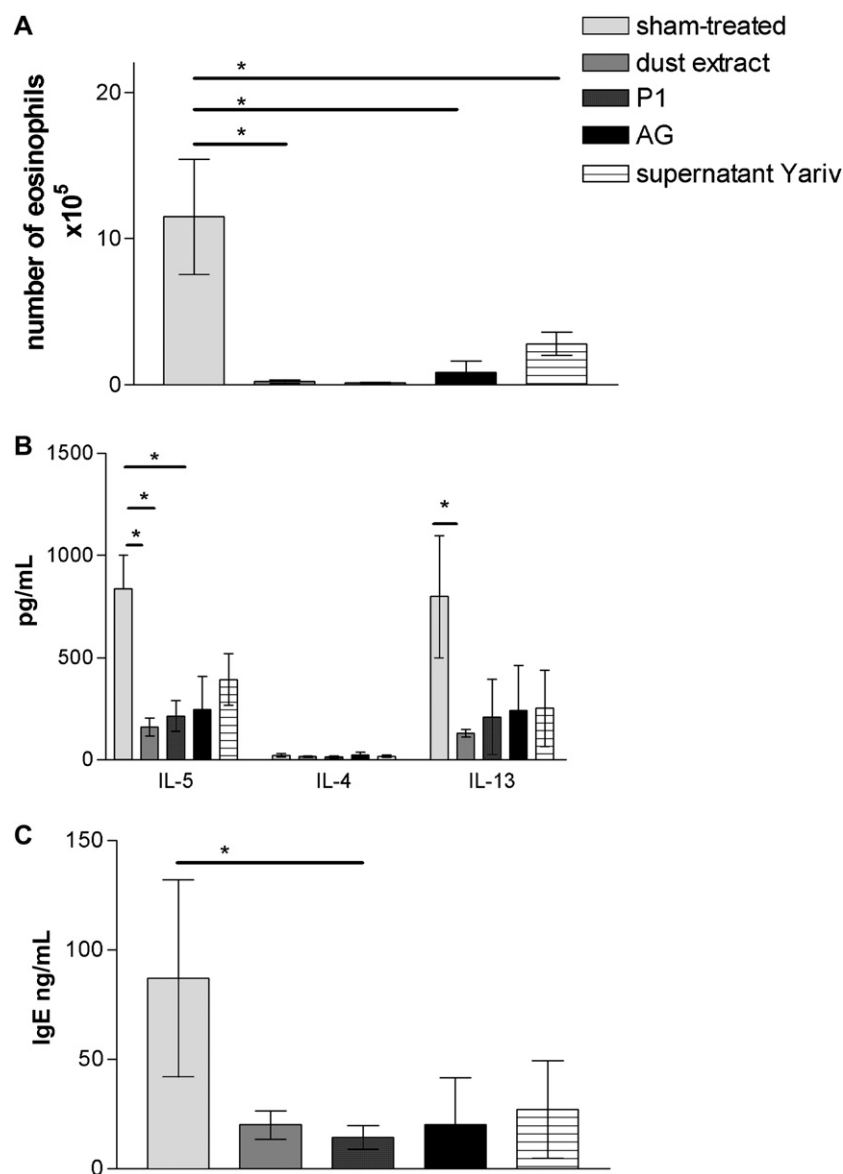


FIG 1. Mice were treated during sensitization and airway challenge with 50 μ g CDE, high-molecular-mass fraction (P1), 1 μ g arabinogalactan (AG) isolated from P1, or supernatant of the Yariv precipitation. Subsequently, mice were evaluated for eosinophilic airway inflammation (A), cytokine production (B), and IgE levels (C). Results are presented as means with SDs ($n = 4$). * $P < .05$.

Arabinogalactan from *A pratensis* protects mice from allergic airway inflammation and sensitization

In a further step, we aimed at identifying the source of arabinogalactan in CDE. Because cowshed dust contains high amounts of plant fibers and several pollen species (data not shown), we assumed that arabinogalactan isolated from CDE is derived from feeding hay. Therefore, *A pratensis*, which is a major component of hay, was collected and extracted, subjected to gel-permeation chromatography, and purified by Yariv precipitation from the high-molecular-mass fraction. To test the potential immune modulating properties of arabinogalactan purified from *A pratensis*, it was used in the OVA mouse model of allergic airway inflammation at 2 different concentrations and compared with an extract of fresh grass.

The results presented in Fig 2 indicate that mice treated either with arabinogalactan in higher amounts (5 μ g) or with whole grass extract were protected from developing allergic airway inflammation and sensitization, as demonstrated by reduced eosinophilic infiltration (Fig 2, A) and IL-5 and IL-13 production (Fig 2, B). Moreover, analysis of local antibody production revealed that IgE antibodies (Fig 2, C) were significantly reduced in mice treated with arabinogalactan from *A pratensis* compared with sham-treated control mice. Arabinogalactan from *A pratensis* did not result in an increased IgG_{2a} production (188 ± 96 ng/mL in arabinogalactan-treated mice vs 690 ± 126 ng/mL in sham-treated mice). Moreover, we did not observe increased IFN- γ production in arabinogalactan-treated mice. This finding indicates that the T_H2 response is suppressed by grass arabinogalactan without induction of a T_H1 response. To investigate

