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This information is current as of February 13, 2012

J Immunol; Prepublished online 8 February 2012;

doi:10.4049/jimmunol.1102488

<http://www.jimmunol.org/content/early/2012/02/08/jimmunol.1102488>

Supplementary Data	http://www.jimmunol.org/content/suppl/2012/02/08/jimmunol.1102488.DC1.html
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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Analysis of NLRP3 in the Development of Allergic Airway Disease in Mice

Irving C. Allen,^{*,†} Corey M. Jania,[‡] Justin E. Wilson,[†] Erin M. Tekeppe,[†] Xiaoyang Hua,[‡] Willie J. Brickey,[†] Mildred Kwan,[§] Beverly H. Koller,^{§,¶} Stephen L. Tilley,^{‡,§} and Jenny P.-Y. Ting^{*,†,§}

The contribution of NLRP3, a member of the nucleotide-binding domain leucine-rich repeat-containing (NLR) family, to the development of allergic airway disease is currently controversial. In this study, we used multiple allergic asthma models to examine the physiologic role of NLRP3. We found no significant differences in airway eosinophilia, histopathologic condition, mucus production, and airway hyperresponsiveness between wild-type and *Nlrp3*^{-/-} mice in either acute (alum-dependent) or chronic (alum-independent) OVA models. In addition to the OVA model, we did not detect a role for NLRP3 in the development of allergic airway disease induced by either acute or chronic house dust mite Ag exposure. Although we did not observe significant phenotypic differences in any of the models tested, we did note a significant reduction of IL-13 and IL-33 in *Nlrp3*^{-/-} mice compared with wild-type controls in the chronic OVA model without added alum. In all of the allergic airway disease models, the NLRP3 inflammasome-associated cytokines IL-1 β and IL-18 in the lung were below the level of detection. In sum, this report surveyed four different allergic asthma models and found a modest and selected role for NLRP3 in the alum-free OVA model. However, this difference did not greatly alter the clinical outcome of the disease. This finding suggests that the role of NLRP3 in allergic asthma must be re-evaluated. *The Journal of Immunology*, 2012, 188: 000–000.

Asthma, a chronic inflammatory disorder with an extensive range of genetic and environmental risk factors, is characterized by airway inflammation, bronchoconstriction, and airway hyperresponsiveness (AHR). These pathologic features clinically manifest as exacerbations of wheezing and breathlessness, which have significant impact on the quality of life. The airway inflammation that occurs during atopic asthma is associated with exposure to either specific allergens, such as house dust mite (HDM) allergen, or nonspecific triggers, such as air pollution (1). Many of these allergens are immunologically non-reactive and do not induce disease unless they are cocontaminated

with pattern-associated molecular patterns (PAMPs), such as LPS, or damage-associated molecular patterns (DAMPs), including uric acid (UA) and ATP (1).

It has been suggested that exposure to low levels of PAMPs and DAMPs is responsible for priming the Th2 immune response to many common allergens (2). Similarly, several studies have identified a potential role for various bacteria and virus species in influencing the development of adaptive immunity and allergy (3). Indeed, mouse studies have indicated that Th2 responses are at least partially dependent upon pattern recognition receptors (PRR), such as TLR4 and MyD88, which are historically associated with the innate immune response to pathogens (4). TLRs represent the archetype transmembrane PRRs and function through extracellular ligand recognition. In addition to the TLRs, the innate immune response is also regulated by the nucleotide-binding domain leucine-rich repeat-containing (NLR) family of PRRs. Although the underlying mechanism for the involvement of TLRs in mediating the adaptive immune response is not clear, one hypothesis suggests that activation of these PRRs by their respective ligands produces a cytokine cascade that induces leukocyte maturation and results in enhanced atopy (5).

The NLRs are cytosolic proteins that sense intracellular PAMPs and DAMPs. To date, >22 distinct NLR family members have been characterized in metazoans, and mutations in several *NLR* genes have been associated with a broad spectrum of human disorders, including atopic diseases. For example, mutations in *NOD1* (*NLRC1*) and *NOD2* (*NLRC2*) are associated with Crohn's disease, and these same polymorphisms have been shown to confer susceptibility to asthma, atopic dermatitis, and increased IgE levels (6). The NLR family can be further categorized into subgroups on the basis of their common proinflammatory or anti-inflammatory properties. One subgroup includes NLRs that are capable of forming a multiprotein complex with the NLR adaptor protein PYCARD (ASC) and caspase-1. This complex is termed the inflammasome and is defined by the specific NLR that pro-

*Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [†]Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [‡]Division of Pulmonary and Critical Care Medicine, Department of Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [§]Center for Environmental Medicine, Asthma and Lung Biology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and [¶]Department of Genetics, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Received for publication August 30, 2011. Accepted for publication January 10, 2012.

This work was supported by National Institutes of Health Grants A1063031, U19-AI067798, U19-AI077437, DK38108, DE016326, and HL068141 (to J.P.-Y.T.) and F32-AI-082895-01, T32-AR007416, and T32-CA009156 (to I.C.A.), as well as the American Cancer Society (to I.C.A.); J.P.-Y.T. is a Sandler Program Award Recipient.

Address correspondence and reprint requests to Dr. Jenny P.-Y. Ting, University of North Carolina School of Medicine, Campus Box 7295, Room 209, Chapel Hill, North Carolina 27599; E-mail address: jenny_ting@med.unc.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: AB/PAS, Alcian-blue/periodic acid-Schiff; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DAMP, damage-associated molecular pattern; G, tissue resistance; HDM, house dust mite; i.n., intranasal; MCh, methacholine; NLR, nucleotide-binding domain leucine-rich repeat containing; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; Raw, airway resistance; UA, uric acid.

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vides specificity to the complex. NLRs in this subgroup sense the intracellular environment and undergo a conformational change following activation that allows the NLR to associate with PYCARD and Caspase-1. Once the inflammasome is formed, caspase-1 becomes activated and cleaves pro-IL-1 β and pro-IL-18 into their mature cytokines. Several different NLRs have been shown to be capable of inflammasome formation in the presence of specific stimuli, including NLRP3 (cryopyrin) and NLRC4 (IPAF). NLR inflammasomes have been most extensively studied in the context of the host innate immune response to specific pathogens, PAMPs, and DAMPs.

NLRP3 is one of the most extensively characterized NLRs owing to its relevance in several human inflammatory disorders and because a diverse range of stimuli are associated with its activation. However, the contribution of the NLRP3 inflammasome to asthma pathogenesis has not been fully elucidated. In humans, genetic studies have revealed two gain-of-function single nucleotide polymorphisms in the *NLRP3* gene that were significantly associated with food-induced anaphylaxis and aspirin-induced asthma (7). Similarly, NLRP3 expression levels have been shown to be significantly upregulated in human nasal epithelial cells during exacerbations of allergic rhinitis and downregulated among patients during pollen season (8). Although the functional significance of these findings is not clear, a significant amount of evidence supports a role for the NLRP3 inflammasome in sensing a variety of agents known to induce asthma exacerbations, including ROS, ATP, LPS, and several bacterial and viral species.

A critical role for the NLRP3 inflammasome in the development of allergic airway inflammation in mice has also been described. *Nlrp3*^{-/-}, *Pycard*^{-/-}, and *Casp-1*^{-/-} mice were found to have significantly attenuated airway inflammation, Ig Ab production, and cytokine release in response to OVA (2, 9). Although the physiologic outcomes of these studies are similar, the underlying mechanisms proposed for NLRP3 inflammasome function are not well defined. The original study by Eisenbarth et al. (2) did not directly examine the underlying mechanism; however, the authors suggest that the NLRP3 inflammasome may directly influence the development of allergic airway inflammation by downregulating IL-1 β , IL-18, and IL-33. It has also been suggested that these inflammasome-mediated cytokines influence Th2 cell proliferation and IgE Ab production, whereas IL-33 can be a potent Th2 stimulus, depending on the milieu (10–12). Similarly, the study by Besnard et al. (9) has indicated that the absence of IL-1 β results in the reduced expression of Th2-associated cytokines, including IL-13, IL-5, and IL-33, which ultimately leads to attenuated allergic airway inflammation.

