Asthma-protective agents in dust from traditional farm environments

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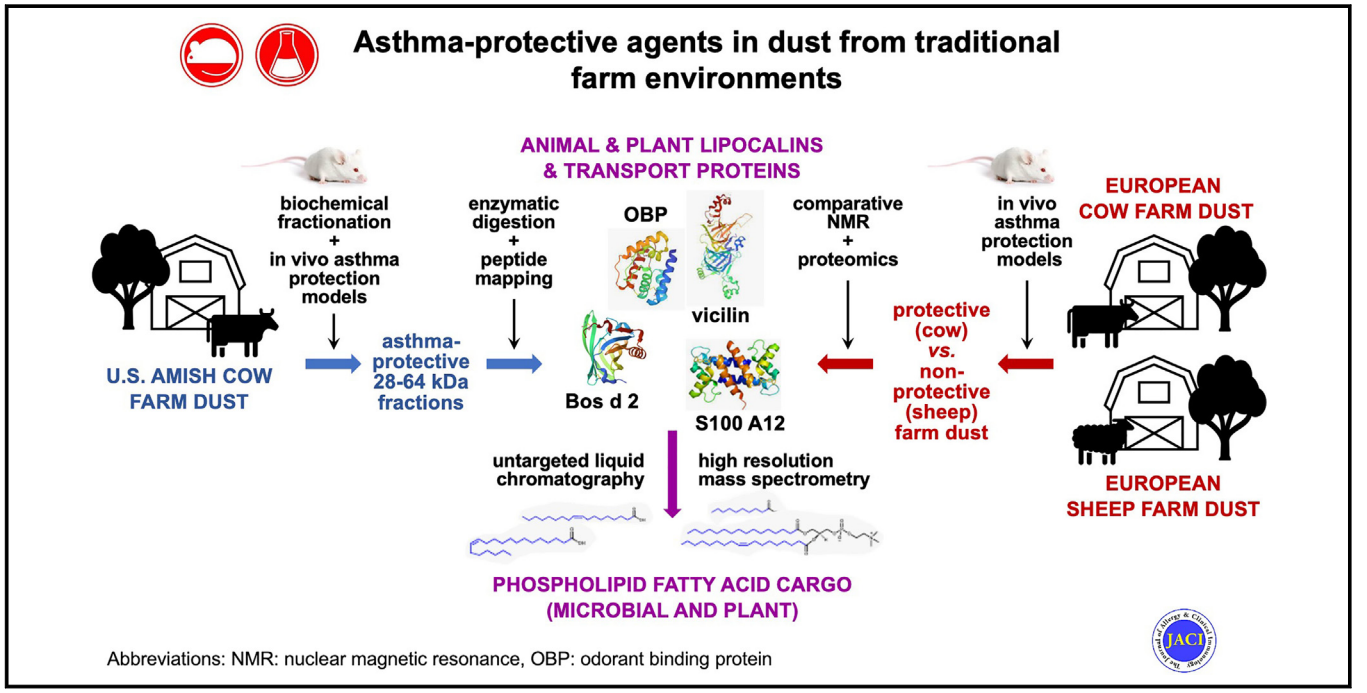
GRAPHICAL ABSTRACT



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Background: Growing up on traditional European or US Amish dairy farms in close contact with cows and hay protects children against asthma, and airway administration of extracts from dust collected from cowsheds of those farms prevents allergic asthma in mice.
Objectives: This study sought to begin identifying farm-derived asthma-protective agents.
Methods: Our work unfolded along 2 unbiased and independent but complementary discovery paths. Dust extracts (DEs) from protective and nonprotective farms (European and Amish cowsheds vs European sheep sheds) were analyzed by comparative nuclear magnetic resonance profiling and differential proteomics. Bioactivity-guided size fractionation focused on protective Amish cowshed DEs. Multiple *in vitro* and *in vivo* functional assays were used in both paths. Some of the proteins thus identified were characterized by in-solution and in-gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis enzymatic digestion/peptide mapping followed by liquid chromatography/mass spectrometry. The cargo carried by these proteins was analyzed by untargeted liquid chromatography–high-resolution mass spectrometry.
Results: Twelve carrier proteins of animal and plant origin, including the bovine lipocalins Bos d 2 and odorant binding protein, were enriched in DEs from protective European cowsheds. A potent asthma-protective fraction of Amish cowshed DEs (≈0.5% of the total carbon content of unfractionated extracts) contained 7 animal and plant proteins, including Bos d 2 and odorant binding protein loaded with fatty acid metabolites from plants, bacteria, and fungi.
Conclusions: Animals and plants from traditional farms produce proteins that transport hydrophobic microbial and plant metabolites. When delivered to mucosal surfaces, these agents might regulate airway responses. (J Allergy Clin Immunol 2023;152:610-21.)

Key words: *Asthma, farm effect, asthma protection, microbial metabolites*

Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
BLG:	β-Lactoglobulin
DE:	Dust extract
EUC:	European cowshed
EUS:	European sheep shed
LPS:	Lipopolysaccharide
NMR:	Nuclear magnetic resonance
OBP:	Odorant binding protein
OVA:	Ovalbumin
PLFA:	Phospholipid fatty acid
SDS-PAGE:	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC:	Size-exclusion chromatography
TEER:	Transepithelial electrical resistance

Asthma, the most common chronic disease of childhood, imposes a societal burden heavier than that associated with tuberculosis and HIV/AIDS combined.[1] However, despite much research, effective prevention remains unavailable. Novel preventive strategies may be based on compelling epidemiologic studies showing that children raised on traditional farms are strongly protected from asthma and allergy compared to nonfarm children.[2] This so-called farm effect observed in rural Europe was largely attributable to the child's early life contact with farm animals, especially cows, and was later corroborated by studies in US Amish and Hutterite farmers, who are similar in genetic ancestry and asthma-associated lifestyles. However, farming is traditional among the Amish but industrialized among the Hutterites. Interestingly, Amish children, who are intensely exposed to cowsheds early in life, show very low asthma and allergy risk, whereas Hutterite children, who live away from farming operations and are moderately exposed to them from school age on, have significantly higher prevalences of these diseases.[3]

These epidemiologic observations were mirrored by experimental studies. When instilled intranasally into mice sensitized with ovalbumin (OVA) or house dust mite, aqueous extracts of Amish, but not Hutterite, farm dust were sufficient to dramatically reduce cardinal phenotypes of allergic asthma: airway hyperresponsiveness (AHR), bronchoalveolar lavage (BAL) eosinophilia, and serum IgE.[3,4] Protection was also observed on airway administration of aqueous extracts of cowshed dust from German, Austrian, or Swiss dairy farms.[5,6] The similarities among findings obtained with farm dust samples collected in distinct, faraway continents underscored the fundamental asthma-protective role of traditional dairy farm exposure.

Notably, farms are heterogeneous in their protective properties. The European PARSIFAL and GABRIEL surveys showed that asthma risk was significantly reduced by a child's contact with cowsheds (especially those also containing straw and/or hay), the child's involvement in haying, and grain cultivation on an animal farm, suggesting that exposure to animal and plant materials was essential for asthma protection. In contrast, children raised on sheep farms were at higher risk of current wheeze and allergic asthma.[7,8]

All these considerations suggested that samples representative of these farm environments would be ideal sources of asthma-protective substances and encouraged us to develop and implement independent but complementary strategies to isolate and characterize these substances. On the one hand, we built on epidemiologic evidence and used nuclear magnetic resonance (NMR) profiling and differential proteomics to compare dust extracts (DEs) from asthma-protective and nonprotective farms (European and Amish cow farms vs European sheep farms). On the other hand, we used stepwise, bioactivity-guided biochemical fractionation to identify asthma-protective components in Amish cowshed DE. Remarkably, these distinct, unbiased discovery paths ultimately converged on asthma-protective DE fractions containing animal and plant transport proteins loaded with microbial and/or plant fatty acid metabolites.

