

NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant

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To cite this article: Besnard A-G, Guillou N, Tschopp J, Erard F, Couillin I, Iwakura Y, Quesniaux V, Ryffel B, Togbe D. NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant. *Allergy* 2011; DOI:10.1111/j.1398-9995.2011.02586.x.

Keywords

allergic inflammation; asthma; IL-1R1; NLRP3 inflammasome.

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Accepted for publication 2 March 2011

DOI:10.1111/j.1398-9995.2011.02586.x

Edited by: Angela Haczku

Abstract

Background: Inflammasome activation with the production of IL-1 β received substantial attention recently in inflammatory diseases. However, the role of inflammasome in the pathogenesis of asthma is not clear. Using an adjuvant-free model of allergic lung inflammation induced by ovalbumin (OVA), we investigated the role of NLRP3 inflammasome and related it to IL-1R1 signaling pathway.

Methods: Allergic lung inflammation induced by OVA was evaluated *in vivo* in mice deficient in NLRP3 inflammasome, IL-1R1, IL-1 β or IL-1 α . Eosinophil recruitment, Th2 cytokine, and chemokine levels were determined in bronchoalveolar lavage fluid, lung homogenates, and mediastinal lymph node cells *ex vivo*.

Results: Allergic airway inflammation depends on NLRP3 inflammasome activation. Dendritic cell recruitment into lymph nodes, Th2 lymphocyte activation in the lung and secretion of Th2 cytokines and chemokines are reduced in the absence of NLRP3. Absence of NLRP3 and IL-1 β is associated with reduced expression of other proinflammatory cytokines such as IL-5, IL-13, IL-33, and thymic stromal lymphopoietin. Furthermore, the critical role of IL-1R1 signaling in allergic inflammation is confirmed in IL-1R1-, IL-1 β -, and IL-1 α -deficient mice.

Conclusion: NLRP3 inflammasome activation leading to IL-1 production is critical for the induction of a Th2 inflammatory allergic response.

Allergic inflammatory response is characterized by the activation of Th2 pathway, which is initiated by the uptake and processing of allergens by professional antigen-presenting cells (APCs). APCs present selected peptides on MHC class II molecules to naive T cells, leading to B-cell activation and T-helper-2 type-response characterized by IL-4, IL-5, and IL-13 production (1, 2).

IL-1 family cytokines, such as IL-1 α and IL-1 β , are involved in multiple aspects of immune and inflammatory responses (3). Both isoforms bind to IL-1R, composed of the IL-1R1 chain and the IL-1 receptor accessory protein

(IL-1RAcP), which is ubiquitously expressed in vertebrate suggesting important and widespread roles (4). There is increasing evidence that IL-1 contributes to allergic diseases by promoting mast cell activation and Th2 cytokine production (5, 6). Indeed, Th2 allergic response and lung inflammation are reduced in IL-1R1-deficient mice (7). Importantly, elevated levels of IL-1 β have been found in the BALF from patients with asthma (8). IL-1 β is synthesized as a precursor (pro-IL-1 β) by blood monocytes, tissue macrophages, and dendritic cells (DC) after activation by proinflammatory stimuli such as IL-1, TNF, or TLR ligands (3) and needs to be processed to be active. Pro-IL-1 β is cleaved by the protease caspase-1, which is activated through a multiprotein complex called inflammasome (9). In contrast, the pro-form of IL-1 α is constitutively present intracellularly in epithelial and endothelial cells and is active in both form (pro- and mature forms) (10).

Abbreviations

BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; DC, dendritic cell; EPO, eosinophil peroxidase; OVA, ovalbumin; mLN, mediastinal lymph nodes; TSLP, thymic stromal lymphopoietin.

The inflammasome is activated by endogenous danger signals such as adenosine triphosphate (ATP) or uric acid crystals released from dying cells or by hypotonic stress (9, 11, 12). A critical role of the NLRP3 inflammasome was recently highlighted in experimental allergic asthma induced in the presence of aluminum hydroxide (alum) adjuvant (13). Given during ovalbumin (OVA) sensitization, alum potentializes the inflammatory response by activating the NLRP3 inflammasome, leading to IL-1 β maturation and humoral adaptive immune response (13–15).

The role of NLRP3 inflammasome in allergic asthma in the absence of aluminium adjuvant, a setting corresponding to naturally occurring disease, remains unclear. In this study, we demonstrate that NLRP3 inflammasome is required for allergen-specific Th2 cell activation in the absence of alum adjuvant and confirm that IL-1R1 signaling is critical for the development of experimental allergic airway inflammation.

Methods

Mice

C57BL/6 wild-type (WT) mice, IL-1R1^{-/-} (backcross 7) (16), IL-1 α ^{-/-} (backcross 9) (17), IL-1 β ^{-/-} (backcross 9) (17), and NLRP3^{-/-} (generated directly on C57BL/6 background) (9) mice were bred in our specific pathogen-free animal facility at CNRS, Orléans, France. Mice were maintained in a temperature-controlled (23°C) facility with a strict 12-h light/dark cycle and were given free access to food and water. Experiments were performed with gender-matched mice aged 8–10 weeks. All protocols were approved by the CNRS institutional animal research ethical committee and complied with the French Government's ethical and animal experiment regulations.

OVA model of allergic airway inflammation

Mice were sensitized subcutaneously twice at days 0 and 7 with 10 μ g ovalbumin (OVA, grade V; Sigma, Lyon, France) without adjuvant and were challenged on days 14–16 with 10 μ g of OVA by intranasal route. Control mice were challenged with saline alone. Twenty-four hours after the last challenge, mice were killed and bronchoalveolar lavage (BAL) was performed. Half of the lung was stored at -80°C for the analysis of eosinophil peroxidase (EPO) enzyme, cytokines and chemokines, and the other half was fixed overnight in buffered 4% formaldehyde solution for histology analysis.

Bronchoalveolar lavage (BAL) and flow cytometry of lung mononuclear cells

Bronchoalveolar lavage was performed by washing the lungs four times with 0.5 ml of saline solution at room temperature. After centrifugation at 400 *g* for 10 min at 4°C, the supernatant (cell-free BAL fluid) was stored at -20°C for cytokine analysis. FACS on BAL and lung mononuclear cells was performed as described in Data S1.

Lung histology

Lungs were fixed in 4% buffered formaldehyde, and 3- μ m sections were stained with hematoxylin and eosin (HE) or with periodic acid Schiff reagent and examined with a Leica microscope ($\times$ 40 magnification). Peribronchial infiltrates and mucus hypersecretion were assessed by a semiquantitative score (0–3) by two observers independently as described before (18).

Pulmonary eosinophil peroxidase (EPO) activity

Eosinophil peroxidase activity was determined in order to estimate the recruitment of eosinophils to the lung parenchyma as described (18).