Contrary to these previous reports, recent data have shown that components of the NLRP3 inflammasome are dispensable in the development of allergic airway disease in mice. *Nlrp3*^{-/-} mice were found to develop Th2 cell-dependent airway inflammation at levels comparable to those in wild-type animals in OVA and HDM Ag models (13). In addition to controversy regarding NLRP3, the role of IL-1 β in human asthma and allergic airway disease in mice has also been a historically divisive issue. Although it is likely that IL-1 β indirectly contributes to asthma through instigating the host innate immune response following PAMP or DAMP activation, which can ultimately result in exacerbations, the direct contribution of IL-1 β to the Th2-mediated immune response and adaptive immunity is unclear. Recent data have demonstrated that mice lacking either IL-1R or MyD88, which function as intermediate signaling molecules associated with the IL-1R, developed normal Th2-mediated cellular immunity and airway inflammation following OVA exposure (13). However, IL-1 α , IL-1 β , and the IL-1R have previously been found to contribute to airway inflammation

following low-dose, alum-free OVA exposures (14). Conflicting data have been generated for IL-18 as well as for IL-1 β . For example, elevations of IL-18 levels have been shown in human patients with asthma and other atopic diseases (15); however, other studies have found reduced IL-18 levels in asthmatic patients (16). Thus, with regard to the extensive conflicting data, it is not surprising that controversy exists in the NLR field regarding the contribution of inflammasome components to the development of allergic airway disease.

In the current study, we tested the hypothesis that NLRP3 contributes to the development of Th2-mediated immune responses in the airway. Specifically, we sought to reconcile the controversy associated with NLRP3 by extensively characterizing the development of acute and chronic allergic airway disease in mice. Using two well-characterized models of OVA and two models of HDM Ag-induced airway disease, we evaluated the development of inflammation, cytokine production, mucus secretion, and AHR in *Nlrp3*^{-/-} mice. Aside from an effect of NLRP3 on IL-13 and IL-33 in one model, we were unable to confirm a role for NLRP3 in the majority of commonly assessed features associated with allergic lung disease.

Materials and Methods

Experimental animals

All studies were conducted with the approval of the Institutional Animal Care and Use Committee for the University of North Carolina at Chapel Hill and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study used *Nlrp3*^{-/-} mice that have been previously described (17). These animals were backcrossed 9–12 generations onto the C57BL/6J background and were maintained in specific pathogen-free animal facilities. All experiments were conducted on age- and sex-matched mice.

Induction of allergic airway inflammation

Allergic airway inflammation was induced by OVA, using two different protocols (Supplemental Fig. 1A, 1B). Acute airway inflammation was induced by sensitizing mice with OVA emulsified in alum. Mice received an i.p. injection of 20 μ g OVA (Grade V; Sigma-Aldrich, St. Louis, MO) emulsified in alum in a total volume of 200 μ l, on days -21 and -7. Airway inflammation was induced via intranasal (i.n.) administration of 1% OVA in saline for 5 d consecutively. Chronic airway inflammation was induced by sensitizing mice with OVA (no alum). Mice received five i.p. injections of 20 μ g OVA in saline every 3 d, beginning 12 d prior to the first i.n. OVA sensitization. Airway inflammation was induced via i.n. administration of 1% OVA in saline once per week for 6 wk. For each model, airway inflammation and disease progression were assessed 24 h following the last i.n. OVA administration.

Allergic airway inflammation was also induced by either acute or chronic HDM challenge (Supplemental Fig. 1C). Mice were exposed i.n. to 0.05 AU/ml purified 50:50 DerP and DerF whole-body extract (Greer Laboratories, Lenoir, NC) in 50 μ l saline for 5 d consecutively, followed by 2 d of rest, for either 2 wk (acute model) or 10 wk consecutively (chronic model). Mice were harvested 24 h following the last i.n. administration of HDM Ag.

Evaluation of airway inflammation

Mice were euthanized and serum was isolated from whole blood to assess total IgE levels. Mice were then perfused with saline. The lungs were lavaged three times with 1 ml saline, and the resultant bronchoalveolar lavage fluid (BALF) was centrifuged to separate the cellular components from the supernatants. Total BALF cellularity was determined using a hemacytometer, and the BALF composition was evaluated morphologically following differential staining (Diff-Quik; Dade Behring). In subsets of mice, lungs were harvested and homogenized for RNA extraction and protein analysis. Protein levels were assessed from serum, cell-free BALF supernatants, and lung homogenates by either ELISA (OptEIA; BD or R&D Biosystems) or Western blot.

Whole lungs were fixed by inflation and submersion in 10% buffered formalin, embedded in paraffin, and then sectioned to reveal the maximum longitudinal view of the main intrapulmonary bronchus of the left lung lobe. Histopathologic study was made using H&E-stained lung sections. The

histologic score for each sample included an evaluation of leukocyte infiltration, epithelial cell hyperplasia and damage, extravasation, perivascular and peribroncheolar cuffing, and an estimate of the area involved with disease. Each of these parameters was scored on a scale of 0 (absent) to 3 (severe) and averaged to generate a semiquantitative histologic score (18).

In addition to inflammation, goblet cell hyperplasia was evaluated on Alcian-blue/periodic acid-Schiff (AB/PAS) reaction-stained lung sections. A 2-mm section located in the middle of the main axial airway was identified and digitally imaged in an effort to consistently observe identical regions across all samples and experiments. The length and area of the AB/PAS-stained regions of the epithelium were assessed using ImageJ software (National Institutes of Health, Springfield, VA), as previously described (19). The data are expressed as the mean volume density ($V_s = \text{nl/mm}^2$ basal lamina + SEM of AB/PAS-positive epithelium).

Measurement of lung function

Mice underwent tracheostomies and were mechanically ventilated (Scireq, Montreal, Canada), as previously described (18). They were exposed to aerosol challenges of increasing concentrations of either methacholine (MCh; Sigma-Aldrich) or saline for 30 s, using an ultrasonic nebulizer (Scireq, Montreal, Canada). Airway reactivity was determined by assessing forced oscillatory mechanics every 10 s for 3 min following each MCh challenge. Like other studies using forced oscillatory mechanics to evaluate allergic airway disease, we focused our analysis on airway resistance (Raw) and tissue resistance (G).

Statistical analysis

All data are presented as the mean $\pm$ SEM. For complex data sets, we used an ANOVA followed by either the Tukey-Kramer honestly significant difference test or Newman-Keuls posttest for multiple comparisons. Single data point comparisons were assessed by the Student two-tailed *t* test. In all cases, $p < 0.05$ was considered statistically significant.

Results

OVA-alum-driven airway inflammation develops normally in *Nlrp3*^{-/-} mice

Previous studies have reported that development of allergic airway disease in response to OVA is attenuated in *Nlrp3*^{-/-} mice (2). As

shown in Fig. 1A, airway challenge with OVA induced a significant increase in leukocyte recruitment to the airways, as evidenced by an increase in total BALF cellularity. Further morphologic assessments of differentially stained BALF samples revealed that the increase in cellularity resulted from an influx of monocytes and eosinophils (Fig. 1B). Our data did not reveal a significant difference between wild-type and *Nlrp3*^{-/-} mice in the number or composition of the BALF cellularity. To further characterize the resulting Th2-mediated immune response to OVA, lungs were harvested for histopathologic examination and lung inflammation was evaluated. Consistent with the results from the BALF assessments, mice sensitized with OVA-alum and challenged with OVA demonstrated significantly increased airway histopathologic changes (Fig. 1C). These data revealed a significant increase in overall airway inflammation in both genotypes; however, we did not observe any major histopathologic differences between the OVA-challenged wild-type and *Nlrp3*^{-/-} mice (Fig. 1D). Together, these data do not support a strong role for NLRP3 in the development of allergic airway inflammation in this OVA model.

