

Research paper

Caspase-11 regulates lung inflammation in response to house dust mites

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A B S T R A C T

Asthma is an inflammatory lung disorder characterized by mucus hypersecretion, cellular infiltration, and bronchial hyper-responsiveness. House dust mites (HDM) are the most prevalent cause of allergic sensitization. Canonical and noncanonical inflammasomes are multiprotein complexes that assemble in response to pathogen or danger-associated molecular patterns (PAMPs or DAMPs). Murine caspase-11 engages the noncanonical inflammasome. We addressed the role of caspase-11 in mediating host responses to HDM and subsequent allergic inflammation using *caspase-11*^{-/-} mice, which lack caspase-11 while express caspase-1. We found that HDM induce caspase-11 expression *in vitro*. The presence of IL-4 and IL-13 promote caspase-11 expression. Additionally, *caspase-11*^{-/-} macrophages show reduced release of IL-6, IL-12, and KC, and express lower levels of costimulatory molecules (e.g., CD40, CD86 and MHCII) in response to HDM stimulation. Notably, HDM sensitization of *caspase-11*^{-/-} mice resulted in similar levels of IgE responses and hypothermia in response to nasal HDM challenge compared to WT. However, analysis of cell numbers and cytokines in bronchiolar alveolar lavage fluid (BALF) and histopathology of representative lung segments demonstrate altered inflammatory responses and reduced neutrophilia in the airways of the *caspase-11*^{-/-} mice. These findings indicate that caspase-11 regulates airway inflammation in response to HDM exposure.

1. Introduction

Asthma is a long-term recurring inflammatory lung disorder characterized by mucus hypersecretion, cellular infiltration, chronic airway inflammation and bronchial hyper-responsiveness [5,85,100]. Different forms of asthma can coexist in some patients including atopic (allergic, Th2-dependent), and non-atopic (non-allergic, Th2-independent). These forms are initiated and regulated by multiple genetic and environmental factors, such as HDM allergens.

House dust mites, such as *Dermatophagoides pteronyssinus*, are an unsurpassed cause of atopic sensitization and the major causes of allergic asthma worldwide [22,92]. A battery of *D. pteronyssinus* allergens, proteases, bacterial lipopolysaccharide (LPS), and chitin from the mite exoskeleton are present in fecal pellets, and decaying mite materials and have potent sensitizing capacities [18,101]. Protease-

dependent effects of mite allergens are associated with breaching of the epithelial layer, breaking tight junctions and stimulating protease-activated receptors. These events lead to increased epithelial permeability and production of chemokines and cytokines, which recruit antigen presenting cells (APCs) into the epithelial layers and promote airway inflammation and remodelling [7,52,53,58,66]. CD4⁺ Th2 cells orchestrate the HDM allergic response via production of IgE against mite allergens and stimulation of inflammation and lung remodelling [83]. It is also now recognized that the innate immune system plays a fundamental role in initiating and shaping the allergic response by programming and maintaining Th2-biased adaptive immunity in response to HDM allergens and their contaminants [47,106].

Innate immune system activation is mediated through pattern recognition receptors (PRRs), which sense PAMPs and DAMPs [54,59]. The PRR families include intracellular NOD-like receptors (NLRs) and

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extracellular Toll-like receptors (TLRs) [59]. Sensing of PAMPs or DAMPs by the NLR protein family, such as NLRP3 or NLRC4, mediates the assembly of inflammasomes [3]. Downstream of inflammasome signaling, IL-1 β and IL-18 are cleaved by active caspase-1 which delineates the canonical inflammasome pathway [20,68,109]. In addition to canonical inflammasome activation, caspase-11 mediates non-canonical inflammasome activation in response to cytosolic LPS [61].

Caspase-11 in mice (or caspase-4/5 in humans) belongs to the family of inflammatory caspases and exhibits 46% similarity to caspase-1 [35,36,55,67,71,74,82,104]. It has been demonstrated that *caspase-11*^{-/-} but not *caspase-1*^{-/-} mice are resistant to septic shock mediated by injection of lethal doses of LPS [61,62]. Caspase-11 is expressed broadly in immune and non-immune cells and its expression in resting cells is low [61,104]. In contrast to other caspases that are regulated by proteolytic cleavage, caspase-11 is regulated at both transcriptional and post-translational levels. Caspase-11 expression is induced through LPS-activated TLR4 signaling via the adaptor TIR-domain-containing adaptor-inducing interferon- β (TRIF) and TRIF-dependent type I interferon (IFN) production [21,23,86,93,104]. In this regard, extracellular LPS and cytokines primarily serve as a priming signal for inducing the expression of caspase-11 and other inflammasome components. On the other hand, intracellular LPS triggers caspase-11-dependent inflammasome activation in the cytoplasm independently of TLR4 [12,24,46,61,62].

A previous study examined the role of caspase-11 in asthma using ovalbumin (OVA) as an allergen [111]. Sensitization of mice was achieved by intraperitoneal injection of OVA and the adjuvant potassium aluminum sulfate (alum), followed 7 days later by airway challenges with OVA. Even though this protocol results in eosinophilic inflammation and induction of Th2 cytokines, it may not fully represent the same conditions experienced by asthmatics where allergen exposure may be more frequent and for much longer periods of time [95]. HDM has been increasingly used as an allergen in mice. Repeated administration of this allergen induces asthma attacks in humans and chronic airway inflammation and tissue remodeling in mice [39]. Adjuvants such as aluminum hydroxide (alum) are known to promote the development of Th2-type responses, and alum has been extensively used in animal models of allergy and asthma [34]. Adjuvant-free sensitization protocols are also effective, but these usually require a greater number of exposures to achieve the proper sensitization and tend to elicit less robust responses to allergen challenge [17].

