

Nucleotide Oligomerization Domain 1 Ligation Suppressed Murine Allergen-Specific T-Cell Proliferation and Airway Hyperresponsiveness

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Abstract

The cytosolic nucleotide oligomerization domain (NOD)-like receptors NOD1 and NOD2 are important contributors to the intracellular recognition of pathogens including *Chlamydomonas pneumoniae*, but little is known about their influence on allergen-induced airway inflammation. In BALB/c mice, we observed that infection with *C. pneumoniae* before systemic sensitization with ovalbumin (OVA) and local OVA airway exposure diminished airway hyperresponsiveness (AHR). Thus, the impact of the NOD1 agonist FK156 and the NOD2 agonist muramyl dipeptide given 6 hours before each sensitization or airway challenge was evaluated regarding AHR, OVA-specific plasma immunoglobulins, bronchoalveolar lavage fluid differentials, and cytokines. Spleen dendritic cells of FK156-treated mice were isolated and cocultured with OVA-specific T cells isolated from DO11.10 mice, and T-cell proliferation was quantified after OVA restimulation. T-cell proliferation was investigated *in vivo* in lungs and lymph nodes of FK156-treated and OVA-exposed DO11.10 mice. FK156, but not muramyl dipeptide, reduced AHR and pulmonary eosinophilic infiltration if given before OVA sensitization or challenge, whereas T-helper (Th)2 cytokines were not diminished. Dendritic cells from FK156-treated mice evoked less OVA-specific T-cell proliferation as

compared with solvent-treated controls. Similarly, antigen-specific T-cell activation in lung tissue was diminished after FK156 treatment. We conclude that NOD1 activation reduced AHR in allergen-induced lung inflammation, which was accompanied by a reduction of allergen-specific T-cell proliferation.

Keywords: nucleotide oligomerization domain 1; allergic airway inflammation; airway hyperresponsiveness; T-cell proliferation

Clinical Relevance

In the present study, we observed that nucleotide oligomerization domain (NOD)1 ligation is able to recapitulate protective effects of *Chlamydomonas pneumoniae* lung infection on allergen-induced airway hyperresponsiveness. Moreover, NOD1 activation reduced pulmonary eosinophilic influx and suppressed allergen-specific T-cell proliferation *in vitro* and *in vivo*. Because systemic NOD1 activation with the synthetic ligand FK156 was beneficial even in the presence of established allergen sensitization, these data may have therapeutic implications.

Allergic asthma is a common, multifactorial respiratory disorder characterized by an inadequate T-helper (Th)2-driven immune

response resulting in eosinophilic airway inflammation, mucus secretion, and bronchoconstriction. Exposure to infections

can promote and prevent the development of allergen-induced airway inflammation. Ligation of specific pattern recognition

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receptors (PRRs) that play a major role in pathogen recognition and initiation of inflammatory and immune responses (1) can alter allergen-induced airway inflammation (2). Whereas administration of various Toll-like receptor agonists has been shown to prevent or attenuate allergic airway inflammation (3–6), the same agonists may cause the opposite effects and induce or enhance allergic responses, depending on the experimental conditions and timing (7–10).

Nucleotide-binding oligomerization domain (NOD) proteins have been implicated in intracellular pattern recognition (11, 12) and may play a role in allergen-evoked Th2 responses (13–16). NOD1 and NOD2 recognize bacterial peptidoglycans. Whereas NOD1 is activated by mesodiaminopimelic acid (meso-DAP) expressed by most gram-negative bacteria, NOD2 is activated by muramyl dipeptide (MDP) conserved in peptidoglycans of basically all bacteria (17). MDP prevented the induction of oral (13) and airway (14) tolerance in mice. NOD2 has been identified as a PRR that can sense viral single-strand RNA and mediate antiviral defense mechanisms after infection with the respiratory syncytial virus (18), which has been linked to asthma susceptibility (19, 20).

NOD1 and NOD2 polymorphisms have been associated with the presence of asthma and elevated serum immunoglobulin E (IgE) levels, indicating that intracellular recognition of specific bacterial products might affect the presence of atopic diseases (15). Moreover, infections with *Chlamydomonas* spp., which are intracellularly recognized by NOD1 and NOD2 (21–23), may alter allergic responses. *Chlamydomonas pneumoniae* infection has been linked to the induction, exacerbation, and/or persistence of asthma (24–27), and *C. pneumoniae*-specific IgE has been associated with disease severity (28). Schröder and colleagues showed that *C. pneumoniae* infection may promote allergic sensitization to human serum albumin through dendritic cell (DC) activation. Airway sensitization depended on the timing of chlamydial infection relative to allergen challenge and the severity of infection. Low-dose infection and antigen exposure within 5 days of infection induced allergic sensitization, whereas high-dose infection or antigen exposure 10 days after infection did not

(29). Experimental data also revealed that exposure to *Chlamydomonas* may suppress the development of allergic responses (30) and that IL-10-producing DCs and natural killer cells play an important role in the infection-mediated inhibition of allergic responses (31). Thus, in the context of bacterial or viral infections, NOD1 and NOD2 might link allergen exposure to alterations of allergic airway disease.

In this study, we investigated whether NOD1 receptor stimulation can recapitulate the protective effects of a *C. pneumoniae* lung infection on allergic airway responses. We show that systemic NOD1, but not NOD2, activation reduced allergen-induced airway hyperresponsiveness (AHR) and pulmonary eosinophilic infiltration if given before OVA sensitization or challenge, whereas Th2 cytokines were not diminished. Furthermore, we demonstrate that NOD1 activation led to reduced MHC class II antigen presentation by spleen-derived DCs and diminished antigen-specific T-cell activation in lung tissue.

Materials and Methods

A detailed description of the methods is provided in the online supplement.

Mice

BALB/c and DO11.10 OVA peptide-specific T-cell receptor transgenic mice and littermates (female, 8–12 wk old) were maintained under specific pathogen-free conditions. Animal procedures were approved by institutional and governmental authorities.

Ovalbumin Sensitization and Allergen Airway Exposure

Mice were sensitized by intraperitoneal injections of 20 μ g ovalbumin (OVA) and 2 mg aluminum-hydroxide [$\text{Al}(\text{OH})_3$] in 110 μ l PBS on Days 0 and 14. Nonsensitized mice received 2 mg $\text{Al}(\text{OH})_3$ in 110 μ l PBS. Mice were exposed to aerosolized 1% OVA in PBS for 20 minutes on Days 28, 29, and 30 and were killed on Day 31.