In addition to lung inflammation, we also evaluated mucus production and goblet cell hyperplasia, which are hallmark features of allergic airway disease progression in mice. These assessments were based on previous studies, which suggested that mucus hypersecretion was attenuated in *Nlrp3*^{-/-} mice (9). In this study, we evaluated AB/PAS-stained sections of inflated lungs, using a highly sensitive scoring system and quantified mucus production along the large conducting airways (represented as V_s) (Fig. 1E). No significant differences in mucus hypersecretion were observed between wild-type and *Nlrp3*^{-/-} mice (Fig. 1F). In addition to direct assessments of mucus production, we also examined *Muc5B* gene transcription. *Muc5B* is one of the primary mucin genes upregulated in mouse models of asthma, and its transcription has been shown to be influenced by several important regulatory pathways. OVA induced a significant increase in *Muc5B* gene

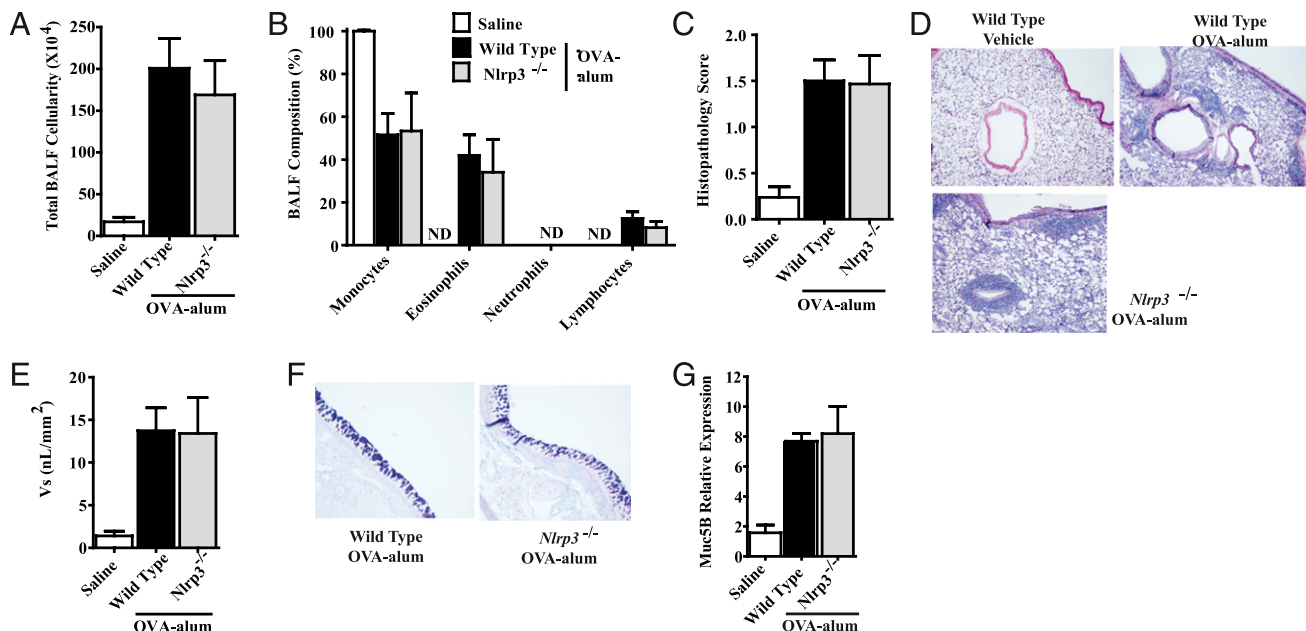


FIGURE 1. NLRP3 is not required for the development of OVA-alum-driven airway inflammation. (A) BALF cellularity from wild-type and *Nlrp3*^{-/-} mice was evaluated following OVA-alum immunization and i.n. OVA challenge. (B) The leukocyte composition of the BALF was evaluated via cellular morphology following differential staining. (C) Lung histopathology was evaluated and scored using a semiquantitative scoring system. (D) H&E-stained sections were evaluated and found to have significant levels of inflammation, concentrated around the airways and vasculature (original magnification $\times 10$). (E) Mucus levels were quantified along the main bronchiole (represented as V_s). (F) AB/PAS staining was used to quantify mucus production (original magnification $\times 20$). Data shown are representative images of AB/PAS⁺ cells. (G) *Muc5B* gene expression in whole lung homogenates was evaluated. Saline, $n = 7$; wild-type, $n = 15$; *Nlrp3*^{-/-}, $n = 9$. Data are representative of five individual experiments.

transcription; however, no significant differences were noted between wild-type and *Nlrp3*^{-/-} mice (Fig. 1G). These data are consistent with the other assessments of mucus production shown in Fig. 1E and 1F and further indicate that NLRP3 does not contribute to mucus hypersecretion in the OVA-alum model.

NLRP3 is not required for Th2 cell immunity following OVA-alum-mediated allergic airway disease

The OVA-alum model is considered a highly robust model of allergic airway inflammation that does not necessarily recapitulate many of the subtle aspects associated with human asthma (20). Both allergic airway inflammation in mice and asthma in humans are mediated by a specific cytokine milieu that includes IL-4, IL-5, and IL-13. Thus, we considered the possibility that NLRP3 could contribute to the gene expression of one or more of these cytokines through either direct or indirect mechanisms, which may not manifest itself as a prominent phenotypic outcome in the OVA-alum-based model. To assess this hypothesis, we evaluated gene expression of these cytokines in whole-lung homogenates. We did not observe any significant differences in the expression of any Th2-associated cytokines that were assessed, including *Il-5*, *Il-13*, or *Ifn-γ* (Fig. 2A). Although we routinely observed a trend toward elevated *Il-4* expression in lungs from *Nlrp3*^{-/-} mice, the increase never achieved statistical significance in any individual study or when pooled together (Fig. 2A). In addition to cytokine expression, we evaluated Th2-associated cytokine protein levels by ELISA in the BALF. In this study, we observed a significant increase in IL-13 following OVA challenge (Fig. 2B). However, no significant differences between *Nlrp3*^{-/-} and wild-type mice were observed. Together, these data do not support a role for NLRP3 in the production of Th2-associated cytokines in the OVA-alum model of allergic airway disease.

It is now widely accepted that NLRP3 mediates innate immune responses through the posttranslational processing of pro-IL-1β and pro-IL-18 into their mature, active forms. Previous studies have suggested that this mechanism is responsible for the contribution of NLRP3 to the development of allergic airway disease (2, 9). To evaluate this hypothesis, we assessed *in vivo* Th1-associated cytokine production in BALF and lung homogenates by ELISA. IL-1β and IL-18 were below the level of ELISA detection for both *Nlrp3*^{-/-} and wild-type mice (data not shown). To further confirm these findings, we homogenized whole lungs following OVA challenge and used Western blot to evaluate IL-1β generation and cleavage. Similar to our ELISA data and consistent with the Th2 skewed immune response, we did not detect either pro or cleaved forms of IL-1β following OVA challenge (Fig. 2C). Thus, it does not appear that NLRP3-associated IL-1β or IL-18 production is

associated with the development of allergic airway disease in this particular model.

*Airway reactivity is normal in OVA-alum-challenged *Nlrp3*^{-/-} mice*

In addition to inflammation, AHR is a cardinal feature of human asthma and mouse models of allergic airway disease. Multiple pathways contribute to the development of AHR, which can also serve as a surrogate assessment for many subtle characteristics associated with airway disease models. For example, small changes in airway inflammation can result in significant effects on airway mechanics. Although we did not detect any noticeable differences in airway inflammation or cytokine responses in *Nlrp3*^{-/-} mice, we considered the possibility that NLRP3 may contribute to an unrecognized biologic pathway that could influence the development of AHR in the OVA model. To test this hypothesis, we evaluated whether the loss of NLRP3 contributed to a change in airway mechanics following exposure to the bronchoconstricting agent MCh. We used a computer-controlled small animal ventilator (flexiVent) to compare changes in Raw and G. These two parameters are commonly used to evaluate AHR associated with allergic airway disease in mice (18). Exposure to MCh resulted in a significant increase in both Raw and G in the OVA-challenged mice. Overall, we observed a modest attenuation in AHR from *Nlrp3*^{-/-} mice; however, the magnitude of these changes was not significant between *Nlrp3*^{-/-} and wild-type animals (Fig. 3A, 3B). Thus, in addition to the lack of a phenotypic difference between *Nlrp3*^{-/-} and wild-type mice in the development of allergic airway inflammation, we also did not note a significant role for NLRP3 in altering lung mechanics during MCh administration.

