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SUPPLEMENTARY MATERIALS

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Supplementary Text
Materials and Methods
Figs. S1 to S13
Table S1
References (*28*–*37*)

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ALLERGY

Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells

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Growing up on a dairy farm protects children from allergy, hay fever, and asthma. A mechanism linking exposure to this endotoxin (bacterial lipopolysaccharide)–rich environment with protection has remained elusive. Here we show that chronic exposure to low-dose endotoxin or farm dust protects mice from developing house dust mite (HDM)–induced asthma. Endotoxin reduced epithelial cell cytokines that activate dendritic cells (DCs), thus suppressing type 2 immunity to HDMs. Loss of the ubiquitin-modifying enzyme A20 in lung epithelium abolished the protective effect. A single-nucleotide polymorphism in the gene encoding A20 was associated with allergy and asthma risk in children growing up on farms. Thus, the farming environment protects from allergy by modifying the communication between barrier epithelial cells and DCs through A20 induction.

Allergic asthma is characterized by eosinophilic airway inflammation, goblet cell metaplasia, and bronchial hyperreactivity (BHR) and is controlled by innate and adaptive immune responses to inhaled allergens such as house dust mites (HDMs), pollen, and fungal spores that signal via pattern recognition receptors (PRRs) on barrier epithelial cells (ECs) and dendritic cells (DCs) (*1*, *2*). In children, allergic sensitization and asthma are strongly influenced by genes and the environment. A dairy farm is one of the strongest protective environments (*3*–*6*). On farms, there is high-level exposure to endotoxin [lipopolysaccharide (LPS)], a cell wall component of Gram-negative bacteria. The protective effect that high levels of environmental endotoxin demonstrate against allergy has also been noticed in nonfarming households, where exposure was measured in dust collected from mattresses or kitchen floors (*7*–*9*). Protection in these environments is influenced by genetic polymorphisms in key PRRs that recognize endotoxin (*10*). A clear mechanism encompassing the complex interactions between a protective environment, genetics, and the immune response to allergens has been lacking.

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To address whether exposure to environmental endotoxin and protection from allergy are causally related, we exposed mice every other day for 2 weeks to a low dose (100 ng) of LPS or to control phosphate-buffered saline (PBS) before HDM sensitization and challenge (Fig. 1A) (see supplementary materials and methods). Sham-protected mice exhibited strong airway eosinophilia and lymphocytosis (Fig. 1B), T helper 2 (T_H2)–dependent HDM allergen–specific immunoglobulin E (IgE) (Fig. 1C), and BHR to methacholine (Fig. 1D). However, mice pretreated with LPS failed to develop all of these canonical asthma features. Protective LPS led to reduced production of the type 2 cytokines interleukin (IL)–5 and IL-13 in mediastinal lymph node (MLN) cells (Fig. 1E), without a shift to T_H1- or T_H17-associated cytokines or to T_H1-dependent serum immunoglobulin G2a (IgG2a) antibodies (Fig. 1, C and E). All of the key asthma features were also suppressed when a single high dose (1 μg) of LPS was given as a preventive regimen 14 days before sensitization (fig. S1, A to E), as well as when chronic low-dose LPS was given before and throughout the entire HDM sensitization and challenge period (fig. S1, F to I).

Sensitization to HDMs depends on various DC subsets that migrate to the MLNs to prime CD4 T cell responses (*11*, *12*). When PBS-treated control mice were exposed to a single dose of HDM, $CD11b^+$ conventional DCs (cDCs), $CD103^+$ cDCs, and monocyte-derived DCs (moDCs) were recruited to the lungs and MLNs (Fig. 2A). In mice receiving preventive LPS, there was less HDM-induced recruitment of both subsets of cDCs, whereas moDCs were unaffected (Fig. 2A). cDCs that migrate to the MLN cells induce T_H2 polarization in HDM-reactive naïve T cells (*12*). To study the primary immune response to HDMs, we adoptively transferred $CD4^+$ HDM-specific 1-DER T cells [that express a transgenic T cell receptor

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specific for the Der p $1_{WAFSGVAAT}$ peptide from HDMs (*12*)], followed by a single administration of HDMs. Prior 2-week treatment with LPS suppressed the proliferation of 1-DER T cells in the MLN cells (Fig. 2B), along with their differentiation into IL-5– and IL-13–secreting T_H2 cells (Fig. 2C).