C. pneumoniae Infection and NOD Receptor Activation

C. pneumoniae (TW-183) was cultured and purified (21). Three days before OVA sensitization, HEp-2 cells containing 5×10^6 inclusion-forming units TW-183 were transtracheally inoculated using

a microsprayer (32). Control mice received HEp-2 cells.

The NOD1 agonist FK156 (800 μ g) (Astellas Pharma Inc., Tokyo, Japan), the NOD2 agonist MDP (500 μ g; Sigma-Aldrich, Steinheim, Germany), or solvent were injected intraperitoneally 6 hours before each sensitization or airway challenge. DO11.10 mice were exposed to aerosolized OVA or PBS on Days 0 and 1, and FK156 was injected intraperitoneally 6 hours before each airway challenge.

Airway Responsiveness in Isolated Perfused and Ventilated Mouse Lungs

Murine lungs were prepared as described previously (33), perfused with 37°C sterile Krebs-Henseleit hydroxyethyl amylopectin buffer (1 ml/min), and ventilated by negative pressure ventilation. After a steady-state period of 20 minutes, NaCl as control and increasing doses of methacholine (MCh) were nebulized (Pari LL). Airway resistance values were determined at the end of the steady-state period and at the respective maximal response. Airway resistance change ($\Delta\text{Res}_{\text{aw}}$) was expressed in cm $\text{H}_2\text{O}/\text{ml}/\text{s}$.

Immunoglobulins

Total and OVA-specific IgE, IgG1, and IgG2a were measured by ELISA (34).

Leukocytes and Cytokines in Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) total cell count and differentials were determined. Cytokines were measured using a multiplex assay (Bioplex; Bio-Rad, Hercules, CA).

Cytokine Quantification *In Vitro*

FK156- or solvent-treated, sensitized BALB/c mice were killed on Day 15. Lymphocytes were isolated from spleen and peribronchial lymph nodes and incubated (35). Cytokines from cell culture supernatants were quantified (Bioplex).

DC Isolation and Flow Cytometric Analysis

Splenic DCs were isolated from FK156- or solvent-treated BALB/c mice (36). For determination of DC activation, CD11c^+ cells were stained with FITC- or R-phycoerythrin-conjugated antibodies (anti-MHC II, CD80, CD86) and analyzed by flow cytometry.

T-Cell Isolation and *In Vitro* T-Cell Proliferation Assay

OVA peptide-specific T cells of DO11.10 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) as described (37) and cocultured with OVA-pulsed (50/100/150 $\mu\text{g/ml}$) DCs from FK156- or solvent-treated BALB/c mice. For proliferation analysis, DO11.10 cells were stained with CD4-APC and analyzed by flow cytometry. The total number of dividing (CD4^+ propidium iodide $^-$ CFSE $^{\text{low}}$) cells was determined.

Lung Histology and *In Vivo* T-Cell Proliferation

FK156- or solvent-treated DO11.10 mice received an intraperitoneal injection of 1 mg bromodeoxyuridine (BrdU) 64 hours after the last challenge. One hour later, lungs and inguinal lymph nodes were removed. Double-label immunohistochemistry for CD4 and BrdU was performed to identify proliferating CD4^+ T cells as described (38). Hematoxylin and eosin-stained lung tissue was examined by light microscopy.

Results

C. pneumoniae Infection before OVA Sensitization Reduced AHR

Transtracheal application of *C. pneumoniae* (TW-183) 3 days before systemic sensitization reduced Th2-induced AHR to MCh application in OVA-sensitized and OVA-challenged mice (O/O) compared with noninfected (HEp-2) O/O-treated control mice (Figure 1).

NOD1 Activation before Sensitization Reduced AHR

Sensitization and challenge with OVA (O/O) resulted in AHR to MCh application as compared with nonsensitized, nonchallenged (P/P) or nonsensitized, OVA-challenged (P/O) mice. Systemic administration of NOD1 agonist FK156 before sensitization (Figure 2A) led to impaired MCh-evoked AHR. In contrast, NOD2 agonist MDP given before sensitization did not reduce AHR (Figure 2A).

NOD1 Activation before Sensitization Did Not Diminish Immunoglobulins and Th2 Cytokines

Sensitization and challenge with OVA increased total and OVA-specific IgE, IgG1,

and IgG2a plasma levels as compared with nonsensitized, nonchallenged mice (Figure 2B). NOD1 and NOD2 activation before sensitization further increased total IgE and OVA-specific IgE plasma levels, whereas OVA-specific IgG1 and IgG2a plasma levels were unaltered (Figure 2B).

To investigate if NOD1 activation affects the Th1 and/or the Th2 response, BALF cytokines were quantified. Sensitization and challenge led to increased levels of cytokines, including IL-4, IL-5, IL-12, IL-13, granulocyte colony-stimulating factor, and eotaxin. Neither NOD1 nor NOD2 activation before sensitization led to altered levels of the cytokines investigated (Figure 2C). Levels of IL-1 β , regulated upon activation, normal T-cell expressed and secreted tumor necrosis factor- α (RANTES), chemokine (C-X-C motif) ligand 1, and tumor necrosis factor- α did not differ significantly among the examined groups (data not shown).

To further determine the effect of NOD1 activation on allergen-induced cytokine production at the single-cell level, spleen and lymph node cells from sensitized and nonsensitized mice were isolated and exposed to OVA *in vitro*, and the cytokine profile in the cell culture

supernatant was examined. Supernatants of splenic and peribronchial lymph node cells of sensitized mice (Figure 2D) showed increased levels of IL-4, IL-6, and IL-10 compared with that seen in nonsensitized mice, whereas levels of IFN- γ were reduced. FK156-treatment before OVA sensitization led to altered *in vitro* Th2 cytokine production with by-trend increased levels of IL-5 and IL-13 and by-trend decreased levels of IL-10 compared with OVA sensitization without treatment (Figure 2D).

NOD1 Activation before Allergen Challenge Reduced AHR

Because NOD2 activation failed to improve Th2-induced AHR, we focused on the effects of NOD1 activation in our further investigations. To analyze whether NOD1 agonism is also able to improve AHR and airway inflammation in previously sensitized mice, which better reflects the clinical situation, FK156 was administered before airway challenge in the following experiments.