In this study, we use *caspase-11*^{-/-} mice and an HDM-mediated allergic inflammation model to delineate the role of caspase-11 *in vivo*. An optimization model with WT mice was used to confirm symptoms of asthma, antibody responses and lung inflammation before experimenting with *caspase-11*^{-/-} mice. We demonstrate that the inflammatory environment of asthma, specifically IL-4 and IL-13 cytokines, promote caspase-11 expression. Furthermore, *caspase-11*^{-/-} macrophages produce lower amounts of KC, IL-6, and IL-12, and express lower levels of costimulatory molecules (e.g., CD40, CD86 and MHCII) in response to HDM stimulation. *In vivo* data show that HDM induce caspase-11 expression in WT mice. Furthermore, HDM promote differential inflammation and cellular infiltration with lessened neutrophilia in the *caspase-11*^{-/-} mice. Therefore, caspase-11 promotes inflammation and neutrophil trafficking in response to HDM exposure.

2. Results

2.1. HDM promote caspase-11 expression in macrophages derived from WT mice.

Since caspase-11 is only expressed upon induction with pro-inflammatory stimuli [93], we tested whether HDM induce caspase-11 expression *in vitro* using primary bone marrow derived macrophages (BMDMs) from WT C57BL/6 mice. In contrast to freshly isolated lung macrophages, dendritic cells or epithelial cells, BMDMs are not activated

or primed. We therefore expect their response will be directed only to HDM stimulation. The BMDMs were treated with different concentrations of HDM for 4 or 24 h, and caspase-11 expression was detected by Western blotting (Fig. 1A). Our data shows that the full-length (43 kDa) caspase-11 is expressed at 4 h post-HDM at all tested concentrations, and stays active up to 24 h post-treatment. Furthermore, the anti-caspase 11 antibody detected a shorter form (38 kDa) of caspase 11 in cells exposed to HDM for 24 hr suggesting that HDM could stimulate a non-canonical signaling of the CARD domain in macrophages (Fig. 1A).

2.2. Allergy-associated cytokines promote caspase-11 expression in macrophages derived from WT mice

Macrophages are categorized into classically- (M1) and alternatively-activated macrophages (M2 or AAM), which reflect the T helper (Th1/Th2) subsets [40,96]. M1 macrophages promote inflammation, whereas M2 macrophages are critical for inflammation resolution [8]. To determine if the inflammatory environment of asthma, and more specifically IL-4 and IL-13 cytokines, promote caspase-11 expression, we treated macrophages with IL-4 or IL-13 and examined caspase-11 expression by Western blotting. Our data demonstrate that macrophage treatment with IL-4 or IL-13 induces the expression of the 43 and the 38 kDa fragments of caspase-11, similar to the treatment with the inflammatory stimuli IL-1 β , IL-1 α and LPS (Fig. 1B & C).

Therefore, the inflammatory environment of asthma, including IL-4 and IL-13 cytokines, induces caspase-11 expression.

2.3. Macrophages derived from *caspase-11*^{-/-} mice show reduced proinflammatory responses and reduced expression of costimulatory molecules in response to HDM treatment

IL-1 β is a pivotal cytokine in asthma pathogenesis. Thus, it plays a central role in HDM-mediated allergic inflammation [52,53,98], and its presence often designates the activation of the inflammasome [21,45,61,86]. We determined the role of caspase-11 in the production of IL-1 β by treating WT or *caspase-11*^{-/-} macrophages with 100 μ g/mL of HDM. In contrast to WT, *caspase-11*^{-/-} macrophages released significantly less IL-1 β (Fig. 2A). Therefore, HDM induced caspase-11 expression and increased IL-1 β release in macrophages derived from WT mice.

HDMs elicit a strong immune response via stimulation of pro-inflammatory and anti-inflammatory cytokines. Increased amounts of proinflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, are found in the sputum and BALF of individuals with asthma. These cytokines amplify inflammation, in part through the activation of NF- κ B and subsequent increased expression of multiple inflammatory genes [11]. IL-4 is released and upregulated in asthma [32,60]. Despite data showing that HDM or IL-4 induced caspase-11 expression, we hypothesized that the caspase-11 response would be different in an environment containing the allergen only (which models the priming phase), or in asthma-like environment where both allergen and IL-4 are present (which represent the effector phase). To examine the role of caspase-11 in response to these environments, we treated macrophages with HDM to model the priming phase or polarized BMDMs toward M2 by stimulating macrophages with IL-4 overnight followed by treatment with IL-4 + HDM for 24 h, modeling the effector phase. Our data demonstrate that *caspase-11*^{-/-} macrophages release significantly less KC, IL-6 and IL-12 at 24 h post-HDM treatment when compared to their WT counterparts (Fig. 2B, C, & D). Treatment with IL-4 in the presence of HDM yields the same trend of responses in *caspase-11*^{-/-} macrophages with significant decrease in the levels of released KC, IL-6 and IL-12 (Fig. 2B, C, & D). Treatment with IL-4 + HDM induced a significant increase in the level of IL-6 in both WT and *caspase-11*^{-/-} macrophages but significantly less in *caspase-11*^{-/-} compared to WT macrophages (Fig. 2C). Therefore, our data demonstrate reduced production of pro-inflammatory mediators (KC, IL-6 and IL-12) from *caspase-11*^{-/-} macrophages as compared to WT in response to HDM.