NLRP3 does not contribute to OVA-mediated allergic airway inflammation in an alum-free model

The use of an alum adjuvant during the immunization phase of the OVA model has been demonstrated to dramatically enhance the cardinal features of allergic airway disease (20). Similarly, NLRP3 has been previously shown to modulate the inflammasome response to alum, although controversy exists regarding the physiologic relevance of these findings (21). Thus, we next sought to evaluate the contribution of NLRP3 during the development of OVA-induced allergic airway inflammation in a chronic model, without the potentially confounding effects of alum (Supplemental Fig. 1B). The alum-free model used in this study has previously been shown to induce allergic airway inflammation that is more closely related to the human disease (20). Mice that were immunized with OVA and received *i.n.* challenges with OVA exhibited a significant increase in airway leukocyte recruitment. However,

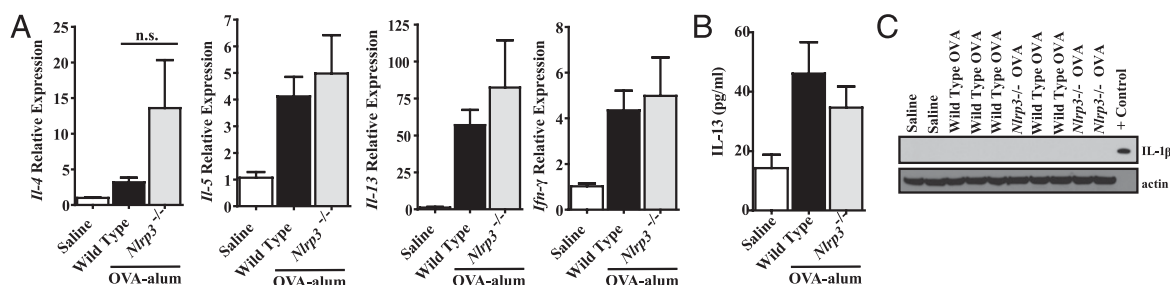


FIGURE 2. NLRP3 does not attenuate Th2-associated cytokine production in the OVA-alum model. (A) The expression of select Th2-associated cytokines in whole lungs following OVA-mediated allergic airway inflammation was determined. (B) IL-13 levels in the BALF were assessed by ELISA. Saline, *n* = 7; wild-type, *n* = 15; *Nlrp3*^{-/-}, *n* = 7. Data are representative of five individual experiments. (C) IL-1β production in whole-lung homogenates was assessed by Western blot. IL-1β and IL-18 were not detected by either Western blot or ELISA (data not shown). Saline, *n* = 2; wild-type, *n* = 5; *Nlrp3*^{-/-}, *n* = 3. Data are representative of three individual experiments.

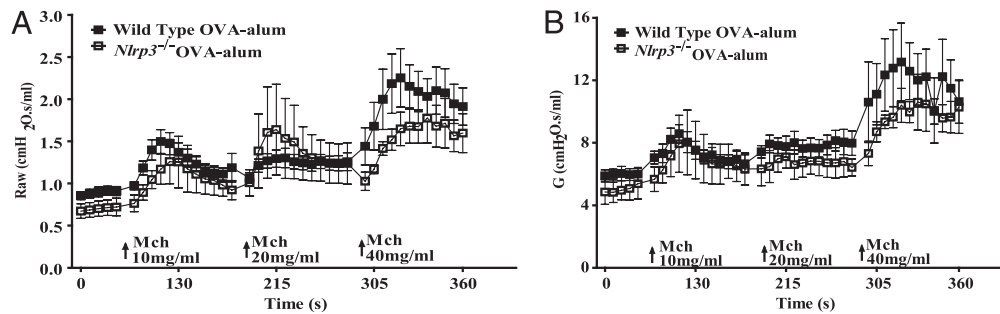


FIGURE 3. NLRP3 does not contribute to OVA-alum mediated AHR. Airway resistance (Raw) (**A**) and tissue damping (G) (**B**) in the airways, in response to MCh, was evaluated in wild-type and *Nlrp3*^{-/-} mice. Wild-type, *n* = 9; *Nlrp3*^{-/-}, *n* = 4. Data are representative of three individual experiments.

no significant differences were detected in total BALF cellularity between *Nlrp3*^{-/-} and wild-type mice (Fig. 4A; Supplemental Fig. 2A). In addition to total cellularity, we evaluated leukocyte populations by differential staining and morphologic examination. Monocytes and eosinophils were the significant leukocyte populations in the airways of both *Nlrp3*^{-/-} and wild-type mice (Fig. 4B). Although we detected a significant difference in the total BALF cellularity between the alum-based model and the alum-free model, no significant differences were observed between the two models in terms of the leukocyte composition of the BALF (Figs. 1A, 1B, 4A, 4B). In addition to the BALF analysis, we performed a histopathologic examination of the lung in the alum-free OVA model. Semiquantitative scoring revealed a significant increase in lung tissue disease in mice that were sensitized and challenged with OVA. However, no significant differences between *Nlrp3*^{-/-} and wild-type mice were detected (Fig. 4C). Closer analysis of the histopathologic examination revealed a significant increase in interstitial lung inflammation, with only moderate levels of airway inflammation (Fig. 4D). This finding was noticeably different from that of the alum-based OVA model,

which included increased airway and vasculature-associated inflammation (Fig. 1D). This observation likely explains the reduced BALF cellularity with increased tissue disease observed in the alum-free OVA model compared with the alum-based models previously described. Regardless of the models used, our data did not reveal a prominent role for NLRP3 in the development of OVA-mediated allergic airway inflammation.

The rapid and acute nature of the traditional OVA-alum model has been shown to occur independently of many critical features associated with human disease (20). However, previous data have indicated that long-term models, such as the alum-free model used in this study, are more appropriate for assessing the contribution of many of the mediators associated with chronic aspects of the disease, including mast cell-dependent enhancement of airway mucus hypersecretion (20). Thus, in addition to OVA-mediated inflammation, we also evaluated mucus hypersecretion in the alum-free model, using AB/PAS staining of lung histologic sections and subsequent mucus volumetric assessments. Mice immunized with OVA and challenged with OVA demonstrated a significant increase in airway mucus production. No significant

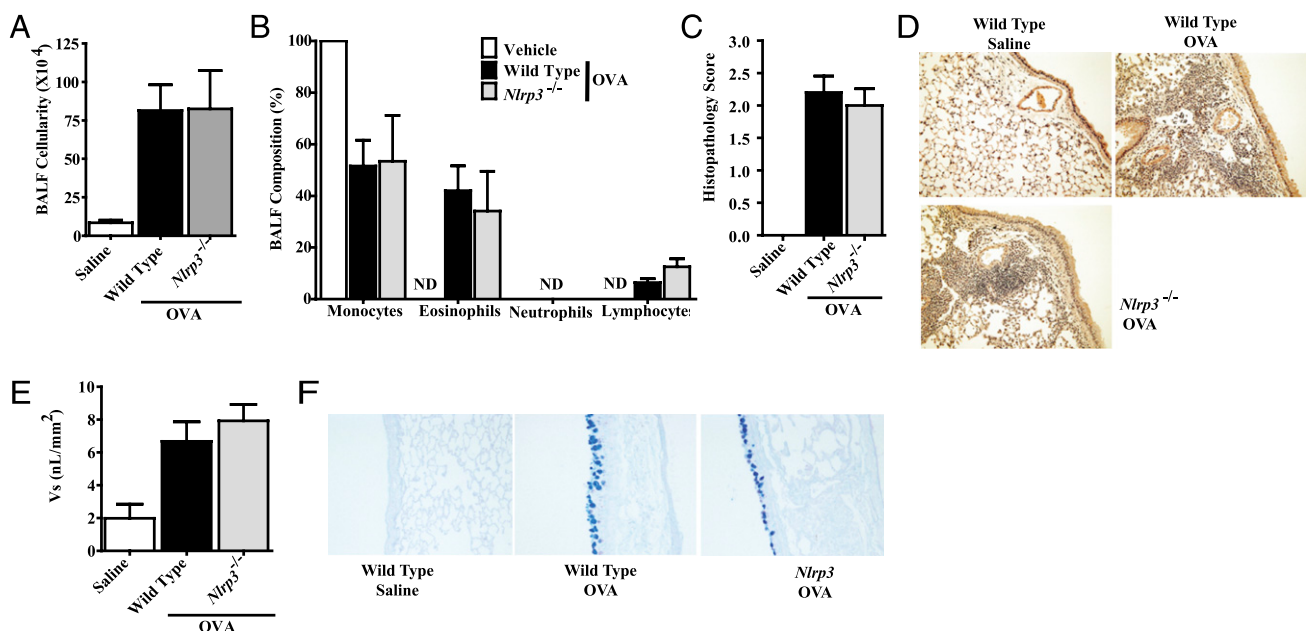


FIGURE 4. NLRP3 is not required for the development of OVA-mediated airway inflammation in a chronic, alum-free model of allergic airway disease. (**A**) BALF cellularity from wild-type and *Nlrp3*^{-/-} mice following 6 wk of i.n. OVA challenge was evaluated. (**B**) The leukocyte composition of the BALF was evaluated via morphology following differential staining. (**C**) Lung tissue disease was evaluated and scored using a semiquantitative scoring system. (**D**) H&E-stained sections were evaluated and found to have significant levels of inflammation, which was more diffuse and interstitial in nature (original magnification $\times 10$). (**E**) Mucus levels along the main bronchiole were quantified (represented as Vs). (**F**) AB/PAS staining was used to assess mucus production (original magnification $\times 20$). Data shown are representative images of AB/PAS⁺ cells in the large conducting airway. Vehicle, *n* = 6; wild-type, *n* = 6; *Nlrp3*^{-/-}, *n* = 6. Data are representative of four individual experiments.