METHODS

Farm dust collection

European farm dust was collected from 9 traditional European cow farms (EUC) in Southern Germany and Austria that did not house sheep. One of these farms was sampled 3 times in different years and seasons (EUC-01, EUC-01.2, and EUC-01.3). Dust was also collected from 2 European sheep-only farms (EUS) in

Austria and Southern Germany. Samples from EUC-01/-04 and EUS-01 were used for *in vivo* bioactivity assessments, NMR profiling, and proteomics analysis. To broaden the database, samples from 2 additional farms (EUC-05/-06) were included in NMR profiling, and samples from 3 additional farms (EUC-07/-09) were included in the proteomics analyses. Dust was collected from stables by sweeping settled dust from ledges, windowsills, and other, higher shelves (at least 1 m above ground) to obtain dry airborne dust. Essentially the same approach was used to collect settled Amish cowshed dust multiple times during different seasons over at least 8 years from a single Amish farm included in and representative of those described in our original study.[3] Dust batches were tested individually and exhibited strikingly consistent biochemical and functional properties over time; they were used interchangeably throughout the project. Extracts of dust collected from 5 Hutterite farms[3] were also used interchangeably. All samples were stored at −20°C before use.

Biochemical analyses of farm DEs

Biochemical analyses aimed at identifying asthma-protective substances contained in European and Amish farm DEs followed 2 unbiased, independent, but complementary discovery paths. DEs from protective and nonprotective farms (European and Amish cowsheds vs European sheep sheds) were analyzed by comparative NMR profiling and differential proteomics. Amish cowshed DEs were deconvoluted by bioactivity-guided size fractionation relying primarily on size-exclusion chromatography (SEC) and enzymatic digestion coupled with functional testing. Details about individual analytical methods are provided in the Methods section in this article's Online Repository available at www.jacionline.org.

Assessments of farm dust sample bioactivity

Multiple *in vivo* and *in vitro* functional assays were used to assess the protective properties of European and Amish farm dust samples. Some farm DE samples were screened for airway-protective activity using the lung γδ T cell induction assay, but all samples were characterized primarily by their ability to inhibit OVA-induced BAL eosinophilia in a classic mouse model of allergic asthma.[9-13] Additional readouts [lung *Il5* mRNA, AHR, and transepithelial electrical resistance (TEER)] focused on specific cardinal phenotypes of allergic asthma (airway constriction and airway epithelial barrier dysfunction, respectively). The most prominent candidate proteins identified in selected asthma-protective samples were characterized by in-solution and in-gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) enzymatic digestion/peptide mapping followed by liquid chromatography/mass spectrometry. The cargo carried by these proteins was analyzed by untargeted liquid chromatography–high-resolution mass spectrometry. Details about individual experimental procedures are provided in the Methods section in the Online Repository.

RESULTS

DEs from European cowsheds but not sheep sheds protect against experimental asthma

Different types of farms differ in their asthma-protective properties. In our European GABRIEL survey, we collected information regarding the type of farming. An effect of exposure to cows and straw on asthma was seen for both dairy farms (adjusted odds ratio = 0.51, 95% confidence interval 0.33-0.77, $P = .002$) and cattle breeding (adjusted odds ratio = 0.51, 95% confidence interval 0.35-0.76, $P < .001$), indicating that protection was conferred by cows, not dairy farming (data not shown). Moreover, exposure to cowsheds but not sheep sheds protected from allergic asthma and wheeze.[7,8] Therefore, we used a mouse model of allergic asthma (Fig 1@, *A*) to directly compare the asthma-protective activity of aqueous extracts of dust collected in cowsheds and sheep sheds. On intranasal administration, an unfractionated European cowshed DE (EUC-01) suppressed OVA-induced BAL eosinophilia as effectively as an Amish cowshed DE with demonstrated asthma-protective properties[3] ($P = .007$ and .0001, respectively; Fig 1, *B*). Autoclaved cowshed DEs, both European (EUC-01) and Amish, were also strongly protective ($P = .02$ and .001, respectively; Fig 1, *B*). When autoclaved EUC-01 DE was fractionated by filtration through membranes with different molecular weight cutoffs, the >10 kDa fraction suppressed OVA-induced BAL eosinophilia at least as effectively as the parental unfractionated EUC-01 DE ($P = .02$ for both treatments: Fig 1, *C*). Comparable results were obtained using autoclaved >10 kDa DE fractions from 3 different European cow farms (EUC-02, $P = .002$; EUC-03, $P = .008$; and EUC-04, $P = .02$: Fig 1, *D*). In contrast, OVA-induced BAL eosinophilia was unaffected on intranasal administration of an autoclaved >10 kDa DE fraction from a sheep farm (EUS-01: Fig 1, *D*). Notably, the effects of distinct farm DE preparations on BAL eosinophilia were confirmed by measuring lung *Il5* mRNA expression, another cardinal phenotype of allergic inflammation, in the same experiments (Fig 1, *E-G*). These results in an experimental asthma model were consistent with the lack of sheep shed–induced asthma protection detected in our epidemiologic studies.[7,8]

The NMR profiles of Amish and European cowshed DEs are distinct from those of sheep shed DEs

The differential ability of cowshed and sheep shed DEs to protect against human and experimental asthma prompted us to compare the biochemical characteristics of these DEs. We focused on 1 Amish cow farm DE, 2 European sheep shed DEs, and 8 European cowshed DEs from 6 different farms, one of which had been sampled 3 times in different years and seasons. One- and 2-dimensional ^{1}H and ^{13}C NMR spectra of cowshed DEs, which allow definition and quantification of coarse atomic environments within molecules (1-dimensional NMR) and confirmation of relevant resonance assignments (2-dimensional NMR),[14] identified protein- and carbohydrate-related chemical environments as major constituents of the >10 kDa fractions, explaining >95% of overall ^{1}H NMR integral (Fig 2, and see Fig E2 in the Online Repository available at www.jacionline.org). Homonuclear and heteronuclear 2-dimensional NMR spectra of cowshed DEs (which reveal correlations between protons separated by 2-4 bonds [see Fig E3, *A*, in the Online Repository] and correlations between directly connected H-C pairs [Fig E3, *B*], respectively) produced distinct cross peaks, most of which were annotated to major random coil peptide-related binding motifs and carbohydrate molecules (Fig E3).[15,16] Contributions of glycoproteins with chemical environments congruent to proteins and carbohydrates could not be excluded but were likely limited