Sensitization and challenge with OVA (OVA/solvent + OVA) induced AHR to MCh compared with nonsensitized, nonchallenged (PBS/solvent + PBS) or sensitized, nonchallenged (OVA/solvent

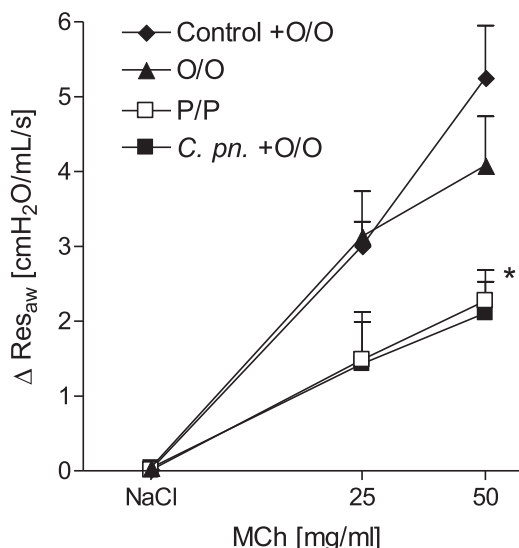


Figure 1. *Chlamydia pneumoniae* (*C. pn.*) infection before ovalbumin (OVA) sensitization reduced airway hyperresponsiveness (AHR). BALB/c mice were systemically sensitized with OVA (or PBS as control) and repeatedly exposed to aerosolized OVA (O/O) or PBS (P/P). HEp-2 cells containing *C. pneumoniae* (TW-183) dissolved in PBS were applied transtracheally 3 days before sensitization. Control mice received HEp-2 cells dissolved in PBS. Airway responsiveness to inhaled methacholine (MCh) was investigated in isolated perfused and ventilated mouse lungs 24 hours after the last OVA challenge. Values are given as mean + SEM ($n = 4$). * $P < 0.05$ *C. pn.* +O/O versus Control +O/O.

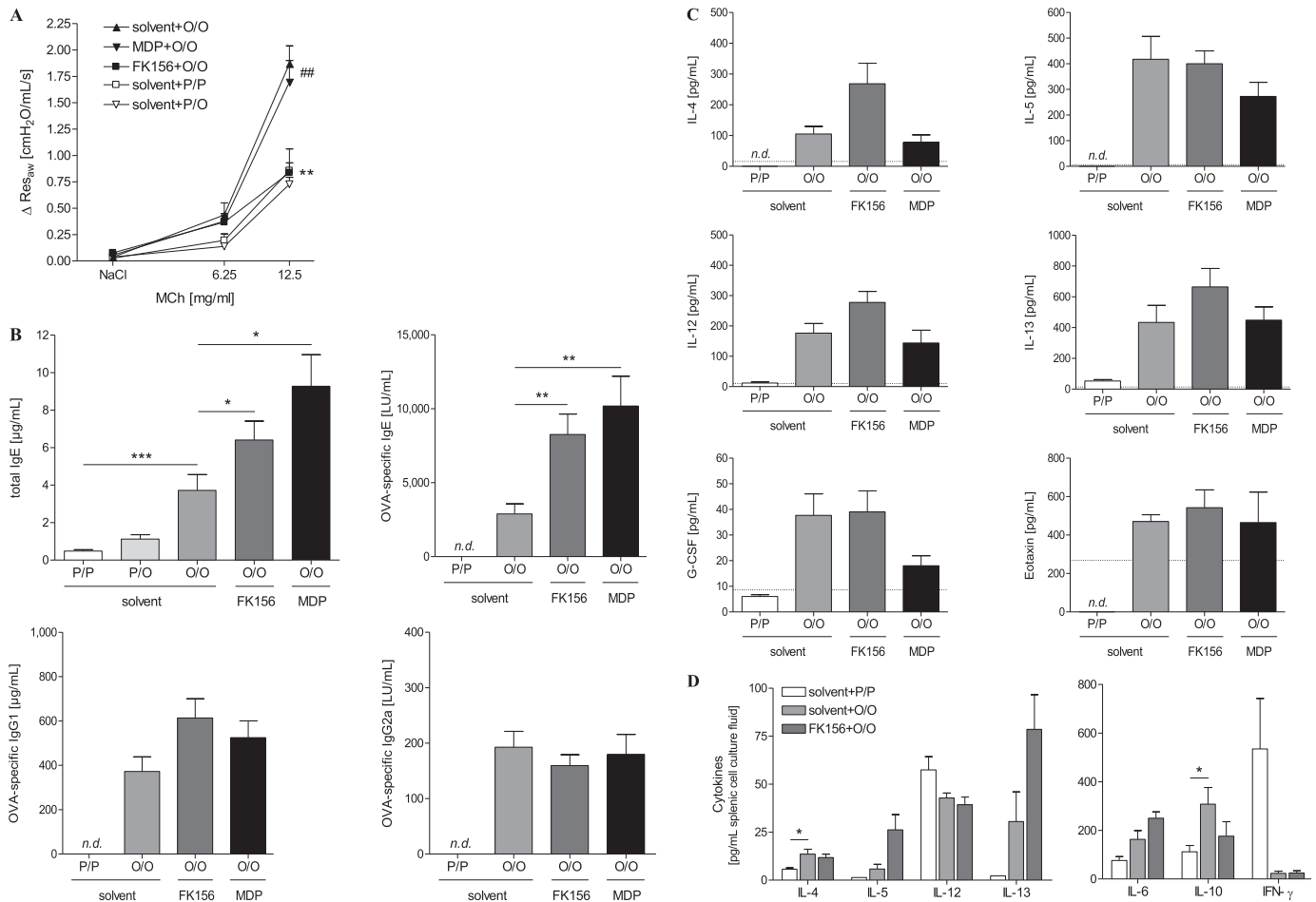


Figure 2. Nucleotide oligomerization domain (NOD)1 activation before OVA sensitization reduced allergen-evoked AHR without diminishing T-helper (Th)2 cytokine production. BALB/c mice were systemically sensitized with OVA and repeatedly exposed to aerosolized OVA. NOD1 agonist FK156 or NOD2 agonist MDP was injected intraperitoneally 6 hours before each sensitization. Control mice received saline applications. Twenty-four hours after the last challenge, airway responsiveness to increasing doses of inhaled MCh was investigated in isolated perfused and ventilated mouse lungs ($n = 5-7$) (A), and blood samples were collected and total immunoglobulin E (IgE) and OVA-specific Ig were quantified (B) (MDP, $n = 4$; FK156, $n = 6$; solvent, $n = 6-9$). (C) BALF cytokines were analyzed (P/P, $n = 3$; O/O, $n = 4$). Dotted lines indicate the detection limit of the cytokine assay. (D) BALB/c mice treated with FK156 or saline before OVA sensitization were killed on Day 15. Lymphocytes were prepared from spleen and peribronchial lymph nodes and incubated with culture medium, concanavalin A (P/P), or OVA (O/O). Cytokine production was measured in supernatants ($n = 5-6$). n.d., not detectable; O/O, OVA/OVA; O/P, OVA/PBS; P/O, PBS/OVA; P/P, PBS/PBS. Values are given as mean + SEM. $^{##}P < 0.01$ solvent + O/O versus solvent + P/P (A); $^{**}P < 0.01$ FK156+O/O versus solvent + O/O (A); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (B–D).