Cytokine measurement and antigen-specific IgE by ELISA

IL-1 β , IL-4, IL-5, IL-6, IL-13, thymic stromal lymphopoietin (TSLP), IL-33, CCL11 (Eotaxin), CCL17 (TARC) concentrations in BAL, cell culture supernatant from mediastinal lymph nodes (mLN) or lung homogenate and OVA-specific IgE in serum were determined by enzyme-linked immunosorbent assay (ELISA), using commercial kits from R&D (Abingdon, UK) according to the manufacturer's recommendations.

Lung mononuclear cells isolation and stimulation

Lung mononuclear cells were isolated from mice 24 h after the last challenge as described (19). Isolated lung mononuclear single cells were plated in round-bottomed 96-well plates (2.10⁶/ml) and restimulated 4 h *in vitro* with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml).

Mediastinal lymph nodes restimulation

Mediastinal lymph nodes cells from OVA- and saline-treated WT and gene-deficient mice were plated in round-bottomed 96-well plates at 1 $\times$ 10⁶ cells/well in IMDM medium supplemented with 5% FCS, L-glutamine (2 μ M), penicillin (100 U/ml), streptomycin (100 μ g/ml) and β -mercaptoethanol (50 nM; all from Invitrogen, Cergy Pontoise, France) and restimulated with OVA (10 mg/ml) for 5 days.

Dendritic cell culture and uptake of OVA-FITC

Bone marrow cells were isolated from femurs of WT and NLRP3^{-/-} mice and differentiated into myeloid DC as described previously (20). At day 10, 100 μ g/ml OVA-FITC (Molecular probes, Cergy Pontoise, France) was added to DC cultures. Cells were incubated for 2 h at 37°C in a humidified incubator with 5% CO₂. Cells were collected, washed three times with cold PBS containing 1% BSA, and analyzed using a FACS-Canto II Flow cytometer (Becton-Dickinson, San Jose, CA, USA) and FLOWJo Software (Tree Star, Inc., Ashland, OR, USA). The markers for the monovariant histogram were

set based on the negative staining control, which was provided by incubating DCs with OVA-FITC at 4°C.

In vivo migration assay

To follow DC migration, 500 µg of OVA-FITC (Molecular probes) was administrated intranasally. Control mice received saline. Twenty-four hours after injection, cells were counted and stained with the following antibodies, PE-labeled CD40 (clone 3/23), APC-labeled CD80 (clone 16-10A1) and biotin-labeled MHCII (clone 2G9) and isotype-matched control. All the antibodies were purchased from BD Biosciences, France. DCs containing OVA were counted in the mLN as CD11c⁺ MHCII⁺ and FITC⁺ cells.

Statistical analysis

The data are presented as the mean ± SEM with $n = 6$ animals per group. The significance of differences between two groups was determined by one-way ANOVA (nonparametric test), followed by Bonferroni comparison test using Prism software. Statistical significance was reported if $P < 0.05$ was achieved.

Results

NLRP3 inflammasome drives allergic airway inflammation

To determine the functional role of NLRP3 in allergic lung inflammation model in the absence of aluminum adjuvant, we immunized WT and NLRP3-gene-deficient (NLRP3^{-/-}) (10 µg, subcutaneous injection) mice with OVA on days 0 and 7 followed by three intranasal challenges on days 14–16. OVA-sensitized and OVA-challenged WT mice showed a robust cell infiltration mostly eosinophils into the lung compared to saline-treated mice (Fig. 1A). By contrast, we found a significant reduction in eosinophil recruitment in BAL and in lung (EPO activity) in OVA-challenged NLRP3^{-/-} mice compared with WT mice (Fig. 1A, B). The reduced leukocyte infiltration seen in OVA-challenged NLRP3^{-/-} mice correlated with diminished IL-1β and IL-6 in lung homogenate (Fig. 1C, D). Th2 cytokines including IL-5 in BAL (Fig. 1E) and TSLP and IL-33 in lung (Fig. 1F, G) were also reduced in NLRP3^{-/-} mice. Diminished IL-13 and IL-4 levels were found in the culture supernatants of mLN from NLRP3^{-/-} mice compared with WT mice after antigen restimulation (Fig. 1H, I). Serum levels of OVA-specific IgE were significantly lower in NLRP3^{-/-} mice than in WT mice, indicating a systemic impairment of the adaptive Th2 response in NLRP3^{-/-} mice (Fig. 1J). The expression of chemokines CCL5 (rantes) and CCL11 (eotaxin), known to play a role in eosinophil influx into the lung, and CCL17 (TARC), which is a Th2 lymphocyte attractive chemokine, were also reduced in lung of OVA-challenged NLRP3^{-/-} mice compared with WT mice (Fig. 1K–M). Therefore, NLRP3 is required to develop an allergic response because inflammatory cell infiltration, cytokine and chemokine production, and specific IgE response were impaired in NLRP3-deficient mice.

Absence of NLRP3 prevents lung inflammation and mucus hypersecretion

Microscopically, the lung tissue of OVA-immunized and OVA-challenged WT mice showed peribronchial eosinophil and lymphocyte recruitment together with mucus hypersecretion and thickening of the epithelium with hyperplasia of smooth muscle and goblet cells (Fig. 2A, B). Consistent with the reduction in lung inflammation, cell recruitment and mucus overproduction were dramatically reduced in OVA-challenged NLRP3^{-/-} mice (Fig. 2A, B).

Reduced pulmonary CD4⁺ IL-13⁺ producing cells in NLRP3-deficient mice

IL-13 is produced by activated CD4⁺ T cells and is critical for the promotion of allergic inflammatory responses by mediating airway hyper-responsiveness and mucus hypersecretion (21). To decipher the importance of NLRP3 inflammasome in IL-13 producing cells in allergic lung inflammation, we isolated and restimulated lung mononuclear cells from OVA-challenged WT and NLRP3^{-/-} mice and restimulated them with PMA/ionomycin for 4 h. Pulmonary CD4⁺IL-13⁺-producing cells were increased in OVA-challenged WT mice compared with saline controls (Fig. 3A, C). By contrast, CD4⁺IL-13⁺ cells were drastically reduced (percentage and absolute numbers) in NLRP3^{-/-} mice (Fig. 3A, C). The majority of IL-13-producing cells were CD4⁺ cells, not CD8⁺ cells as shown by the representative dot plot and histograms (Fig. 3B, D).

Thus, the absence of allergic lung inflammation in NLRP3^{-/-} mice is associated with a dramatic reduction in recruitment and activation of CD4⁺IL-13⁺ cells into the lung.