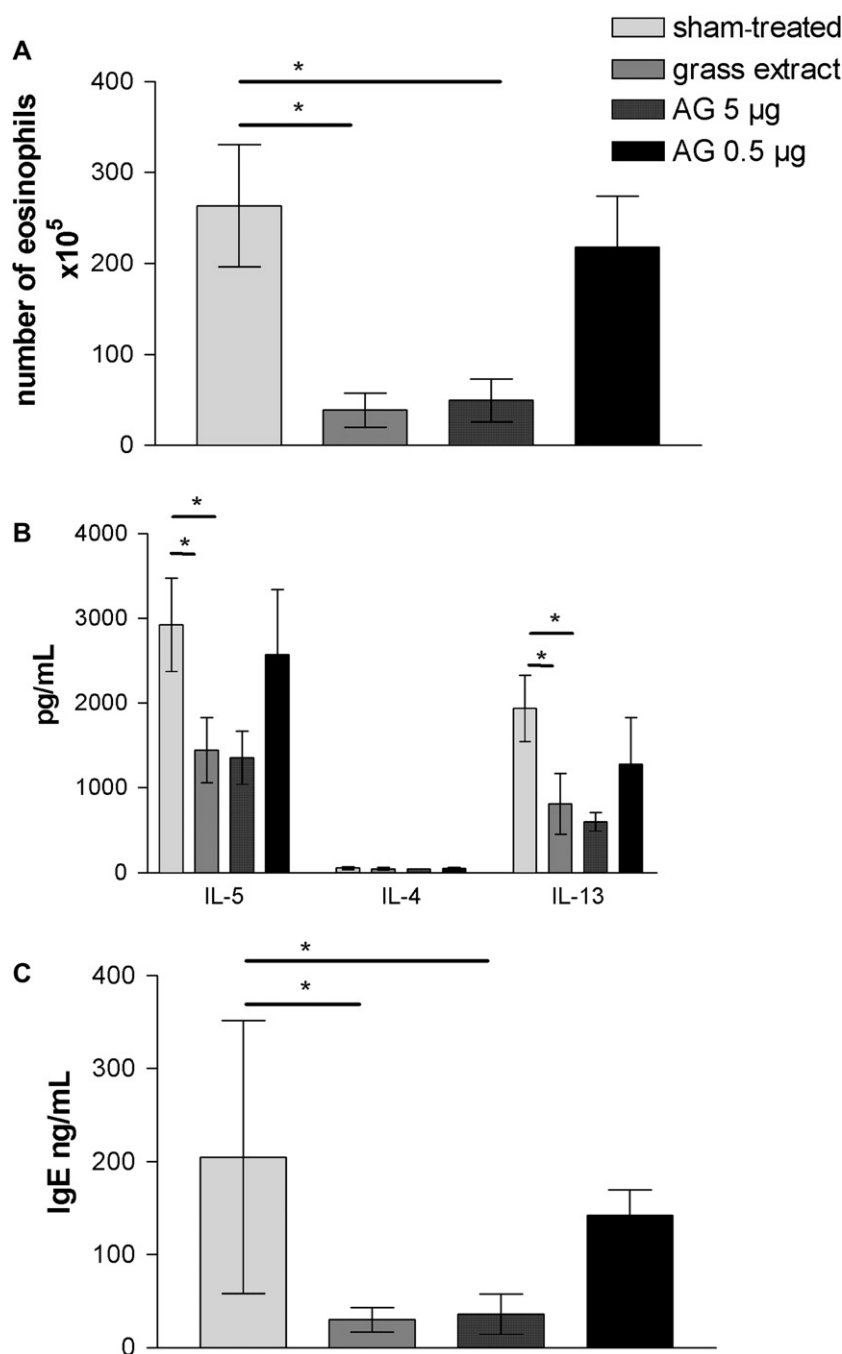


FIG 2. Mice were treated during sensitization and airway challenge with either extract from *A pratensis* or arabinogalactan (AG) isolated from this extract. Subsequently, mice were evaluated for eosinophilic airway inflammation (**A**), cytokine production (**B**), and IgE production in BAL fluid (**C**). Results are presented as means with SDs ($n = 4$). * $P < .05$.

potential contamination with LPS, its concentration in the arabinogalactan preparation was assessed in a Toll like receptor-4/MD-2/CD14 transfected cellular assay and was found to be 0.06% (wt/wt). With each application of 5 μg arabinogalactan, mice thus also received 3 ng LPS. Control experiments showed that LPS applied in such concentration resulted only in marginal, nonsignificant immunomodulatory activity in the OVA mouse model (see this article's Fig E2 in the Online Repository at www.jacionline.org).

To find out whether allergy protective activity is not restricted to arabinogalactan isolated from *A pratensis*, we further tested arabinogalactan from other sources. We observed that arabinogalactan from another grass species, *H lanatus*, showed allergy protective activity comparable to arabinogalactan from *A pratensis* (data not shown). These findings indicate that arabinogalactans from different grass species have allergy-protective properties in common.

Inhalation of arabinogalactan from grass protects from induction of airway hyperreactivity and goblet cell metaplasia in mice

To investigate whether arabinogalactan isolated from grass protects not only from sensitization and eosinophilic airway inflammation but also from induction of airway hyperreactivity, we measured lung function in spontaneously breathing orotracheally intubated mice. Data presented in Fig 3, A, show that with increasing concentration of methacholine, airway resistance in sham-treated mice rose to maximum values at $4 \text{ cm H}_2\text{O} \times \text{s} \times \text{mL}^{-1}$ with 25 mg/mL methacholine, whereas arabinogalactan-treated animals exhibited significantly lower airway resistance at all methacholine concentrations tested.

Moreover, lung histology revealed that goblet cell metaplasia and mucus production were alleviated in lungs of arabinogalactan-treated mice (Fig 3, B).

These results indicate that intranasal application of arabinogalactan in a mouse model of asthma results not only in inhibition of sensitization and eosinophilia but also in inhibition of important features of asthma like airway hyperreactivity and goblet cell metaplasia.

Arabinogalactans from gum arabic or from larch exhibit no allergy-protective activity

Next, we determined whether allergy protection properties of arabinogalactan from grasses were specific for these arabinogalactans or a general feature also found in arabinogalactans from different species. In particular, arabinogalactan from larch was found to have immunomodulatory activity in several instances.^{17,18} Therefore, arabinogalactans from gum arabic and from larch wood, respectively, were given intranasally in the OVA mouse model. Fig 4, A-C, shows that arabinogalactans from these 2 tree species were not able to reduce eosinophilia, IL-5 production of splenocytes, and IgE in BAL fluid of OVA-sensitized mice. These findings indicate that in contrast with arabinogalactan from grass, those from gum arabic or larch wood exhibited no allergy protective activity at all.

Arabinogalactan reduces the capacity of BMDCs to sensitize mice

We previously demonstrated that CDE treatment of BMDCs reduced the ability of BMDCs to induce eosinophilic airway inflammation in naive mice.²³ Moreover, we demonstrated that this effect partially depended on autocrine IL-10 production. To test whether arabinogalactan mediates this effect, we treated BMDCs with 10 μg grass arabinogalactan on day 6 and subsequently pulsed them with OVA on day 8 (AG/BMDCs).

Grass arabinogalactan induced IL-10 production by BMDCs (Fig 5, A) *in vitro*. Furthermore, mice sensitized with AG/BMDCs showed reduced airway eosinophilia (Fig 5, B) and reduced IL-4 production (Fig 5, C), indicating a diminished capability of $\text{T}_\text{H}2$ induction. However, IL-5 and IL-13 production by lymphocytes from mice sensitized with AG/BMDCs was not reduced (Fig 5, C).