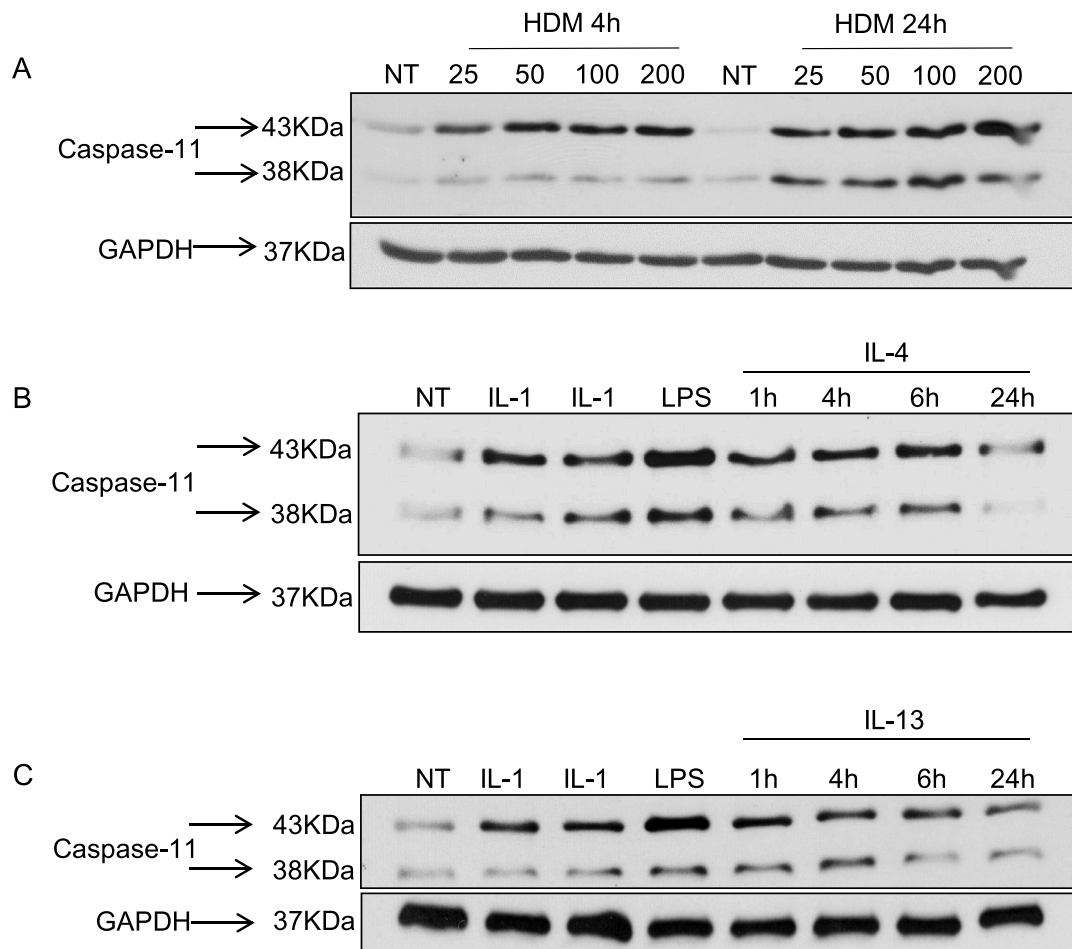


Fig. 1. HDMS, IL-4 and IL-13 cytokines promote caspase-11 expression in macrophages derived from WT mice. WT macrophages were plated at 2×10^6 cells/well. Cells were either not treated (NT) or treated with different concentrations of HDM (25, 50, 100 and 200 $\mu\text{g/ml}$) for 4 or 24 h. Caspase-11 expression was examined by Western blotting and GAPDH was used as a loading control (A). WT macrophages were treated with several inflammatory stimuli (IL-1 β , IL-1 α or LPS) or stimulated with different concentrations of IL-4 (5 ng/mL) (B) or IL-13 (20 ng/mL) (C) for 1, 4, 6 or 24 h and compared to NT samples. Western blot membranes were immunoblotted with caspase-11 and GAPDH was used as a loading control. Representative blots are shown from one of two independent experiments ($n = 2$).

Macrophages are involved in antigen presentation to immune effector cells, providing two specific signals to achieve optimum activation of T cells [76]. The CD28 and CD40L molecules on T cells are known to interact, respectively, with costimulatory-molecules CD80/CD86, and CD40 on APCs [41,44]. We used flow cytometry to examine CD86 and CD40 expression, 24 h following HDM or HDM + IL-4 treatment, in WT and *caspase-11*^{-/-} macrophages. *Caspase-11*^{-/-} macrophages showed significantly reduced levels of CD86, CD40 and MHCII 24 h post-HDM + IL-4 stimulation (Fig. 2E & F). In asthmatic animals, the M2 phenotype of macrophages is typically associated with high level of arginase-1 and family proteins chitinase-like Ym1 [15,51,112]. Under HDM + IL-4 stimulation, *caspase-11*^{-/-} macrophages expressed higher levels of arginase-1 and Ym1 but lower levels of the M1 marker *Cxcl10*, when compared to macrophages treated with HDM only (Fig. 2G, H & I). A significant change in the level of arginase was seen in WT or *caspase-11*^{-/-} macrophages treated with HDM, or treated with HDM + IL-4 (Fig. 2G). A significant change in the level of Ym1 was seen in *caspase-11*^{-/-} macrophages treated with HDM, or treated with HDM + IL-4 (Fig. 2H). No significant change in the level of CXCL10 was seen between WT treated with HDM, or treated with HDM + IL-4. Moreover, no significant change in the level of CXCL10 was seen between *caspase-11*^{-/-} macrophages treated with HDM, or treated with HDM + IL-4. Taken together, *caspase-11*^{-/-} macrophages express lower levels of costimulatory molecules and higher levels of arginase-1 and Ym1 associated with M2 phenotype.