The recruitment of lung DCs after allergen exposure depends on the chemokine CCL20, as well as their maturation on granulocyte-macrophage colony-stimulating factor (GM-CSF) (*2*, *12*–*14*), which programs DCs to induce T_H2 cell polarization (*2*, *15*). After receiving protective LPS in vivo, the lung levels of GM-CSF and CCL20 protein induced by HDMs were reduced (Fig. 2D). As ECs are the predominant source of these cytokines (*2*, *13*, *15*), we flow-sorted $EpCAM^+CD31^-Sca1^-CD45^-$ bronchial ECs 2 hours after they received HDMs in vivo. In the PBS pretreated group, HDM induced the mRNA of GM-CSF and of CCL20, yet this response was strongly blunted in the LPS-protected group (Fig. 2E). Similar data were found for IL-33 mRNA, another pro-T_H2 cytokine made by ECs in response to HDMs (fig. S2) (*2*, *15*).

These findings demonstrate that LPS protection blunts the innate immune response to HDMs, which we and others previously found to be driven by Toll-like receptor 4 (TLR4) on ECs (*2*, *15*, *16*). TLR4 signaling in ECs leads to nuclear translocation of NF-κB, inducing not only proinflammatory genes but also attenuators of signaling such as A20 (encoded by *Tnfaip3*). A20 is a ubiquitin-modifying enzyme that attenuates NF-κB activation by deubiquitinating key signaling intermediates downstream of TLR, IL-1 receptor, and tumor necrosis factor–family receptors (*17*–*19*). The mRNA for *Tnfaip3* was induced in sorted lung ECs 2 hours after in vivo exposure to a single HDM or LPS injection, and the effect was more pronounced for LPS (Fig. 3A). To address whether A20 mediated the protective effects of LPS, we generated mice lacking A20 selectively in lung ECs by crossing *Tnfaip3*$^{fl/fl}$ mice with mice that displayed lung-specific Cre recombinase expression under the tetracycline-inducible control of the *Ccsp* promoter (*Ccsp*-rtTA × Tet_{O7}Cre mice, referred to as *Tnfaip3*$^{EC\text{-}KO}$ mice) (*20*, *21*). We treated these mice with doxycycline from birth onward (fig. S3, A and B) and observed accelerated nuclear translocation of the p65 subunit of NF-κB in lung ECs 2 hours after HDM exposure in vivo, as compared with control *Tnfaip* $^{fl/fl}$ *Ccsp*Cre^- mice (*Tnfaip3*$^{EC\text{-}WT}$) (fig. S3C). Whereas LPS suppressed the salient features of asthma in *Tnfaip3*$^{EC\text{-}WT}$ mice, this effect was completely lost in *Tnfaip3*$^{EC\text{-}KO}$ mice (Fig. 3, B to D). The suppression of lung CCL20 and GM-CSF protein was less effective in *Tnfaip3*$^{EC\text{-}KO}$ mice receiving LPS (Fig. 3E) yet was still present to some extent, suggesting the existence of other molecular mechanisms by which LPS suppresses asthma. However, the HDM-induced recruitment of DCs to the lungs and MLNs was no longer inhibited by low-dose LPS treatment in *Tnfaip3*$^{EC\text{-}KO}$ mice (Fig. 3F), and asthma was no longer suppressed, which suggests that A20 is an important player in LPS-mediated protection.

