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REFERENCES

1. GINA Science Committee. Global Strategy for Asthma Management and Prevention GINA . 2016. Available at: http://ginasthma.org/wp-content/uploads/2016/04/GINA-2016-main-report_tracked.pdf. Accessed April 19, 2017.
2. Mallol J, Crane J, von Mutius E, Odhiambo J, Keil U, Stewart A, et al. The International Study of Asthma and Allergies in Childhood (ISAAC) phase three: a global synthesis. *Allergol Immunopathol (Madr)* 2013;41:73-85.
3. Kuschnir MCC, Bloch KV, Szklo M, Klein CH, Barufaldi LA, Abreu Gde A, et al. ERICA: prevalence of metabolic syndrome in Brazilian adolescents. *Rev Saude Publica* 2016;50:11s.
4. Perez MK, Piedimonte G. Metabolic asthma: is there a link between obesity, diabetes, and asthma? *Immunol Allergy Clin North Am* 2014;34:777-84.
5. Cottrell L, Neal WA, Ice C, Perez MK, Piedimonte G. Metabolic abnormalities in children with asthma. *Am J Respir Crit Care Med* 2011;183:441-8.
6. Forno E, Han Y-Y, Muzumdar RH, Celedón JC. Insulin resistance, metabolic syndrome, and lung function in US adolescents with and without asthma. *J Allergy Clin Immunol* 2015;136:304-11.e8.
7. Bloch KV, Szklo M, Kuschnir MCC, Abreu Gde A, Barufaldi LA, Klein CH, et al. The study of cardiovascular risk in adolescents—ERICA: rationale, design and sample characteristics of a national survey examining cardiovascular risk factor profile in Brazilian adolescents. *BMC Public Health* 2015;15:94.
8. Lai CKW, Beasley R, Crane J, Foliaki S, Shah J, Weiland S, et al. Global variation in the prevalence and severity of asthma symptoms: phase three of the International Study of Asthma and Allergies in Childhood (ISAAC). *Thorax* 2009;64:476-83.
9. Alberti KGMM, Zimmet P, Shaw J. Metabolic syndrome—a new world-wide definition: a Consensus Statement from the International Diabetes Federation. *Diabet Med* 2006;23:469-80.

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Farm dust reduces viral load in human bronchial epithelial cells by increasing barrier function and antiviral responses



To the Editor:

Early-life respiratory infections caused by rhinoviruses (RVs) and/or respiratory syncytial virus can lead to recurrent wheezing and constitute one of the most dominant risk factors for the development of asthma.¹ Multiple studies show that growing up on farms is associated with protection against allergies, rhinitis, and asthma.² Similarly, farm children also suffer less from virus-induced wheezing in the first years of life.² An increased susceptibility to RVs and respiratory syncytial virus could be related to a weakened epithelial barrier function facilitating viral penetration and/or reduced antiviral responses, such as, a lower IFN production.^{1,3} Interestingly, polymorphisms were identified in genes involved in regulation of the epithelial (barrier) function and linked to a higher susceptibility to develop allergies and asthma.⁴ Furthermore, epithelial cells isolated from patients with asthma demonstrate a defective barrier function,

characterized by a lower transepithelial electrical resistance, higher permeability, and reduced expression of adhesion molecules,⁵ suggesting that a reduced epithelial barrier function may also contribute to the pathophysiology of asthma.^{3,5} In the gut, epithelial recognition of commensal microbes by Toll-like receptors (TLRs) is crucial in maintaining intestinal homeostasis and integrity.⁶ We hypothesized that microbe-rich farm dust decreases susceptibility to RV infection in human airway epithelium by increasing barrier function, and maintaining mucosal tolerance.

All experimental procedures were performed as described in this article's Online Repository at www.jacionline.org. Farm dust extract pre-exposure (18 hours) of undifferentiated primary bronchial epithelial cells (PBECs) followed by RV1b infection caused a significant reduction in viral load at 48 hours postinfection compared with medium, as shown by viral RNA and titer (Fig 1, A). Despite lower viral load, farm dust exposure significantly increased expression of the antiviral IFN-stimulated gene viperin, whereas the expression of other IFN-stimulated genes was not significantly different (Fig 1, B; see Fig E1, A, in this article's Online Repository at www.jacionline.org). This effect could not be attributed to a significant enhancement of production of IFN- α , IFN- β , and IFN- λ by farm dust in RV-infected cells (Fig E1, B), nor to increased responsiveness of farm dust-treated cells to IFN as shown by the inability of farm dust to modulate IFN- β -induced expression of viperin mRNA (Fig E1, C). Interestingly, induction of transcriptional activator IFN regulatory factor (IRF)-1, which regulates viperin expression via IFN-independent pathways,⁷ was increased by farm dust exposure (Fig 1, B). These findings suggest that enhanced viperin expression during treatment of infected cells with farm dust is independent of IFN pathways and mediated by IRF-1 (Fig 1, B).⁷ In addition to enhanced antiviral defenses, the reduction in viral load upon farm dust treatment may also be explained by the reduced binding and subsequent receptor-mediated endocytosis of RV1b. This is supported by our finding of decreased cell-associated RV1b RNA directly after viral exposure in farm dust-treated cells (Fig 1, C), which may have resulted from an increased barrier resistance. Indeed, after 2 hours of farm dust exposure, we observed a dose- and time-dependent increase in resistance (see Fig E2, A, in this article's Online Repository at www.jacionline.org), reaching a maximal 1.7-fold increase compared with medium. In intestinal epithelium, TLR2 was identified to modulate the formation of intercellular junctions.⁶ Similarly, we observed that TLR2-ligand Pam3CSK4 also increased resistance in PBECs to a similar extent as farm dust (Fig E2, A). These observations were supplemented by a reduced transport of macromolecules (fluorescein isothiocyanate-dextran) across the epithelial layer (Fig 1, D, and Fig E2, B), indicating that farm dust exposure affects both ionic and macromolecular permeability. Interestingly, while Pam3CSK4 strongly increased barrier resistance, the TLR4-ligand LPS did not affect permeability (Fig E2, C). This inability may result from the absence of MD-2 in human airway epithelial cells and the lack of TLR4-mediated signaling. We next studied farm dust in the context of recovery from epithelial injury by exposing fully differentiated air-liquid interface (ALI) PBEC cultures to cigarette smoke followed by farm dust. The initial decrease in barrier activity observed at 1 hour after cigarette smoke exposure could not be prevented by farm dust, but recovery was enhanced as shown by an increased transepithelial electrical resistance at 4 hours (Fig E2, D). Thus, farm dust not only improves airway epithelial barrier