2.4. HDM sensitization and challenge promotes differential allergic inflammation and antibody responses in WT and *caspase-11*^{-/-} mice

Recently, an acute model of HDM-mediated experimental allergic lung inflammation was used to delineate the role of caspase-1 in asthma [73]. However, mice used in these studies lack both caspase-1 and caspase-11 and thus, it is not clear if the phenotype observed is due to the lack of caspase-1, caspase-11, or both. Our results above showed altered inflammatory cytokine responses to HDM in *caspase-11*^{-/-} macrophages *in vitro*. Thus, to study the role of caspase-11 in HDM-mediated inflammation, *in vivo*, WT mice were sensitized with alum + 100 μg HDM on day 0 and 7 intra-peritoneally. Mice were then challenged intranasally with HDM (25 $\mu\text{g/dose}$). Control WT mice were sensitized and challenged with phosphate-buffered saline (PBS) (Supplementary Fig. 1A). Optimization experiments were performed initially to determine the number of HDM challenges needed to provoke asthma symptoms. Self-isolation, low activity and drop in body temperature after each challenge were used as basic measurements to evaluate signs of allergic reaction in mice (Supplementary Fig. 1B). This model of HDM administration was used to ensure a robust IgE response before we sacrifice the animals [6,43,48]. Further, we aimed to mimic aspects of chronic allergic asthma including eosinophilic inflammation, airway remodelling and airway hyperresponsiveness (AHR). Our data suggest that HDM administration mediated successive and daily drop in body temperatures, which confirmed the validity of our model. Moreover,