In the absence of allergen exposure, baseline production of GM-CSF did not differ between *Tnfaip3*$^{EC\text{-}KO}$ and *Tnfaip3*$^{EC\text{-}WT}$ mice (Fig. 3E). However, the production of GM-CSF after HDM injection was much higher in *Tnfaip3*$^{EC\text{-}KO}$ mice. Recently, GM-CSF was found to be a crucial determinant of allergen recognition threshold in the lung (*14*). To address whether the loss of A20 would alter the allergen recognition threshold, we lowered the dose of sensitizing HDM by a factor of 10, in the absence of preventive LPS (fig. S4A). Whereas a 100-ng dose of HDM extract did not induce asthma features in *Tnfaip3*$^{EC\text{-}WT}$ mice, it did so in *Tnfaip3*$^{EC\text{-}KO}$ mice (fig. S4, B to F). Thus, in the induced absence of A20, sensitivity to inhaled HDM allergen is increased, and asthma runs a more severe course.

In addition to LPS, dust from dairy farms also contains peptidoglycan components of the wall of Gram-positive bacteria, extracellular polysaccharides from fungi, cowshed-derived bacteria such as *Acinetobacter Iwoffii* F78, and plant-derived polysaccharides (*5*). We therefore also performed

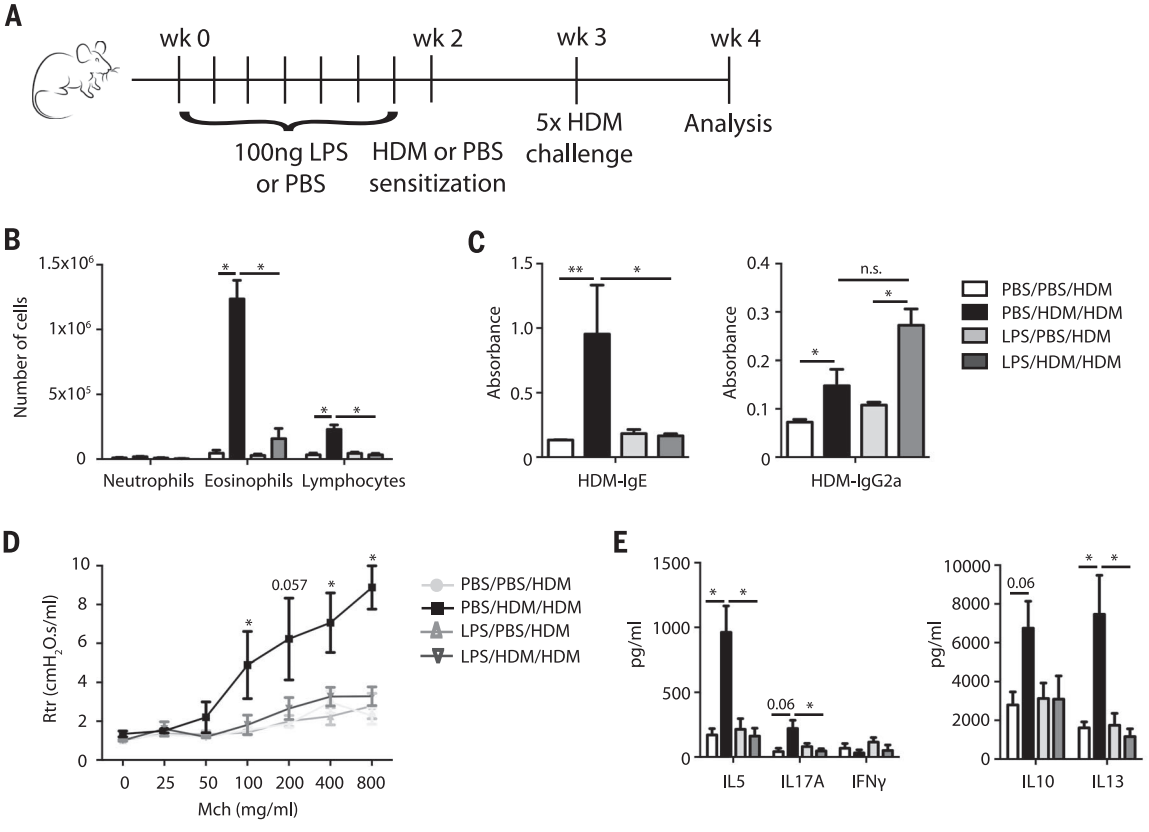

Fig. 1. Chronic LPS preexposure protects mice from asthma development. (**A**) Experimental setup illustrating the dosing regimen of LPS and the various controls. (**B**) Bronchoalveolar lavage differential cell counts of mice that received a chronic low dose of LPS or control PBS before sensitization and challenge with HDMs. To control for sensitization to HDMs, some mice were sham-sensitized to PBS. (**C**) Serum levels of HDM-specific IgE and IgG2a. (**D**) Bronchial hyperreactivity was measured after exposure to increasing doses of methacholine (Mch) using flexiVent (SCIREQ). (**E**) Cytokine production by MLN cells restimulated with HDMs for 3 days ex vivo. Data are representative of three independent experiments, with at least $n = 5$ mice per group. IFNγ, interferon-γ. In (B) to (E), error bars indicate SEM. *P* values reflect the Mann-Whitney *U* test: $^*P < 0.05$, $^{**}P < 0.01$. n.s., not significant.