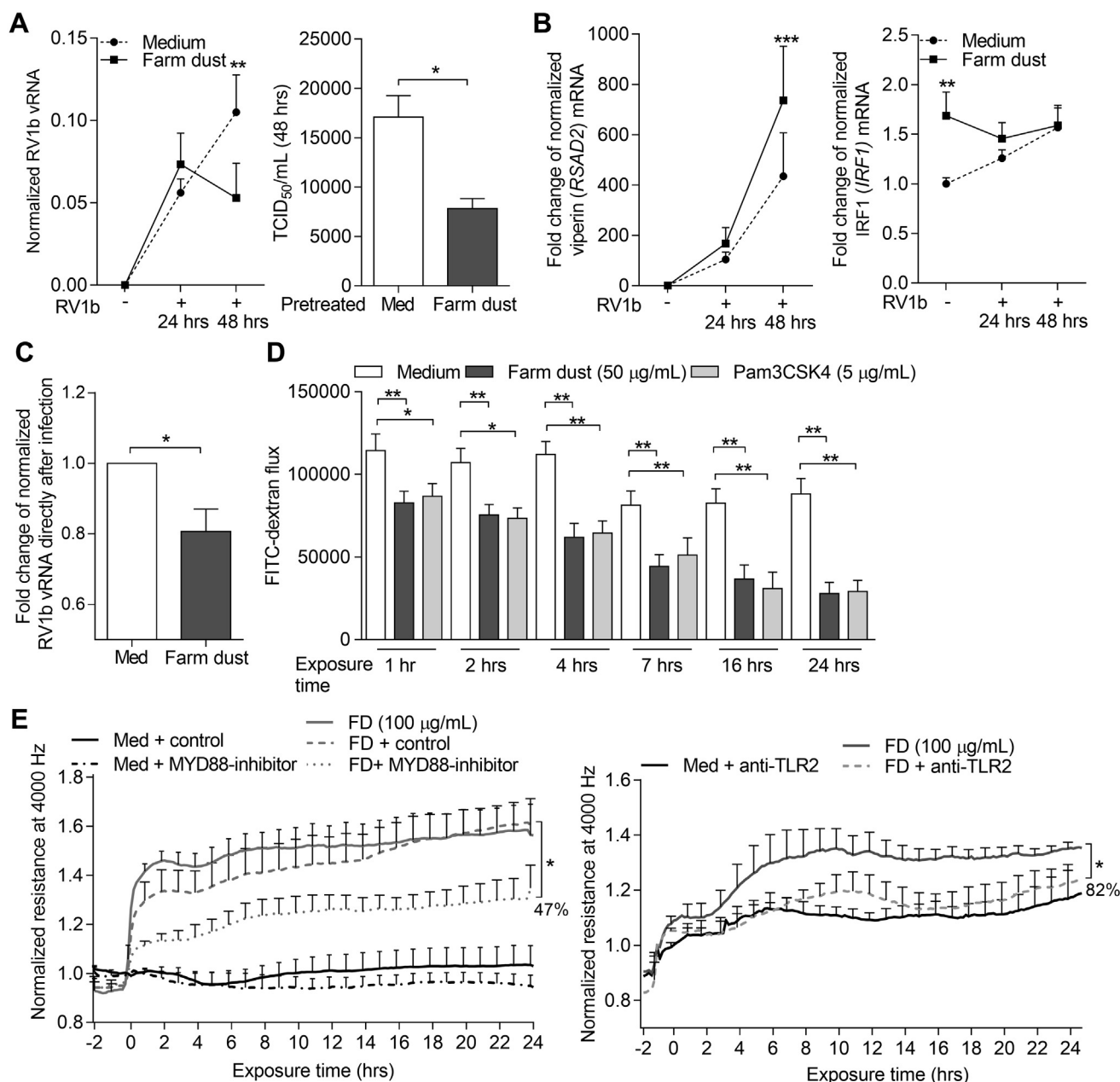


FIG 1. Farm dust reduces viral infection of bronchial epithelial cells: role of farm dust-induced antiviral response and barrier-enhancing effect. Viral RV1b copy numbers (vRNA) in the cell lysate and the TCID₅₀/mL in the supernatant after farm dust pre-exposure (**A**) ($n = 4$, Mann-Whitney test). **B**, Viperin and IRF-1 mRNA expression in farm dust-treated and RV1B-infected cells set at 1 before infection ($n = 5$, Sidak multiple comparisons test). **C**, vRNA expression directly after RV1b infection ($n = 4$, Mann-Whitney test). **D**, After exposure, FITC-dextran was applied apically and fluorescence in basal medium was measured after 1 hour ($n = 5$, Wilcoxon matched pairs test). **E**, Anti-hTLR2 antibodies, MyD88-inhibitor, or Pepinh-control was mixed with farm dust and applied to PBECs cultured in 8WE10 arrays to measure resistance (ECIS) (both $n = 4$ donors, 2-way ANOVA with Sidak multiple comparisons test). ECIS, Electric cell-substrate impedance sensing; FD, farm dust; FITC, fluorescein isothiocyanate; Med, medium; TCID, tissue culture infective dose. Data are shown as mean $\pm$ SEM.

function but also accelerates recovery of an epithelial layer following epithelial injury.

To investigate whether microbial components in farm dust improve the barrier function, we blocked MyD88, a downstream molecule in TLR signaling. An MyD88 inhibitor caused 70% versus 47% reduction in Pam3CSK4- or farm dust-induced

resistance compared with controls (Fig 1, E; see Fig E3, A, in this article's Online Repository at www.jacionline.org). Blood cells from farm children have increased TLR expression, of which TLR2 and CD14 showed the strongest association with farming.² In line with this, farm dust increased TLR2 mRNA expression in PBECs, although to a lesser extent than in Pam3CSK4-exposed