+ PBS) mice. Systemic administration of NOD1 agonist FK156 before challenge (OVA/FK156 + OVA) diminished AHR to a level comparable to those seen in both control groups (Figure 3A), whereas total IgE plasma levels were unaffected (Figure 3B).

To rule out a direct effect of NOD1 activation on airway smooth muscle contraction, isolated lungs of naive mice were continuously perfused with FK156 (10 μ M) starting 15 minutes before MCh application, which had no effect on airway responsiveness compared with controls (see Figure E1 in the online supplement).

NOD1 Activation before Allergen Challenge Reduced Airway Inflammation

OVA-sensitized and OVA-challenged mice (O/O) showed a dramatic increase in BALF eosinophils compared with P/O-treated mice. NOD1 activation before allergen challenge led to a reduced increase of BALF eosinophils, whereas BALF neutrophils were increased. BALF macrophages and lymphocytes were not significantly altered (Figure 3C).

Similar to FK156 treatment before sensitization, the BALF cytokine profile was unaltered after FK156 treatment before challenge (Figures 3D and E2).

Lung histology of DO11.10 mice exposed to aerosolized OVA showed peribronchial and perivascular inflammatory infiltrates characterized by a predominance of eosinophils. Treatment with FK156 before OVA exposure led to reduced inflammatory infiltrates. No infiltrates were detected in PBS-exposed control mice (Figure 3E).

NOD1 Activation Led to Reduced MHC Class II Antigen Presentation by Spleen-Derived DCs and Decreased T-Cell Proliferation

To investigate if systemic NOD1 activation leads to DC maturation, splenic CD11c⁺