experiments using dust samples collected from farms in Germany (*22*). A low dose of farm dust extract (100 ng) was given prophylactically every other day for 2 weeks before induction of HDM asthma in wild-type (WT) mice. As in the prophylactic treatment with LPS, farm dust extracts suppressed the salient features of asthma (fig. S5, A to D). The farm dust suppressed the levels of HDM-induced GM-CSF mRNA in sorted lung ECs (fig. S5E). Whereas farm dust suppressed asthma features in *Tnfaip3*$^{EC\text{-}WT}$ mice (Fig. 3G), this was not the case in *Tnfaip3*$^{EC\text{-}KO}$ mice (Fig. 3H), demonstrating that farm dust also mediates protection via epithelial A20.

We next validated our findings in humans. Normal human bronchial ECs (NHBECs) were grown to confluence and differentiated in air-liquid interface (ALI) cultures. In vitro pre-exposure of NHBECs to LPS suppressed the HDM-induced production of IL-1α and GM-CSF (Fig. 4A). We also collected endobronchial biopsies from healthy controls and patients with moderate to severe asthma (table S1 lists the clinical characteristics). Primary bronchial ECs were grown to confluence and then differentiated to ALI cultures. The ALI cultures of selected asthmatic patients (n = 3) were stimulated for 1 week with 100 ng of LPS or control PBS added every other day to the apical side of the ALI culture. In the PBS-treated group, HDM induced the production of GM-CSF and IL-1α on the basolateral side of the culture, and this was significantly reduced by LPS pretreatment (Fig. 4B). The mRNA levels of A20 were measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and were significantly reduced in ALI-cultured ECs of mild (n = 12) and severe asthmatics (n = 14) compared with healthy controls (n = 12) (Fig. 4C). These effects were confirmed on protein levels (Fig. 4D). Thus, levels of A20 are lower in the barrier ECs of asthmatics, as compared with healthy controls.

We finally studied whether well-known polymorphisms in the human *TNFAIP3* gene (located at position 6q23 in the genome) were associated with various allergic diseases in the population of the GABRIELA study (table S2), a cross-sectional,