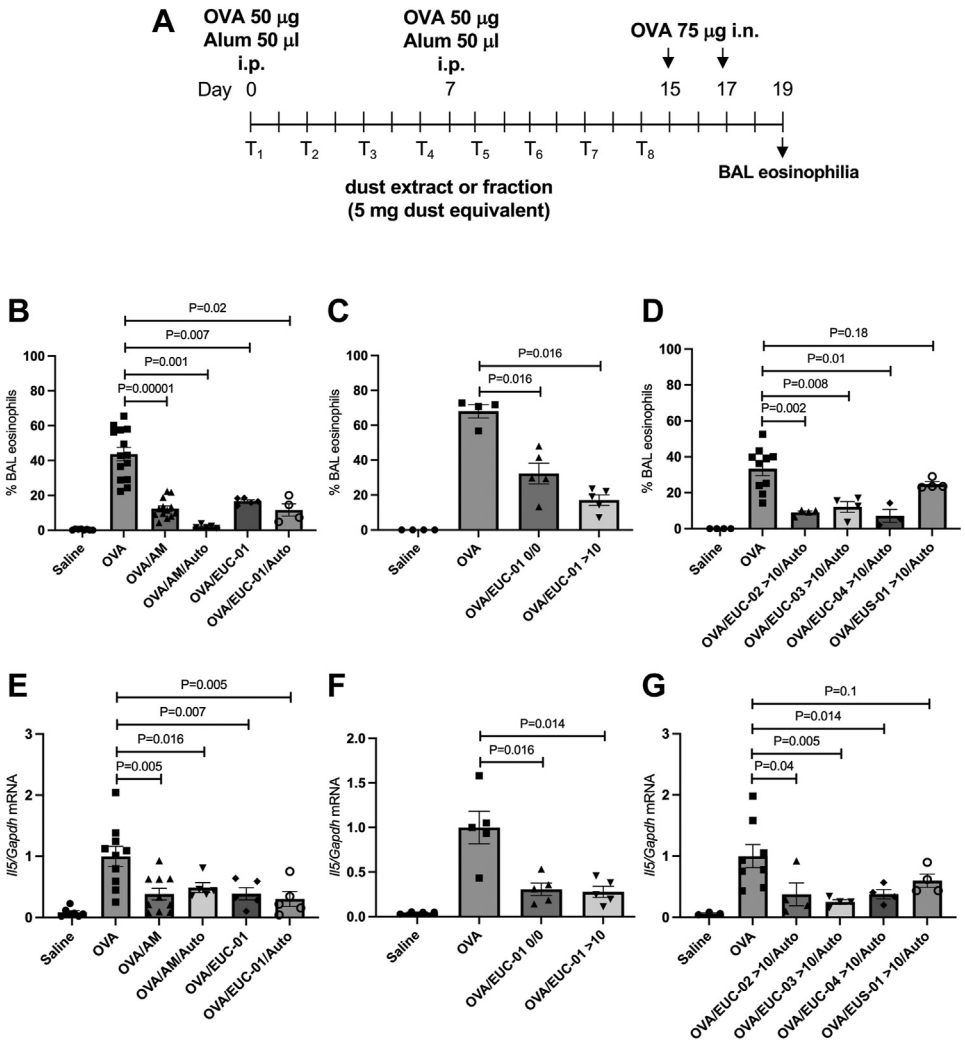

FIG 1. DEs from European cowsheds but not sheep sheds protect against experimental asthma. **A,** Balb/c mice were immunized with OVA + alum intraperitoneally (i.p.) (days 0 and 7) and challenged intranasally (i.n.) with OVA (days 15 and 17). DEs (5 mg of dust equivalent) were administered i.n. 8 times. BAL eosinophilia was assessed at day 19. **B,** BAL eosinophilia in mice receiving OVA (n = 14), OVA + Amish (AM) cowshed DE (n = 13), or OVA + EUC-01 DE (n = 5). Autoclaved (auto) samples were also tested (n = 5 mice per group for OVA + AM cowshed DE and n = 4 for OVA + EUC-01 DE). **C,** BAL eosinophilia in mice receiving OVA (n = 4), OVA + unfractionated (0/0) EUC-01 DE (n = 5), or OVA + EUC-01 DE fraction >10 kDa (n = 5). **D,** BAL eosinophilia in mice receiving OVA (n = 10), OVA + autoclaved >10 kDa DE fractions EUC-02 (n = 4), EUC-03 (n = 3), or EUC-04 (n = 3), or OVA + EUS-01 (n = 4). Data (means ± SEMs) are from 3 independent experiments. **E-G,** Lung *Il5* mRNA levels in all samples tested in *(B-D).* Data were analyzed by unpaired 2-tailed *t* test *(B, E-G)* or Wilcoxon 2-sample test (*C* and *D*) after assessing normality of value distributions by Shapiro-Wilk test.

because integral ratios of carbohydrates and proteins varied considerably in individual cowshed DE (Fig 2 and Fig E2). Likewise, lipopolysaccharides (LPS) were not dominant in cowshed DEs because the ^{1}H NMR resonance related to polymethylene $(CH_2)_n$ units at $\delta_H \sim 1.2$ ppm was virtually absent (Fig 2). Small molecules did not produce relevant sharp ^{1}H NMR resonances in cowshed DEs, but those could have experienced line broadening because of tight association with proteins.[17] In general, nonprotective sheep shed DEs showed consistently higher relative proportions of carbohydrates and lower proportions of peptides/proteins than cowshed DEs (Fig 3, and see Fig E4 in the Online Repository).

^{1}H NMR spectra of cowshed DEs > 10 kDa from the 1 Amish and the 4 European dairy farms included in Fig 1 showed analogous curvature of protein-related resonances indicative of analogous structural features, with some variance in relative abundance (Fig 2). Including cowshed DEs from 2 additional European dairy farms did not change our results (Fig 2 and Fig E2). Because β-lactoglobulin (BLG) was recently reported to have allergenic or tolerogenic properties depending on the presence (holo-BLG) or absence (apo-BLG) of cargo (zinc),[18] we also compared the NMR spectra of cowshed DEs to the spectra of holo- and apo-BLG as well as autoclaved and nonautoclaved commercially available BLG.[19] These comparisons pointed to a

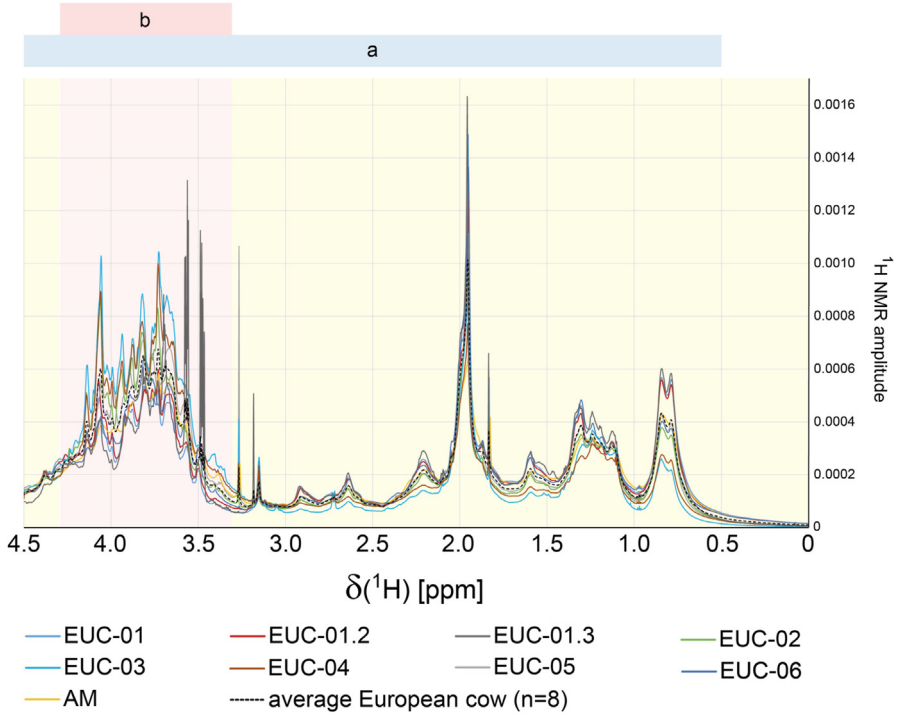

FIG 2. NMR spectra of European and Amish cowshed DEs reveal presence of random coil peptides/proteins and carbohydrates. Area-normalized ^{1}H NMR spectra (800 MHz, D_2O) show section of aliphatic peptide side chains, OCH, and CCH units of carbohydrates. Eight European cowshed DEs (EUC-01, EUC-01.2, EUC-01.3, EUC-02/06) and 1 Amish (AM) cowshed DE *(yellow line)* are shown individually. Average ^{1}H NMR spectrum of 8 European cowshed DEs is shown by *dotted black line.* All cowshed DEs show closely related curvature of ^{1}H NMR spectra, corroborating the rather congruent structural main features that primarily originate from amino acid side chains (δ_H ~ 0.5-4.5 ppm for aliphatic, *blue shade "a"*) and subordinate, from OCH and $HOCH_2$ units in carbohydrates (δ_H ~ 3.2-4.5 ppm, *red shade "b"*). Entire ^{1}H NMR spectrum, also showing aromatic side chains (δH ~ 6.5-8.2 ppm), is shown in Fig E2.

marginal contribution of BLG derivatives to the cowshed DEs used in our study (see Fig E5 in the Online Repository available at www.jacionline.org).