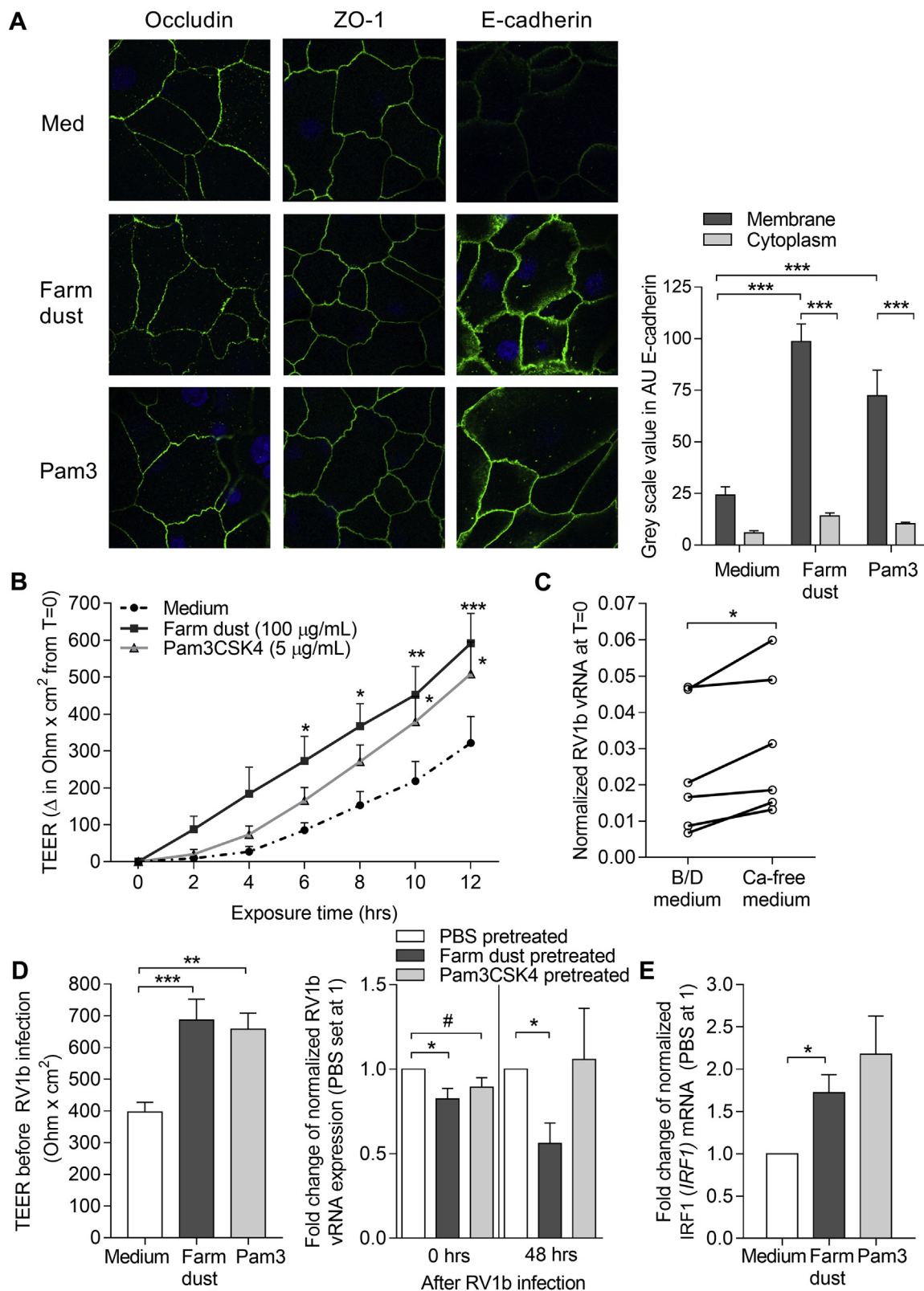


FIG 2. Enhanced E-cadherin-mediated barrier function after farm dust exposure reduces viral load in differentiated PBECS. **A**, Expression of AJC using confocal microscopy. The graph shows the gray value of E-cadherin in the cell-cell junction area over the intensity in the cytoplasm ($n = 5$). **B**, TEER of ALI-PBECs was reduced to less than 200 Ohm $\times$ cm² ($T = 0$) using calcium-free medium. TEER was measured every 2 hours in the presence of stimuli. Data are depicted as difference in TEER compared with the TEER at $T = 0$ ($n = 5$, 2-way ANOVA with Dunnett multiple comparisons test). **C**, ALI-PBECs were infected with RV1b after calcium- or mock-treatment (B/D medium). vRNA was measured from lysed cells ($n = 6$, Wilcoxon matched pairs test). **D**, Calcium-treated PBECs were incubated with farm dust until the TEER was significantly higher compared with PBS-treated samples. The cells were lysed for RV1b vRNA directly (0 hour) and 48 hours postinfection (48 hours). **E**, IRF-1 analysis directly after RV1b infection. PBS-treated samples were set at 1 ($n = 5$ -6, Wilcoxon matched pairs test). **AJC**, Apical junctional complex; **Med**, medium; **TEER**, transepithelial electrical resistance. Data are shown as mean $\pm$ SEM.

cells (Fig E3, B). Blocking TLR2 by antibodies resulted in an 81% reduction in farm dust–induced resistance, which was similar for Pam3CSK4 conditions (Fig 1, E, and Fig E3, C). Next to TLRs, the ubiquitin-editing enzyme A20 was implicated as an important player in farm dust–mediated protection against asthma.⁸ However, because A20 mRNA was not induced here, our data do not suggest a role for A20 in farm dust–induced protection against RV infections or increased barrier function (see Fig E4 in this article's Online Repository at www.jacionline.org). Instead, they rather point to a protective effect of a TLR2 agonist in farm dust, which also links to associations with TLR2 polymorphisms found in asthma and other allergic diseases.⁹

Next, we evaluated the effect of farm dust on apical junctional complex proteins in undifferentiated PBECs, in which intercellular junctions are relatively less well established compared with ALI-PBECs. Farm dust increased the gene expression of tight junction occludin and adherens junction E-cadherin after 2 hours, but only increased the translocation of E-cadherin to the cell-cell contact regions (Fig 2, A; see Fig E5 in this article's Online Repository at www.jacionline.org). To assess whether E-cadherin contributes to the enhanced barrier activity, ALI-PBECs were transferred to calcium-free medium to disrupt the barrier integrity by the dissociation of (calcium-dependent) E-cadherin from the apical junctional complex. Stepwise junctional reassembly was measured after adding back calcium-containing medium. This approach was taken because the high epithelial barrier activity of intact well-differentiated ALI-PBECs could not be further enhanced by farm dust or Pam3CSK4 (data not shown). In the presence of farm dust or Pam3CSK4, the recovery of the barrier function was significantly accelerated compared with PBS, indicating that E-cadherin contributes to the farm dust–induced barrier resistance (Fig 2, B). Interestingly, disruption of epithelial barrier function of ALI-PBECs using calcium depletion before RV1b exposure enhanced viral RV1b RNA levels (Fig 2, C). Farm dust accelerated the repair of this disrupted barrier, which was accompanied by a decreased viral load, suggesting that an increased barrier function reduces the passage of RV and thereby limits receptor-mediated endocytosis by, for example, basal cells (Fig 2, C and D). Because farm dust induces IRF-1, but does not affect IFN- α , IFN- β , or IFN- λ in both undifferentiated and differentiated PBECs, this suggests that IRF-1 may contribute to the effect of farm dust in controlling RV1b infections once the virus has entered the cell (Fig 2, E; see Fig E6 in this article's Online Repository at www.jacionline.org), although the exact signaling pathways remain to be elucidated.

Collectively, our data show that farm dust exposure reduces RV infection in primary epithelial cells, which is related to its barrier-enhancing effect and IFN-independent induction of antiviral responses. These findings may explain the protective farm effect against the development of asthma in epidemiological studies.