These findings demonstrate that grass arabinogalactan reduced the ability of DCs to induce eosinophilia and polarization of IL-4-producing $\text{T}_\text{H}2$ lymphocytes. However, grass arabinogalactan had no impact on induction of IL-5 production by T cells, at least in the tested concentration. To test whether autocrine

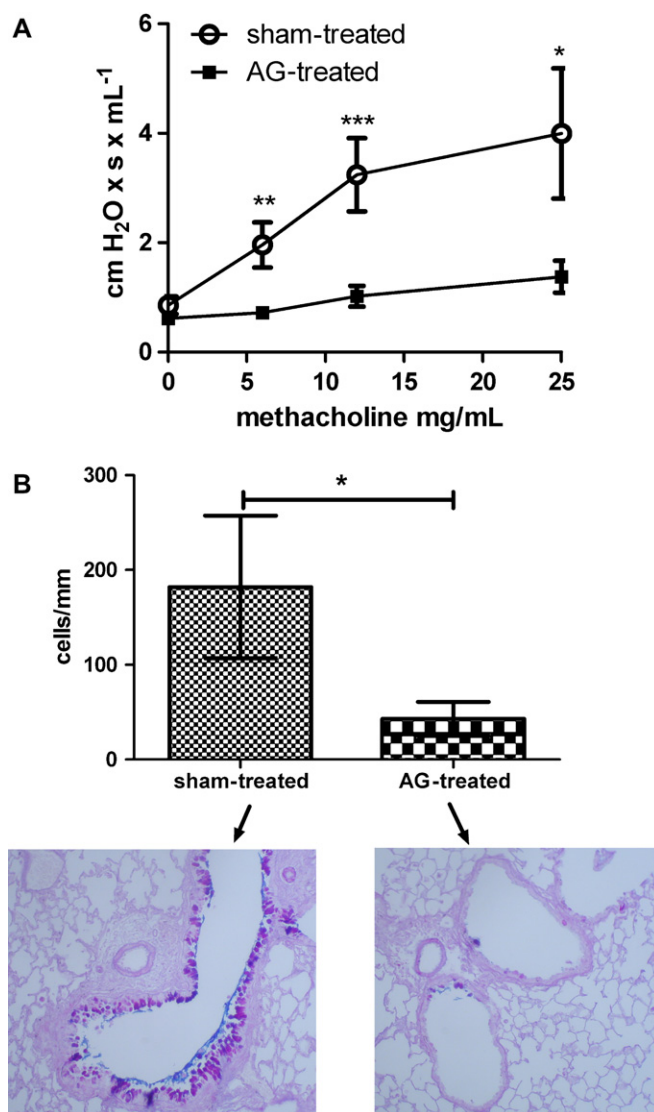


FIG 3. Mice were either sham-treated ($n = 10$) or treated by intranasal application of 5 μg arabinogalactan (AG; $n = 10$). Airway hyperreactivity was measured invasively. Means $\pm$ SEMs are shown * $P < .05$; ** $P < .01$; *** $P < .001$ (A). Subsequently, lung sections were stained by Alcian blue/periodic acid-Schiff reagent (B). Goblet cells were counted in 4 different lung slices from each mouse and given as cells per millimeter basement membrane. Means $\pm$ SDs are shown ($n = 5$).

IL-10 production is involved in this process, we used either an isotype control or an IL-10 neutralizing antibody with DCs in addition to stimulation with arabinogalactan. Subsequently, these cells were used to sensitize mice via the airways. Animals that were sensitized with arabinogalactan-treated cells in the presence of an isotype control antibody showed no change compared with arabinogalactan alone. In contrast, the sensitizing capacity of DCs that were treated with arabinogalactan in the presence of IL-10 neutralizing antibodies was partially restored. Mice sensitized with these cells showed an increase of eosinophilia up to 52% of the number of cells found in animals sensitized with OVA-primed DCs in the absence of arabinogalactan (see this article's Fig E3 in the Online Repository at www.jacionline.org).

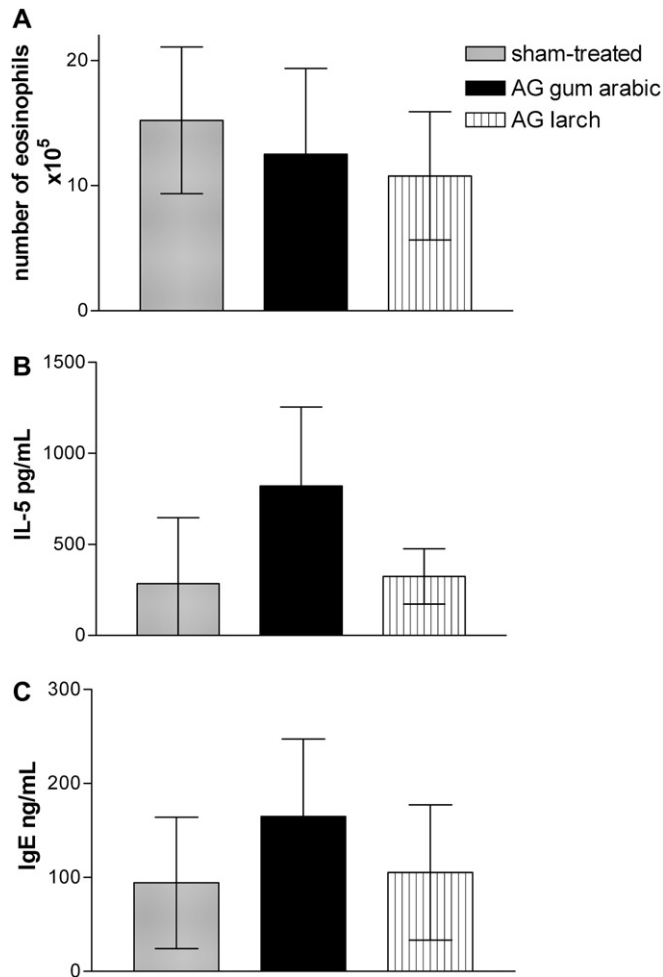


FIG 4. Mice were treated by inhalation of arabinogalactan (AG) isolated from gum arabic or larch during sensitization and airway challenge with OVA. Subsequently, mice were evaluated for eosinophilic airway inflammation (A), IL-5 production (B), and IgE production in BAL fluid (C). Results are presented as means with SDs ($n = 4$).

Analysis of the composition of monosaccharides and linkage analysis of arabinogalactan from *A pratensis*

To analyze the structure of allergy-protective arabinogalactan in more detail, we subjected arabinogalactan from *A pratensis* to monosaccharide composition and linkage analysis. As expected, isolated arabinogalactan was predominantly composed of 28% arabinose, 67% galactose, 2% rhamnose, 1% mannose, 1% xylose, and 1% glucose with traces of fucose and N-acetylglucosamine.

Linkage analysis showed a broad spectrum of different linkage types such as, most frequently, terminal arabinose and 3,6-linked galactose, indicating that grass arabinogalactan is highly branched and the saccharide chains are capped with arabinose.