DC migration into draining lymph nodes is reduced in the absence of NLRP3

Airway DC were known to sample antigen in the lung and migrate to draining lymph nodes to stimulate naive T cells (22, 23). To determine whether the absence of NLRP3 could have an effect on DC migration *in vivo*, OVA-FITC was administered intranasally to WT and NLRP3^{-/-} mice and the uptake and transport of OVA-FITC complex were analyzed in the draining mLN. The total number of DC in draining mLN was counted 24 h after the OVA-FITC administration. The number of DC (MHCII⁺CD11c⁺) was greatly enhanced in OVA-FITC-sensitized WT mice compared with OVA-FITC-sensitized NLRP3^{-/-} mice (Fig. 4A). Interestingly, we observed a significant reduction in OVA-FITC uptake by CD11c⁺ cells from NLRP3^{-/-} mice compared with WT mice (Fig. 4B), and this was accompanied by a strong decrease in the expression of the costimulatory molecules CD40 and CD80 in NLRP3-deficient mice compared to WT mice (Fig. 4D–I). Importantly, OVA uptake and processing were not affected in NLRP3-deficient bone marrow-derived DC *in vitro* (Fig. 4C). Therefore, DC antigen processing and activation are impaired in NLRP3-deficient mice, which may explain the reduced Th2 response in these mice after OVA sensitization and challenge.

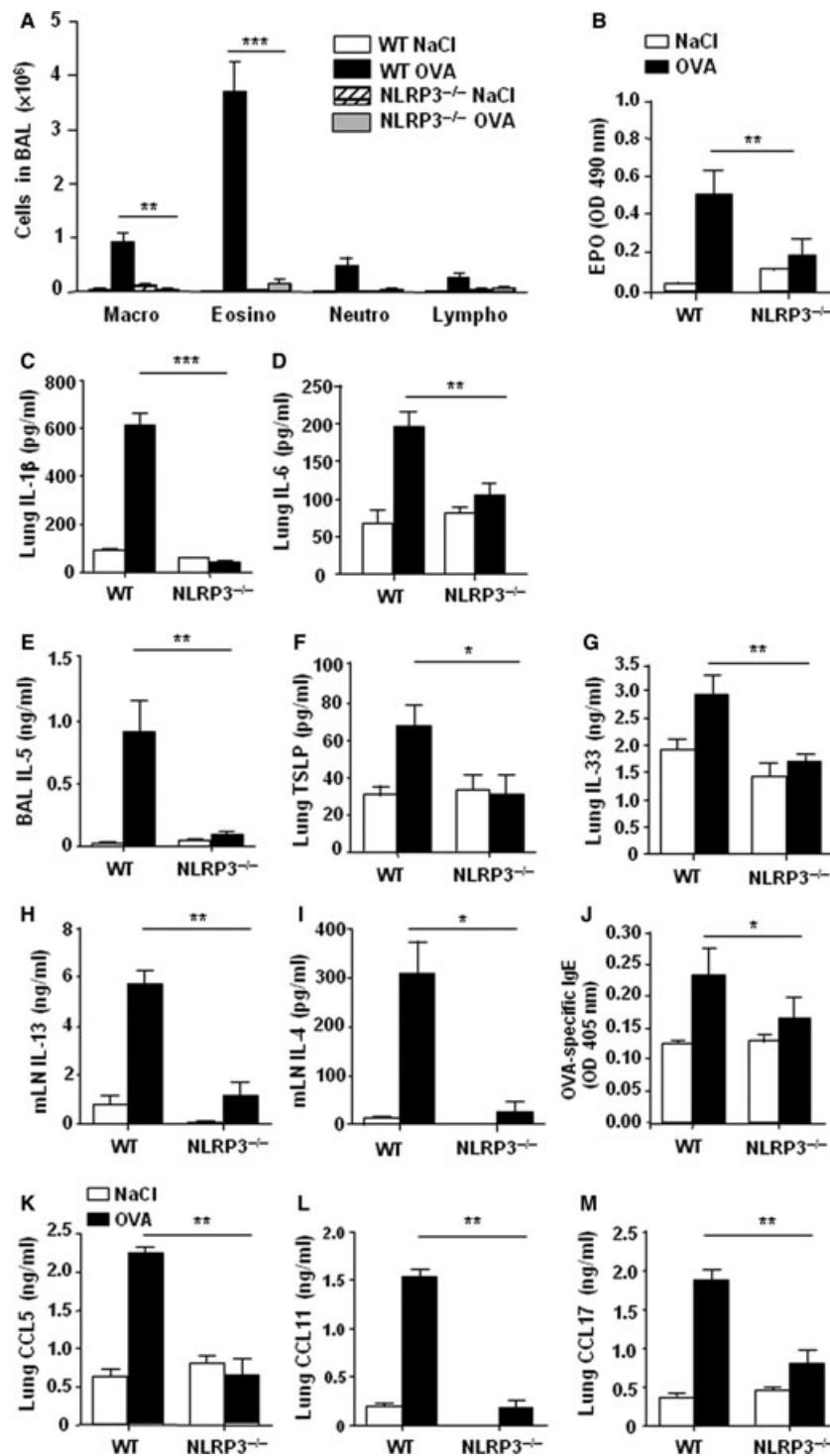


Figure 1 NLRP3 mediates allergic inflammation. BALF cell differential counts of wild-type and NLRP3^{-/-} mice sensitized by s.c. injection of ovalbumin (OVA) on days 0 and 7 and exposed on days 14–16 to intranasal OVA challenges (A). Eosinophil recruitment to the tissue was estimated by measuring eosinophil peroxidase activity in the lung tissue homogenates given in optical density (OD 490 nm) values in (B). Th2 cytokine and chemokine production in

lung, bronchoalveolar lavage, and mediastinal lymph nodes and OVA-specific serum IgE levels was determined by ELISA (C–M). The IgE levels are expressed as OD at 405 nm. These experiments were performed 3 times. One representative experiment is shown. Values are the mean ± SEM of 8–10 mice per group. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Both IL-1 α and IL-1 β are critical for the development of pulmonary Th2 response

As the NLRP3 inflammasome is crucial for IL-1 β production, we investigated the role of IL-1R1 signaling. Furthermore, we explored the contribution of IL-1 α , which is released independently of NLRP3 independent in the development of allergic lung inflammation. Airway inflammation was significantly reduced in OVA-sensitized and OVA-challenged IL-1R1-deficient mice, but also in mice deficient in IL-1 β or IL-1 α . Eosinophil recruitment in BAL (Fig. 5A) and lung (Fig. 5B) as well as IL-1 β , TSLP, and IL-33 levels in the lung and IL-5 production in the BAL was significantly impaired (Fig. 5C–F). IL-5 and IL-13 production in mLN cell culture supernatants was lower in OVA-challenged IL-1 α ^{−/−}, IL-1 β ^{−/−}, and IL-1R1^{−/−} mice compared with WT mice (Fig. 5G, H). Furthermore, the levels of the Th2-specific chemokines, CCL17 and CCL11 in the lung (Fig. 5I, J) were also reduced in IL-1 α ^{−/−}, IL-1 β ^{−/−}, and IL-1R1^{−/−} mice.