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REFERENCES

1. A.S.FeldmanY.HeM.L.MooreM.B.HershensonT.V.HartertToward primary prevention of asthma: reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthmaAm J Respir Crit Care Med19134-44.
2. E.von MutiusThe microbial environment and its influence on asthma prevention in early lifeJ Allergy Clin Immunol137680-9.
3. H.H.SmitsL.E.van der VlugtE.von MutiusP.S.HiemstraChildhood allergies and asthma: new insights on environmental exposures and local immunity at the lung barrierCurr Opin Immunol4241-7.
4. F.MoheimaniA.C.HsuA.T.ReidT.WilliamsA.KicicS.M.StickThe genetic and epigenetic landscapes of the epithelium in asthmaRespir Res17119.
5. S.N.GeorasF.RezaeeEpithelial barrier function: at the front line of asthma immunology and allergic airway inflammationJ Allergy Clin Immunol134509-20.
6. M.T.AbreuToll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal functionNat Rev Immunol10131-44.
7. K.J.HelbigM.R.BeardThe role of viperin in the innate antiviral responseJ Mol Biol4261210-9.
8. M.J.SchuijsM.A.WillartK.VergoteD.GrasK.DeswarteM.J.EgeFarm dust and endotoxin protect against allergy through A20 induction in lung epithelial cellsScience3491106-10.
9. M.S.KormannR.FerstlM.DepnerN.KloppS.SpillerT.IlligRare TLR2 mutations reduce TLR2 receptor function and can increase atopy riskAllergy64636-42.

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METHODS

Bronchial epithelial cell culture

Human PBECs were obtained from macroscopically normal bronchial tissues of patients undergoing resection surgery for lung cancer at the Leiden University Medical Center, The Netherlands. Only those patients with a prebronchodilator FEV₁/forced vital capacity ratio of 0.7 or more were included to exclude patients with chronic obstructive pulmonary disease (according to Global Initiative for Chronic Obstructive Lung Disease guidelines). Epithelial cells were obtained by enzymatic digestion, and expanded in culture.^{E1} PBECs at passage 2 (4000/well) were seeded on coated 8WE10 arrays for electric cell-substrate impedance sensing (ECIS) measurements, or 40,000 PBECs per well were seeded on coated semipermeable Transwell inserts with 0.4 μ m pore size (Corning Costar, Tewksbury, Mass) in 50% Dulbecco modified Eagle medium (Invitrogen) and 50% Bronchial Epithelial Cell Growth Medium (Lonza, Verviers, Belgium) (B/D) media supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, BEGM SingleQuot (all Lonza), 1 mM HEPES, 15 μ g/mL BSA (Sigma, St Louis, Mo), and 15 ng/mL retinoic acid (Lonza) at 37°C with 5% CO₂. After reaching confluence, the Transwell-seeded cells were either used as undifferentiated (submerged) PBECs or cultured at ALI for 2 to 2.5 weeks to allow mucociliary differentiation. Both undifferentiated and differentiated ALI-PBECs were exposed to different stimuli diluted in B/D medium including supplements as described earlier without additional retinoic acid supplementation.

Farm dust collection and extraction

Farm dust was collected from the stables of traditional German farms 50 km around Munich, which mainly housed cows. Farm dust was stored at –80°C before use. Twenty milliliter of isotonic saline solution (0.9% NaCl in water [w/v]) was added to 1 g of farm dust and shaken for 2 hours at 1000 rpm at room temperature (RT). Next, the samples were centrifuged at 2500g for 5 minutes at RT. The supernatant was filtrated through cellulose filters to remove insoluble material, filtered through a polyethersulfone 0.22- μ m membrane, and dialyzed for 24 hours against sterile water using a cellulose ester dialysis membrane from Spectra/Por (MWCO 3,500, Breda, The Netherlands). After dialysis, sucrose was added (final concentration 5% w/v) to stabilize proteins during the subsequent freeze-drying process and a second sterile filtration was performed using a 0.22- μ m polyethersulfone membrane. Under sterile conditions, 3 mL of the resulting farm dust extract was filled into a 5-mL glass vial and freeze-dried. The lyophilized farm dust extract was stored at 5°C. Upon reconstitution with sterile water, the samples were aliquoted in 10- to 50- μ L portions and stored at –80°C. Farm dust extract aliquots were thawed only once and the remainders were discarded. The indicated concentrations were based on the weight (in mg) of the original farm dust samples. The optimal concentration was determined for each batch using the ECIS assay (optimal concentration for all batches varied between 50 and 200 μ g/mL). Throughout this article, the term “farm dust” refers only to the water-soluble components of the farm dust extract.

Assessment of barrier function

For ECIS measurements (Applied Biophysics, Troy, NY), 8WE10 ECIS arrays, containing integrated gold-film electrodes, were coated with PureCol (30 μ g/mL, Advanced BioMatrix, Carlsbad, Calif), fibronectin (10 μ g/mL, isolated from human plasma), and BSA (10 μ g/mL) diluted in PBS at 37°C for 2 to 24 hours. Passage 2 PBECs were grown to 80% confluence (mostly in 4 days) in 400 μ L B/D medium and the arrays were placed in the ECIS machine. Using a low frequency (4000 Hz), the current predominantly flows paracellularly and between adjacent cells (through tight junctions). When the resistance was stable and between 1000 and 1500 Ohm, B/D medium (300 μ L/well) was replaced 6 hours before the addition of different stimuli. Hundred milliliter medium with farm dust (12.5–200 μ g/mL) or Pam3CSK4 (5 μ g/mL, Invivogen, San Diego, Calif) as positive control was applied (in duplicate) to minimize disturbance in the resistance measurements. Resistance was recorded every 10 minutes for 24 hours. In 15% of the donors, no barrier-enhancing effect of Pam3CSK4 (and also of farm dust) was observed, and the results from these experiments were

therefore not included in the analysis of the results. Anti-hTLR2 antibodies (10 μ g/mL, Invivogen), MyD88-inhibitor (5 μ M pepinh-MYD, Invivogen), or Pepinh-control (5 μ M) was mixed with farm dust or Pam3CSK4 in solution, and next 100 μ L/well was applied to the undifferentiated PBECs cultured in 8WE10 arrays. Pilot studies showed that at 5 μ M, the MyD88 inhibitor showed minimal nonspecific inhibition and cell death (data not shown).

To assess transport of macromolecules, undifferentiated PBECs were exposed apically to farm dust dissolved in 500 μ L medium. After 1 to 24 hours of exposure, farm dust was removed and 1 mg/mL fluorescein isothiocyanate (FITC)-dextran (4 kDa, Sigma-Aldrich, St Louis, Mo) was applied apically. After 1 hour, FITC-dextran levels (485/525 nm) were measured in the basal medium using Wallac 1420 VICTOR2 (PerkinElmer, Mass). The FITC-dextran assay could not be applied to ALI-PBECs because no leakage is measured because of a high barrier resistance, and this resistance was not affected by Pam3CSK4 or farm dust exposure. To be able to evaluate effects of farm dust on differentiated epithelial cells, we conducted barrier-decreasing treatments with ALI-PBECs such as cigarette smoke (CS) exposure and the calcium-switch model.

CS exposure

ALI-PBECs were exposed to air or to whole CS generated from 3R4F reference cigarettes for 5 minutes (University of Kentucky, Lexington, Ky) as described in detail by Amatngalim et al.^{E2} After the exposure, farm dust or Pam3CSK4 in PBS (500 μ L/well) was applied apically and transepithelial electrical resistance (TEER) was measured hourly using an electrometer EVOM2 (World Precision Instruments, Sarasota, Fla). The TEER measurement before exposure was set at 0 (baseline) due to donor variation (845–1065 Ohm $\times$ cm²). Data are shown as the difference in Ohm $\times$ cm² between CS- and air-exposed cells compared with the baseline resistance (n = 4, 2-way ANOVA with Dunnett multiple comparisons test).