differences between *Nlrp3*^{-/-} and wild-type mice, however, were observed (Fig. 4E, 4F). Like the data presented for the OVA-alum-based model, these data also indicate that NLRP3 does not significantly contribute to mucus production in the chronic alum-free OVA model.

NLRP3 attenuates IL-13 and IL-33 in the alum-free OVA model

Previous studies have shown that NLRP3 mediates mast cell-affiliated IL-1 β production, which was found to be associated with neutrophil recruitment and vascular leakage (22). Because the alum-free model is mast cell dependent, we next considered the possibility that NLRP3-mediated IL-1 β /IL-18 production may contribute to an increased Th1-associated cytokine response in the chronic OVA model. To evaluate this hypothesis, we assessed IL-1 β levels by Western blot and ELISA and IL-18 levels by ELISA in lung homogenates. However, in all cases, both cytokines were below the level of detection for the assays (data not shown). This finding is in contrast to a previous publication, which suggested that NLRP3 mediates allergic airway disease in an alum-free OVA model through the NLRP3 inflammasome-IL-1 β axis (9).

The contribution of IL-1 β to the development of allergic airway inflammation is controversial. However, a previous study also suggested an indirect role for NLRP3 in mediating the production of Th2-associated cytokines and IgE in the alum-free OVA model (9). That study used a short-term alum-free OVA exposure model; thus, we hypothesized that we would see increased Th2 cytokine differences between *Nlrp3*^{-/-} and wild-type mice in our chronic alum-free OVA model. We observed a significant increase in total serum IgE levels in OVA-immunized and -challenged mice. However, we did not note any significant differences in total IgE between *Nlrp3*^{-/-} and wild-type animals (Fig. 5A; Supplemental Fig. 2C). Similarly, OVA-immunized and -challenged mice demonstrated a significant increase in BALF IL-13 levels. Unlike the levels observed in the acute OVA model, the IL-13 increase was significantly attenuated in the *Nlrp3*^{-/-} mice compared with the wild-type animals (Fig. 5B). This finding was unexpected because of the lack of phenotypic differences observed in the chronic OVA model for *Nlrp3*^{-/-} mice. However, owing to the robust nature of IL-13, it is possible that the concentration of this particular cytokine is likely sufficient to drive the Th2-associated inflammatory response and not manifest itself as an observable phenotype in the *Nlrp3*^{-/-} mice in this model.

In an effort to determine the underlying cause of reduced IL-13 levels in *Nlrp3*^{-/-} mice, we also assessed IL-33 levels in the BALF. IL-33 is a potent Th2-associated cytokine, depending on the milieu, and a previous study has suggested a role for NLRP3 in

its posttranslational processing (23). Of particular relevance to our findings, IL-33R-deficient mice have demonstrated attenuated elements of allergic airway disease in the alum-free OVA model, and it was suggested that their phenotype was associated with attenuated dendritic cell production of Th2 cytokines, including IL-13 (9). In this study, we assessed IL-33 levels in the BALF, using ELISA, and found that cytokine levels were increased in wild-type mice (Fig. 5C; Supplemental Fig. 2D). However, in *Nlrp3*^{-/-} mice, IL-33 levels were significantly decreased compared with levels in wild-type animals (Fig. 5C).

NLRP3 does not contribute to HDM Ag-induced allergic airway inflammation

Robust airway inflammation is a recognized limitation of OVA-based models and may obscure subtle phenotypes associated with minor regulators of Th2-associated responses. Similarly, OVA-based models lack or circumvent many relevant disease processes associated with asthma causation. Thus, we considered the possibility that our models lacked the sensitivity to detect any potential direct or indirect contributions of NLRP3 to the development of allergic airway disease. To evaluate this possibility, we used an HDM model, which has proven to be sensitive to subtle phenotypic differences between genetically manipulated mouse lines and physiologic characteristics relevant to human asthma (13). HDM Ag was administered five times per week via i.n. administration for 2 wk to evaluate acute exposure effects or for 10 wk to evaluate chronic reactions (Supplemental Fig. 1C). We observed a significant increase in total BALF cellularity following both acute and chronic administration of HDM Ag (Fig. 6A). Subsequent analysis of BALF cellularity revealed a significant influx of monocytes, eosinophils, and lymphocytes following the acute model. However, chronic exposure to HDM resulted in a significant shift to a predominantly monocyte and lymphocyte influx, without significant populations of granulocytes (Fig. 6B). In both acute and chronic models, no significant differences in BALF cellularity were detected between *Nlrp3*^{-/-} and wild-type mice (Fig. 6A, 6B). Lung histopathologic examination revealed a significant increase in peribronchiolar and perivascular inflammation following both acute and chronic HDM Ag exposure models (Fig. 6C, 6D, respectively). Histopathologic scoring confirmed that both acute and chronic HDM Ag administration resulted in a significant increase in airway disease, which was attenuated, compared with the OVA-based models. However, in both HDM models, no significant differences in lung tissue disease were detected between *Nlrp3*^{-/-} and wild-type mice (Fig. 6E). The pathologic score for *Nlrp3*^{-/-} mice was lower than that for controls in the acute model, but this was not statistically significant. The HDM Ag data confirm our previous observations from the OVA-based models that NLRP3 does not appear to exert a dramatic effect on the development of overt allergic airway inflammation in mice.

The acute administration of HDM Ag resulted in recruitment of monocytes, eosinophils, and lymphocytes to the airways, which suggests a Th2-associated cytokine profile. Because we identified significant differences in IL-13 and IL-33 levels between wild-type and *Nlrp3*^{-/-} mice that were subjected to the alum-free OVA model, we next investigated these cytokines in the acute HDM model. Lungs were harvested, and the transcription levels of several Th2-affiliated cytokines were assessed. HDM administration resulted in increased transcription of *Il-4*, *Il-5*, *Il-13*, and *Ifn- γ* . However, no significant differences in the transcription of these cytokines were observed between *Nlrp3*^{-/-} and wild-type mice (Fig. 7A). In addition to gene transcription, we also assayed the levels of BALF IL-13 and total serum IgE by ELISA. HDM ad-

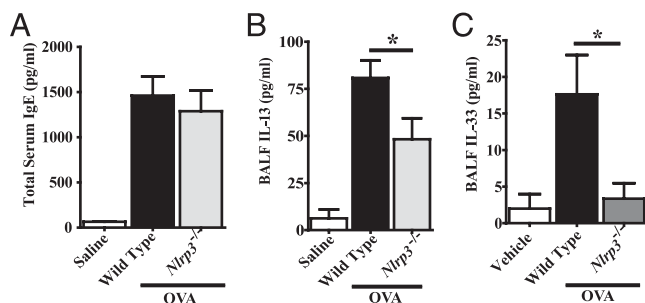


FIGURE 5. IL-33 was significantly attenuated in *Nlrp3*^{-/-} mice in the chronic alum-free OVA model. (A) Total serum IgE was evaluated by ELISA. (B and C) IL-13 and IL-33 levels in the BALF were assessed by ELISA. IL-1 β and IL-18 were not detected by either Western blot or ELISA (data not shown). Vehicle, *n* = 6; wild-type, *n* = 6; *Nlrp3*^{-/-}, *n* = 6. Data are representative of four individual experiments. **p* < 0.05.