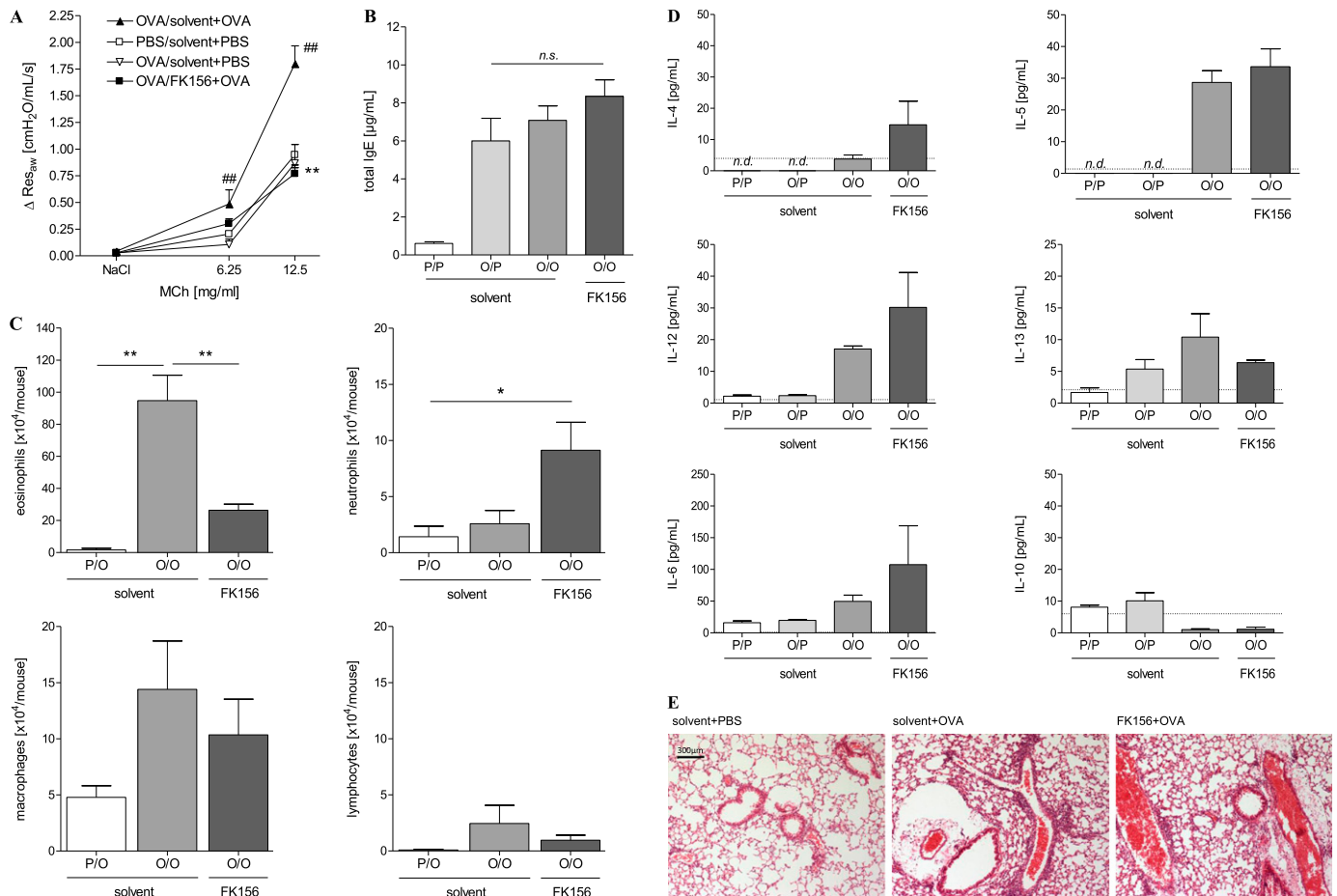


Figure 3. NOD1 activation before OVA challenge reduced Th2-induced AHR and allergic airway inflammation. BALB/c mice were systemically sensitized with OVA and repeatedly exposed to aerosolized OVA once daily on three consecutive days. NOD1 agonist FK156 was injected intraperitoneally 6 hours before each airway challenge. Control mice received saline applications. Twenty-four hours after the last challenge, airway responsiveness to increasing doses of inhaled MCh was investigated in isolated perfused and ventilated mouse lungs ($n = 5-6$) (A); blood samples were collected, and total IgE was measured by ELISA ($n = 3-6$) (B); BALF was performed, and BALF leukocytes were analyzed ($n = 4-5$) (C); and BALF cytokines were quantified ($n = 3-4$) (D). Values are given as mean + SEM. ### $P < 0.01$ OVA/solvent + OVA versus PBS/solvent + PBS (A), ** $P < 0.01$ OVA/FK156 + OVA versus OVA/solvent + OVA (A); * $P < 0.05$ and ** $P < 0.01$ (B). Dotted lines indicate the detection limit of the cytokine assay. (E) Hematoxylin and eosin-stained lung sections of FK156- or solvent-treated DO11.10 mice 72 hours after the last challenge. FK156 treatment reduced OVA-induced peribronchial and perivascular inflammatory infiltrates.

DCs were isolated from FK156- and solvent-treated mice, and surface expression of MHC II, CD80 and CD86 was determined by FACS analysis. DCs from FK156-treated mice showed a distinctly higher expression of MHC II and CD86 compared with DCs from solvent-treated animals, whereas the increase in CD80 expression due to NOD1 activation was moderate (Figure 4A). To assess antigen presentation, DCs from FK156- or solvent-treated BALB/c mice were pulsed with OVA, cocultured with CFSE-labeled OVA-specific T cells, and T-cell proliferation, visualized as a progressive dilution of the CFSE fluorescent marker, was quantified (Figure 4B). DCs led to

increased T-cell proliferation when pulsed with OVA, but this effect was reduced after treatment of donor mice with FK156 (Figures 4B and 4C).

NOD1 Activation Results in Diminished Antigen-Specific T-Cell Activation in Lung Tissue

To confirm the effect of NOD1 activation on T-cell proliferation, FK156-treated and OVA-exposed DO11.10 mice received BrdU, and T-cell proliferation in lungs and inguinal lymph nodes was studied. Double-label immunohistochemistry of DO11.10 mice exposed to OVA showed an increase in proliferating T cells in lung tissue compared with PBS-exposed control mice.

Treatment with FK156 before OVA exposure led to reduced T-cell proliferation in lung tissue (Figures 5A and 5B). No increase in T-cell proliferation due to OVA exposure or FK156 treatment was seen in control inguinal lymph nodes (Figure 5C).

Discussion

In this study, we show that systemic NOD1 activation before systemic OVA sensitization or before local OVA challenge reduced AHR and pulmonary eosinophilic influx in a murine model of allergen-induced lung inflammation. These

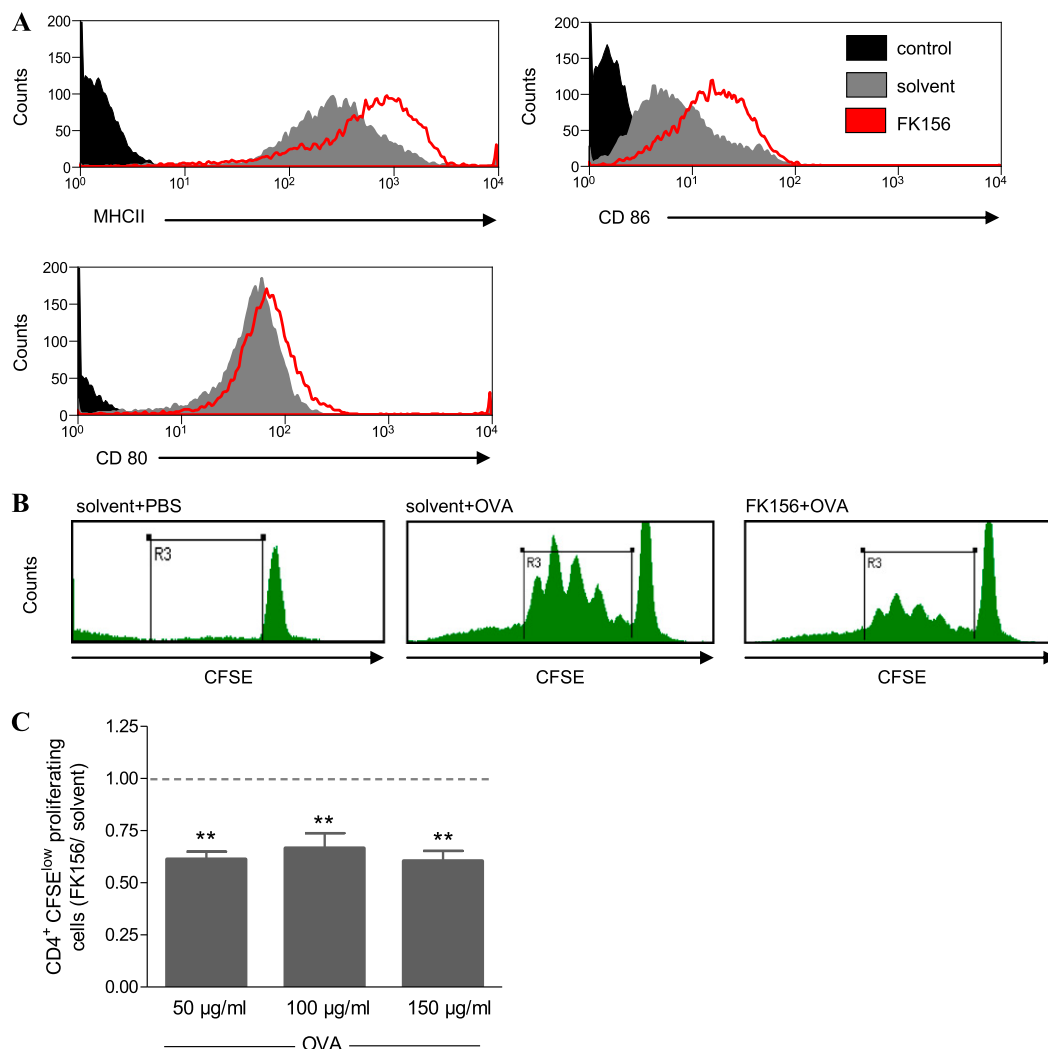


Figure 4. Antigen presentation and T-cell stimulation by spleen-derived dendritic cells (DCs). (A) CD11c⁺ DCs were isolated from spleen of FK156- and solvent-treated BALB/c mice, and surface expression of MHC class II, CD80 and CD86 was determined by FACS analysis. Control group comprised DCs solely stained with CD11c. (B) Representative carboxyfluorescein succinimidyl ester (CFSE) proliferation profile resulting from coculture of OVA-pulsed spleen DCs and DO11.10 cells. (C) Spleen DCs of mice treated with FK156 or solvent as control were incubated with OVA as indicated and cocultured with CFSE-labeled DO11.10 cells. Proliferation assays were performed in triplicate by flow cytometry, and the results were normalized to levels of solvent-treated controls (dotted line). Values are given as mean + SEM from three or four independent experiments. ** $P < 0.01$ FK156 versus solvent treatment.

protective effects were associated with a reduction of allergen-specific T-cell proliferation.