The peribronchial eosinophil and lymphocyte recruitment, together with mucus hypersecretion seen in lung tissue of

OVA-immunized and OVA-challenged WT mice was dramatically reduced in the absence of IL-1R1, IL-1 β , and IL-1 α (Fig. S1A, B).

Therefore, the findings obtained in mice deficient in IL-1R1 and IL-1 β are consistent with an implication of NLRP3 activation pathway in the OVA-induced allergic response. The fact that absence of IL-1 α also abrogates allergic inflammation may be related to the impaired IL-1 β level seen in IL-1 α ^{−/−} mice. Thus, IL-1 β and IL-1 α , through IL-1R1 signaling, are essential in OVA-induced allergic lung inflammation and mucus overproduction.

Disruption of IL-1R1 and IL-1 β genes reduced IL-13-producing cells and airway inflammation

To investigate the importance of IL-13-producing cells in the IL-1R1-mediated allergic response, we isolated and stimulated lung mononuclear cells from OVA-challenged WT, IL-1R1^{−/−}, and IL-1 β ^{−/−} mice with PMA/ionomycin for 4 h, followed by intracellular staining of IL-13. Reduced frequency and absolute number of CD4⁺IL-13⁺ cells were

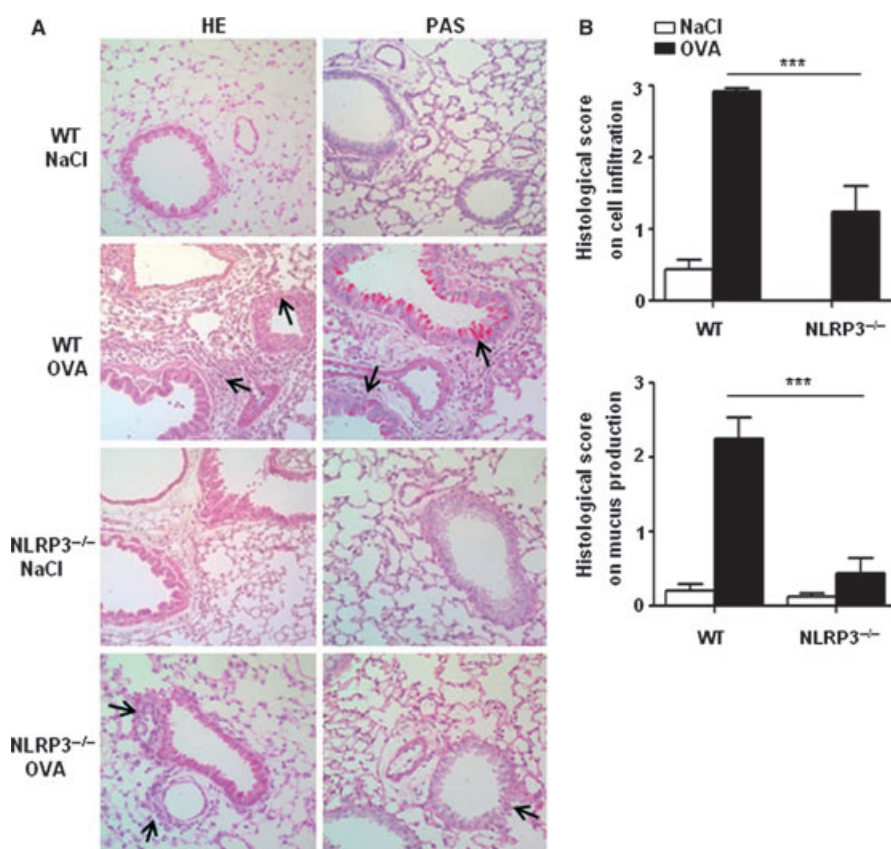


Figure 2 Reduced lung inflammation after ovalbumin (OVA) exposure in NLRP3^{−/−} mice. Lung sections from saline- and OVA-sensitized and saline- and OVA-challenged wild-type and NLRP3^{−/−} mice were stained with hematoxylin & eosin (HE) to visualize cell recruitment and with periodic acid Schiff reagent to visualize mucus (A),

as shown by the arrows. The extent of peribronchial and perivascular inflammation was scored by two independent observers (B). Magnification is $\times 40$. These experiments were performed three times. One representative experiment is shown. Values are the mean $\pm$ SEM of six mice per group (B). *** $P \leq 0.001$.