In addition, results of 1D (Fig 6, A) and 2D (COSY, TOCSY) nuclear magnetic resonance (NMR) spectra of arabinogalactan of *A pratensis* showed data similar to those published for arabinogalactan isolated from the grass species *Phleum pratensis*.²¹ As shown, administration of arabinogalactan from larch does not result in allergy protection. Structural differences between the 2 molecules may contribute to these differences. We therefore

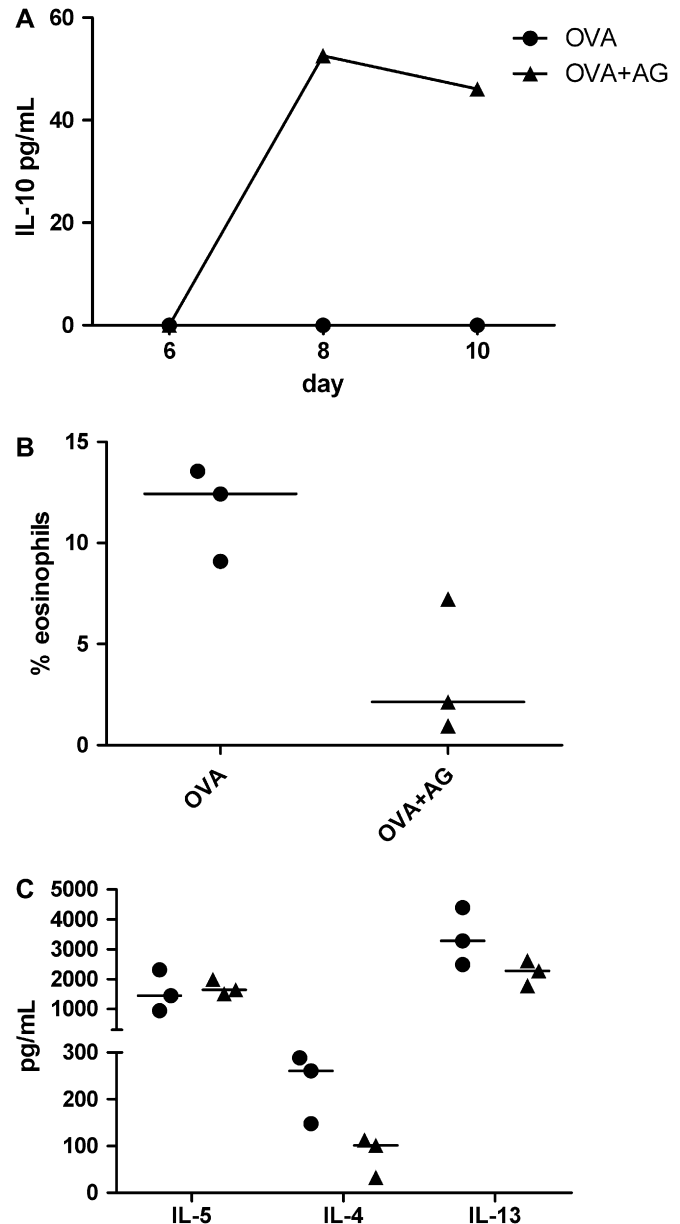


FIG 5. Analysis of activity of arabinogalactan (AG) from *A pratensis* on mouse BMDCs. A, IL-10 production of BMDCs *in vitro* after stimulation with 10 μ g/mL AG. B and C, AG-treated or untreated BMDCs were used to sensitize mice via the airways. After challenge of mice, they were evaluated for eosinophilic airway inflammation (B) and cytokine production of splenic lymphocytes (C). Median is shown.

subjected arabinogalactan from larch wood to 1D NMR (Fig 6, B). The spectrum confirmed the presence of arabinose and galactose in larch arabinogalactan. However, the data also indicate structural differences.

Cleaving of arabinose by mild oxalic acid hydrolysis resulted in arabinogalactan with reduced protective activity

We further analyzed whether immunomodulation mediated by arabinogalactan depends on sugar moieties that are located on the surface of the arabinogalactan molecule and whether the immune response is altered when terminal arabinose residues are cleaved

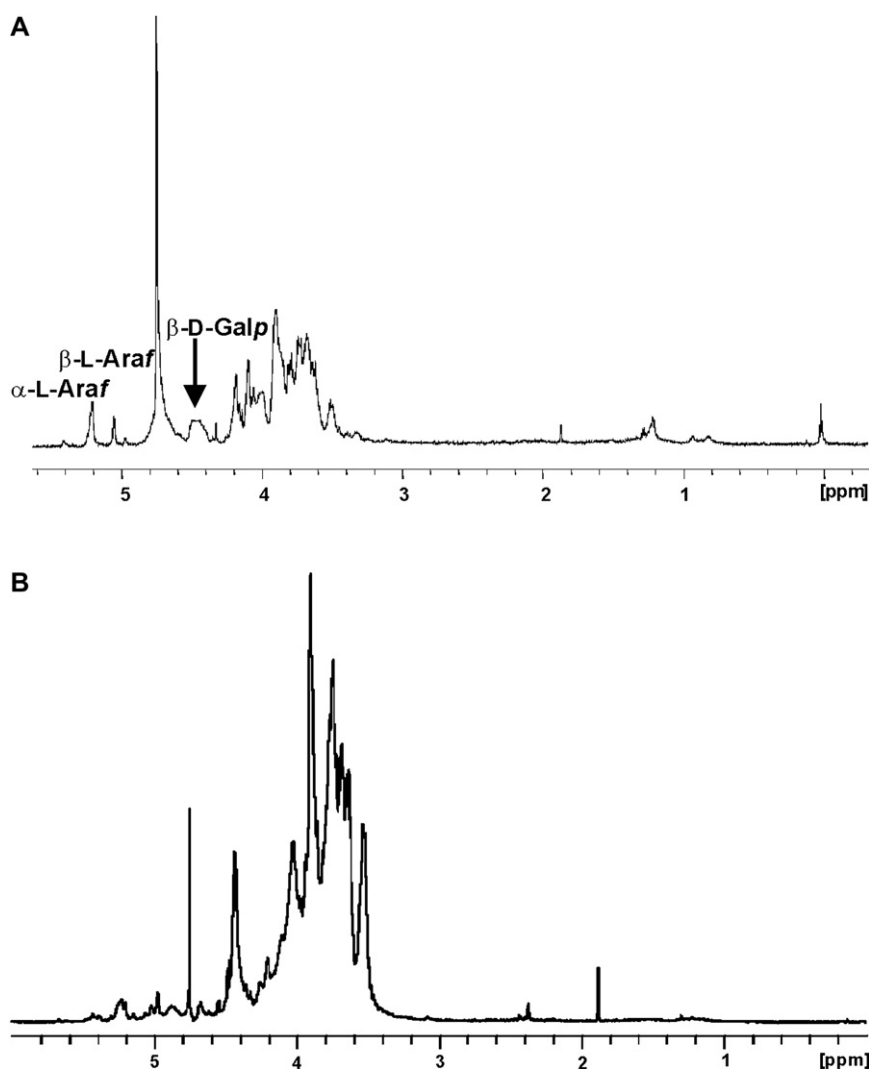


FIG 6. One dimensional hydrogen-1 NMR spectrum of AG from *A pratensis* (27°C, deuterated water) (A) and of arabinogalactan (AG) from *Larix* (B). The anomeric protons of α- L-Araf, β-L-Araf, and β-D-Galp residues are labeled.

from the macromolecule. Therefore, arabinogalactan from *A pratensis* was subjected to mild oxalic hydrolysis using 12.5 mmol/L oxalic acid at 60°C for 18 hours. Gas chromatographic analysis revealed that predominantly arabinose residues were cleaved because of this treatment (data not shown). When the residual polysaccharide with reduced content of arabinose was used to treat mice during sensitization and challenge, the ability of arabinogalactan to alleviate eosinophilic airway inflammation (Fig 7, A) and sensitization (Fig 7, C) in mice was reduced, whereas production of IL-5 was unaltered (Fig 7, B). These results indicate that arabinose residues are critically involved in arabinogalactan-mediated immunomodulation.