Proteomic analyses identify candidate asthma-protective molecules in European cowshed DEs

^{1}H NMR spectra revealed consistently higher relative proportions of carbohydrates and lower proportions of peptides/proteins in sheep shed DEs compared to cowshed DEs. Autoclaving did not affect these ratios. These findings, combined with our functional data (Fig 1), prompted us to perform differential proteomic analyses of cow versus sheep shed DEs with autoclaved and nonautoclaved extracts. Differential quantitative mass spectrometry focused on DE samples from 7 different cowsheds and 2 different sheep sheds, including the European sheds tested in Fig 1. Two DEs from 1 European cow farm sampled in 2 different years and seasons were also included. We acquired 2 and 1 measurements for autoclaved and nonautoclaved DE samples, respectively.

A total of 1800 proteins were identified in the autoclaved DEs and 100 proteins in the nonautoclaved DEs. Proteins identified only by a single peptide hit were excluded from further analysis. In all, 25 proteins were identified and quantified in both autoclaved and nonautoclaved samples. For the proteins that exhibited a >2-fold intensity enrichment in cowshed over sheep shed DEs in at least 5 of 7 cowshed DEs, we also calculated average enrichment ratios (ie, the ratio of average protein abundance in cowshed vs sheep shed DEs) and their *P* values. Table I shows the 12 proteins of animal and plant origin that were significantly and strongly overrepresented in protective cowshed versus nonprotective sheep shed DEs. BLG was found in both cowshed and sheep shed DEs, but on average, it was not overrepresented in cowshed DEs (data not shown), suggesting that although it is a signature of farm animal exposure, it is neither more abundant in nor unique to cowsheds. Comparable results were obtained limiting the analysis to DE samples tested in the asthma mouse model (EUC-01/EUC-04 vs EUS-01). Of the 12 asthma-protective protein candidates identified in European farm dust samples, 2 [odorant binding protein (OBP) and the allergen Bos d 2 (dander major allergen BDA20)] were bovine lipocalins, whereas 5 (NPC intracellular cholesterol transporter, protein S100-A7/8/12, and peptidoglycan recognition protein) were transport proteins.

Characterization of protective activity in Amish cowshed DEs

To maximize the robustness of our results, we undertook an independent, stepwise, bioactivity-guided biochemical deconvolution of Amish farm dust samples with demonstrated asthma-protective activity.[3,4] First, dust extraction methods were compared by treating unfractionated Amish cowshed dust using solvents (water, methanol, chloroform, methylene chloride,

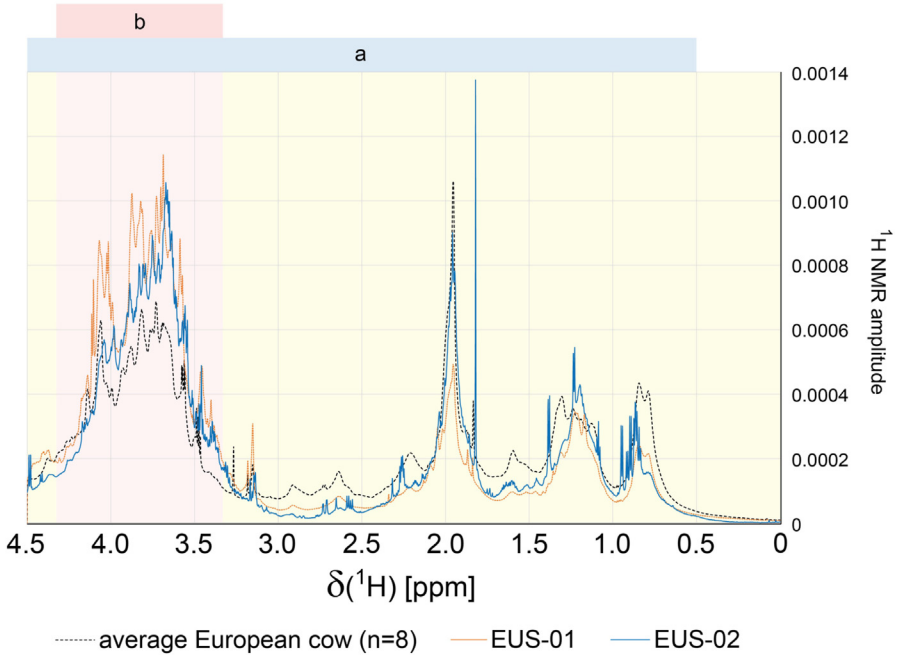

FIG 3. Comparative NMR spectra of sheep shed versus cowshed DEs. Area-normalized ^{1}H NMR spectra (800 MHz, D_2O) show section of aliphatic peptide side chains, and OCH and $HOCH_2$ units of carbohydrates, OCH, and CCH units. Two sheep shed DEs (EUS-01 and EUS-02) are compared to computed average of 8 European cowshed DEs. DEs derived from sheep sheds show higher relative proportions of carbohydrates, comprising OCH and $HOCH_2$ units ($\delta_H \sim$ 3.3-4.3; *red shade "b"*) together with lower proportions of peptide $CONHC\alpha H$ units and lower proportions of peptides/proteins [$\delta_H \sim$ 0.5-4.5 ppm ($C_{sp3}H$), *blue shade "a"*] than cowshed DEs. Entire ^{1}H NMR spectrum, also showing aromatic side chains ($\delta_H \sim$ 6.5-8.2 ppm), is shown in Fig E4.

hexane) with different polarity indices. To monitor the recovery of protective substances, we developed the lung γδ T-cell induction bioassay, which identifies protective DEs based on their ability to expand γδ T cells in the mouse lung on airway administration (see the Methods section and Fig E1 in the Online Repository available at www.jacionline.org). Amish cowshed dust samples extracted once with water strongly induced γδ T cells, whereas a second and third aqueous extraction or other solvents did not yield significant bioactivity. Moreover, activity was unaffected by heating at 80°C or sterile filtration (see Fig E6, *A,* in the Online Repository). To further investigate the nature of active substances contained in aqueous Amish cowshed DEs, samples were incubated with enzymes targeting proteins or carbohydrates. Enzymes were subsequently removed by SEC, and samples were autoclaved. Addition of 2 serine proteases (proteinase K and porcine trypsin) reduced Amish cowshed DE activity by over 60%, whereas addition of both α- and β-galactosidase or β-galactosidase alone had negligible effects. Endotoxin depletion by adsorption on a poly-(ε-lysine) resin also failed to affect activity (Fig E6, *B*). In combination, these studies suggested that heat-stable proteins rather than endotoxin or carbohydrates are responsible for the protective bioactivity of Amish cowshed DEs.