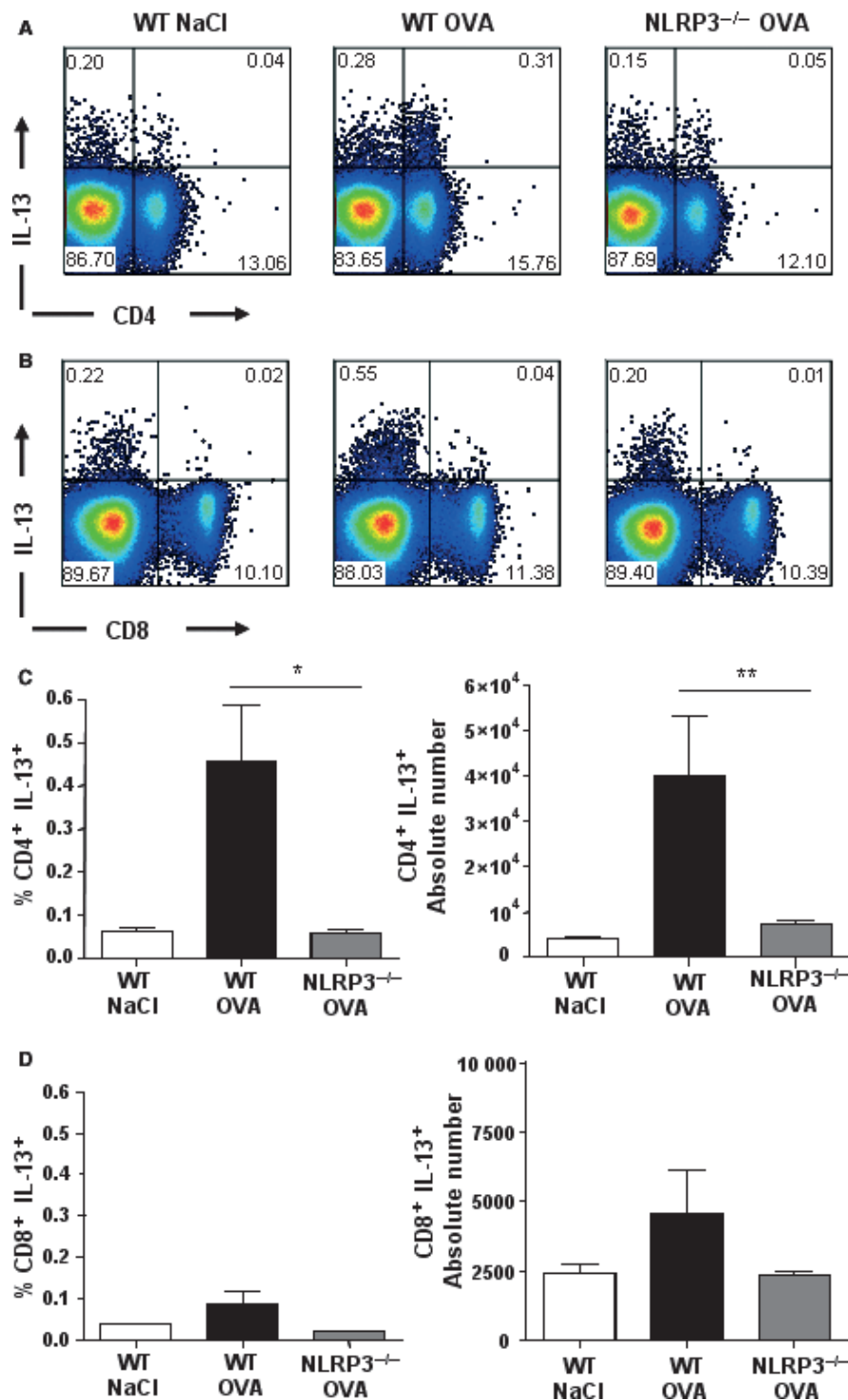


Figure 3 Reduced IL-13 production in NLRP3^{-/-} mice after ovalbumin (OVA) exposure. OVA-sensitized wild-type and NLRP3^{-/-} mice were challenged three times with OVA. Twenty-four hours after the third challenge, lung mononuclear cells were isolated and restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of Brefeldin A followed by extracellular staining of TCRβ,

CD4, and CD8α, and intracellular staining of IL-13. Representative dot plots (A, B), frequency and cell numbers of CD4⁺ IL-13⁺ (C), and CD8α⁺ IL-13⁺ (D) cell populations are given. These experiments were performed three times. Values are the mean ± SEM of six mice per group. **P* ≤ 0.05; ***P* ≤ 0.001.

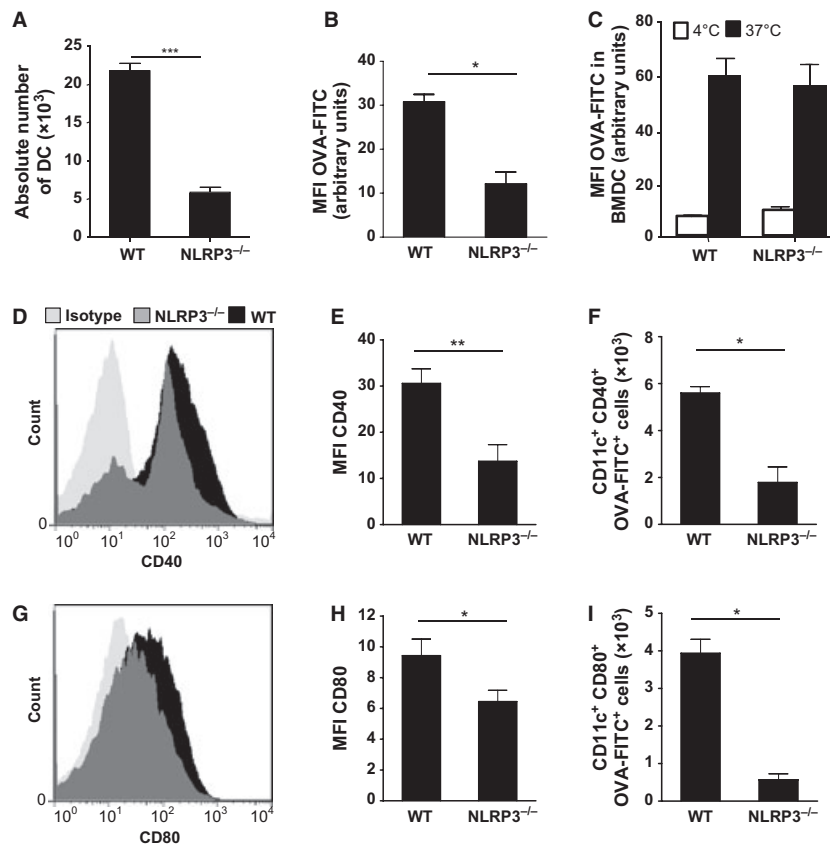


Figure 4 Reduced dendritic cell (DC) migration in mediastinal lymph nodes (mLN) of NLRP3-deficient mice. Ovalbumin (OVA)-FITC was administered in the lung of wild-type (WT) and NLRP3^{-/-} mice by intranasal route, DC antigen uptake and migration were followed twenty hours later in mLN. Absolute numbers of DC (MHCII⁺CD11c⁺) (A), mean of fluorescence intensity (MFI) of OVA-

FITC (B), histograms, MFI and absolute numbers of OVA-FITC⁺ DC cells expressing CD40 (D-F) or CD80 (G-I) were analyzed in mLN. *In vitro* OVA-FITC uptake on bone marrow-derived dendritic cells (BMDC) from WT and NLRP3^{-/-} mice was analyzed (C). These experiments were performed three times. Values are the mean ± SEM of six mice per group. **P* ≤ 0.05.

found in OVA-challenged IL-1R1^{-/-} and IL-1β^{-/-} mice (Fig. 6A, B). Therefore, IL-1R1 signaling is important for IL-13 expression by CD4⁺ T cells in the lung.

Discussion

Here, we investigated the role of NLRP3 inflammasome and IL-1R1 signaling in OVA-induced allergic lung inflammation in the absence of alum adjuvant in order to avoid any overlapping effect of this strong NLRP3 activator. We report that NLRP3 deficiency causes impaired Th2 response and allergic lung inflammation with lower eosinophil influx in the airways, and pulmonary Th2 cytokine and chemokine production. These effects were accompanied by diminished DC migration to draining lymph nodes and IL-13-producing CD4⁺ T-cell recruitment in the lung of NLRP3-deficient mice. Impaired allergic responses were documented in OVA-challenged mice deficient in IL-1α, IL-1β, or IL-1R1.