Calcium-switch model

Calcium-free S-MEM (Life Technologies, Carlsbad, Calif) was applied to ALI-PBECs to break down the integrity of the intercellular junctions until background levels (180–200 Ohm $\times$ cm²) were reached. Next, the basal medium was substituted by B/D medium and farm dust or Pam3CSK4 was applied apically. TEER was measured every 2 hours.

Human RV infection

RV1b was propagated in H1 HeLa cells (American Type Culture Collection, Manassas, Va). Viral titers in PBEC supernatants were determined by 50% tissue culture infective dose (TCID₅₀/mL) using HeLa cells. Before infection, all cells were placed in “infection” medium (B/D medium without hydrocortisone, bovine pituitary extract, epidermal growth factor, and bovine serum albumin) for 18 hours.^{E3} To which farm dust or Pam3CSK4 was added apically. PBECs were stimulated with IFN- β (25 IU/mL), or infected with RV1b (100 μ L apically) at a multiplicity of infection of 0.5 for 1 hour at RT. The RV1b-infected cells were washed to remove residual viral particles and these cultures were replenished with “infection” medium.

ALI-PBECs were first treated according to the “calcium-switch model” and the barrier function was reconstituted with B/D medium basally, and farm dust apically. After 8 to 10 hours, cells were infected with RV1b as described above. To reduce interdonor variability in the response to farm dust and to allow evaluation of an increased barrier activity to RV infection, only those cultures (6 out of 9 donors) showing an additional increase of 250 Ohm $\times$ cm² (range from 255 to 664 Ohm $\times$ cm²) after farm dust exposure compared with PBS were used.

Quantitative RT-PCRs

Total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Leiden, The Netherlands) and quantified using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Del). cDNA was generated from 1 μ g of RNA using M-MLV polymerase, oligo(dT) primers,

and RNAsin (all Promega) for each sample. Quantitative PCR assays were run on a CFX-384 Real-Time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) with use of specific primers (from Primerbank) and SensiFAST SYBR green (Bioline, Luckenwalde, Germany). Reference genes *RPL13A* and *ATP5B* were used for normalization. For arbitrary gene expression, the Bio-Rad CFX manager 3.0 software was used according to the relative standard curve method.

Immunofluorescence

Immunofluorescent labeling of cells was performed by rinsing the PBEC-containing membranes with PBS, followed by fixing for 15 minutes in 3.7% paraformaldehyde. Next, the cells were washed with PBS and fixed for 10 minutes in cold methanol at 4°C. After blocking the filters with 1% BSA 0.3% Triton-X-100 in PBS (PBT) for 10 minutes at RT and washing with PBS, the membranes with PBECs were detached from the Transwell using a razor blade and transferred to 24-well tissue plates for antibody staining. The cells were first incubated with the primary antibody (1 hour, RT) in PBT, washed with PBS, and followed by a second incubation (30 minutes, RT) with 4'-6-diamidino-2-phenylindole, dihydrochloride and donkey antimouse IgG secondary AlexaFluor488 antibody (ThermoFisher, Waltham, Mass). Next, the PBEC-containing membranes were placed on coverslips and were mounted with Vectashield Mounting Medium (Vector Labs, Burlingame, Calif) and immunofluorescence was measured using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). To quantify the fluorescence intensity, confocal settings were optimized for the farm dust

extract-treated samples for each individual donor using 63× original magnification with 3× zoom. Confocal images were processed using the Leica Application Suite Advanced Fluorescence software (Leica Microsystems). The gray value of E-cadherin in the cell-cell junction area over the intensity in the cytoplasm was determined from 2 Transwell inserts per stimulus using ImageJ. For every picture, the gray value of the membrane and cytoplasm of 4 cells was analyzed.

Statistical analysis

Statistical analysis was performed in GraphPad prism (La Jolla, Calif). Data are represented as mean $\pm$ SEM, and *P* value of less than .05 was considered statistically significant.

REFERENCES

- E1. Mertens TC, Hiemstra PS, Taube C. Azithromycin differentially affects the IL-13-induced expression profile in human bronchial epithelial cells. *Pulm Pharmacol Ther* 2016;39:14-20.
- E2. Amatngalim GD, van Wijck Y, de Mooij-Eijk Y, Verhoosel RM, Harder J, Lekkerkerker AN, et al. Basal cells contribute to innate immunity of the airway epithelium through production of the antimicrobial protein RNase 7. *J Immunol* 2015;194:3340-50.
- E3. Porter JD, Watson J, Roberts LR, Gill SK, Groves H, Dhariwal J, et al. Identification of novel macrolides with antibacterial, anti-inflammatory and type I and III IFN-augmenting activity in airway epithelium. *J Antimicrob Chemother* 2016;71:2767-81.