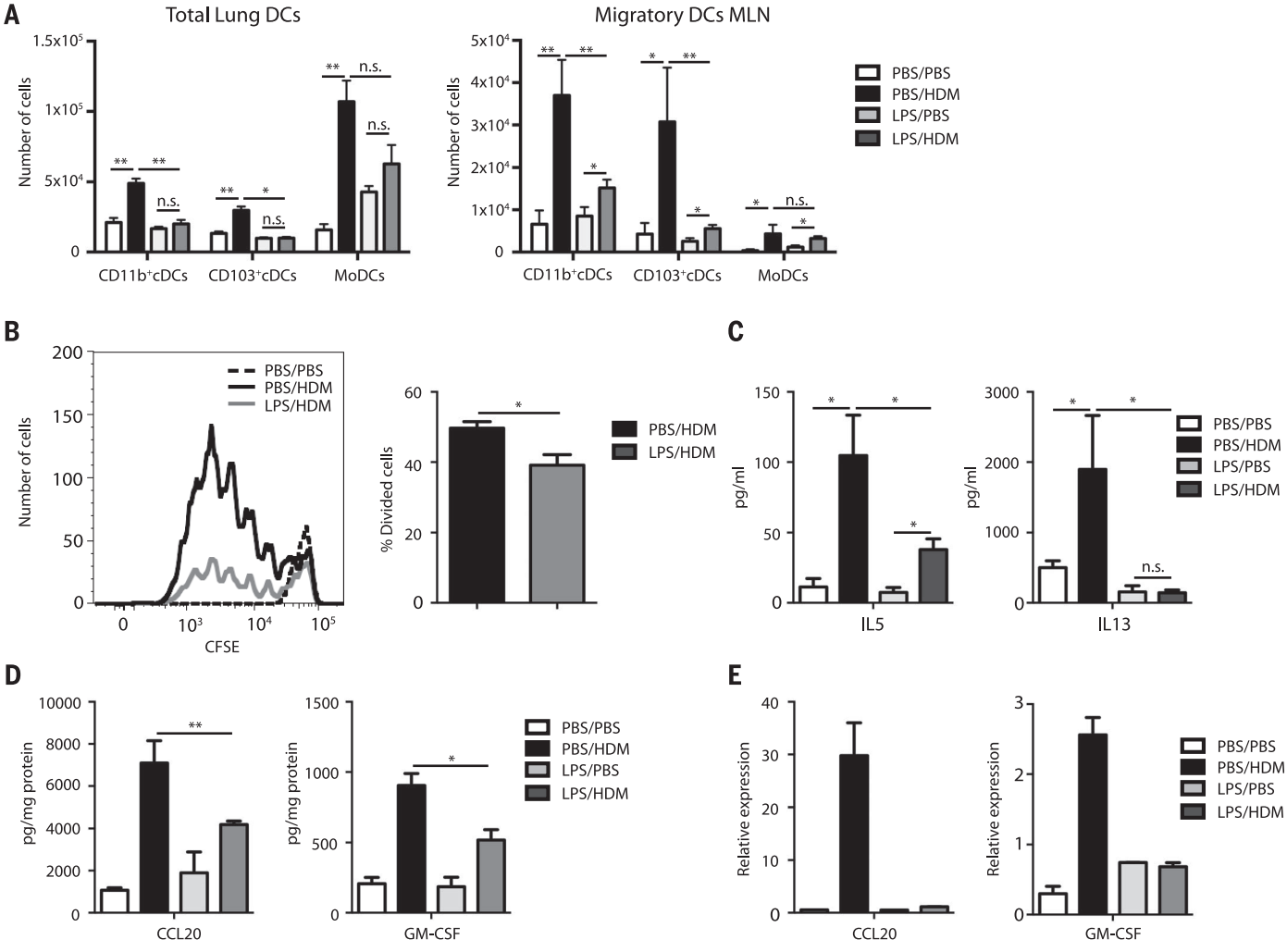

Fig. 2. Mechanism of suppression of asthma by chronic low-dose LPS inhalation. Mice received a chronic low dose of LPS or control PBS before sensitization to HDM extract. Some mice were sham-sensitized to PBS. (**A**) Recruitment of DC subsets was measured in the lungs and draining MLNs 24 hours after administration of HDM extract. (**B**) Proliferation of adoptively transferred 1-DER T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), measured 3 days after a single injection of HDM. Some mice received protective low-dose LPS or control PBS, and some were sham-sensitized. (**C**) Cytokine production from the MLNs of mice receiving 1-DER T cells, collected 3 days after HDM injection. (**D**) Chemokine and cytokine levels measured in lung homogenates taken 2 hours after HDM sensitization. Some mice received protective low-dose LPS or control PBS, and some mice were sham-sensitized. (**E**) mRNA levels of the same chemokines and cytokines measured in epithelial cell adhesion molecule (EpCAM$^+$) lung ECs sorted 2 hours after HDM sensitization. Mice were treated as described in (D). Data are representative of two [(A) to (D)] and four (E) independent experiments with n = 5 mice per group [(A) to (D)] and n = 6 to 8 mice per group (E). In all panels, error bars indicate SEM. P values reflect the Mann-Whitney U test: *P < 0.05, **P < 0.01. n.s., not significant.