The exact mechanism by which NLRP3 and IL-1α/β signaling deficiencies reduce the recruitment of inflammatory cells is not well documented. Here, we demonstrate that the release

of eosinophils (CCL11) and Th2 lymphocytes (CCL17) chemoattractant was strongly reduced in mice deficient in NLRP3, IL-1α, IL-1β, and IL-1R1, suggesting that NLRP3 and IL-1α/β signaling pathways may participate in the control of chemokine gradients necessary for cell recruitment. Moreover, reduction in inflammation in NLRP3-deficient mice could be related to an alteration of DC functions. DC are known to be crucial for allergic lung inflammation because they contribute to the Th2 lymphocyte recruitment into the airways and trigger local Th2 lymphocytes effector cytokine production (23). While antigen uptake and activation of myeloid DC was not affected *in vitro*, the migration and activation of antigen-loaded DC to mLN was strongly reduced in NLRP3-deficient mice. Therefore, impairment of DC function affected Th2 cell accumulation and cytokine and chemokine productions in the lung of NLRP3-deficient mice.

Moreover, TSLP level in the lung was reduced in mice deficient in NLRP3, IL-1α, IL-1β, and IL-1R1. These data could be linked to the defective function of DC because TSLP is known to induce upregulation of OX40L on DC, which in turn promotes Th2 cell differentiation (24). TSLP

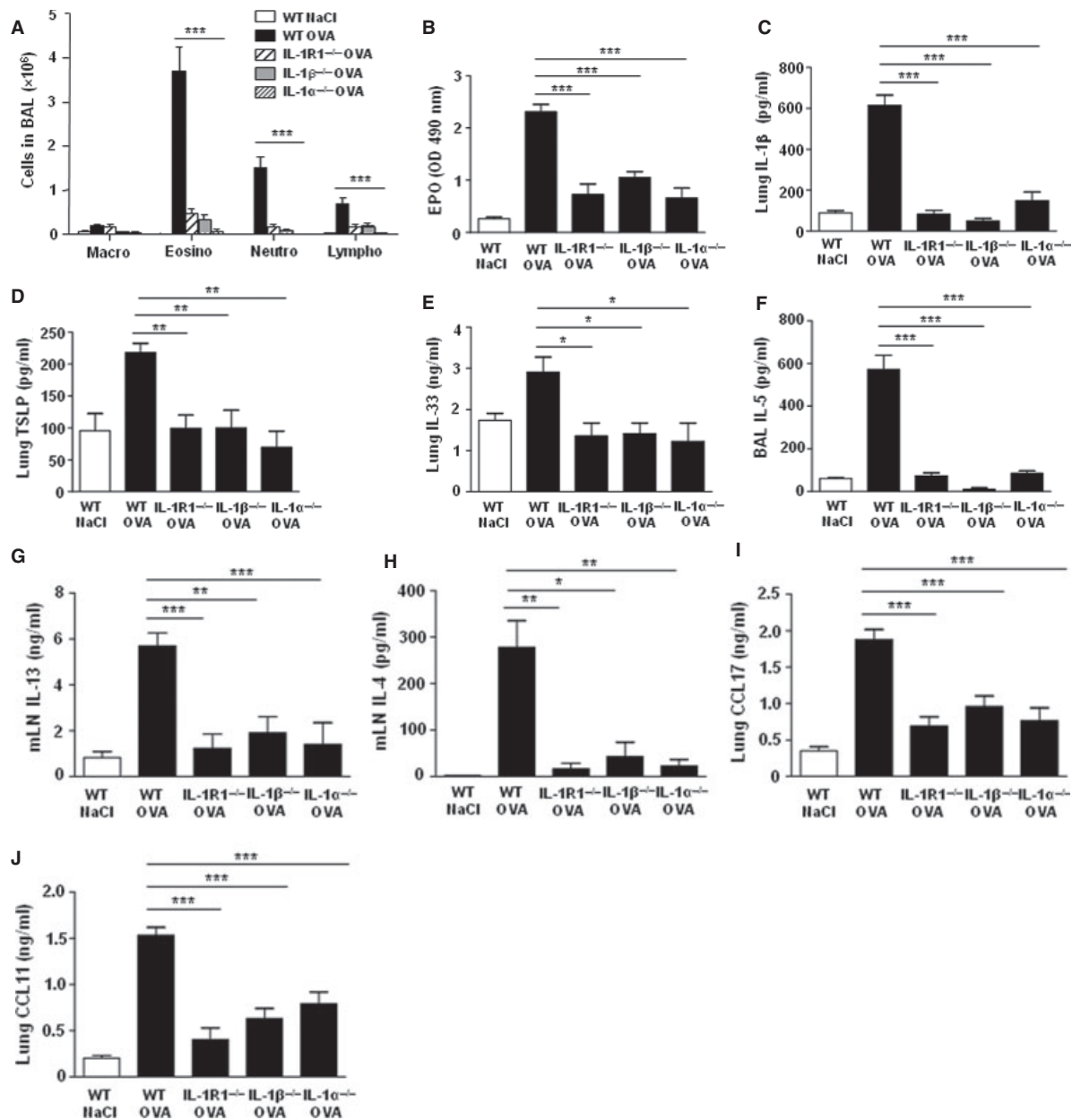


Figure 5 IL-1R1 signaling is critical during allergic response. BALF cell differential count, measured by flow cytometry, of wild-type, IL-1R1^{-/-}, IL-1β^{-/-} and IL-1α^{-/-} mice sensitized by subcutaneous injection of ovalbumin (OVA) on days 0 and 7 and exposed on days 14–16 to intranasal OVA challenges (A). Eosinophil recruitment into the tissue was estimated by measuring eosinophil peroxidase activity in the lung tissue homogenates given in optical density (OD

490 nm) values (B). Th2 cytokine and chemokine production in lung, bronchoalveolar lavage, mediastinal lymph nodes, and OVA-specific IgE levels in serum were determined by ELISA (C–J). The IgE levels are expressed as OD at 405 nm. These experiments were performed three times. One representative experiment is shown. Values are the mean ± SEM of six mice per group. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

can also directly influence CD4⁺ T-cell polarization, inducing Th2 cell differentiation and IL-4 synthesis (25).