We also examined the effect of NOD2 activation on the development of allergic airway disease. In contrast to NOD1 stimulation, NOD2 activation did not reduce AHR or inflammation in our model. This finding is in agreement with a study of Duan and colleagues, who showed that NOD1 and NOD2 have differential activity, with only NOD2 ligation blocking airway tolerance in a murine model of Th2-induced airway disease (14). An association of NOD1 to the development of

inflammatory airway disease was also shown by Bogefors and colleagues, demonstrating down-regulated NOD1 expression in the human nasal epithelium during pollen season among patients with allergic rhinitis (39).

Several studies have emphasized that the timing and the severity of infection or PRR stimulation relative to allergen sensitization and/or exposure is critical for modulation of the immune response (14, 29, 40–42). In our study, the effect of systemic NOD1 receptor stimulation was independent of the timing relative to antigen sensitization or exposure. NOD1

activation before sensitization and before challenge reduced AHR. NOD1 activation before allergen challenge also reduced pulmonary inflammation. These data have therapeutic implications because systemic NOD1 activation may be beneficial even in established sensitization to an allergen. Moreover, the NOD1-specific agonist FK156 is a synthetically manufactured agent.

To further analyze the impact of NOD1 activation on the allergic phenotype, we determined immunoglobulins in plasma and quantified cytokine production by splenic and PBLN cells *in vitro* and from

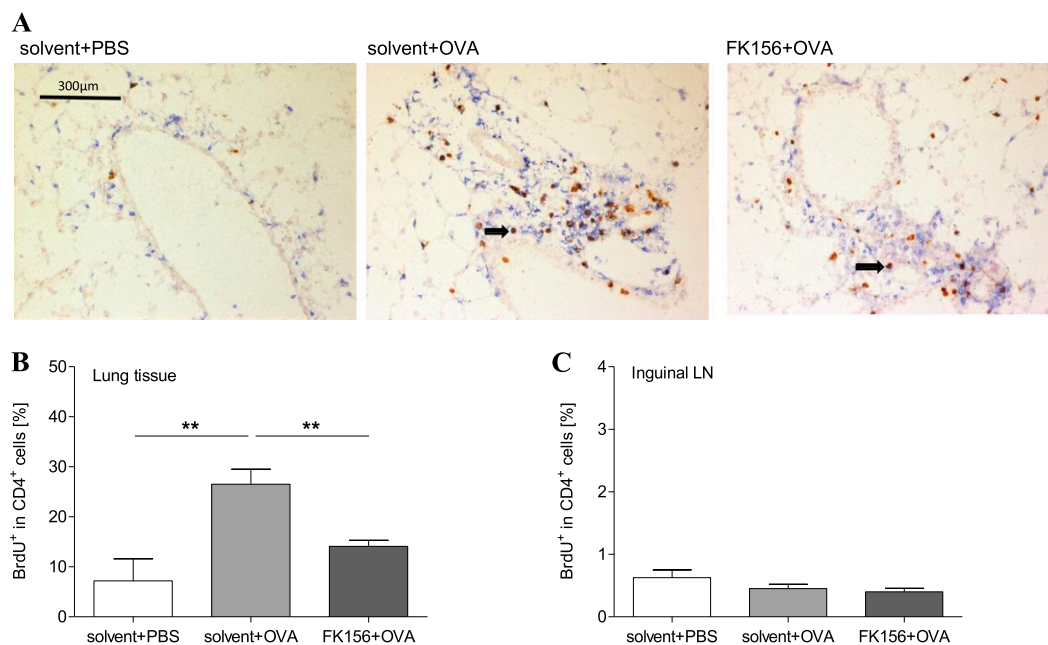


Figure 5. NOD1 activation resulted in diminished antigen-specific T-cell activation in lung tissue. DO11.10 mice were exposed to aerosolized OVA or PBS on Days 0 and 1, and the NOD1 activator FK156 was applied 6 hours before each airway challenge. Sixty-four hours after the last challenge, mice received BrdU, and 1 hour later, the lungs and inguinal lymph nodes (LN) were removed. (A) Representative lung tissue sections were stained with anti-CD4 (blue) and anti-BrdU (light brown). Arrows indicate double-labeled cells (dark brown). (B and C) Percentage of BrdU⁺ cells in the CD4⁺ cell population determined on lung tissue sections (B; $n = 10$) and on cytoplots of inguinal lymph nodes (C; $n = 6-8$ per group, except for solvent + PBS group [$n = 2$]). Values in B and C are given as mean + SEM. ** $P < 0.01$ versus indicated group.

BALF supernatant. After NOD1 activation, plasma immunoglobulin levels and Th2 cytokines were unaltered or increased. NOD1 activation before OVA sensitization evoked elevation of IgE plasma levels and by trend of the Th2 cytokine IL-13 *in vivo* and *in vitro*. Although these findings are consistent because IL-13 induces IgE (43), the discrepancy between FK156-induced elevation of IL-13 and IgE on the one hand, and FK156-induced reduction of eosinophilic airway inflammation and AHR on the other, is difficult to explain because IL-13 has been linked to eosinophil recruitment and AHR (44). Because NOD1 receptor ligation can effectively suppress eosinophilic airway inflammation but not secretion of the eosinophil chemoattractants IL-5, IL-13, chemokine (C-X-C motif) ligand 1, RANTES, and eotaxin, it may be speculated that NOD1 activation can affect expression of adhesion molecules, their ligands, and/or cytokine receptors on eosinophils. Furthermore, NOD1 activation could inhibit mast cell degranulation and thereby diminish AHR because NOD1 is expressed and functional in mast cells (45).