The asthma-protective activity of Amish cowshed DEs resides within the 28-64 kDa range

Aqueous Amish cowshed DEs were then fractionated by SEC, collecting fractions every 1.5 minutes (Fig 4, *A*). The 18 samples thus recovered were initially pooled into 4 groups of consecutive fractions (F-I, A-E, J-N, O-S), dried, and mass adjusted to the original concentration (100 mg/mL of dust equivalents). The asthma-protective activity of these samples was assessed *in vivo* by measuring their ability to inhibit OVA-induced BAL eosinophilia on intranasal administration. Fig 4, *B,* shows that compared to an unfractionated Amish cowshed DE control, essentially all the protective bioactivity was contained in the A-E fraction pool (molecular weight, 22.4-64 kDa), which strongly ($P = .001$) suppressed OVA-induced BAL eosinophilia. A complex pattern emerged when the 5 fractions included in the A-E pool were tested individually. Fractions A and B almost completely abrogated OVA-dependent BAL eosinophilia (P = 9.6E-09 and 3.1E-08, respectively), whereas fractions C and D induced less intense, but still significant, suppression ($P = .007$ and 2.4E-05, respectively). Fraction E was inactive (Fig 4, *C*). Interestingly, fractions B, C, and D, but not A and E, significantly inhibited OVA-dependent AHR, another cardinal asthma phenotype (Fig 5, *A*). These data show that all Amish cowshed dust fractions within the 28-64 kDa molecular weight range (fractions A to D) were active *in vivo* but had distinct protective properties (see Table E1 in the Online Repository available at www.jacionline.org).

Fraction B selectively boosts human airway epithelial barrier function

To further characterize the protective activity of individual Amish cowshed DE fractions (Fig 5, *C*) on early innate events that initiate allergic lung inflammation, we measured the ability of fractions A-E to boost human epithelial airway barrier function,[20,21] assessed as TEER.[12] TEER-enhancing activity resided selectively in fraction B (51.5-42 kDa), which was several times

TABLE I. Proteins identified in European cowshed DEs by differential proteomic analysis

UniProt accession no.	Description	Average intensity (abundance) of: Cowshed DEs	Sheep shed DEs	Enrichment ratio*	*P* value
P07435	Odorant-binding protein OS = *Bos taurus* OX = 9913 PE = 1 SV = 2	1.35×10^8	1.81×10^5	746 (752)	.00000
Q28133	Allergen Bos d 2 OS = *Bos taurus* OX = 9913 PE = 1 SV = 1	2.57×10^9	5.05×10^6	509 (142)	.00000
P79105	Protein S100-A12 OS = *Bos taurus* OX = 9913 GN = S100A12 PE = 1 SV = 3	2.47×10^7	4.37×10^4	564 (1201)	.00000
Q8SPP7	Peptidoglycan recognition protein 1 OS = *Bos taurus* OX = 9913 GN = PGLYRP1 PE = 1 SV = 1	6.83×10^6	2.37×10^4	289 (686)	.00000
Q28050	Protein S100-A7 OS = *Bos taurus* OX = 9913 GN = S100A7 PE = 1 SV = 1	7.58×10^8	6.53×10^6	116 (39)	.00000
A0JNP2	Secretoglobin family 1D member OS = *Bos taurus* OX = 9913 GN = SCGB1D PE = 3 SV = 2	5.58×10^6	5.07×10^4	110 (135)	.00000
P79345	NPC intracellular cholesterol transporter 2 OS = *Bos taurus* OX = 9913 GN = NPC2 PE = 1 SV = 1	1.71×10^6	2.14×10^4	80 (121)	.00000
P28782	Protein S100-A8 OS = *Bos taurus* OX = 9913 GN = S100A8 PE = 1 SV = 2	1.88×10^6	2.68×10^4	70 (236)	.00000
P80416	Cystatin-A OS = *Bos taurus* OX = 9913 GN = CSTA PE = 1 SV = 1	1.03×10^7	2.19×10^5	47 (24)	.00123
P01083	Alpha-amylase inhibitor 0.28 OS = *Triticum aestivum* OX = 4565 GN = IMA1 PE = 1 SV = 3	2.18×10^6	5.47×10^4	40 (32)	.00863
P79124	Short palate, lung and nasal epithelium carcinoma-associated protein 2A OS = *Bos taurus* OX = 9913 GN = SPLUNC2A PE = 2 SV = 2	5.80×10^6	1.60×10^5	36 (23)	.02024
P93693	Serpin-Z1B OS = *Triticum aestivum* OX = 4565 PE = 1 SV = 1	1.63×10^6	5.01×10^4	33 (32)	.04456

*Average intensity (abundance) of a given protein in all cowshed DEs divided by average intensity (abundance) of that protein in all sheep shed DEs. Ratio-based significance was calculated by PERSEUS v1.6.7.0. Parenthetical values represent enrichment ratios for DE samples tested in asthma mouse model (EUC-01/EUC-04 vs EUS-01).

more active than all other fractions and its parent unfractionated Amish cowshed DE ($P = .0001$, Fig 5, *B*). Therefore, unlike other fractions, fraction B not only significantly suppressed OVA-induced BAL eosinophilia and AHR (Fig 4, *C,* and Fig 5, *A*) but also supported a fundamental innate airway response (Table E1). Remarkably, fraction B represented as little as ≈0.5% of the total carbon content of unfractionated Amish cowshed DE (see Fig E7 in the Online Repository available at www.jacionline.org).

Identification of candidate proteins in fraction B

Because fraction B protected against multiple pillars of allergic lung inflammation (Table E1), and because enzymatic digestion of unfractionated Amish cowshed DEs had suggested that protein structures were important for bioactivity (Fig E6), we next characterized the proteins contained in fraction B. In-solution and in-gel SDS-PAGE enzymatic digestion/peptide mapping identified 7 proteins (Table II). Among them were 4 plant proteins: provicilin, vicilin, β-conglycinin, MATH domain and coiled-coil domain-containing protein At3g58400, the first 3 of which have storage function. Another protein (IFN-γ from dog) is an immune mediator. Most importantly, the 2 most prominent proteins in fraction B were the bovine lipocalins OBP and Bos d 2, which were also the most enriched proteins in DEs from protective European cowsheds (Table I). The presence of Bos d 2 and OBP in fraction B was confirmed by enzymatic digestion and peptide mapping after immunoprecipitation with specific antibodies (see Fig E8 in the Online Repository available at www.jacionline.org).

Characterization of cargo transported by Bos d 2 and OBP

Because Bos d 2 and OBP are transport proteins whose functional properties depend on their cargo, substances carried by Bos d 2 and/or OBP isolated from fraction B were characterized by untargeted liquid chromatography–high-resolution mass spectrometry. These analyses identified a complex mixture of fatty acid metabolites likely originating primarily from microbes and/or plants (Table III). These metabolites were associated with, and thus appeared to be transported by, both OBP and Bos d 2.