multiphase, population-based survey of the farm effect on asthma and allergic disease in children aged 6 to 12 years in four central European countries. Detailed data on asthma, farming exposure, and specific IgE levels have been collected from a random sample of 1707 children. From a previous genome-wide association study (GWAS), the exonic single-nucleotide polymorphism (SNP) rs2230926 and the intronic SNP rs610604 were available (*23*). We found that SNP rs2230926 (T>G), which resulted in a phenylalanine-to-cysteine switch at amino acid position 127 (Phe^{127}→Cys^{127}) in exon 3 of *TNFAIP3*, was associated with increased risk of asthma [odds ratio (OR) = 1.76] and eczema (OR = 2.18) across the entire sample population. We also noticed that there was a gene-by-environment interaction for this SNP with farming: the protective effect that growing up on a farm exerted against asthma was much stronger in children with the G allele [adjusted OR = 0.14 (0.05 to 0.39)] as compared to those with the T allele [OR = 0.73 (0.66 to 0.80); *P* for interaction = 0.030]. The rs2230926 SNP causes a mutation in the functional DUB domain in A20 and has been linked to several autoimmune disorders, such as systemic lupus erythematosus (*24*, *25*). The intronic SNP rs610604 was unrelated to the health outcomes.

The hygiene hypothesis states that the rise in allergy and asthma that has been observed in affluent countries since the Second World War is caused by reduced "infectious pressure" from the Western lifestyle environment (*26*). The mechanism behind this association has been linked to an imbalance in the immune system, favoring pathogenic T_H2 immunity (*27*, *28*) in the absence of counterbalancing T_H1 immunity, natural killer T cells, or regulatory T cells that are often induced by infections (*26*, *29*). We have provided evidence that environmental protective factors can also influence the threshold for allergen recognition, by suppressing the activation of ECs and DCs via induction of the ubiquitin-modifying enzyme A20. This regulatory mechanism is also seen in the gut, where colonizing microbiota induce the expression of A20 shortly after birth, thus dampening overt