IL-33 belongs to the IL-1 cytokine family and was known to promote Th2 responses and enhance allergic inflammation (26). IL-33 can be cleaved by caspase-1 after SER₁₁₁ residue to its biological active form (27) or after ASP₁₇₈ residue to

an inactive form and by caspase-3 (28). However, IL-33 cleavage is controversial, and the exact link between NLRP3 inflammasome and IL-33 remains to be elucidated (27–29). We found here low levels of IL-33 in saline- and OVA-treated NLRP3-deficient mice compared with saline- and OVA-treated WT mice, suggesting that NLRP3 inflamma-

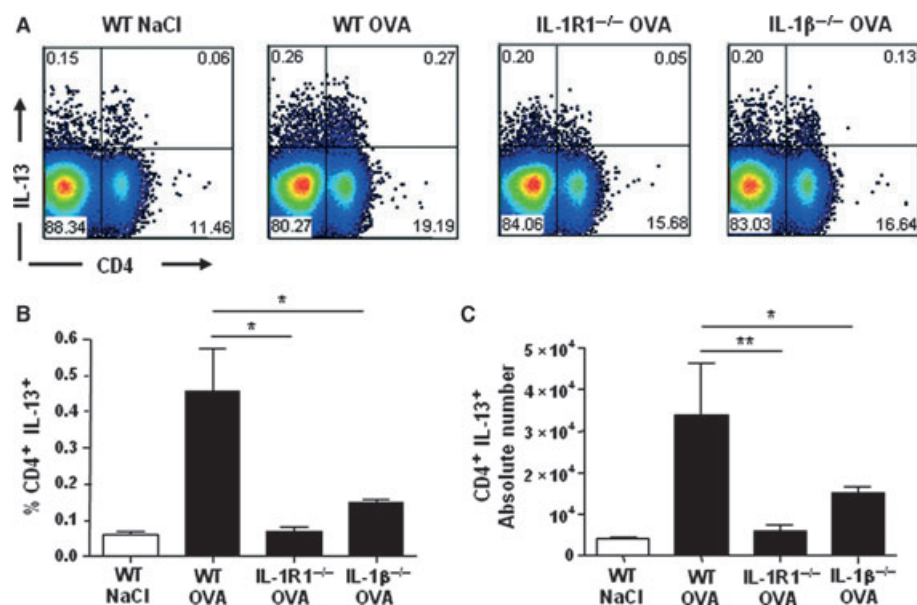


Figure 6 IL-1 α and IL-1 β signaling is necessary for IL-13-producing CD4⁺ T-cell recruitment in the lung. IL-13 producing T-cell subsets in lung of ovalbumin (OVA)-sensitized and OVA-challenged wild-type, IL-1 β ^{-/-}, and IL-1R1^{-/-} mice were assessed by flow cytometry. Lung mononuclear cells were stained and isolated and restimulated with PMA (50 ng/ml) and ionomycin (750 ng/ml) in the presence of Brefeldin A followed by extracellular staining of TCR β

and CD4 and intracellular staining of IL-13. The frequency and absolute cell number of CD4⁺IL-13⁺ (A, C) cell populations are given. Representative dot plot, frequency, and absolute number of IL-13⁺CD4⁺ cells gated on TCR β ⁺ cell populations (A–C) were shown. These experiments were performed three times. Values are the mean $\pm$ SEM of six mice per group. * $P \leq 0.05$; ** $P \leq 0.01$.

some may affect IL-33 production during allergic lung inflammation. This effect might be indirect, through IL-1 pathway because we found reduced IL-33 levels in mice deficient in IL-1 α , IL-1 β , and IL-1R1 compared with WT mice. These data suggest a correlation between the low level of IL-33, which is known as Th2 cytokine, and the inhibition of allergic asthma in the absence of IL-1 α , IL-1 β , or IL-1R1.

It is well accepted that endotoxin contamination of OVA is necessary to induce Th2 responses to inhaled antigens in mouse model of allergic asthma (30). LPS binding to TLR4 leads to NF κ B activation and pro-IL-1 β synthesis. When OVA is administered with alum, the cytotoxic effect of this adjuvant induces cell death and release of danger signals, such as ATP or uric acid crystals, leading to inflammasome activation and maturation of IL-1 β (13, 15). In our alum-free model, we observed an increase in ATP levels in the BAL of OVA-immunized and OVA-challenged mice compared with saline control (Fig. S2). Likewise, elevated ATP levels are found in the airways of patients with asthma and chronic obstructive pulmonary disease (31, 32). These observations suggest that cell death occurs in the lung during established asthma and is sufficient to induce NLRP3 inflammasome activation even in the absence of adjuvant.

The role of IL-1 α in inflammation is being revisited. As IL-1 α is active as immature form, it is considered as an alarmin, which is released by injured cells (3). In a model of

sterile inflammation induced by dead cells, antibody neutralization of IL-1 α significantly reduced the inflammatory response, whereas IL-1 β neutralization did not (12).

The interconnection between IL-1 α and IL-1 β is well documented, and there is increasing evidence showing that their productions are linked. A recent study showed *in vitro* that IL-1 α -deficient macrophages produce less IL-1 β than WT macrophages (33). By contrast, IL-1 α expression was strongly reduced in the brain of IL-1 β -deficient mice treated with turpentine (17). In addition, other studies suggest that IL-1 α production may be caspase-1 or NLRP3 inflammasome dependent (34, 35). Here, we showed a significant reduction in IL-1 β production in OVA-sensitized and OVA-challenged IL-1 α -deficient mice, suggesting that IL-1 β production may be in part under the control of IL-1 α .

In conclusion, using an adjuvant-free mouse model of allergic asthma, we document a critical role of NLRP3 inflammasome and IL-1 signaling in driving lung inflammation. We demonstrate here that NLRP3 inflammasome activation, occurring during allergic lung inflammation, contributes to DC functions and the induction of Th2 response. NLRP3 disruption strongly affects IL-1 signaling, which is crucial for asthma pathogenesis.

In addition, we showed that the impaired allergic inflammation in IL-1R1-deficient mice is attributable to not only IL-1 β but also IL-1 α signaling. Further investigations are required to determine the respective role of these cytokines in

experimental asthma. Therefore; the NLRP3/IL-1 pathway may represent an interesting therapeutic target in allergy.

Acknowledgments

This work was supported by the 'Fondation pour la Recherche Médicale' (FRM allergy DAL 2007 0822007) and the 'Fond européen de développement régional' (FEDER Asthme 1575-32168).

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Figure S1. Diminished lung inflammation after OVA exposure in IL-1R1, IL-1 β and IL-1 α -deficient mice.

Figure S2. Release of ATP during allergic airway inflammation in WT mice.

Data S1. Methods.