DISCUSSION

Recent studies suggest that living in rural environments, for instance those found in China[22] and South Africa,[23] protects children from asthma and allergy because of reduced exposure to risk factors. However, our work over the years has shown that children who grow up on traditional farms in close contact with cows and hay are actively protected against asthma,[2] as highlighted by the demonstration that airway administration of cowshed DEs from these farms was sufficient to prevent allergic asthma in mice.[3-6] The current study combined complementary, unbiased analytical strategies and multiple functional assays to begin identifying asthma-protective agents in dust from US Amish and European farms. Our analyses converged on asthma-protective fractions, primarily one in the 51.5-42 kDa range, that represented as little as ≈0.5% of the total carbon content of unfractionated Amish cowshed DE. This fraction was highly protective both *in vitro* and *in vivo,* and contained several transport proteins of animal and plant origin loaded with microbial and/or plant metabolite cargo. Among these proteins, 2 bovine lipocalins (OBP and Bos d 2) were also enriched in protective European cowshed DEs. In contrast, putative protective substances identified in other farm studies (the lipocalin BLG,[17,18] hay-derived arabinogalactan,[24] endotoxin[6]) did not appear to be critical for asthma protection in our hands. In this regard, we note that in our 2001 multicenter ALEX farm study, microbial exposures were assessed by measuring indoor endotoxin levels[25] because 16S rRNA sequencing had not yet been developed. These measurements could not specifically target LPS and were therefore a rather crude

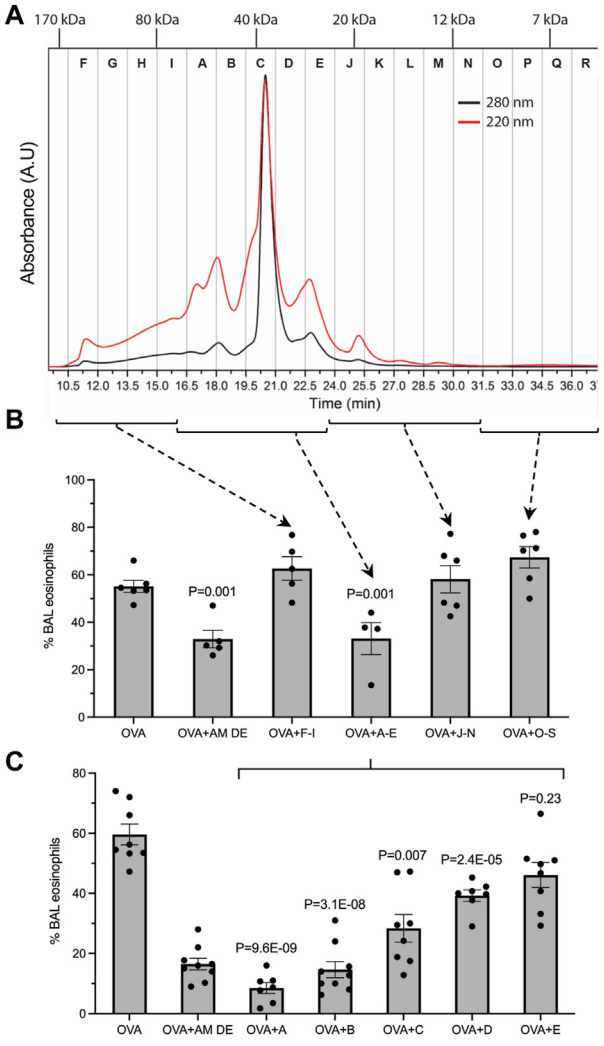

FIG 4. Protective activity of Amish cowshed DEs resides within the 28-64 kDa range. **A,** Aqueous Amish cowshed DEs were fractionated by SEC collecting fractions (named as *above graph*) every 1.5 minutes. *Red line* and *black line* represent spectrophotometric readings at 220 and 280 nm, respectively. **B,** Fractions were pooled into 4 consecutive groups (F-I, 6-16.5 minutes; A-E, 16.5-24 minutes; J-N, 24-31.5 minutes; O-S, 31.5-40 minutes) and mass adjusted to original concentration (100 mg/mL of dust equivalents). Fraction bioactivity was assessed by comparing percentages of BAL eosinophils in mice treated with OVA or OVA + fraction as in Fig 1, *A*. Shown are mean percentages of BAL eosinophils ± SEs (8-9 mice per group from 2 independent experiments for each fraction pool). *C,* Effects of fractions A-E (each mass adjusted to 100 mg/mL of dust equivalent) on OVA-induced BAL eosinophilia. Shown are mean percentages of BAL eosinophils ± SEs (8-9 mice per group from 2 independent experiments for each fraction). Unfractionated Amish (AM) cowshed DE served as positive control. Differences in percentage BAL eosinophilia between mice treated with OVA and OVA + fraction were assessed by unpaired 2-tailed *t* test after evaluating normality of value distribution by Shapiro-Wilk test.

proxy for all types of microbial exposures. Our current findings indicate that the protective activity of cowshed DEs resides in components other than LPS. However, we cannot rule out the possibility that minute amounts of LPS contributed to the effects we observed. As for BLG, this protein was found in both cowshed and sheep shed DEs and was not overrepresented in cowshed DEs. Moreover, BLG was not found in the most active Amish fraction, fraction B, but we cannot exclude its presence in the other active Amish fractions (fractions A, C, and D). Finally, NMR spectroscopy showed that peptides were more abundant in cowshed than in sheep shed DEs, prompting us to focus our analyses on proteins rather than carbohydrates. Therefore, an additional effect of arabinogalactan cannot be completely excluded.

Bos d 2 is a lipocalin produced by cattle sweat glands and is found exclusively in the skin. OBP, also a lipocalin, is produced in the mammalian nasal mucosa and binds a variety of chemical odorants and substances. Both proteins bind hydrophobic molecules and cell surface receptors, and form complexes with soluble macromolecules.[26] Interestingly, the function of these proteins seems to be determined by the properties of their ligands. Lipocalins can bind and transport long-chain fatty acids[27] that protect them against heat- and chaotrope-induced denaturation, modulate their sensitivity to chemical and physical denaturation, and prevent their hydrolysis.[28] On the one hand, the properties of fatty acid–bound lipocalins might help explain why autoclaved cowshed DEs were as protective as their nonautoclaved counterparts. On the other hand, Amish cowshed dust treated with methanol and chloroform, which extract fatty acids, was not active, suggesting that free fatty acids *per se* may not be sufficient for protection.

The multiple fatty acids associated with Bos d 2 and OBP likely derive from plants and microbes and belong to the phospholipid fatty acid (PLFA) fraction, an essential part of cell membranes. Plants and microbes synthetize fatty acids of different chain lengths and composition to maintain cell membrane integrity and cellular function in response to their immediate environmental conditions.[29] After cell death, PLFA are rapidly degraded by digestion of the hydrophilic glycerol 3-phosphate head,[30] but the remaining fatty acids are stable and can persist for decades.[31] Therefore, the high proportion of fatty acid metabolites found in our samples is not surprising.

The synthetic pathways for PLFA-derived fatty acids are highly conserved between bacteria and eukaryotes even though the catalytic entities reside in markedly different protein arrangements.[32] Overall, fatty acids synthesized by bacteria are similar to those found in eukaryotic cells, except that bacterial acids tend to be shorter, they generally lack polyunsaturation, and the monoenoic C_{18} acids have different double bond positions.[32] The taxonomic resolution of PLFA-derived fatty acids is consequently relatively low.[33] Unique reactions occur in lactic acid bacteria, which produce conjugated linoleic acid-based derivates[34] potentially yielding the various octadecadienoic acid-based metabolites found in our samples. Lactobacilli form bioactive linolenic acid metabolites,[35] which might explain the presence of 9-hydroperoxy-10E,12,15Z-octadecatrienoic acid in Amish cowshed DEs. However, polyunsaturated fatty acids with 18:2 and 18:3 structures (octadecadienoic and octadecatrienoic acid derivates) might also derive from fungi, in which they act as cell wall constituents.[36] Furthermore, several bacteria, including streptococci, metabolize octadecenoic acid (oleic acid).[37] Interestingly, oleic acid–derived metabolites, which are abundant in our samples, were also detected in methanotrophic bacteria, important regulators of the cowshed ecosystem.[38]

Fatty acids are also components of storage lipids from microbes and plants.[32] Palmitic acid is an important intermediate for the subsequent transformation of other fatty acids.[39] Hexadecanoic acid–based metabolites in our samples might thus derive from plant or microbe storage pools, but a more precise definition is impossible because of the broad distribution of palmitic acid across kingdoms of life. Myristic acid (tetradecanoic acid and