To investigate whether further mechanisms contribute to the diminishing effects on allergic airway inflammation by NOD1 activation, we analyzed the impact of NOD1 activation on antigen-specific T-cell stimulation by spleen-derived DCs. DCs play a pivotal role in the development of allergic Th2 responses to inhaled antigens (46). On antigen recognition and uptake, DCs process antigen, mature, migrate to the T-cell area of draining lymph nodes and present antigen-MHC complexes (46). Additional T-cell stimulation by costimulatory molecules on T cells and DCs then induces T-cell proliferation, followed by secondary immune responses like AHR (47). Activated DCs were shown to be no longer capable of presenting newly encountered antigens via MHC II *in vitro* or *in vivo* (48). For immunomodulation with CpG oligodeoxynucleotides, it has been demonstrated that CpG (via TLR-9 activation) inhibits the Th2 response by rendering pulmonary antigen-presenting cells unable to present antigen to Th2 cells. This loss of antigen-presenting cell function correlated with reduced expression of costimulatory molecules (49), which are crucial for induction of T-cell proliferation and subsequent AHR (47, 50). In

agreement, we found that systemic NOD1 activation led to phenotypic maturation of splenic DCs as determined by expression of MHC class II and CD86. NOD1-induced DC maturation diminished OVA-evoked CD4⁺ T-cell proliferation *in vitro* likely through reduced presentation of new antigens. To overcome the limitations of *in vitro* experiments, we determined T-cell proliferation *in vivo* and observed that NOD1 activation diminished T-cell proliferation in lung tissue.

In summary, we found that NOD1 activation reduced allergen-induced AHR and eosinophilic lung inflammation if given before OVA sensitization or challenge. This protective effect was accompanied by down-regulation of antigen presentation by DCs leading to reduced antigen-specific T-cell stimulation. Thus, specific activation of the NOD1 receptor may provide a novel therapeutic strategy for allergic airway disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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NOD1 Ligation Suppressed Murine Allergen-Specific T Cell Proliferation and Airway Hyperresponsiveness

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Online Data Supplement

Supplemental Detailed Materials and Methods

Mice

All mice were maintained under specific pathogen free (SPF) conditions on an ovalbumin (OVA)-free diet. Wildtype (WT) mice (BALB/c) were purchased from Charles River Laboratories (Sulzfeld, Germany). DO11.10 OVA peptide-specific T cell receptor transgenic mice and littermates (BALB/c) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred under SPF conditions. Female mice aged 8-12 weeks were used for all experiments. All animal procedures were approved by institutional and governmental authorities (LAVES, AZ: 33.9-42502-04-06/1098).

Ovalbumin sensitization, allergen airway exposure

BALB/c mice were systemically sensitized by intraperitoneal (i.p.) injections of 20 µg OVA (grade V; Sigma-Aldrich, Steinheim, Germany) and 2 mg aluminium hydroxide (Al(OH)₃; SERVA Electrophoresis, Heidelberg, Germany) in 110 µl phosphate buffered saline (PBS) on days 0 and 14 (OVA-treated group). Non-sensitized mice received 2 mg Al(OH)₃ in 110 µL of PBS (control group). On days 28, 29 and 30 mice were exposed to aerosolized 1% (wt/vol) OVA in PBS or PBS as diluent control for 20 min. All mice were sacrificed on day 31.

Chlamydomonas pneumoniae infection and NOD receptor activation

C. pneumoniae strain TW-183 (VR2282; ATCC, Rockville, Md) was cultured and purified as previously described(E1). Briefly, TW-183 was propagated to high titers in cyclohexamide-treated HEp-2 cells. Infected monolayers were harvested, sonicated for 30 s, and cellular debris was removed by centrifugation at 500 *g* for 10 min at 4°C. Purified elementary bodies were stored at -75°C until use. Three days prior to OVA sensitization, mice were anesthetized by i.p. ketamine (80 mg/kg body weight) and xylazine (25 mg/kg body weight), and 25 µl PBS containing 5x10⁶ inclusion forming units (IFU) TW-183 were transtracheally inoculated using a microsyringe(E2). Control mice received HEp-2 cells dissolved in 25 µl of PBS.

The NOD1 agonist FK156 (800 µg; Astellas Pharmaceutical Co., Tokyo, Japan) or the NOD2 agonist muramyl dipeptide (MDP; 500 µg; Sigma-Aldrich) or solvent (saline) as control were injected i.p. 6 hours before each sensitization or 6 hours before each airway challenge.

Further, DO11.10 mice were exposed to aerosolized OVA or PBS on days 0 and 1, and the NOD1 agonist FK156 was i.p. applied 6 hours before each airway challenge.

Airway responsiveness in isolated perfused and ventilated mouse lung (IPML)

24 hours after the last challenge, murine lungs were prepared as described previously(E3). Briefly, anesthetized mice were tracheotomized and ventilated. After cannulation of pulmonary artery and left atrium, lungs were perfused with 37°C sterile Krebs-Henseleit hydroxyethyl amylopectin buffer (1 ml min⁻¹; Serag-Wiesner, Naila, Germany) in a non-recirculating fashion, and left atrial pressure was adjusted at +2.2 cmH₂O. Lungs were ventilated by constant negative pressure (P_{exp} -4.5, P_{ins} -9.0 cmH₂O, 90 breaths/min) in a humidified chamber. Airway resistance (Res_{aw}) was continuously monitored. After a steady state period of 20 min, NaCl as control and increasing doses of methacholine (MCh) were nebulized (Pari LL nebulizer; Hugo Sachs Electronics, March-Hugstetten, Germany) for 1 min each at 8 min intervals. Airway resistance values were determined at the end of the steady state period and at the respective maximal response. The difference in airway resistance (ΔRes_{aw}) was expressed in cmH₂O/ml/s.

The direct effect of NOD1 agonist FK156 on MCh-evoked airway responsiveness was investigated in isolated lungs of naïve mice by continuous perfusion with FK156 (10 µM) containing buffer starting 15 min prior to MCh application.

Plasma levels of immunoglobulins

Blood samples were drawn from the inferior V. cava 24 hours after the last challenge. Plasma was prepared and total and OVA-specific IgE, IgG1 and IgG2a were measured by ELISA as previously described(E4).