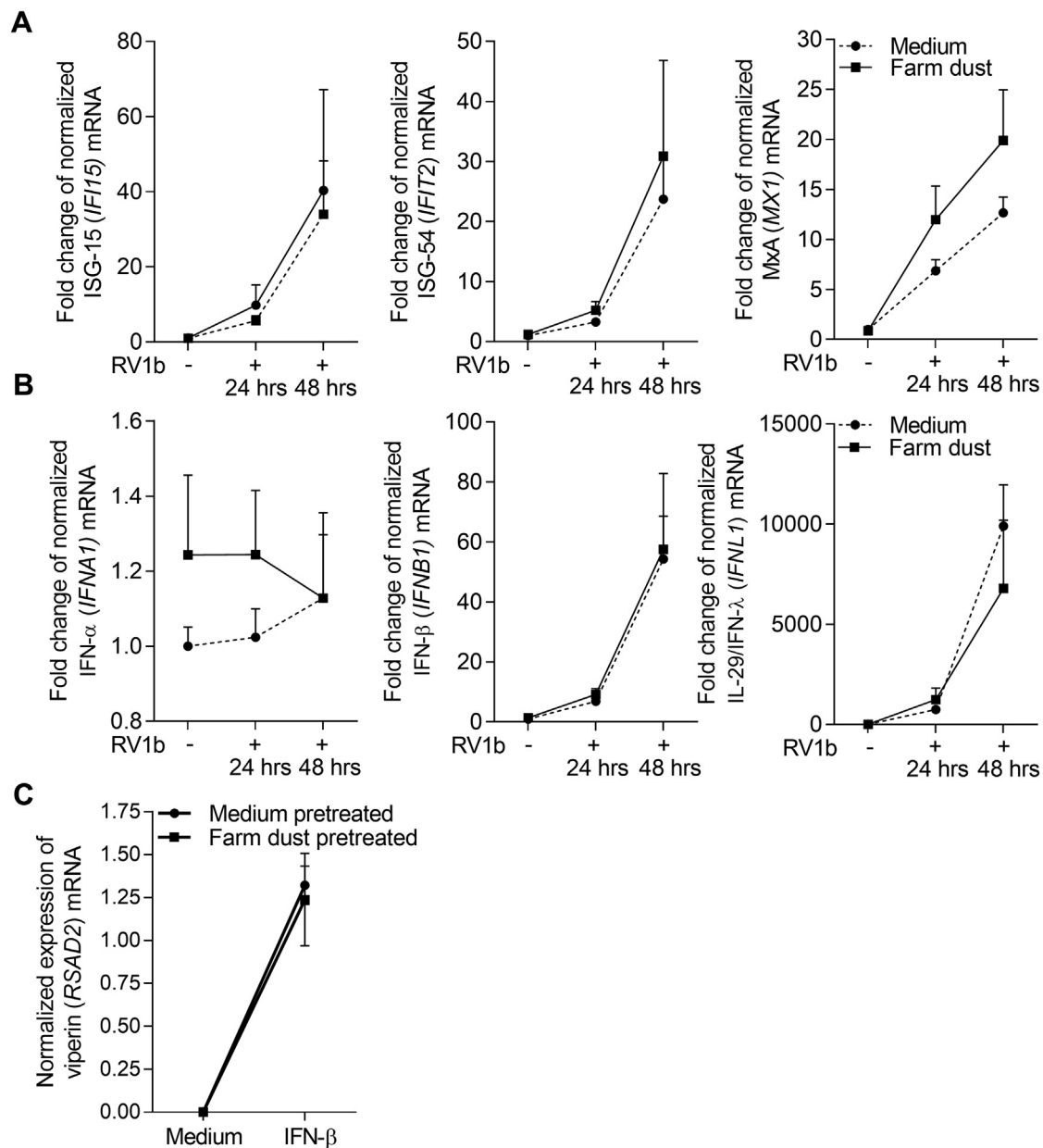


FIG E1. Pre-exposure to farm dust enhances antiviral responses. **A**, Undifferentiated PBECs were exposed apically to 500 μ L “infection” medium or medium containing farm dust (100 μ g/mL) for 18 hours in duplicate. The cells were washed and infected apically with RV1b (100 μ L/well) at multiplicity of infection 0.5 for 1 hour at RT. The cells were lysed 24 or 48 hours postinfection to analyze ISG-15, ISG-54, and MxA mRNA expression after 24 and 48 hours postinfection. The mRNA expression before infection was set at 1 ($n = 5$ donors, Sidak multiple comparisons test). **B**, Expression of IFN- α , IFN- β , and IFN- λ mRNA. **C**, Undifferentiated PBECs were exposed to farm dust apically for 24 hours. Next, the cells were washed and stimulated with 100 μ L/well IFN- β (25 IU/mL, in PBS) for 48 hours. Data shows viperin mRNA expression ($n = 2$ donors, Student t test). ISG, IFN-stimulated gene. Data are shown as mean $\pm$ SEM.