DISCUSSION

Cowshed dust represents an important component of the farm environment that protects children from development of allergies and asthma. We present evidence that cowshed dust contains relevant amounts of plant arabinogalactan contributing to the

immunomodulatory activity of these extracts shown *in vitro* and *in vivo*. This is the first report describing arabinogalactan as an anti-allergic substance protecting from sensitization, allergic lung inflammation, and airway hyperreactivity in a mouse model of asthma.

In the search for an underlying mechanism, we have found that stimulation of BMDCs with arabinogalactan from *A pratensis* induced IL-10 production. Furthermore, we can show that neutralization of IL-10 partially restored the sensitizing capacity of BMDCs, indicating that autocrine activity of IL-10 on tolerization of DCs may be part of the process involved in allergy protection.²³ Consequently, the question arises which cellular receptor is involved in activating IL-10 production of DCs. In this context, it is interesting to note that IL-10 was found to be produced by DCs after stimulation of lectin receptors by microbial sugar molecules—for example, on activation of mannose receptor by mycobacterial lipoarabinomannan or after ligation via cross-linking antibodies.²⁴ In addition, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin was

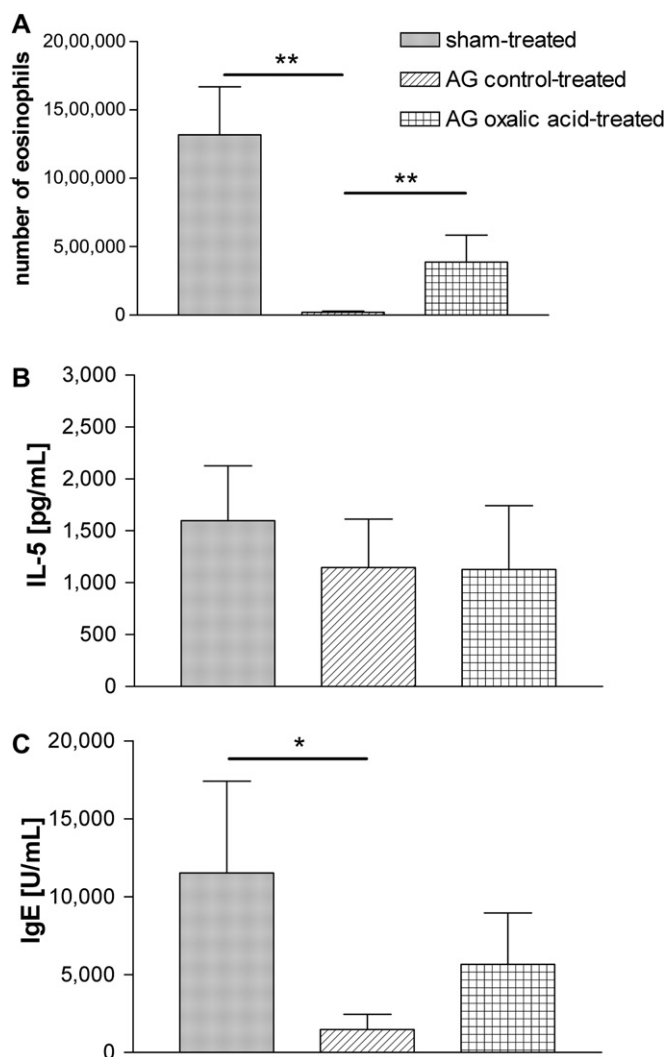


FIG 7. Analysis of activity of arabinogalactan (AG) treated with oxalic acid at 60°C, 18 hours, or control treated by omitting oxalic acid. Mice were treated by inhalation of AG during sensitization and airway challenge. Subsequently, mice were evaluated for eosinophilic airway inflammation (A), IL-5 production (B), and OVA-specific IgE in BAL fluid (C). Means with SDs are shown (n = 5). * $P < .05$; ** $P < .01$.

demonstrated to be an important regulatory receptor that is able to downregulate DC function. Activation of DC-SIGN by mycobacterial polysaccharides is supposed to be a suitable stimulus, and production of IL-10 seems to be involved.²⁵⁻²⁷ Therefore, it is tempting to speculate that arabinogalactan from grass shares structural similarities with polysaccharides derived from microbes and therefore binds to receptors that also bind the microbial counterparts. Interestingly, Chen et al²⁸ have recently shown that arabinogalactan isolated from the medical herb *Lycium barbarum* activates mouse DCs *in vitro*, confirming the existence of receptors on DCs for plant polysaccharides.

Indeed, we have found that arabinogalactan modulates the behavior of BMDCs. When cells treated *in vitro* with arabinogalactan were instilled intranasally, they had reduced T_H2 -inducing capacity measured by IL-4 production of splenocytes and reduced eosinophilia. However, in contrast with experiments in which arabinogalactan was administered intranasally, we found no reduction of IL-5 and IL-13 production. This shows that, although

nasal arabinogalactan treatment led to downregulation of splenocyte cytokine production as a systemic effect, arabinogalactan mainly acts locally, which is further confirmed by the observation that bronchial lymph nodes of those mice sensitized with arabinogalactan-treated DCs were smaller than those of untreated animals (data not shown). Therefore, we conclude that arabinogalactan reduces immune stimulation in the local compartment and downregulates DC function. However, when instilled intranasally into mice, it may activate further regulatory mechanisms. One potential mechanism also activated by arabinogalactan *in vivo* might be complement via the lectin pathway. Activation of complement by arabinogalactan was already shown for arabinogalactan from other plant species.¹⁹ Interestingly, recent publications have shown that release of C5a because of activation of complement downregulates allergic sensitization.^{29,30}

Besides arabinogalactan there are other substances involved in CDE-mediated immunomodulating activity. This can be concluded from the fact that precipitation of arabinogalactan from the high-molecular-mass fraction of CDE still resulted in residues that showed antiallergic activity. In addition, there is evidence for many microbial products included in CDE such as LPS and muramic acid (indicating the presence of bacterial peptidoglycan) that were demonstrated to be allergy-protective. However, arabinogalactan in CDE amounts to 13% of total substance, making it a relevant molecule, whereas LPS is only found in very low concentrations in CDE. Notably, neither in CDE nor in arabinogalactan was LPS found in concentrations sufficient to induce a T_H1 response, showing that LPS does not play an important role for the protective activity of these preparations. However, because it is known from the literature that stimulation of glyco-receptors can modulate TLR signaling, the possibility must be considered that these low concentrations of LPS may be involved in the arabinogalactan-mediated protective immune response.²⁹ These interactions will be subject of further investigations.