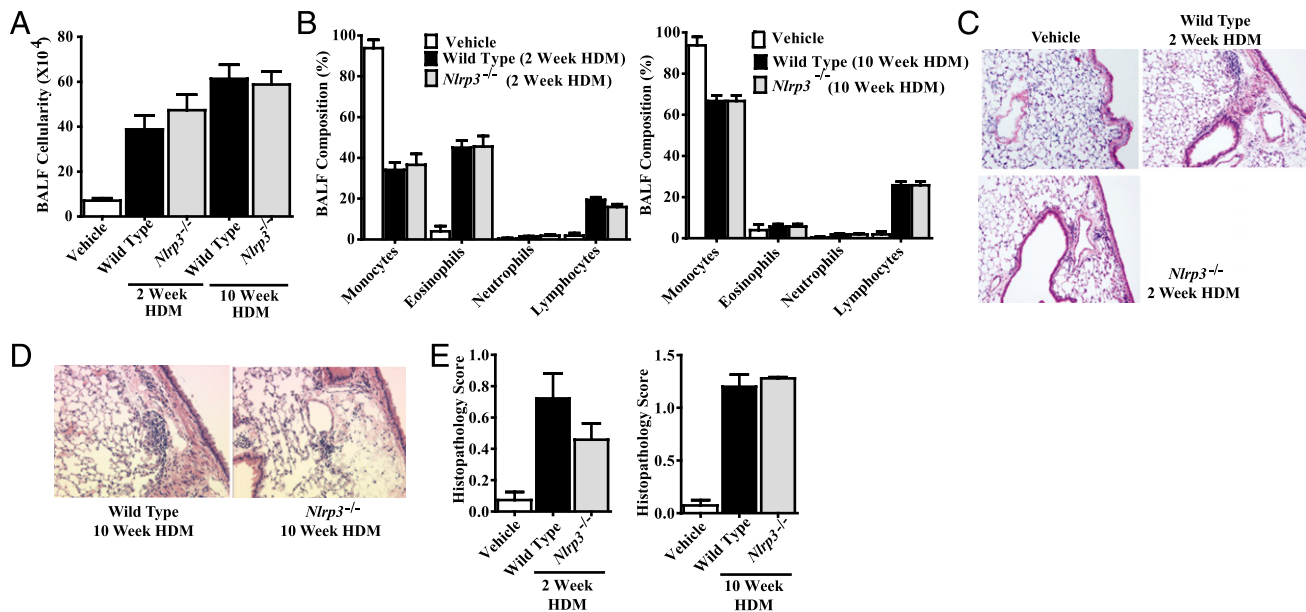


FIGURE 6. NLRP3 is not required for the development of HDM-induced allergic airway disease. (A) BALF cellularity from wild-type and *Nlrp3*^{-/-} mice was evaluated following either acute (2 wk) or chronic (10 wk) HDM challenges. (B) Leukocyte composition of the BALF was evaluated via morphology following differential staining. (C and D) H&E-stained sections were evaluated and found to have low levels of inflammation following either 2 wk (C) or 10 wk (D) of HDM exposure (original magnification $\times 10$). (E) Lung histopathology was evaluated and scored using a semiquantitative scoring system. Vehicle, $n = 6$; wild-type 2 wk, $n = 7$; *Nlrp3*^{-/-} 2 wk, $n = 7$; wild-type 10 wk, $n = 7$; *Nlrp3*^{-/-} 10 wk, $n = 7$. Data are representative of eight individual experiments.

ministration resulted in a significant increase in both IL-13 and IgE, with no significant differences observed between *Nlrp3*^{-/-} and wild-type mice (Fig. 7B, 7C). Along with these common Th2 cytokines, we also evaluated IL-1 β , IL-18, and IL-33. However, all of these cytokines were found to be below the level of detection in lung homogenates and BALF, which were assessed by Western blot and/or ELISA (data not shown). In addition to routine genotyping of our mouse colony, we also confirmed genotypes and assessed *Nlrp3* expression in the lungs of our animals at the completion of the HDM challenge. As expected, we did not observe *Nlrp3* transcript in *Nlrp3*^{-/-} mice, and levels of *Nlrp3* were not increased in wild-type mice following HDM administration (Fig. 7D). These data are consistent with a recent publication suggesting that NLRP3 does not contribute to either OVA-mediated or HDM-mediated allergic airway inflammation in mice (13).

Previous reports have revealed that exogenous administration of IL-18 and IL-33 exacerbates AHR (24, 25). Although we did not observe measurable increases in either IL-18 or IL-33 in the HDM

model, we considered the possibility that NLRP3 may contribute to other subtle mediators of AHR. To evaluate this possibility, we directly assessed airway reactivity in *Nlrp3*^{-/-} and wild-type mice following the conclusion of the acute HDM challenge model. We observed a significant increase in Raw and G in mice that were challenged with HDM (Fig. 8A, 8B). Although *Nlrp3*^{-/-} mice show modestly reduced scores, these physiologic assessments did not uncover statistically significant differences in airway reactivity between *Nlrp3*^{-/-} and wild-type mice (Fig. 8A, 8B). Thus, NLRP3 does not result in detectable differences in the development of either airway inflammation or airway reactivity in response to HDM administration (Fig. 9).

Discussion

Asthma is a complex disease process that relies on a diverse range of biologic pathways. Recent publications have suggested that components of the NLRP3 inflammasome contribute to allergic airway inflammation through the regulation of IL-1 β (2, 9). However, other publications have reported contrary findings (13).

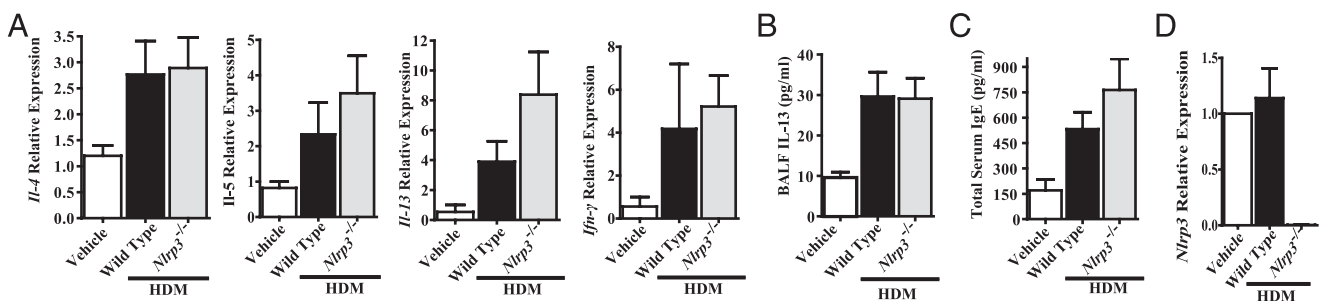
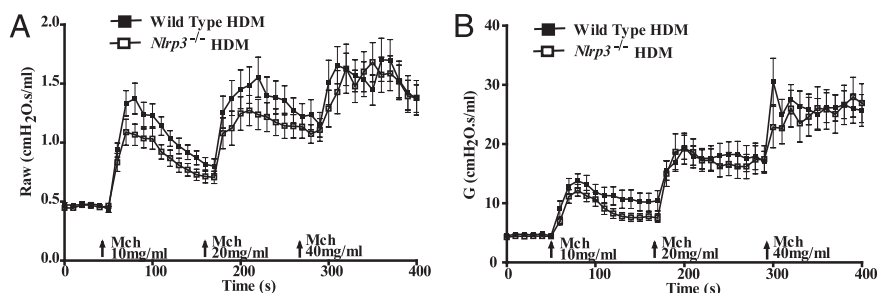


FIGURE 7. NLRP3 does not attenuate Th2-associated cytokine production following acute HDM administration. (A) The expression of select Th2 associated cytokines from whole lungs following 2 wk of i.n. HDM challenges was determined. (B) IL-13 levels in the BALF were assessed by ELISA. (C) Total IgE levels in the serum were assessed. (D) *Nlrp3* expression from whole lungs was evaluated by RT-PCR. IL-1 β , IL-18, and IL-33 were not detected by either Western blot or ELISA from BALF or whole-lung homogenates (data not shown). Vehicle, $n = 6$; wild-type 2 wk, $n = 7$; *Nlrp3*^{-/-} 2 wk, $n = 7$; wild-type 10 wk, $n = 7$; *Nlrp3*^{-/-} 10 wk, $n = 7$. Data are representative of eight individual experiments.