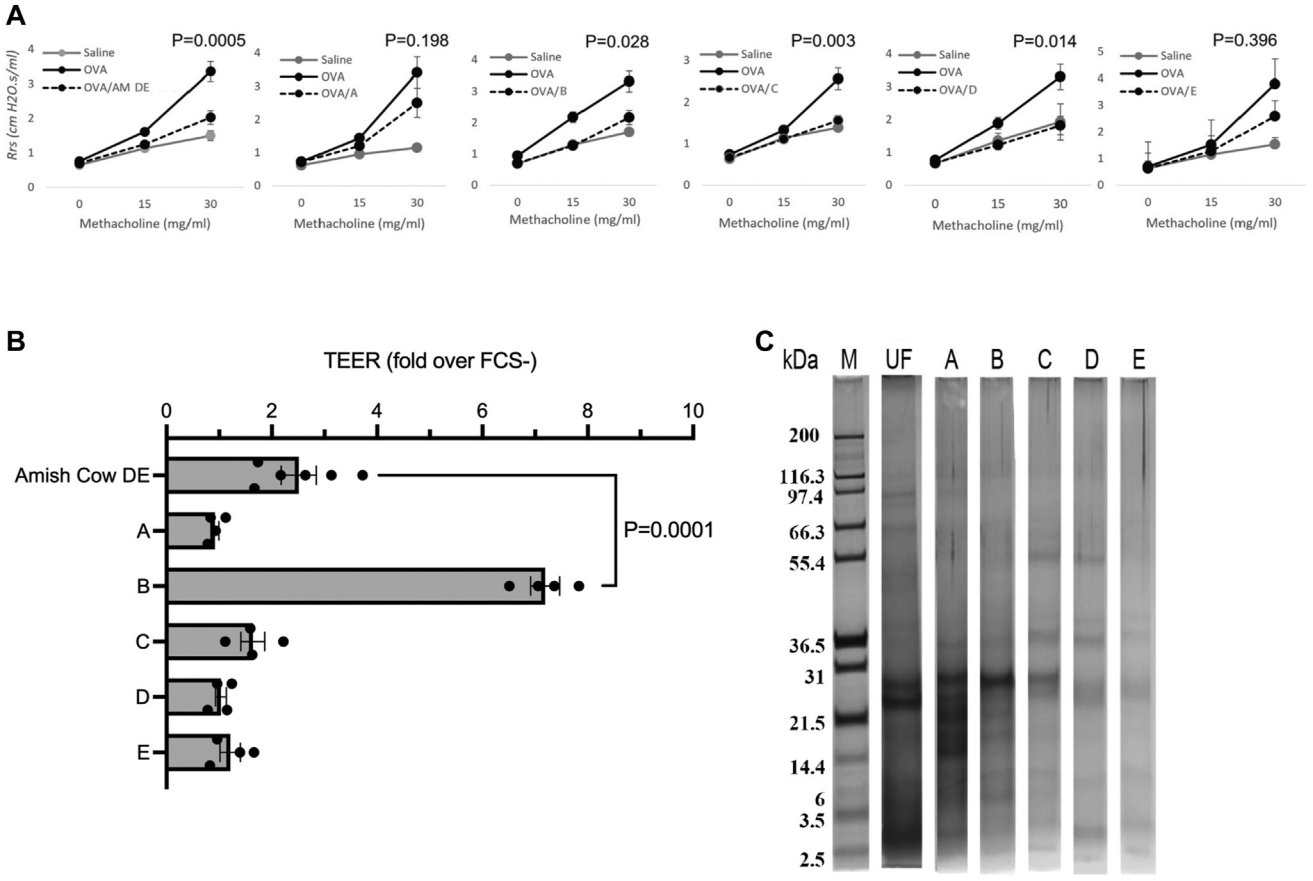

FIG 5. Fraction B inhibits OVA-induced AHR and selectively boosts human airway epithelial barrier function. **A,** Balb/c mice were sensitized with OVA (50 μg) + alum (6 mg) intraperitoneally (i.p.) (days 0 and 7) and treated intranasally (i.n.) with Amish cowshed DE fractions A-E (5 mg dust equivalent/treatment) 8 times over 14 days (Fig 1, *A*). Airway resistance was measured at day 19 after i.n. OVA challenge (100 μg, day 15-17) and methacholine (0-30 mg/mL) nebulization. Data (means ± SEMs) are from 1 of 2 experiments, 4-5 mice per treatment group. AHR differences between OVA- and OVA + DE fraction–treated mice were assessed by unpaired 2-tailed *t* test after assessing normality of sample value distribution by Shapiro-Wilk test. **B,** TEER was measured in human bronchial epithelial cells (16HBE14o−) cultured for 48 hours with fractions A-E or unfractionated Amish cowshed DE (2 mg dust equivalents per well) and was expressed as fold increase in sample-treated over serum-starved (FCS$^-$) cells. TEER differences between cultures were assessed by unpaired 2-tailed *t* test after evaluating normality of sample value distribution by Shapiro-Wilk test. **C,** Silver staining of SDS-PAGE (4-12% reducing gel) of unfractionated (UF) Amish cowshed DE and fractions A-E (10 μg dust equivalent per lane). *FCS,* Fetal calf serum; *M,* molecular weight markers.

TABLE II. Proteins identified in fraction B of Amish cowshed DEs using bioactivity-guided fractionation

UniProt accession no.	Description
P07435	Odorant-binding protein OS = *Bos taurus* OX = 9913 PE = 1 SV = 2
Q28133	Allergen Bos d 2 OS = *Bos taurus* OX = 9913 PE = 1 SV = 1
P42161	IFN-γ OS = *Canis lupus familiaris* OX = 9615 GN = IFNG PE = 2 SV = 2
P02854	Provicilin (fragment) OS = *Pisum sativum* OX = 3888 PE = 1 SV = 1
P02856	Vicilin, 14 kDa component OS = *Pisum sativum* OX = 3888 PE = 1 SV = 1
P0DO15/ P0DO16	β-Conglycinin α subunit 1 and 2 OS = Glycine max OX = 3847 GN = CG-3 PE = 1 SV = 1
Q9M2H6	MATH domain and coiled-coil domain–containing protein At3g58400 OS = *Arabidopsis thaliana* OX = 3702 GN = At3g58400 PE = 4 SV = 2

related derivates) is a saturated fatty acid commonly found in milk.[40] Thus, metabolites from diverse sources—bacteria, plants, milk—might contribute to the asthma-protective properties of cowshed DEs.

Taken together, our results hint at the intriguing possibility that traditional farm environments provide both metabolites that calibrate human immune function and transport proteins that deliver those metabolites to human mucosal cells, thereby

TABLE III. Probable structures of fatty acids associated with Bos d 2 and OBP in asthma-protective fraction B of Amish cowshed DE