We present evidence that arabinogalactans from CDE and the common feeding grass *A pratensis* show antiallergic activity in animal model. Because hay is stored before and distributed during the foddering process in the cowsheds and because *A pratensis* is found in relevant amounts in hay, we propose that these materials constitute the source of arabinogalactan in CDE. Arabinogalactan has been shown to be a major part of grass pollen that leads to a specific antibody response in men.²¹ Interestingly, the concentration of grass pollen in the air of cowsheds correlates well with allergy protection as observed in children.¹⁴ Thus, it is tempting to speculate that arabinogalactan also has immunomodulating properties in human beings.

Because there are reports showing that pollen-associated molecules possess some adjuvant T_H2 -promoting properties, it is surprising that exposure to high concentrations of pollen is associated with protection rather than with sensitization.^{31,32} However, the published experiments were performed with birch pollen, and there might be an important difference between the allergy-protective activity of arabinogalactan from tree pollen and that from grass pollen. Notably, arabinogalactans from the tree species *Larix* spp. and that from *Acacia senegal* were not protective in our experimental setting.

This could be a result of structural differences because we demonstrated that arabinogalactan from *A pratensis* is a highly branched molecule with a β -(1→6)-linked D-Galp backbone, whereas arabinogalactan from larch contains a β -(1→3)-linked D-Galp backbone. Moreover, in contrast with arabinogalactan

from larch, which contains arabinose side chains that were (1 → 3)-linked, we and Brecker et al²¹ have shown that grass arabinogalactan also contains (1 → 5)-linked L-Araf.^{33,34} We present some evidence that arabinose side chains are important for immunomodulating activity because reducing the arabinose content of arabinogalactan by mild oxalic acid treatment resulted in diminished allergy-protective capacity. Thus, our work suggests that allergy-protective properties are a specific attribute of structural characteristics from grass arabinogalactan, and it is the first time that this could be demonstrated.

In summary, arabinogalactan as isolated from grass can be identified in extracts from cowsheds, which have earlier been demonstrated to have allergy protective effects. This arabinogalactan is found in high concentrations in protective farm environments and shows significant immunomodulating capacity in a standard mouse model for allergic asthma and on mouse DCs. Our data strongly suggest that arabinogalactan significantly contributes to the allergy-protective effect of traditional farms.

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Clinical implications: Our study suggests that arabinogalactan from grass significantly contributes to the allergy-protective effect of traditional farms. This finding may lead to the development of new allergy-protective drugs.

REFERENCES

- Adler A, Tager I, Quintero DR. Decreased prevalence of asthma among farm-reared children compared with those who are rural but not farm-reared. *J Allergy Clin Immunol* 2005;115:67-73.
- Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;347:869-77.
- Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;358:1129-33.
- van Strien RT, Engel R, Holst O, Bufe A, Eder W, Waser M, et al. Microbial exposure of rural school children, as assessed by levels of *N*-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004;113:860-7.
- Roy SR, Schiltz AM, Marotta A, Shen Y, Liu AH. Bacterial DNA in house and farm barn dust. *J Allergy Clin Immunol* 2003;112:571-8.
- Broide D, Schwarze J, Tighe H, Gifford T, Nguyen MD, Malek S, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054-62.
- Gerhold K, Blümchen K, Bock A, Seib C, Stock P, Kallinich T, et al. Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 2002;110:110-6.
- Hopfenspirger MT, Agrawal DK. Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-sensitized mice. *J Immunol* 2002;168:2516-22.
- Saito K, Yajima T, Nishimura H, Aiba K, Ishimitsu R, Matsuguchi T, et al. Soluble branched beta-(1,4)glucans from *Acetobacter* species show strong activities to induce interleukin-12 *in vitro* and inhibit T-helper 2 cellular response with immunoglobulin E production *in vivo*. *J Biol Chem* 2003;278:38571-8.
- Zuany-Amorim C, Sawicka E, Manlius C, Le Moine A, Brunet LR, Kemeny DM, et al. Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T cells. *Nat Med* 2002;8:625-9.
- Debary J, Gam H, Hanuszkiewicz A, Dickgreber N, Blümer N, von Mutius E, et al. *Acinetobacter lwoffii* and *Lactococcus lactis* strains isolated from farm cowsheds possess strong allergy-protective properties. *J Allergy Clin Immunol* 2007;119:1514-21.
- Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;196:1645-51.
- Peters M, Kauth M, Schwarze J, Körner-Rettberg C, Riedler J, Nowak D, et al. Inhalation of stable dust extract prevents allergen induced airway inflammation and hyperresponsiveness. *Thorax* 2006;61:134-9.
- Sudre B, Vacheyrou M, Braun-Fahrlander C, Normand AC, Waser M, Reboux G, et al. High levels of grass pollen inside European dairy farms: a role for the allergy-protective effects of environment? *Allergy* 2009;64:1068-73.
- Fincher GB, Stone BA, Clarke AE. Arabinogalactan-proteins: structure, biosynthesis and function. *Ann Rev Plant Physiol* 1983;34:47-70.
- Qi W, Fong C, Lampion DTA. Gum arabic glycoprotein is a twisted hairy rope. A new model based on *O*-galactosylhydroxyproline as the polysaccharide attachment site. *Plant Physiol* 1991;96:848-55.
- Currier NL, Leitenyi D, Miller SC. Effect over time of in-vivo administration of the polysaccharide arabinogalactan on immune and hemopoietic cell lineages in murine spleen and bone marrow. *Phytomedicine* 2003;10:145-53.
- Kelly GS. Larch arabinogalactan: clinical relevance of a novel immune-enhancing polysaccharide. *Altern Med Rev* 1999;4:96-103.
- Nergard CS, Kiyohara H, Reynolds JC, Thomas-Oates JE, Matsumoto T, Yamada H, et al. Structures and structure-activity relationships of three mitogenic and complement fixing pectic arabinogalactans from the malian antiulcer plants *Cochlospermum tinctorium* A. Rich and *Vernonia kotschyana* Sch. Bip. *Ex Walp. Biomacromolecules* 2006;7:71-9.
- Schepetkin IA, Faulkner CL, Nelson-Overton LK, Wiley JA, Quinn MT. Macrophage immunomodulatory activity of polysaccharides isolated from *Juniperus scopulorum*. *Int Immunopharmacol* 2005;5:1783-99.
- Brecker L, Wicklein D, Moll H, Fuchs EC, Becker WM, Petersen A. Structural and immunological properties of arabinogalactan polysaccharides from pollen of timothy grass (*Phleum pratense* L.). *Carbohydr Res* 2005;340:657-63.
- Yariv J, Lis H, Katchalski E. Precipitation of arabic acid and some seed polysaccharides by glycosylphenylazo dyes. *Biochem J* 1967;105:1c-2c.
- Gorelik L, Kauth M, Gehlhar K, Bufe A, Holst O, Peters M. Modulation of dendritic cell function by cowshed dust extract. *Innate Immun* 2008;14:345-55.
- Chieppa M, Bianchi G, Doni A, Del Prete A, Sironi M, Laskarin G, et al. Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol* 2003;171:4552-60.
- Appelmek BJ, den Dunnen J, Driessen NN, Ummels R, Pak M, Nigou J, et al. The mannose cap of mycobacterial lipaarabinomannan does not dominate the Mycobacterium-host interaction. *Cell Microbiol* 2008;10:930-44.
- Gringhuis SI, den Dunnen J, Litjens M, van het Hof B, van Kooyk Y, Geijtenbeek TBH. C-Type lectin DC-SIGN modulates toll-like receptor signalling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB. *Immunity* 2007;26:605-16.
- Koppel EA, Ludwig IS, Hernandez MS, Lowary TL, Gadikota RR, Tuzikov AB, et al. Identification of the mycobacterial carbohydrate structure that binds the C-type lectins DC-SIGN, L-SIGN and SIGNR1. *Immunobiology* 2004;209:117-27.
- Chen Z, Lu J, Srinivasan N, Kwong Huat Tan B, Ha Chan S. Polysaccharide-protein complex from *Lycium barbarum* L. is a novel stimulus of dendritic cell immunogenicity. *J Immunol* 2009;182:3503-9.
- Köhl J, Baelder R, Lewkowich IP, Pandey MK, Hawlisch H, Wang L, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest* 2006;116:783-96.
- Zhang X, Lewkowich IP, Köhl G, Clark JR, Wills-Karp M, Köhl J. A protective role for C5a in the development of allergic asthma associated with altered levels of B7-H1 and B7-DC on plasmacytoid dendritic cells. *J Immunol* 2009;182:5123-30.
- Gutermuth J, Bewersdorff M, Traidl-Hoffmann C, Ring J, Mueller MJ, Behrendt H, et al. Immunomodulatory effects of aqueous birch pollen extracts and phytoprostanes on primary immune responses *in vivo*. *J Allergy Clin Immunol* 2007;120:293-9.
- Traidl-Hoffmann C, Mariani V, Hochrein H, Karg K, Wagner H, Ring J, et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. *J Exp Med* 2005;201:627-36.
- Chandrasekaran R, Janaswamy S. Morphology of Western larch arabinogalactan. *Carbohydr Res* 2002;337:2211-22.
- Odonmazig P, Ebringerova A, Machova E, Alföldi J. Structural and molecular properties of the arabinogalactan isolated from Mongolian larchwood (*Larix dahurica* L.). *Carbohydr Res* 1994;252:21-24.