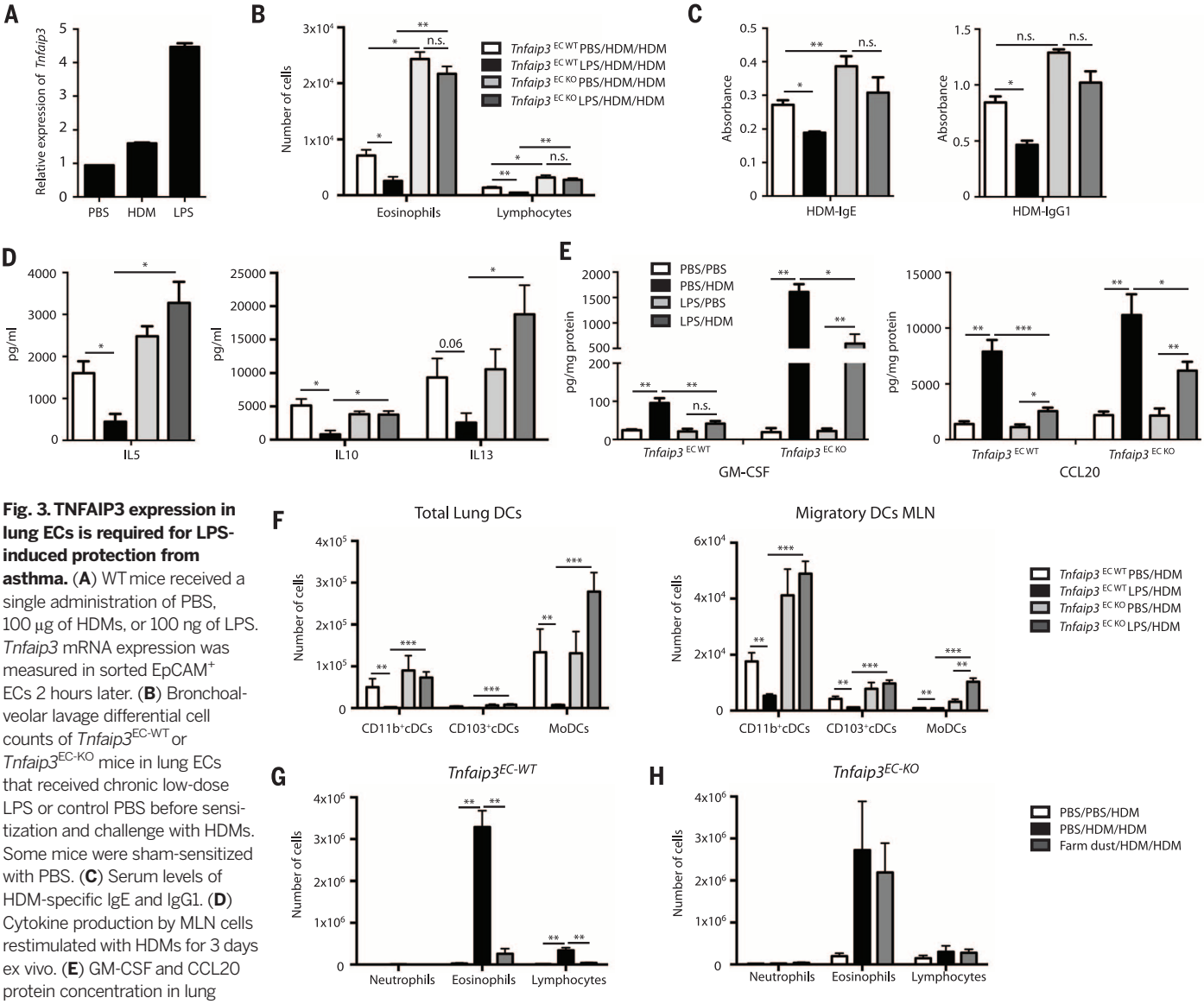

Fig. 3. TNFAIP3 expression in lung ECs is required for LPS-induced protection from asthma. (**A**) WT mice received a single administration of PBS, 100 μg of HDMs, or 100 ng of LPS. *Tnfaip3* mRNA expression was measured in sorted $EpCAM^+$ ECs 2 hours later. (**B**) Bronchoalveolar lavage differential cell counts of $Tnfaip3^{EC\text{-}WT}$ or $Tnfaip3^{EC\text{-}KO}$ mice in lung ECs that received chronic low-dose LPS or control PBS before sensitization and challenge with HDMs. Some mice were sham-sensitized with PBS. (**C**) Serum levels of HDM-specific IgE and IgG1. (**D**) Cytokine production by MLN cells restimulated with HDMs for 3 days ex vivo. (**E**) GM-CSF and CCL20 protein concentration in lung homogenates of mice preexposed to chronic LPS 14 days before a single HDM inhalation. (**F**) Mice received a chronic low dose of LPS or control PBS before sensitization to HDM extract. Some mice were sham-sensitized to PBS. Recruitment of DC subsets was measured in the lungs and draining MLNs 24 hours after the HDM extract was administered. (**G** and **H**) Bronchoalveolar lavage differential cell counts for $Tnfaip3^{EC\text{-}WT}$ (G) or $Tnfaip3^{EC\text{-}KO}$ (H) mice that received chronic low-dose farm dust or control PBS before sensitization and challenge with HDMs. Data are representative of two independent experiments with five to eight mice per group. In all panels, error bars indicate SEM. *P* values reflect the Mann-Whitney *U* test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. n.s., not significant.