FIGURE 8. NLRP3 does not contribute to HDM-mediated AHR. Airway resistance (Raw) (A) and tissue damping (G) (B) in response to MCh was evaluated in wild-type and *Nlrp3*^{-/-} mice that were subjected to subchronic (2 wk) HDM administration. Wild-type, *n* = 37; *Nlrp3*^{-/-}, *n* = 30. Data shown were combined from three individual experiments.



In this article, like the Kool et al. (13) study, we present data suggesting that NLRP3 does not significantly contribute to the development of OVA- or HDM-mediated allergic airway inflammation in mice.

Several alternative explanations exist for the variation in findings between these studies. Many publications cite technical differences that could result in conflicting data. For example, subtle differences in the preparation or type of Ag and timing differences in the model have been proposed as confounders that account for the variation in findings between the three previous reports (13). Although these arguments are certainly valid, our data suggest that technical differences likely do not fully explain the conflicting data. Our studies were conducted separately in two independent laboratories (J.T. and S.T.), by independent investigators. The data presented throughout this article and in the Eisenbarth et al. (2) study were generated using *Nlrp3*^{-/-} mice originally described by Sutterwala et al. (17). These animals were originally backcrossed four generations onto the C57BL/6 background; we additionally backcrossed our animals to C57BL/6 for at least nine generations. The animals used by Besnard et al. (9) and Kool et al. (13) were

previously described by Martinon et al. (29). Thus, it is possible that differences observed between these studies are associated with variations in the generation of *Nlrp3*^{-/-} mice. However, this possibility is unlikely, as the Besnard et al. study and Kool et al. study used animals from the same source but observed differences in phenotype. In addition to the previously described mice, we analyzed a third *Nlrp3*^{-/-} strain that was generated de novo at the University of North Carolina at Chapel Hill by B.H.K and maintained on a 129S6 genetic background. Assay of mice in the acute HDM model, like the original *Nlrp3*^{-/-} animals in our hands, did not reveal any significant differences between University of North Carolina *Nlrp3*^{-/-} mice and wild-type animals (Supplemental Fig. 3). We also generated *Nlrp3*^{-/-} mice through backcrossing the C57BL/6 animals to 129SvEv for 12 generations and evaluated airway inflammation following OVA-alum challenge. Like our findings for animals on the C57BL/6 background, NLRP3 deficiency did not affect the development of allergic airway inflammation in mice on the 129SvEv background (Fig. 9A–H). Together, these data indicate that technical differences or potential issues associated with the original *Nlrp3*^{-/-} mice cannot

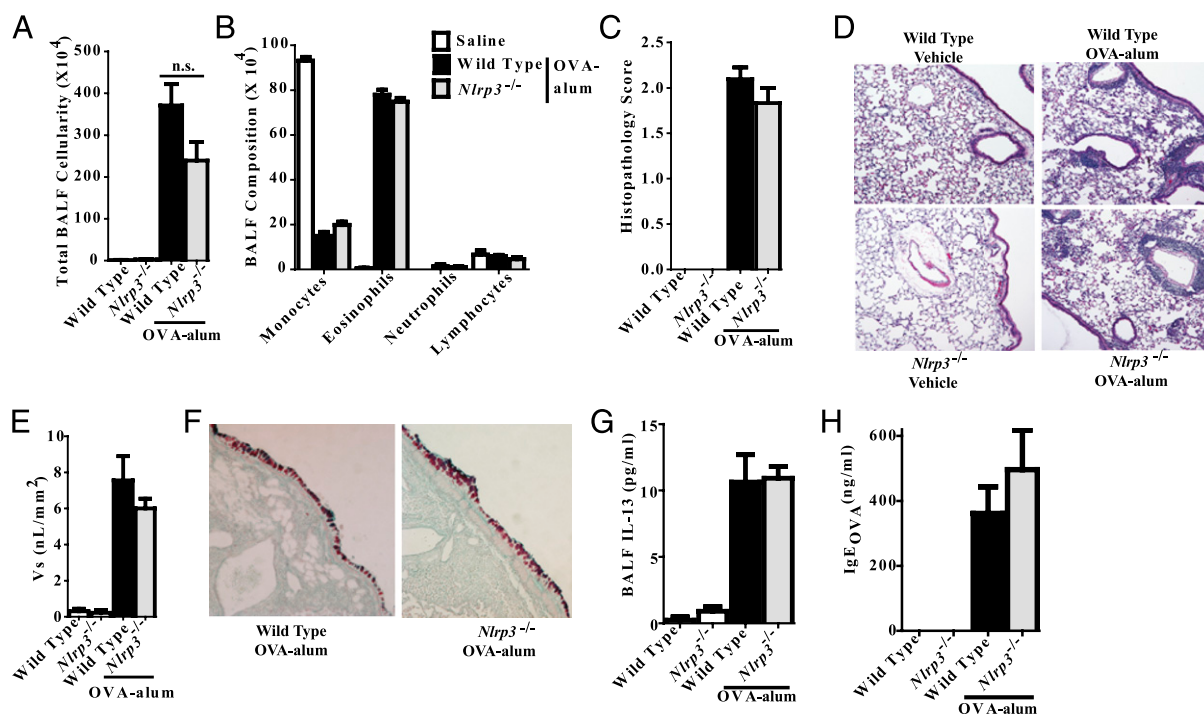


FIGURE 9. NLRP3 is not required for the development of OVA-alum-driven airway inflammation in 129SvEv mice. (A) BALF cellularity from wild-type and *Nlrp3*^{-/-} mice was evaluated following OVA-alum immunization and i.n. OVA challenge. (B) BALF cellularity was evaluated via cellular morphology following differential staining. (C) Lung tissue disease was evaluated and scored. (D) H&E-stained sections were evaluated and found to have significant levels of inflammation (original magnification $\times 10$). (E) Mucus levels were quantified along the main bronchiole (represented as Vs). (F) AB/PAS staining was used to quantify mucus production (original magnification $\times 20$). Data shown are representative images of AB/PAS⁺ cells. (G and H) BALF levels of IL-13 and serum levels of OVA specific IgE were assessed by ELISA. Wild-type saline, *n* = 4; *Nlrp3*^{-/-} saline, *n* = 3; wild-type OVA-alum, *n* = 8; *Nlrp3*^{-/-} OVA-alum, *n* = 4. Data are representative of two individual experiments.

fully explain the differences in phenotypic outcomes between studies.

As an alternative explanation, it is possible that the composition of the host microbiome, which is dependent upon each individual mouse facility, could contribute to the diverse phenotypic outcomes observed for *Nlrp3*^{-/-} mice. Several studies have suggested that oral treatment of mice with the probiotics *Bifidobacterium* and *Lactobacillus* results in decreased airway inflammation and hyperresponsiveness (26, 27). Thus, it is clear that differences in the host gut microbiome can have profound effects on disease outcome. More directly, several studies have associated various bacterial and viral species with the development of asthma. Interestingly, studies assessing differences in respiratory bacteria have revealed that atypical bacteria in the lungs have the ability to subvert the host response from a Th1-mediated response to a Th2-mediated response for protection (28). It is fascinating to speculate that differences in the microbiome may also contribute to differences in the NLRP3-deficient mice, which have already been shown to have altered immune responses to a variety of bacterial and viral species.

Our data argue against a strong contribution of NLRP3 to the development of Th2-mediated allergic airway disease. However, the role of NLRP3 in the recognition of PAMPs and DAMPs associated with asthma exacerbations in the context of allergic lung disease is currently undetermined. Human asthma is characterized by low levels of sustained airway inflammation, which contributes to acute phases of bronchoconstriction. Acute exacerbations can be induced by a diverse range of stimuli, including exposure to endotoxin, ozone, environmental pollutants, cigarette smoke, and viral or bacterial respiratory infections. In this article, we describe a variety of models of Th2-mediated airway inflammation that could be used to better characterize the contribution of NLRP3 in the recognition of agents leading to asthma exacerbation. A similar strategy was recently used to evaluate the contribution of NLRP3 in the recognition of UA in the context of Th2-mediated immunity (13). UA is a potent inducer of neutrophilic inflammation *in vivo* and has been shown to activate the NLRP3 inflammasome (29). In the context of lung disease, Kool et al. (13) revealed that airway HDM exposure results in the release of UA, which was found to promote Th2 cell-dependent allergic inflammation by amplifying the generation of pro-Th2 cytokines by dendritic cells. UA was found to be both necessary and sufficient to induce Th2-mediated immune responses in mice following either OVA or HDM exposure; however, this response was not dependent upon components of the NLRP3 inflammasome (13). Thus, although this study confirmed previous observations that UA crystals mixed with OVA induce the production of mature IL-1 β through a mechanism dependent upon the NLRP3 inflammasome, the Th2 cell-inducing capacity of UA was shown to occur through a mechanism independent of NLRP3 and IL-1 signaling. On the basis of these data, the authors proposed a novel pathway associated with UA-mediated inflammation, which occurred independently of UA recognition by the NLRP3 inflammasome.