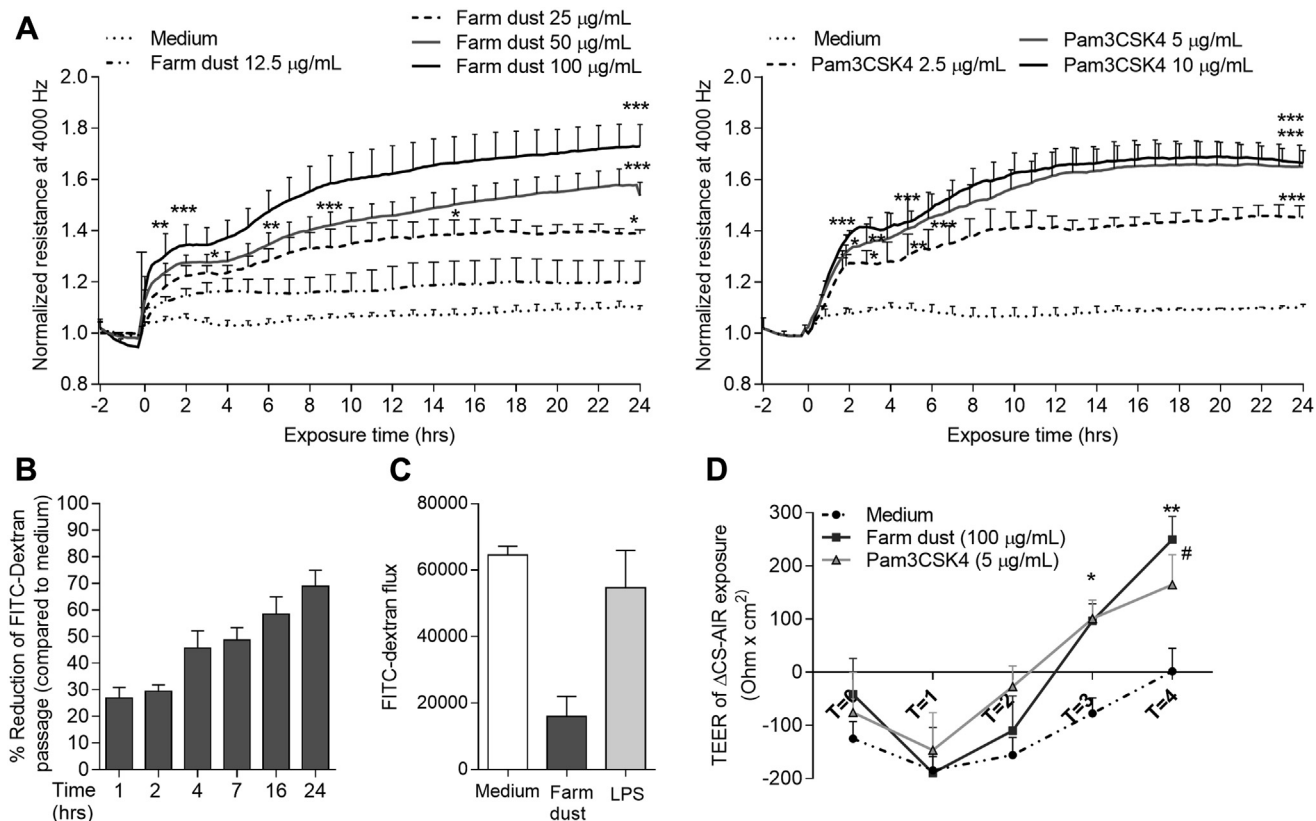


FIG E2. Farm dust increases barrier function in undifferentiated bronchial epithelial cells. PBECs cultured in 8WE10 arrays were exposed to farm dust (12.5–100 $\mu\text{g/mL}$) or Pam3CSK4 (2.5–10 $\mu\text{g/mL}$) in duplicate (**A**). Resistance was recorded every 10 minutes for 24 hours using the ECIS. Significant differences are shown in the graph as $*P < .05$, $**P < .01$, and $***P < .001$ ($n = 3$ –7 donors, 2-way ANOVA with Dunnett multiple comparisons test). **B**, Undifferentiated PBECs were incubated with farm dust or medium (control) in duplicate for 1, 2, 4, 7, 16, and 24 hours. Farm dust or medium was removed and 500 μL FITC-dextran (1 $\mu\text{g/mL}$) was applied apically for 1 hour. Next, the fluorescence was measured in basal medium samples. The fluorescence intensity of the farm dust-treated samples was divided by the medium-treated samples for each time point. Data are shown as percentage in reduction of fluorescence intensity (485/525 nm) after farm dust treatment compared with medium-treated samples ($n = 5$ donors). **C**, PBECs were incubated as described in Fig E2, **B**, in the presence of 10 $\mu\text{g/mL}$ LPS ($n = 2$ donors). **D**, CS-exposed ALI-PBECs were incubated with farm dust or Pam3CSK4 and TEER was measured every hour. Data are shown as the difference between CS- and air-exposed cells compared with the baseline resistance ($n = 4$, 2-way ANOVA with Dunnett multiple comparisons test). Data are shown as mean $\pm$ SEM.

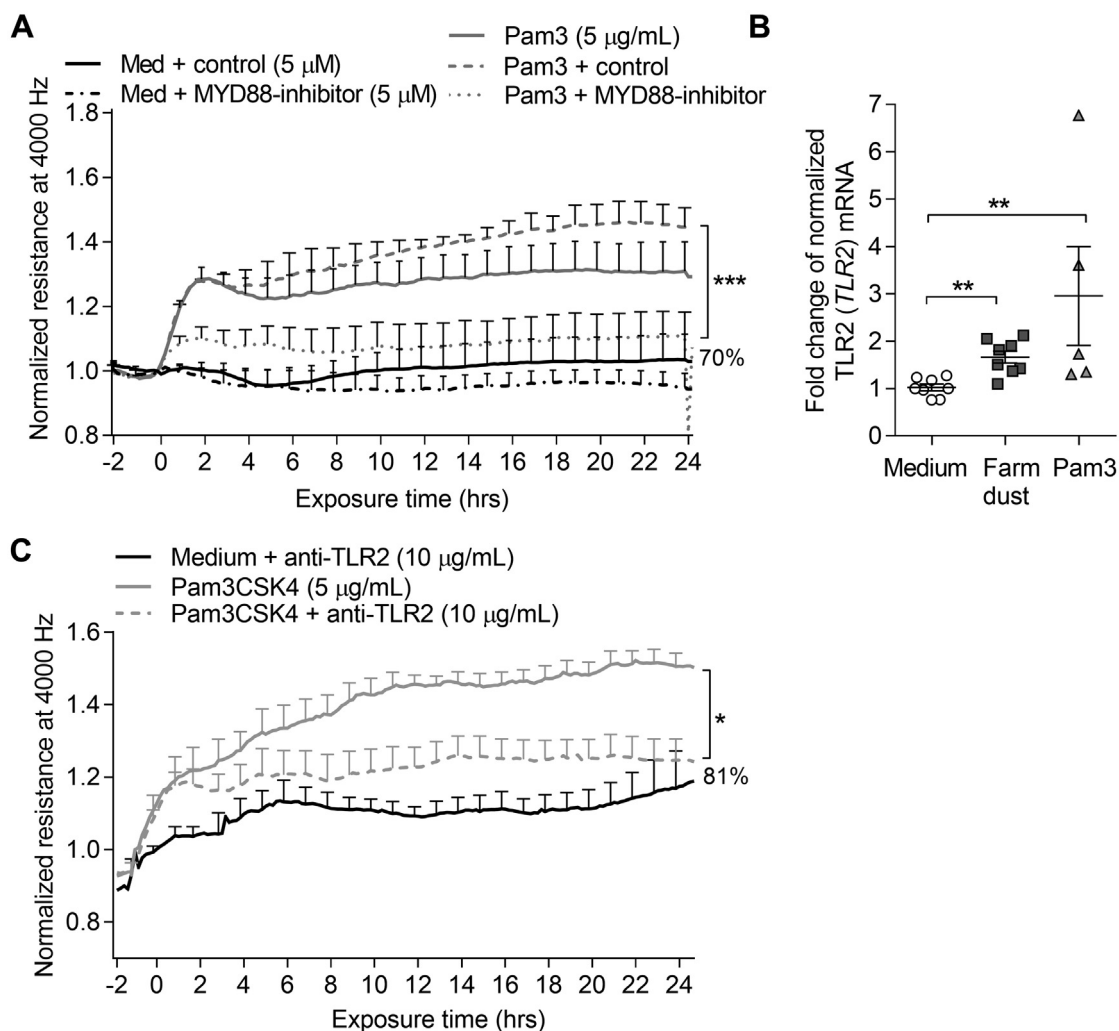


FIG E3. TLR2 signaling in farm dust is important for the enhanced barrier function. **A**, The MyD88-inhibitor (5 μ M pepinh-MYD, Invivogen) or Pepinh-control (5 μ M) was mixed with Pam3CSK4 (Pam3) in solution, and next 100 μ L/well was applied to the undifferentiated PBECs cultured in 8WE10 arrays. Pilot studies showed that at 5 μ M, the MyD88 inhibitor showed minimal nonspecific inhibition and cell death (data not shown). The resistance over time was recorded using the ECIS. 2-way ANOVA with Sidak multiple comparisons test was used (both $n = 4$ donors). **B**, Undifferentiated PBECs were exposed to farm dust or Pam3CSK4 for 2 hours. The fold change of the expression of TLR2 mRNA after 2 hours of incubation is presented ($n = 8$ donors for medium- and farm dust-treated samples and $n = 5$ for Pam3CSK4). The 1-way ANOVA with Dunnett multiple comparisons test was used. **C**, Anti-hTLR2 antibodies (10 μ g/mL, Invivogen) were added to Pam3CSK4 and added to undifferentiated PBECs in the ECIS ($n = 4$ donors, 2-way ANOVA with Sidak multiple comparisons test). Data are shown as mean $\pm$ SEM.

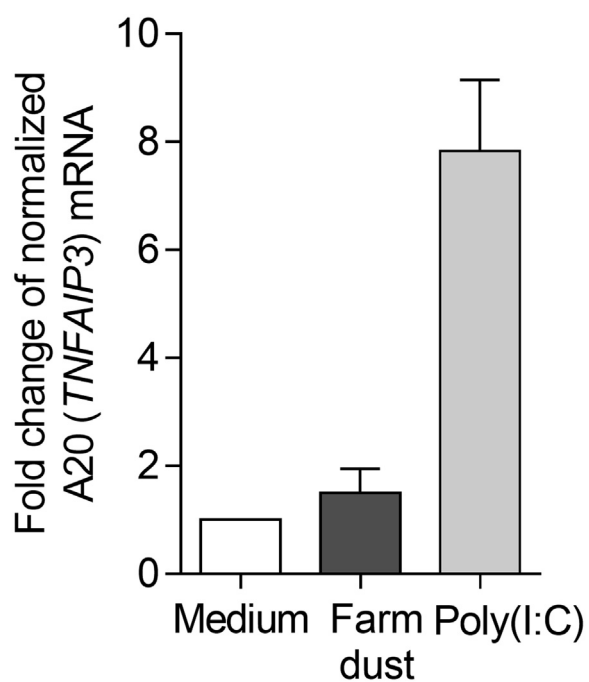


FIG E4. A20 gene expression in bronchial epithelial cells after farm dust exposure. Undifferentiated PBECs were incubated with farm dust, medium, or Poly(I:C) (20 μ g/mL) as control for 24 hours. The expression of A20 was measured using RT-quantitative PCR (n = 4 donors). Data are expressed as fold increase compared with medium (set at 1). Data are shown as mean $\pm$ SEM.