METHODS

Determination of LPS concentration in AG preparations

Ultrapure *Escherichia coli* LPS was a generous gift from Dr Ulrich Zähringer (Forschungszentrum Borstel, Borstel, Germany). CD14/MD-2/toll like receptor-4 transfected human embryonic kidney 293 cells and control cells were purchased from InvivoGen (Cayla, France). Cells (2×10^5) were cultured in 96-depot cell culture plates in Dulbecco modified Eagle medium containing 10% FCS. A standard curve was generated by stimulating cells with *E coli* LPS from 1 pg/mL to 100 ng/mL for 24 hours and subsequently measuring IL-8 production in tissue culture supernatants. This standard curve was used to determine TLR4 stimulating capacity in arabinogalactan preparations expressed as *E coli* LPS equivalent in nanograms per milliliter.

Gel-permeation chromatography

Freeze-dried extracts from CDE, hay, or grass were dissolved at 100 mg/mL in isotonic sodium chloride solution. Subsequently, insoluble particles were removed by filtration through a 0.22- μ m sterile filter. One milliliter was applied on a column Superdex 200 prep grade (16 $\times$ 60 cm) (GE Healthcare Europe GmbH, Munich, Germany) and eluted with 0.9% NaCl solution. Fractions with 1 mL volume were collected, and the eluent was monitored continuously with a 280-nm detector. The sugar content was quantified in the collected fractions by colorimetric resorcin reaction. Arabinogalactan was detected by an arabinogalactan-specific sandwich ELISA.

Arabinogalactan-specific sandwich ELISA

Arabinogalactans are large polysaccharide molecules with repetitive structure motifs on the surface. Because of this multiple epitope structure, we developed a sandwich ELISA by using the same arabinogalactan-specific mAb as catcher and detector. Briefly, ELISA plates (Nunc Maxisorb, Nunc GmbH&Co KG, Langensfeld, Germany) were coated with 3 μ g/mL purified MAC207 monoclonal catcher antibody (MAC207 cell culture supernatant was purchased from Plantprobes, Leeds, UK) for 16 hours. After blocking plates with 1% BSA, diluted samples and arabinogalactan from gum arabic (2-fold dilutions from 500 to 5 ng/mL) as standard were incubated for 1 hour. Subsequently, plates were washed, and 0.3 μ g/mL biotinylated MAC207 was used as detection antibody. The amount of bound detection antibody was measured by using streptavidin-conjugated peroxidase and specific substrate (TMB reagent; BD Biosciences, Heidelberg, Germany).

Sugar composition analysis of isolated arabinogalactans

Arabinogalactan (200 μ g) was mixed with 10 μ g arabinol as an internal standard. In addition, a standard mixture containing 10 μ g each of arabinol, arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose, *N*-acetylgalactosamine, and *N*-acetylglucosamine was prepared. The mixtures were methanolized (methanolic HCl, 80°C, 16 hours), and the products were lyophilized, trimethylsilyl-derivatized, and analyzed by combined gas chromatography-mass spectrometry.

Sugar linkage analysis of isolated arabinogalactans

Arabinogalactan (200 μ g) permethylated by using sodium hydroxide and methyl iodide with the samples shaken and sonicated over 2 hours.^{E1} Subsequently, samples were reduced with sodium borodeuteride and finally acetylated by using acetic anhydride. The partially methylated alditol acetates were then examined by gas chromatography-mass spectrometry. A standard mixture of partially methylated alditol acetates was also run under the same conditions.

NMR spectroscopy

One-dimensional and 2-dimensional NMR spectroscopy recordings were performed as described elsewhere.^{E2}

Briefly, NMR spectroscopy experiments were carried out after hydrogen to deuterium exchange of the samples utilizing 99.9% ²H₂O. Two dimensional Homonuclear proton, proton correlation (COSY) and total correlation (TOCSY) spectroscopy were recorded at 27°C with a Bruker DRX Avance 600 MHz spectrometer (operating frequencies 600.31 MHz for proton NMR, 150.96 MHz for carbon-13 NMR, and 243.01 MHz for phosphorus-31 NMR) applying standard Bruker software (Bruker GmbH, Rheinstetten, Germany). Chemical shifts were reported relative to an internal standard of acetone δ_H 2.225.