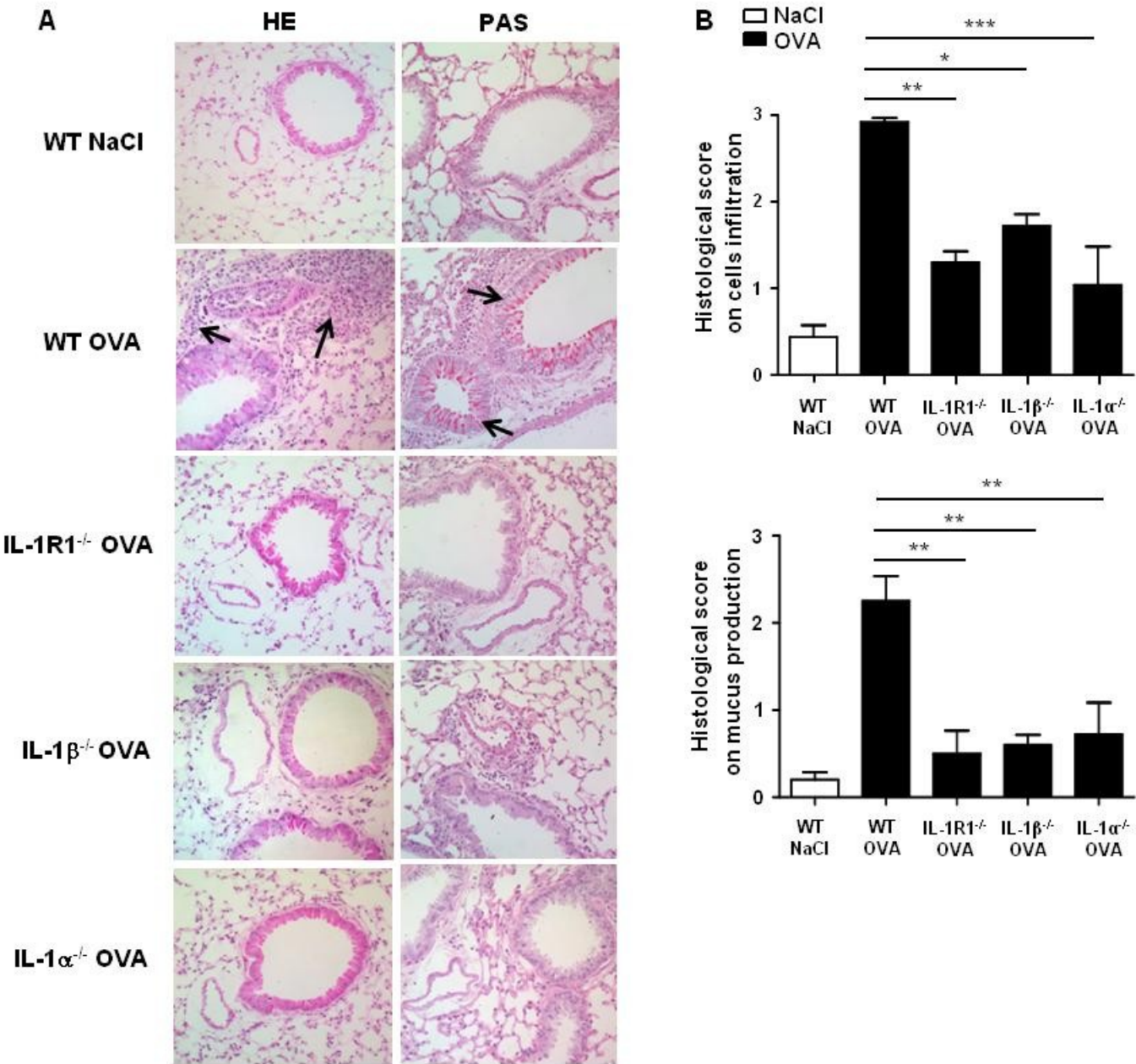
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ONLINE SUPPLEMENTARY FIGURES

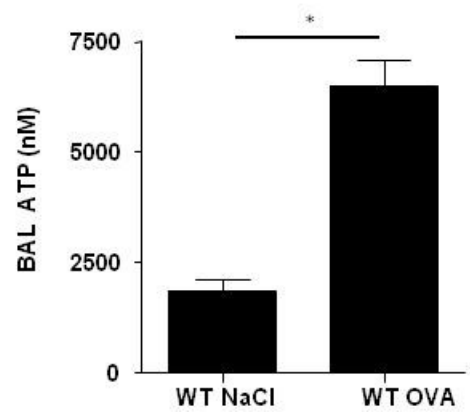
Fig.S1



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ONLINE SUPPLEMENTARY FIGURES

Fig.S2



Critical role of NLRP3 inflammasome in allergic lung inflammation in the absence of aluminium adjuvant

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ONLINE SUPPLEMENTARY INFORMATION

DATA S1: ONLINE SUPPLEMENTARY METHODS

Flow cytometry analysis on BAL fluid and lung mononuclear cells

Cells from BAL were stained with the following antibodies: PerCP- labeled MHCII (clone 2G9), PE-labeled Siglec F (clone E50-2440), FITC-labeled CD3e (clone 145-2C11), FITC-labeled B220 (clone RA3-6B2), PE-cy7-labeled GR1 (clone RB6-8C5), APC-labeled CD11c (clone N418). To prevent a specific binding to FcR, 2.4.G2 blocking purified antibody was used. All the antibodies were purchased from BD Biosciences, France. Eosinophils were identified as non auto fluorescent SSC^{high} expressing Siglec F receptor, and lacking expression of MHCII, B220 and CD3e as described (31). In some experiments, cell suspensions from lung were restimulated in vitro for 4h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml; both from Sigma-Aldrich) in presence of Brefeldine A (GolgiPlug, BD Biosciences, France). After 4h, cells were stained with biotin-labeled TCR β (clone H57-597), V450-labeled CD4 (clone RMA-5) and APC-Cy7-labeled CD8a (clone 53-6.7). Cells were permeabilized for 20 min with cytofix/cytoperm kit (BD Biosciences, France) and stained with Alexa 647-labeled IL-13 (clone Ebio13A, eBioscience). Samples were analyzed on a BD CANTO II flow cytometer. Fluorescence data were acquired by using DIVA software (BD Bioscience, France) and analyzed using FlowJO software (Treestar).

ATP measurements in BALF of asthmatic mice.

To measure ATP levels in BALF, fresh BAL samples were centrifuged at 4 °C and heated at 55°C. Then ATP levels were measured using ATPlite assay (Perkin Elmer, Waltham, MA) according to instructions, but with the cell lysis step omitted to avoid any contaminating intracellular ATP. Luminescence was measured with a luminometer (Victor 3V, Perkin Elmer).

Supplementary figure legend

Fig.S1 . Diminished lung inflammation after OVA exposure in IL-1R1, IL-1 β and IL-1 α -deficient mice

Lung sections from OVA-sensitized and challenged WT and IL-1R1, IL-1 β and IL-1 α -deficient mice were stained with hematoxylin & eosin to visualize cell recruitment and with periodic acid schiff reagent (PAS) to visualize mucus (**A**), as shown by the arrows. The extend of peribronchial and perivascular inflammation and mucus production was scored by two independent observers (**B**). Magnification is x 40. Values represent the mean $\pm$ SEM of 6 mice per group. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant.

Fig. S2. Release of ATP during allergic airway inflammation in WT mice.

ATP levels in the BALF of OVA sensitized and challenged WT mice and saline controls. Values represent the mean $\pm$ SEM of 6 mice per group. * $P \leq 0.05$