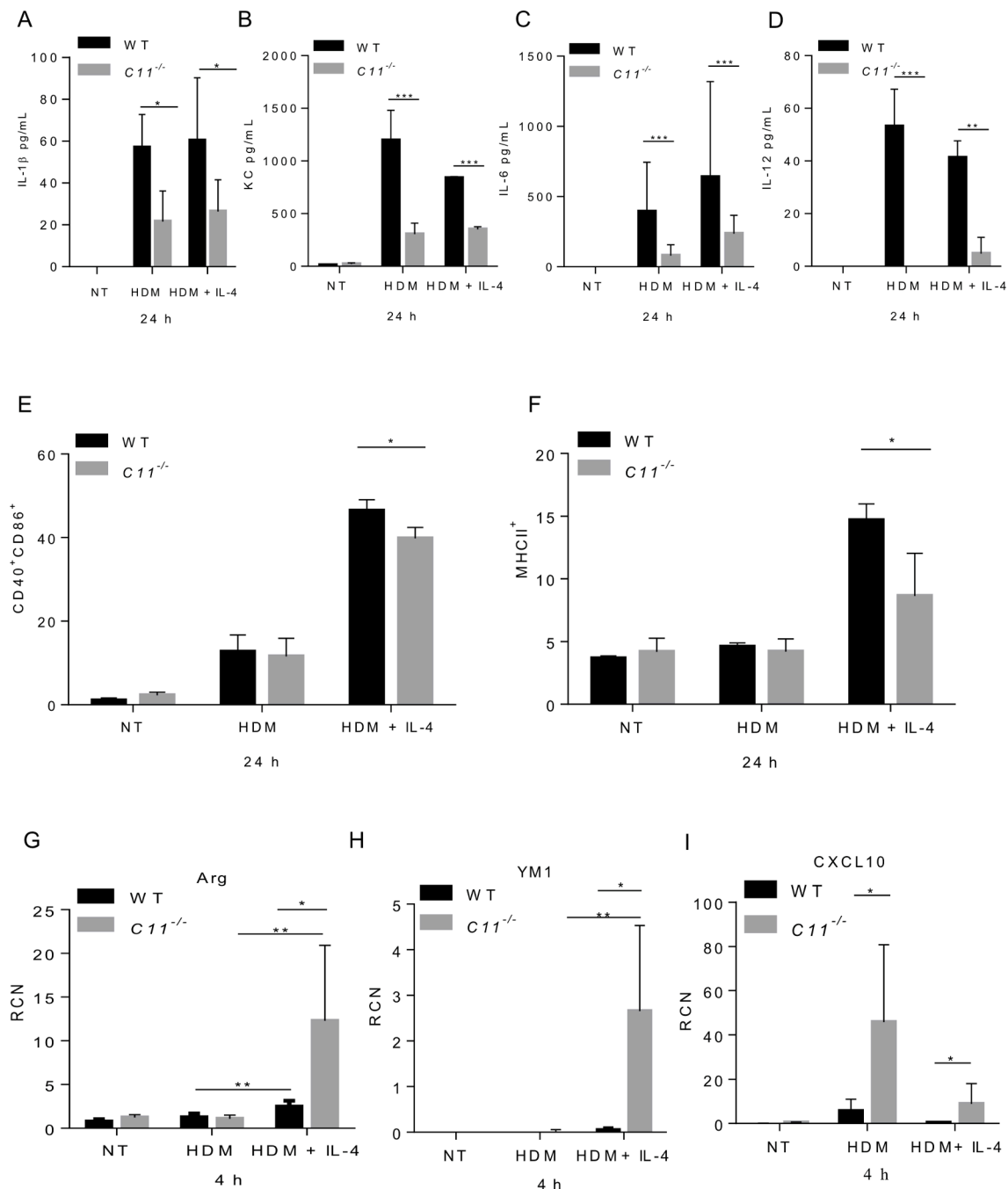


Fig. 2. *Caspase-11*^{-/-} macrophages show reduced release of proinflammatory cytokines and reduced expression of costimulatory molecules post-HDM treatment. Level of IL-1β released in the supernatants of WT or *caspase-11*^{-/-} macrophages treated with 100 μg/mL of HDM after 24 h (A). Data represent the mean ± SD (n = 4) obtained from four independent experiments. Multiple t-tests performed for statistical analysis, * p < 0.05. Concentrations of keratinocyte-derived protein chemokine (KC) (B), IL-6 (C), and IL-12 (D) released in supernatants from WT and *caspase-11*^{-/-} macrophages either un-treated (NT), treated with HDM (100 μg/mL), pre-stimulated with IL-4 (5 ng/mL) then treated with HDM and IL-4 for 24 h. Data represent the mean ± SD (n = 4) obtained from four independent experiments. Multiple t-tests performed for statistical analysis, ** p < 0.01, *** p < 0.001. Expression of costimulatory molecules CD40 and CD86 (E), and MHCII (F) by flow cytometry 24 h post-HDM treatment. Data represent the mean ± SD (n = 3) obtained from three independent experiments. Multiple t-tests performed for statistical analysis, * p < 0.05. WT and *caspase-11*^{-/-} macrophages either NT, treated with HDM (100 μg/mL), pre-stimulated with IL-4 (5 ng/mL) then treated with HDM and IL-4 for 4 h. Expression of M2 (Arg1, YM1) or M1 (CXCL10) markers by RT-qPCR 4 h post-treatment (G, H & I). RCN on the Y axis represents relative copy number. Data represent the mean ± SD (n = 3) obtained from three independent experiments. Multiple t-tests performed for statistical analysis, * p < 0.05.

lungs derived from mice challenged with HDM showed an increase resistance to increasing concentrations of methacholine challenge (Supplementary Fig. 1C). Allergic reaction was evaluated by measuring total level of IgE in the serum and BALF of WT mice. HDM-challenged mice show elevated levels of total IgE in sera and BALF as compared to the PBS control (Supplementary Fig. 1D & E). Our results show a

significant increase in the HDM-specific IgG1 and IgG2A isotypes in the serum (Supplementary Fig. 1F & G). We further demonstrate that HDM induce caspase-11 expression *in vivo* in WT mice (Supplementary fig. H). Thus, we successfully induced systemic and lung inflammation in HDM-sensitized and challenged WT mice.

To investigate the role of caspase-11 in mediating allergic lung

inflammation, *caspase-11*^{-/-} and WT mice were sensitized with alum + 100 µg HDM on day 0 and 7 intra-peritoneally. Then, mice were challenged with 15 doses of HDM intranasally (25 µg/dose) (Fig. 3A). Both WT and *caspase-11*^{-/-} mice displayed low activity and self-isolation, which were consistent clinical signs of allergy. *Caspase-11*^{-/-} mice showed significantly more prominent drops in body temperature on challenges 9, 13 and 14 as compared to WT mice. However, no significant difference was seen after challenge 15 which corresponds to day 35 (Fig. 3B). Further, markers of allergy were evaluated in sera and BALF from WT and *caspase-11*^{-/-} mice challenged with HDM. IgE antibody is normally present at low level in the plasma and is mainly produced by plasma cells in mucosal-associated lymphoid tissues. Atopic conditions, such as asthma, elevate serum levels of total IgE and thus drive the disease. Continuous exposure to HDM allergens leads to continuous production of HDM-specific IgE as well as continued maintenance of allergic inflammation in the airways [101,102]. Therefore, we examined IgE levels in the sera of WT and *caspase-11*^{-/-} mice before sensitization (day 0) and after sensitization with HDM and alum (day 14, day 28, and 24 h after the last challenge on day 35). Our data show that sera and BALF of WT and *caspase-11*^{-/-} mice exhibit similar total IgE levels, (Fig. 3C, D). Sera-derived from WT and *caspase-11*^{-/-} had similar levels of HDM-specific IgG1 on day 14, 28 and 35 (Fig. 3G). However, BALF derived from *caspase-11*^{-/-} show significantly higher levels of total IgA on day 28 and 35 (Fig. 3F). There was no difference in HDM-specific IgA titers in the BALF on day 35 (Fig. 3E). Our data showed that BALF derived from *caspase-11*^{-/-} mice contain elevated level of IgA as compared to WT. No significant differences were seen in total IgE, lung function or body temperature.

2.5. *Caspase-11*^{-/-} mice show altered cellular infiltration in their BALF in response to HDM

Development of allergic responses requires activation of mast cells, basophils, and eosinophils [103]. Therefore, we examined infiltration into the BALF of WT and *caspase-11*^{-/-} mice on day 35. Our data show a significant decrease in the total cellular infiltration into the BALF of *caspase-11*^{-/-} mice compared to WT (Fig. 4A). BALF was also analyzed by flow cytometry. Our data demonstrate that *caspase-11*^{-/-} mice exhibit a significant increase in eosinophils (CD11b⁺SiglecF⁺), total T cells (CD3⁺), T helper cells (CD3⁺CD8⁻) (Fig. 4F, I & K). However, no significant difference was observed in myeloid cells (CD11b⁺), alveolar macrophages (CD11c⁺F480⁺), neutrophils (CD11b⁺Ly6G⁺), NK cells (CD49b⁺), B cells (CD19⁺), cytotoxic T cells (CD3⁺CD8⁺) and mast cells (Ckit⁺) (Fig. 4B-K) in BALF. Therefore, *caspase-11*^{-/-} mice show differential cellular infiltration in their BALF compared to WT in response to HDM.