In conclusion, we have characterized the development of allergic airway disease in *Nlrp3*^{-/-} mice, using a variety of models typically employed as surrogates for human asthma. Because allergic airway inflammation proceeds in a typical fashion in *Nlrp3*^{-/-} mice, the animal models described in this article will allow us to explore the potential contribution of NLRP3 to host immune responses to agents inducing airway exacerbations in the inflamed lung, such as PAMPs, DAMPs, environmental irritants, and biologic agents. Such data both would be biologically relevant and have direct translational value in understanding the disease processes associated with asthma exacerbations.

Acknowledgments

We thank Millenium Pharmaceuticals and Dr. Richard Flavell (Yale University) for supplying *Nlrp3*^{-/-} mice, as well as the members of the University of North Carolina Center for Environmental Medicine, Asthma and Lung Biology for supplying resources and project support for the work.

Disclosures

The authors have no financial conflicts of interest.

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Supplemental Data

Supplemental Figure 1. Schematics representing the 4 allergic airway disease models utilized in this study. **A)** The OVA-alum model of acute airway inflammation. **B)** The alum free, sub-chronic OVA model of allergic airway inflammation. **C)** The sub-chronic (2 week) and Chronic (10 week) HDM model of allergic airway inflammation.

Supplemental Figure 2. OVA mediated airway inflammation in a sub-chronic, alum free model of allergic airway disease. Mice were subjected to one of the following conditions: 1) PBS/PBS or vehicle challenge (mice received i.p. injections of PBS and i.n. challenges with PBS); 2) OVA/PBS (mice were sensitized with OVA and challenged with saline i.n.); 3) PBS/OVA (mice were sensitized with PBS and i.n. challenged with OVA); 4) OVA/OVA wild type (mice were sensitized with OVA and i.n. challenged with OVA). **A)** BALF cellularity was evaluated. **B)** Lung histopathology was evaluated and scored utilizing a semi-quantitative scoring system. **C)** Serum levels of total IgE and **D)** BALF levels of IL-33 were evaluated by ELISA, respectively. Vehicle, n=6; wild type PBS/OVA, n=3; wild type OVA/PBS, n=3; wild type OVA/OVA, n=6. Data are representative of 4 individual experiments.

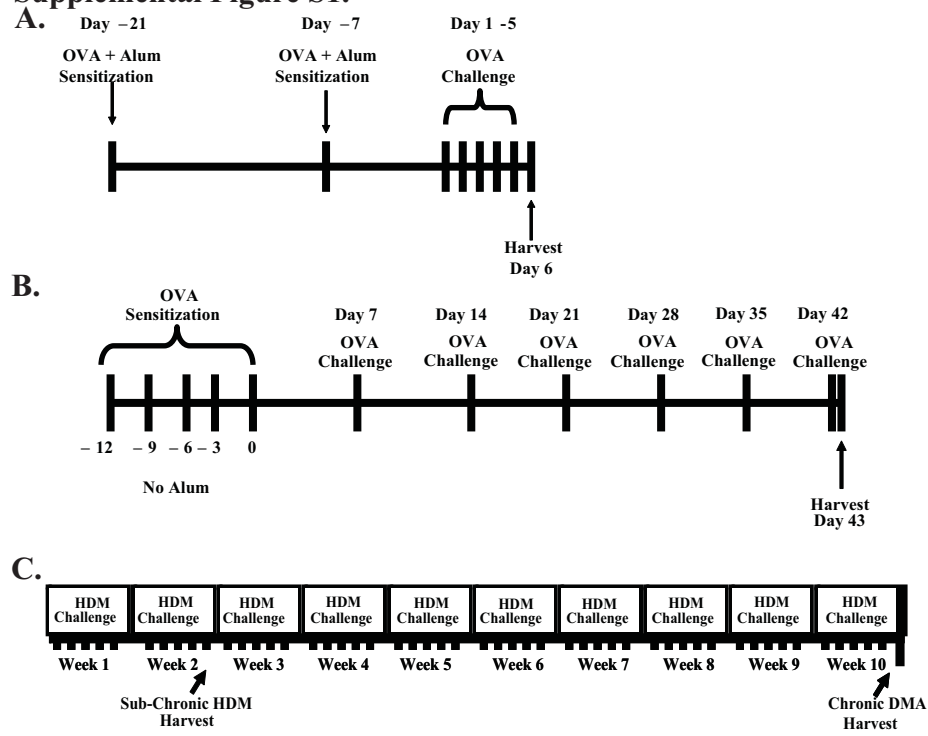
Supplemental Figure 3. Acute HDM induced airway inflammation in *de novo* generated *Nlrp3*^{-/-} mice. *De novo* *Nlrp3*^{-/-} mice were generated by disrupting the entire *Nlrp3* gene, including all exons and introns, with a selectable marker gene using a standard replacer type targeting vector. The mutation was maintained on the 129S6 genetic background. Comparisons of co-isogenic mice, revealed no difference in the development of allergic airway disease, including exposure to acute HDM antigen. **A)** Total BALF cellularity was evaluated. **B)** The

cellular composition of the BALF was evaluated via morphology following differential staining.

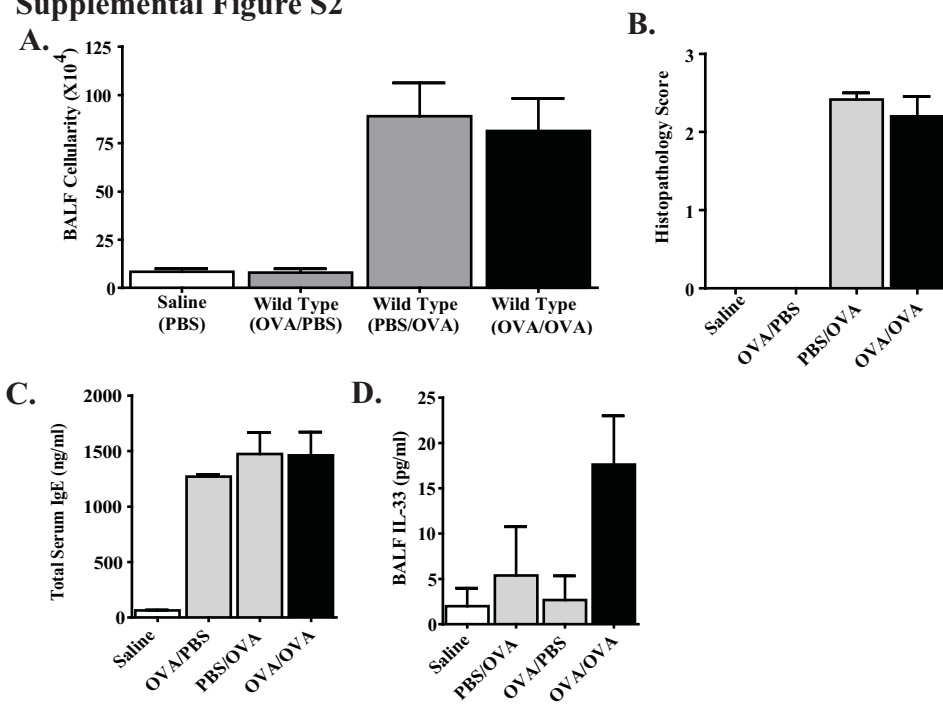
C) BALF levels of IL-13 and **D)** serum levels of total IgE were evaluated by ELISA,

respectively. Wild Type, n=33; *Nlrp3*^{-/-}, n=27.

Supplemental Figure S1.



Supplemental Figure S2



Supplemental Figure S3.