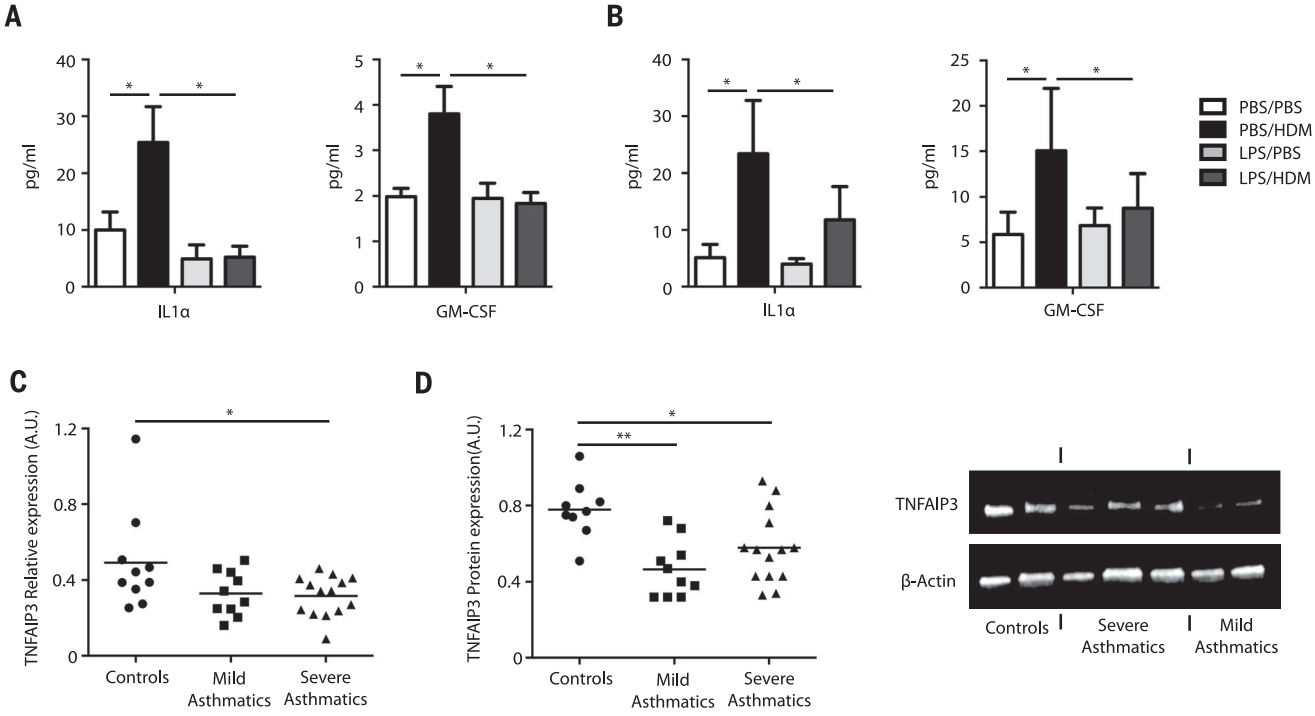

Fig. 4. Role of TNFAIP3 in human asthma. (**A**) Human IL-1α and GM-CSF concentrations were measured in ALI cultures set up from normal human bronchial ECs. Cells were exposed overnight to 100 ng of LPS or PBS. After 2 weeks, cells were stimulated with HDM extract or PBS, and cytokines were measured 24 hours later. (**B**) ALI cultures were also set up from ECs obtained by bronchial brushing of human asthmatics. These cultures were exposed to 100 ng of LPS for 1 week before stimulation with HDMs or PBS. Cytokines were measured 24 hours later. (**C** and **D**) Endobronchial biopsies were collected from healthy controls and from patients suffering from mild or severe asthma. Bronchial ECs were grown in ALI cultures, and mRNA levels of *TNFAIP3* were measured by qRT-PCR (C) and Western blot (D). A.U., arbitrary units. In (A) and (B), error bars indicate SEM. *P* values reflect the Mann-Whitney *U* test (A), the Friedman test with Dunn's post-test (B), and the Kruskall-Wallis test with Dunn's post-test [(C) and (D)]: $*P < 0.05$, $**P < 0.01$.

inflammation to commensals (*30*). Combined with the fact that a recent GWAS identified several SNPs in the TNFAIP3 interacting protein (TNIP-1) as associated with asthma, our finding that *TNFAIP3* SNPs are linked to asthma in children growing up on farms lends further support to the importance of this protective pathway (*31*, *32*). Future studies on the mechanism of the hygiene hypothesis should incorporate the effects of the environment on the activation threshold of structural cells of the airways, as they are often the drivers of innate immunity to allergens (*33*).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/349/6252/1106/suppl/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 and S2

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