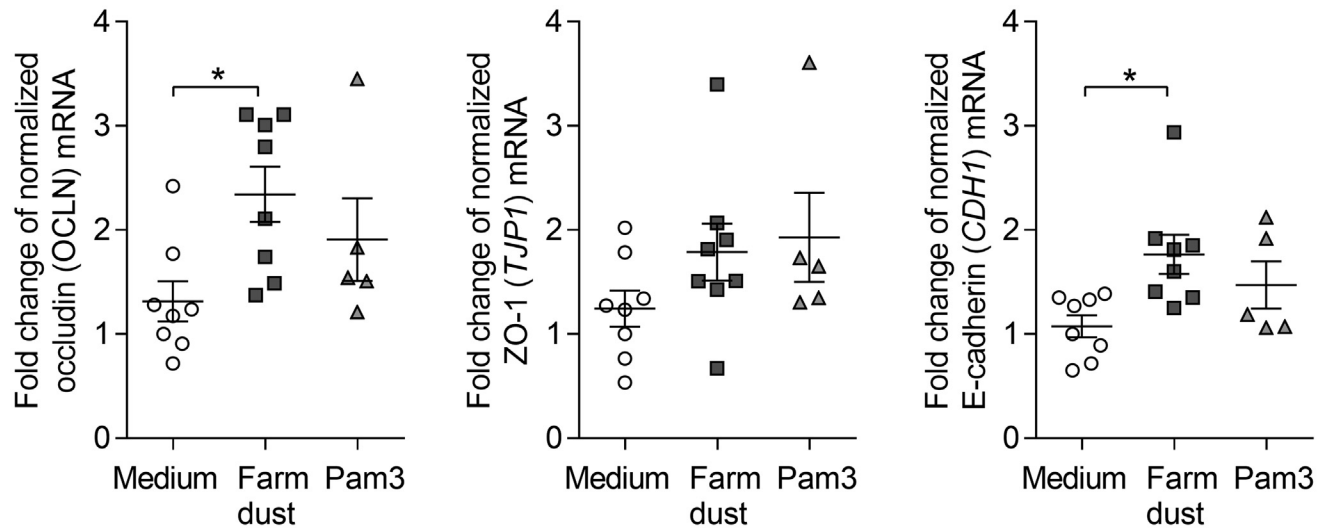


FIG E5. Farm dust affects the expression of apical junctional complex (AJC) proteins in bronchial epithelial cells. Occludin, ZO-1, and E-cadherin mRNA expression was assessed after 2 hours of medium (control), Pam3CSK4 (5 μ g/mL), or farm dust (50 and 100 μ g/mL) exposure. Data are expressed as fold change of normalized mRNA expression compared with $T = 0$ ($n = 8$ for medium- and farm dust-treated and $n = 5$ Pam3-treated samples, 1-way ANOVA Kruskal Wallis test). ZO-1, Zonula occludens-1. Data are shown as mean $\pm$ SEM.

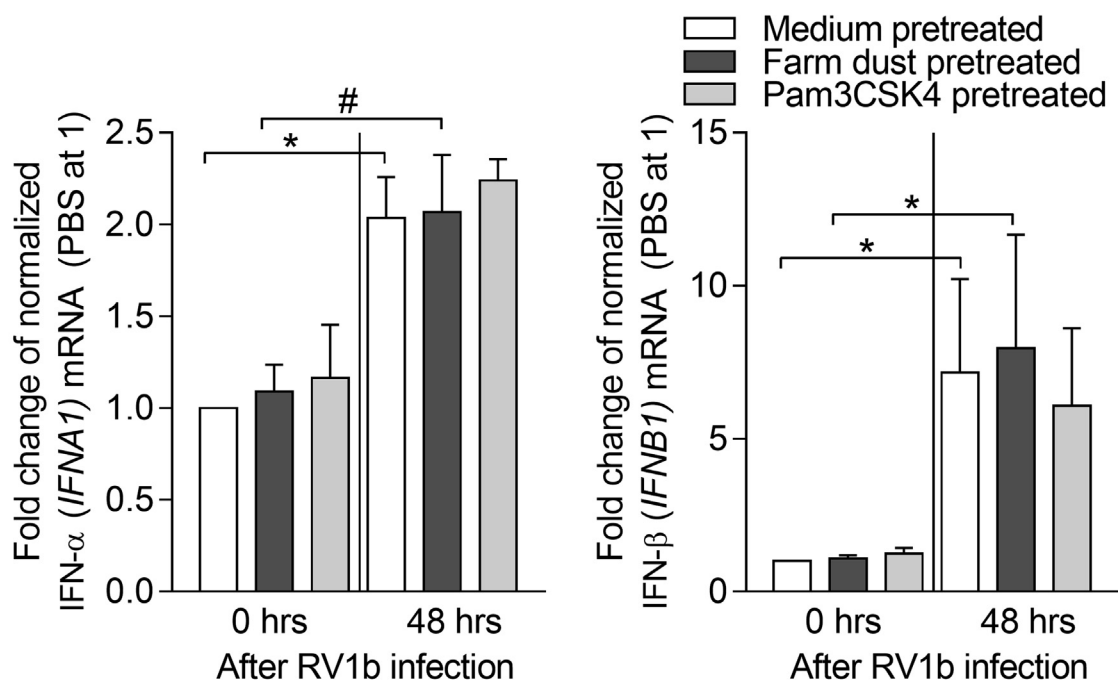


FIG E6. IFN responses are not affected by farm dust exposure. ALI-PBEC cultures were treated with calcium-free medium to disrupt barrier function. Next, calcium-containing medium was added basally to the culture and incubated apically with farm dust, Pam3CSK4, or PBS until the TEER was significantly higher in farm dust-treated samples compared with PBS-treated samples. At this time point, cells were infected with RV1b and cell lysates were obtained directly ($T = 0$) or 48 hours postinfection ($T = 48$ hours). Expression of IFN- α , IFN- β , and IFN- λ mRNA was obtained using quantitative PCR. IFN- λ was below detection limits (data not shown). PBS-treated samples were set at 1 ($n = 6$, Wilcoxon matched pairs test). Data are shown as mean $\pm$ SEM.