2.6. *Caspase-11*^{-/-} mice show reduced histologic signs of lung inflammation but similar mucus production compared to WT mice

Asthma is often sustained by allergic exposure, which leads to bronchial hyper-responsiveness and acute bronchoconstriction in response to specific and non-specific triggers. HDMs cause bronchoconstriction in asthma patients and induce an inflammatory response in the lungs due to the release of cytokines, chemokines and additional mediators. Lungs of both WT and *caspase-11*^{-/-} mice were similarly resistant to increasing concentrations of methacholine measured by Flexivent (Fig. 5A). To determine the effect of chronic HDM challenge on lung pathology in mice, lung tissues were collected 24 h after the last challenge. Serial lung sections were stained with hematoxylin & eosin (H&E) and Periodic Acid Schiff (PAS). HDM challenge for 35 days resulted in significantly reduced inflammatory cell infiltration in *caspase-11*^{-/-} lungs as compared to WT, but similar levels of PAS staining which indicate mucus production in bronchiolar epithelium (Fig. 5B). Semi-quantitative scoring of inflammation in lung sections from *caspase-11*^{-/-} mice demonstrates reduced neutrophils, macrophages and

lymphocytes when compared to lung tissues from WT mice (Fig. 5C). Mucus hypersecretion was also examined by measuring the expression level of *muc5ac* in lungs of WT and *caspase-11*^{-/-} mice by RT-qPCR (Fig. 5D). WT and *caspase-11*^{-/-} mice express similar levels of *muc5ac* in their lungs (Fig. 5E). These data show that *caspase-11*^{-/-} lungs have reduced infiltration of inflammatory cells into the lung tissue without an accompanying decrease in bronchiolar mucus production.

2.7. BALF from *caspase-11*^{-/-} mice show differential levels of Th1 and Th2 cytokines compared to WT

HDM allergens initiate and sustain allergic inflammation. T cells play a role in the initiation and perpetuation of inflammation [87,108]. In particular, Th2 cells are known to be involved in controlling IgE production because of their ability to produce IL-4 and IL-13, and influence function of eosinophils through production of IL-5 [91]. In atopic individuals, allergen-specific-Th2 cytokines, particularly IL-4, IL-5, IL-9, and IL-13, orchestrate and amplify the CD4⁺ Th2 response. In addition, Th2 and Th1 chemokines, including CCL11 and CXCL1, trigger the extravasation and accumulation of eosinophils and neutrophils to perpetuate the allergic inflammation of the airways [90,113]. Furthermore, pro-inflammatory Th1 cytokines, such as IL-1β, IL-1α and IL-6, exacerbate lung inflammation [52,53,98,105]. Selected cytokines (TGF-β, IL-17 and IL-10) are involved in tissue remodeling [13]. Therefore, we examined levels of Th1, Th2 and Th17 cytokines in sera and BALF samples of WT and *caspase-11*^{-/-} mice. In HDM-treated mice, the levels of KC, TNFα and INFγ were reduced in the BALF of *caspase-11*^{-/-} mice on day 35 (Fig. 6A, B & C). The level of IL-33 (Fig. 6D), but also those of IL-4, IL-5 and IL-10 were reduced in the BALF of *caspase-11*^{-/-} mice (Fig. 6E, F & H). Furthermore, IL-17A levels were significantly lower in the BALF of *caspase-11*^{-/-} mice (Fig. 6G). Finally, the levels of IL-13, IL-1α and IL-1β were below the detection level of the multiplex assay. Thus, our data show that *caspase-11*^{-/-} mice exhibit a reduction in the level of Th1, Th2, and Th17 cytokines in BALF.

3. Discussion

HDM is one of the most common aeroallergens, inducing sensitization in approximately 85% of patients with asthma [42], and playing a pivotal role in initiating and perpetuating lung inflammation in patients with asthma [50,75,89]. Clinically, there is a strong correlation between the level of HDM exposure and sensitization, which is a strong predictor for asthma [16,30]. Activation of the inflammasome in the lung can be triggered by stimuli such as HDM in the asthmatic airway [73]. However, the role of the noncanonical inflammasome in asthma has not been elucidated. Herein, we used *caspase-11*^{-/-} mice and macrophages from these mice to dissect whether caspase-11 plays a regulatory role in HDM induced allergic inflammation.

The study of macrophages in allergic asthma has focused on the role of alternatively activated macrophages (M2) mediated by exposure to IL-4/IL-13. This macrophage subset positively correlated with the severity of airway inflammation in many studies [31]. Adoptive transfer of *in vitro* differentiated IL-4/IL-13-stimulated macrophages into the lungs of allergic mice showed that these macrophages actively contribute to the exacerbation of the disease and are not just bystanders [77]. These findings were later confirmed by a few other studies [27,64,78], showing that alternatively activated macrophage enhanced the allergic inflammatory responses in lung tissue. Our data show that *caspase-11*^{-/-} macrophages treated with IL-4 expressed more markers of M2 cells than WT. Ym1 is overexpressed in asthma and aggravates lung injury [28,79]. Even though macrophages express a stronger M2 phenotype, *caspase-11*^{-/-} macrophages exhibit a significant reduction in expression of co-stimulatory molecules CD86, CD40 and MHCII 24 h post-treatment. Co-stimulation by macrophages is required to enhance the release of Th2 cytokines associated with asthma and B cells that will switch to IgE production. Our *in vivo* data show reduced IL-4 and other