Leukocyte and cytokine analysis in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed twice using 800 µl PBS 24 hours after the last challenge. Total cell count was determined using a Neubauer hemacytometer, and leukocytes were differentiated using cytospot preparations stained with Diff-Quick (Medion Diagnostics, Dürdingen, Switzerland). A total of 400 cells/individual was counted in a blinded manner. Cytokines from BAL fluid supernatant were measured using a cytokine multiplex assay (Bioplex[®]; Bio-Rad, Hercules, CA).

Cytokine quantification in vitro

To determine the effect of NOD1 activation on allergen-induced cytokine production at single cell level, sensitized BALB/c mice treated with FK156 or solvent (saline) prior to sensitization were sacrificed at day 15. Lymphocytes (predominance >95%) were prepared from spleen and peribronchial lymph nodes by density gradient centrifugation (Lympholyte-M; Cedarlane Laboratories, Hornby, Ontario, Canada) and incubated with culture medium, concanavalin A (2.5 µg/ml; Sigma-Aldrich) or OVA (50 µg/ml, grade VI; Sigma-Aldrich) as described (E5). At optimal time points(E5), cytokines from cell culture supernatants were measured using a cytokine multiplex assay (Bioplex[®]; Bio-Rad, Hercules, CA). Detection limits were 1.00 pg/ml for IL-4, 0.46 pg/ml for IL-5, 0.19 pg/ml for IL-6, 1.29 pg/ml for IL-10, 0.64 pg/ml for IL-12, 0.93 pg/ml for IL-13, and 0.63 ng/ml for IFN-γ.

Dendritic cell (DC) isolation and flow cytometric analysis

Splenic DCs were isolated from BALB/c mice that had been treated with FK156 or solvent 16 hours previously, as described(E6). DCs were labeled with FITC-conjugated anti-CD11c (BD

Biosciences, Heidelberg, Germany) and fluorescent-activated cell sorting was done on a FACSCaliburTM (Becton Dickinson, Heidelberg, Germany) instrument. Sorts were usually 95-98% pure. For determination of DC activation state, CD11c⁺ cells were stained with FITC- or PE-conjugated antibodies (anti-MHC II, CD80, CD86, Biolegend, London, UK) and analyzed by flow cytometry.

T cell isolation and in vitro T cell proliferation assay

OVA peptide-specific T cells from lymph nodes of DO11.10 mice were enriched by negative depletion using magnetic separation and an antibody cocktail containing anti-erythrocytes, anti-MHC II, anti-Gr-1, anti-B220 and anti-CD19. Enriched T cells were labeled with CFSE as described(E7) and cocultured with OVA-pulsed (50/100/150 µg/ml; Biosyntan, Berlin, Germany) DCs from BALB/c mice treated with FK156 or solvent as control. For proliferation analysis after 64 hours of culture, DO11.10 cells were stained with CD4-APC (BD Biosciences), and analyzed by flow cytometry. The total number of dividing (CD4⁺ propidium iodide⁻ CFSE^{low}) cells was determined at least in duplicate and normalized to solvent-treated control.

Lung histology and measurement of in vivo T cell proliferation

64 hours after the last challenge, FK156- or solvent-treated DO11.10 mice received a single i.p. injection of 1 mg Bromodeoxyuridine (BrdU; Sigma-Aldrich). One hour after BrdU injection, inguinal lymph nodes and the lungs were removed. Cytospots were prepared for lymph nodes as described(E8), and lung tissue was processed for paraffin embedding. Four-µm-thick paraffin sections of lung tissue were subjected to H/E staining and examined by light microscopy for histologic changes. Further, double-label immunohistochemistry for CD4 and BrdU was performed on cytopots and lung sections to identify proliferating CD4⁺ T cells as described before(E8). Briefly, an anti-BrdU antibody (Roche Diagnostics GmbH,

Mannheim, Germany) was applied first and detected with the avidin-biotin-peroxidase system (Vector Laboratories, Burlingame, CA, USA) with DAB as the chromogen, an anti-CD4 antibody (BD Biosciences) was then applied and visualized using an APAAP system (DAKO, Hamburg, Germany) and Fast Blue (Sigma, St. Louis, USA) as the chromogen. Controls (omitting first antibody, isotype antibodies) were negative.

Data analysis

Data are expressed as mean and SEM. Data were analyzed by Student's *t* test or by Mann-Whitney *U* test, adjusted for multiple comparisons by the Bonferroni-Holm test procedure(E9).

Supplemental References

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Supplemental Figure Legend

Figure E1

The NOD1 activator does not directly reduce methacholine (MCh)-evoked airway smooth muscle contraction. Airway responsiveness to increasing doses of inhaled MCh was quantified in isolated perfused and ventilated lungs of naïve BALB/c mice. 10 μ M FK156 or solvent were added to the perfusate 15 min before MCh application. Airway resistance values were determined at the end of a steady state period as well as at the maximum level of resistance increase and expressed as delta airway resistance ($\Delta \text{Res}_{\text{aw}}$). Values are given as mean + SEM; n=6 per group.

Figure E2

NOD1 activation prior to OVA challenge did not alter BALF supernatant levels of KC, RANTES, IL-2, IL-1 β and IFN γ . BALB/c mice were systemically sensitized with OVA and repeatedly exposed to aerosolized OVA once daily on three consecutive days. NOD1 agonist FK156 was injected i.p. 6 hours prior to each airway challenge. Control mice received saline applications. 24 hours after the last challenge, BALF cytokines were quantified, n=5-6. *Dotted lines* indicate the detection limit of the cytokine assay. Abbreviations: P/P, PBS/PBS; P/O, PBS/OVA; O/P, OVA/PBS; O/O, OVA/OVA. Values are given as mean + SEM.

Supplemental Figure

Figure E1

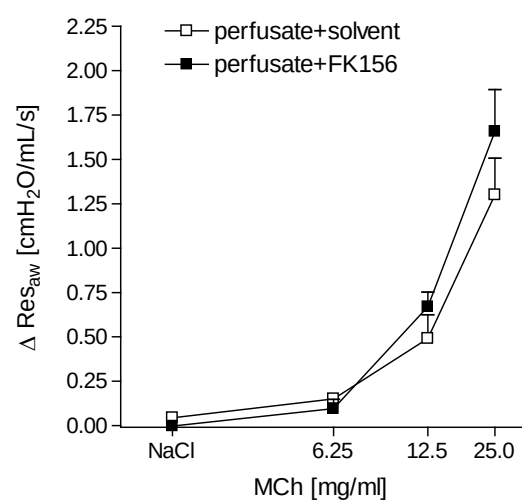


Figure E2