Measurement of IgG_{2a} and IgE

Levels of IgE and IgG_{2a} were assessed in supernatant of BAL fluid. Immunoglobulin concentration was measured by sandwich ELISA (optEIA ELISA sets; BD Biosciences)

In vitro cytokine production of mouse lymphocytes

Spleens were harvested 3 days after the second aerosol challenge. Restimulation of splenocytes was performed as described previously.^{E3} Briefly, single cell suspensions were prepared by mechanical disruption, and erythrocytes were lysed. Lymphocytes were cultured at a concentration of 5×10^6 /mL in complete tissue culture medium (RPMI 1640 with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, all from Biochrom, Berlin, Germany) and restimulated with 50 μ g/mL endotoxin-free OVA (Profos, Regensburg, Germany). After 48 hours of culture, supernatants were removed and stored at -80°C until analysis.

Measurement of cytokines in cell culture supernatants

Levels of IL-4, IL-5, and IL-10 were assessed by using optEIA kits, and IL-13 was measured with an ELISAsset purchased from eBioscience (San Diego, Calif) according to the manufacturer's instructions.

BAL

Two days after the last OVA challenge, lungs were lavaged via a tracheal tube with 2×1 mL PBS, and leukocytes in the lavage fluid were counted. After centrifugation, BAL fluid was frozen for further analysis. Cytospin slides of BAL cells were stained with a fast-staining procedure (HAEME-Schnellfärbung; Labor+Technik Eberhard Lehmann, Berlin, Germany) according to the manufacturer's instructions. The percentages of eosinophils, lymphocytes, and macrophages in BAL samples were determined by light microscopy. At least 300 cells per sample were differentiated by a blind investigator.

Measurement of airway responsiveness

Twenty-four hours after the last challenge, airway resistance was measured in anesthetized, orotracheally intubated, spontaneously breathing animals as described previously.^{E4} Hyperreactivity of airways was determined by provocation with methacholine aerosol for 1 minute followed by a recording period of 3 minutes at increasing concentrations (0, 6, 12, and 25 mg/mL).

REFERENCES

- E1. Ciucanu I, Kerek F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res* 1984;131:209-17.
- E2. Hanuszkiewicz A, Kaczyński Z, Lindner B, Goldmann T, Vollmer E, Debarry J, et al. Structural analysis of the capsular polysaccharide from *Acinetobacter lwof-fii* F78. *Eur J Org Chem* 2008;36:6183-8.
- E3. Peters M, Kauth M, Schwarze J, Körner-Rettberg C, Riedler J, Nowak D, et al. Inhalation of stable dust extract prevents allergen induced airway inflammation and hyperresponsiveness. *Thorax* 2006;61:134-9.
- E4. Glaab T, Mitzner W, Braun A, Ernst H, Korolewitz R, Rohlfeld JM, et al. Repetitive measurements of pulmonary mechanics to inhaled cholinergic challenge in spontaneously breathing mice. *J Appl Physiol* 2004;97:1104-11.

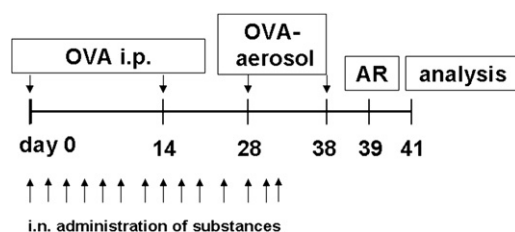


FIG E1. Depiction of treatment protocol. *OVA i.p.*, Systemic immunization with OVA adsorbed in aluminum hydroxide. *OVA-aerosol*, Challenges with 1% OVA aerosol via the airways. *AR*, Measurements of airway responsiveness. *Analysis*, Harvest of BAL cells, serum, and splenocytes for analysis. *i.n.*, Intranasal.

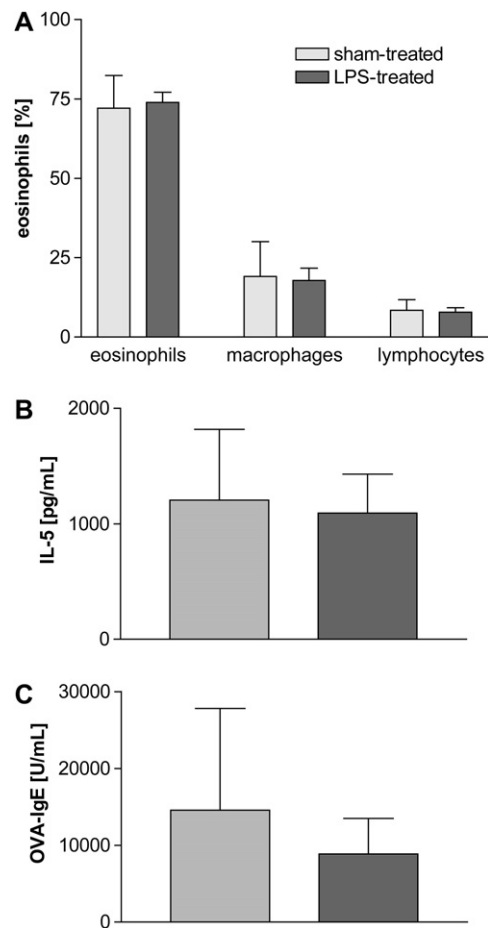


FIG E2. LPS was applied to mice intranasally during sensitization and airway challenge with ovalbumin. Subsequently mice were evaluated for eosinophilic airway inflammation (**A**), IL-5 production (**B**), and IgE production in BAL fluid (**C**). Results are presented as means with SDs calculated from 4 mice per group.

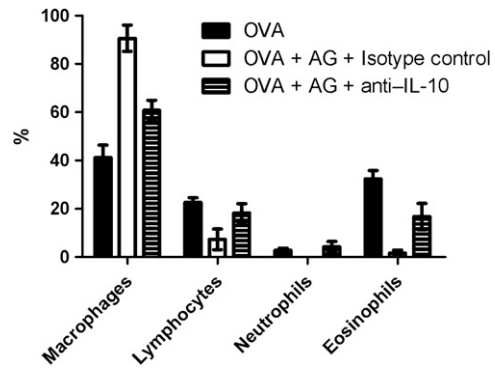


FIG E3. Neutralization of produced IL-10 partially restores sensitizing capacity of arabinogalactan (AG)-treated BMDCs. BMDCs were treated with AG, and released IL-10 was blocked by a IL-10-neutralizing antibody (OVA + AG + anti-IL-10). Control cells were treated with AG in the presence of an isotype control antibody. Subsequently, cells were used to sensitize mice via the airways. Three days after challenge with OVA aerosol, all mice were evaluated for eosinophilic airway inflammation.