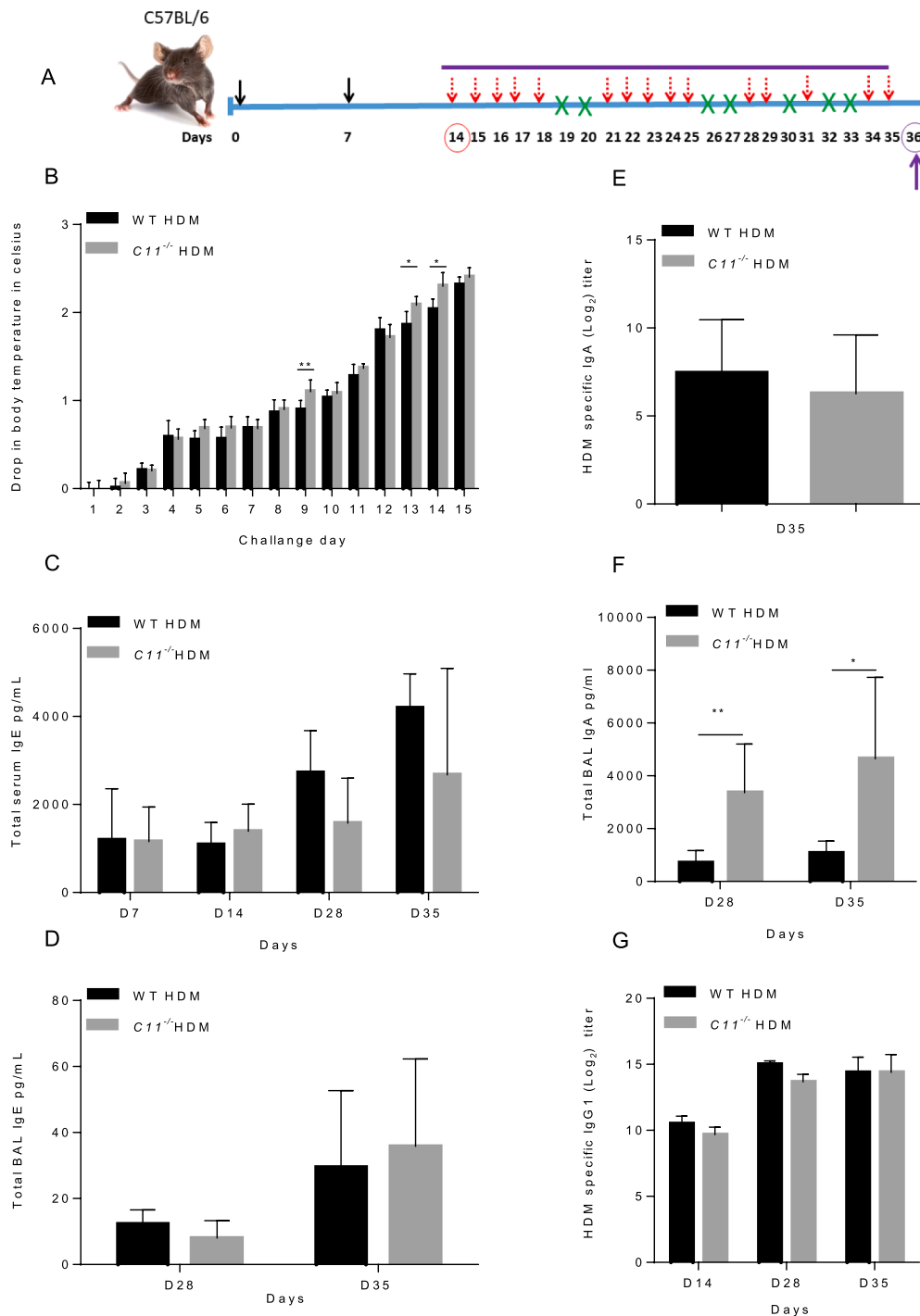


Fig. 3. HDM sensitization and challenge promotes differential allergic inflammation and antibody response in live WT and *caspase-11*^{-/-} mice. Schematic of HDM model (A), five WT and five *caspase-11*^{-/-} mice were sensitized with alum + 100 µg HDM on day 0 and 7 intra-peritoneally (solid arrows). Mice were challenged with 15 doses of HDM intranasally (25 µg) as indicated (dashed arrows). Days without a challenge are indicated by (X) (A). The experiment was done twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment and lead to similar results. Drop in surface body temperature after intra-nasal challenges with HDM was measured before and thirty minutes after each challenge (B). Data indicates the difference in body temperature before and after each challenge. The experiment was done twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment and lead to similar results. Data represent the mean $\pm$ SD (n = 5) obtained from the second *in vivo* experiment. Two-way ANOVA performed for statistical analysis, * p < 0.05, ** p < 0.01, the experiment was performed twice. Total IgE level was quantified in serum (C) and BALF samples (D) collected before sensitization (day 0), after sensitization with HDM and alum (day 14), day 28, and 24 h after the last challenge (day 35). HDM-specific IgA was quantified on day 28 and 35 (E), and total IgA level was quantified in BALF on day 35 (F). HDM-specific IgG1 was quantified in serum samples collected before sensitization (day 0), after sensitization with HDM and alum (day 14), day 28, and 24 h after the last challenge (day 35) (G). Day 0, 14 and (28, 35) represent naïve, sensitized, and sensitized + challenged mice. The experiment was done twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment and lead to similar results. Data represent the mean $\pm$ SD (n = 5) obtained from the second *in vivo* experiment. The experiment was performed twice and lead to similar results. Multiple t-tests performed for statistical analysis, * p < 0.05, ** p < 0.01, the experiment was performed twice.