Formula	Exact mass	Mass error (ppm)	RT (min)	Abbreviation	IUPAC name	Lipid Maps ID (LMFA)	Pub Chem ID	Main class*	Subclass*	Other database	Bos d 2	OBP
$C_{18}H_{36}O_2$	284.2712	−1.24	8.12	FA 18:1;O2	(Z)-11-hydroperoxy octadec-12-enoic acid	02000116	53477454	Octade canoids	Unsaturated fatty acids		+	+
$C_{18}H_{30}O_4$	310.2141	−0.95	7.36	FA 18:3;O2	(10E,12E,15Z)-9-hydroperoxy octadeca-10,12,15-trienoic acid	02000108	5282864				+	+
$C_{18}H_{36}O_4$	316.2616	0.87	5.97	FA 18:0;O2	9,12-dihydroxyo ctadecanoic acid	02000143	316306		Hydroxy fatty acids		+	+
$C_{18}H_{32}O_4$	312.2297	−1.04	5.39	FA 18:0;O2	(E)-9-hydroxy-10-oxooctadec-12-enoic acid	02000170	5282967				+	+
$C_{18}H_{32}O_4$	312.2299	−0.58	4.92	FA 18:0;O2	(Z)-13-hydroxy-12-oxooctadec-9-enoic acid	02000017	16061052				+	−
$C_{18}H_{36}O_5$	332.2568	1.42	4.56	FA 18:0;O3	(9R,10S)-9,10,18-trihydroxy octadecanoic acid	02000006	12311165				+	+
$C_{18}H_{34}O_5$	330.2409	0.86	4.15	FA 18:1;O3	(E)-9,10,13-trihydroxyoctadec-11-enoic acid	02000168	5282965			C14835†	+	+
$C_{18}H_{32}O_3$	296.2354	0.97	6.79	FA 18:0;O	(E)-12-oxooctadec-10-enoic acid	02000265	5312910		Oxo fatty acids		+	+
$C_{18}H_{30}O_3$	294.2110	1.63	7.07	FA 18:3;O	(Z)-11-[3-[(Z)-pent-2-enyl]oxiran-2-yl]undec-9-enoic acid	02000040	16061061		Epoxy fatty acids	HMDB 0010200‡	+	−
$C_{18}H_{32}O_5$	328.2246	−1.14	7.36	FA 18:2;O3	(E)-11-hydroperoxy-11-(3-pentyloxiran-2-yl)undec-9-enoic acid	02000106	5282862				−	+
$C_{16}H_{30}O_3$	270.2197	0.75	5.74	FA 16:1;O	3-oxohexadecanoic acid	01060051	5282997	Fatty acids and conjugates	Oxo fatty acids	HMDB 0010733‡	+	
$C_{16}H_{18}O_2$	242.1304	−0.96	3.12	FA 16:7	(6E,8E,14E)-hexadeca-6,8,14-trien-10,12-diynoic acid	01030704	9543613		Unsaturated fatty acids		+	−
$C_{18}H_{28}O_2$	276.2087	−0.87	5.84	FA 18:4	octadeca-9,12-diynoic acid	01030540	1931				+	
$C_{16}H_{32}O_2$	256.2407	1.86	7.02	FA 16:0	6-ethyltetradecanoic acid	01020169	5282696		Branched fatty acids		−	+
$C_{18}H_{36}O_2$	284.2713	−0.72	8.20	FA 18:0	11,15-dimethylhexadecanoic acid	01020175	5282701				−	+

IUPAC, International Union of Pure and Applied Chemistry; *RT,* retention time.
*Classification as reported in Lipid Maps (lipidmaps.org).
†Kyoto Encyclopedia of Genes and Genomes (KEGG; genome.jp/kegg).
‡Human Metabolome Database (hmdb.ca).

regulating airway responses. There is precedent for the notion that environmental signals affect immune balance by modulating endogenous innate immune responses. One compelling example is the molecular network linking LPS and other bacterial polysaccharides to the host's LPS binding protein, soluble and membrane CD14, and immune tolerance. In this case, the microbial environment relies on evolutionarily conserved mammalian transport proteins to deliver decipherable signals to innate immune cells that require those signals for their maturation and tolerogenic function.[41,42]

Our farming model might be somewhat different because LPS does not appear to be directly involved and transport functions

might be predominantly provided by nonhuman (animal and plant) rather than human proteins. Nevertheless, all these processes might be architecturally similar in that environmental cues ultimately converge on innate immune signaling pathways. Indeed, the asthma-protective activity of Amish cowshed DEs was completely Myd88/Trif dependent.[3] Of note, the identification of plant proteins and metabolites in asthma-protective DEs may help explain the link between exposure to vegetation and asthma protection—the biodiversity hypothesis[43]—and the protective role of agricultural activities in our epidemiologic farm studies.[7,8] It is also notable that Bos d 2, an allergen, was consistently detected in asthma-protective DEs. Because Bos d 2 is a lipocalin with transport functions, it is tempting to speculate that its allergenicity or lack thereof may depend on the quality and quantity of microbial metabolite cargo this molecule carries when it encounters immune cells. Thus, some lipocalin allergens might represent transport molecules that no longer carry the tolerogenic metabolite load they originally evolved to deliver.

We acknowledge that despite (or because of) their novelty, our results should be interpreted with caution. Asthma-protective activity resided in more than 1 dust fraction. Bos d 2 and OBP are the most prominent, but not the only, components of the fraction we focused on (fraction B), and their role in protection remains to be conclusively determined. Moreover, differential proteomics of cowshed versus sheep shed DEs identified other candidates besides these 2 lipocalins, suggesting that additional protective substances may remain uncharacterized. Finally, the sources of the metabolites associated with protection and the mechanisms underlying their potential effects are unknown. However, we find it remarkable that not only Bos d 2 and OBP but also other proteins identified in Amish and European cowshed DEs (β-conglycinin, vicilin, peptidoglycan recognition protein, S100A7, S100A8, and S100A12) have storage or transport properties, regardless of their animal or plant origin.[44-50] Thus, distinct analyses conducted in different laboratories on asthma-protective substances from 2 continents converge on a common theme: animals and plants from traditional farms produce proteins that transport hydrophobic microbial and plant metabolites. When delivered to mucosal surfaces, these agents might regulate airway responses.

DISCLOSURE STATEMENT

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Disclosure of potential conflict of interest: F. D. Martinez, D. Vercelli, S. A. Snyder, E. von Mutius, V. Pivniouk, and M. Marques dos Santos are inventors in PCT/US2021/016918 "Therapeutic Fractions and Proteins From Asthma-Protective Farm Dust." E. von Mutius, B. Rankl, F. Bracher, C. Müller, A. Walker, J. Merl-Pham, and S. M. Hauck are inventors in PCT EP21189353 "Proteins Identified From Barn Dust Extract for the Prevention and Treatment of Diseases." E. von Mutius, F. Bracher, C. Müller, and D. Vercelli are inventors in PCT application PCT/EP2019/085016 "Barn Dust Extract for the Prevention and Treatment of Diseases." E. von Mutius is inventor of the following patents: EP2361632 "Specific Environmental Bacteria for the Protection From and/or the Treatment of Allergic, Chronic Inflammatory and/or Autoimmune Disorders," EP1411977 "Composition Containing Bacterial Antigens Used for the Prophylaxis and the Treatment of Allergic Diseases," and EP1637147 "Stable Dust Extract for Allergy Protection." E. von Mutius has received honoraria as expert from AstraZeneca, HiPP, OM Pharma, and Böhringer Ingelheim International. The rest of the authors declare that they have no relevant conflicts of interest.

Content described herein is the subject of US Patent Application Serial Nos. 17/413,468 and 17/439,112 and their foreign equivalents.

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Key messages

- **Epidemiologic studies have consistently shown that children who grow up on traditional farms in close contact with cows and hay are protected against asthma. Moreover, airway administration of cowshed DEs prevents allergic asthma in mice. However, the substances responsible for these effects remain elusive.**
- **Dust from US Amish and European farms was characterized by combining complementary, unbiased analytical strategies and multiple functional assays. We identified asthma-protective fractions in Amish cowshed DEs. One of these fractions (51.5-42 kDa) contained animal and plant transport proteins loaded with microbial and/or plant metabolite cargo. Two of these proteins, the bovine lipocalins OBP and Bos d 2, were also enriched in protective European cowshed DEs.**
- **We speculate that animals and plants from traditional farms produce proteins that transport hydrophobic microbial and plant metabolites. When delivered to mucosal surfaces, these agents might regulate airway responses.**

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