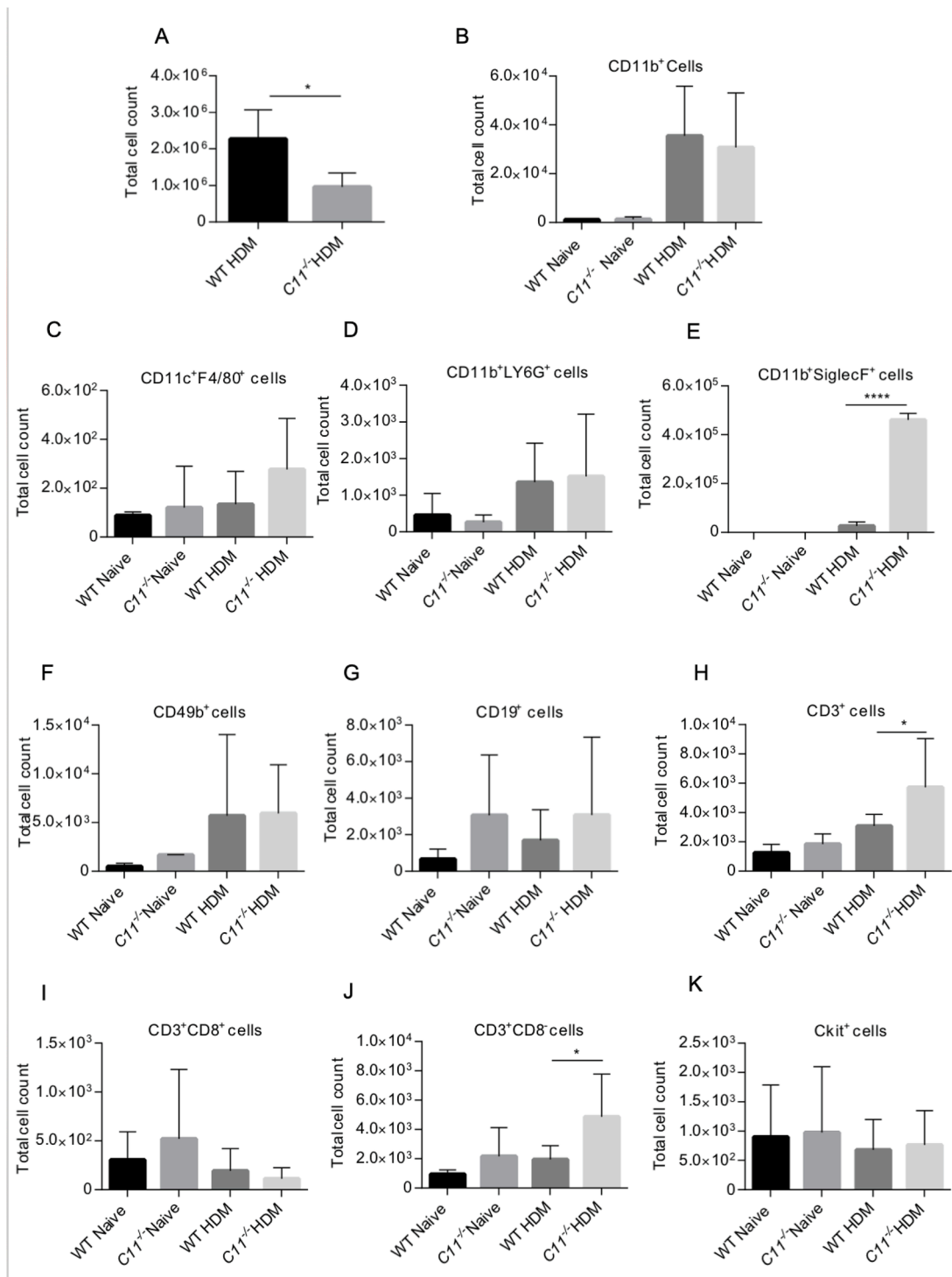


Fig. 4. WT and *caspase-11*^{-/-} mice show differential cellular infiltration in their BALF in response to HDM. Immune cells in bronchial alveolar lavage fluids (BALF) of WT or *caspase-11*^{-/-} mice sensitized and challenged with HDM. Twenty-four hours following the last HDM challenge, cells were isolated by centrifugation and counted with a hemocytometer (A). The experiment was done twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment and lead to similar results. Data represent the mean $\pm$ SD (n = 4) per group obtained from the second *in vivo* experiment. Multiple t-tests performed for statistical analysis, * p < 0.05. Specific cell populations in the BALF were determined by staining cells with several markers followed by flow cytometry. Flow markers are (CD11b⁺), (CD11c⁺F480⁺), (CD11b⁺Ly6G⁺), (CD11b⁺SiglecF⁺), (CD49b⁺), (CD19⁺), CD3⁺, (CD3⁺CD8⁺), (CD3⁺CD8⁺) and (C-kit⁺) (B-K). The experiment was performed twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment. Data represent the mean $\pm$ SD (n = 9) per group pooled from the two experiments. Multiple t-tests performed for statistical analysis, * p < 0.05, ** p < 0.01, *** p < 0.001.

Th2 cytokines in the BALF of *caspase-11*^{-/-} mice. Therefore, we speculate that reduced Th2 cytokines in *caspase-11*^{-/-} lungs are associated with reduced M2 phenotypes and asthma features.

In our model, *caspase-11*^{-/-} and WT mice exhibit similar drop in body

temperature upon allergen challenge, which is consistent with similar IgE level and similar lung functions. However, *caspase-11* BALF contain a significant increased number of eosinophils despite lower IL-5 levels. It has been demonstrated that eotaxin (an important eosinophil-specific

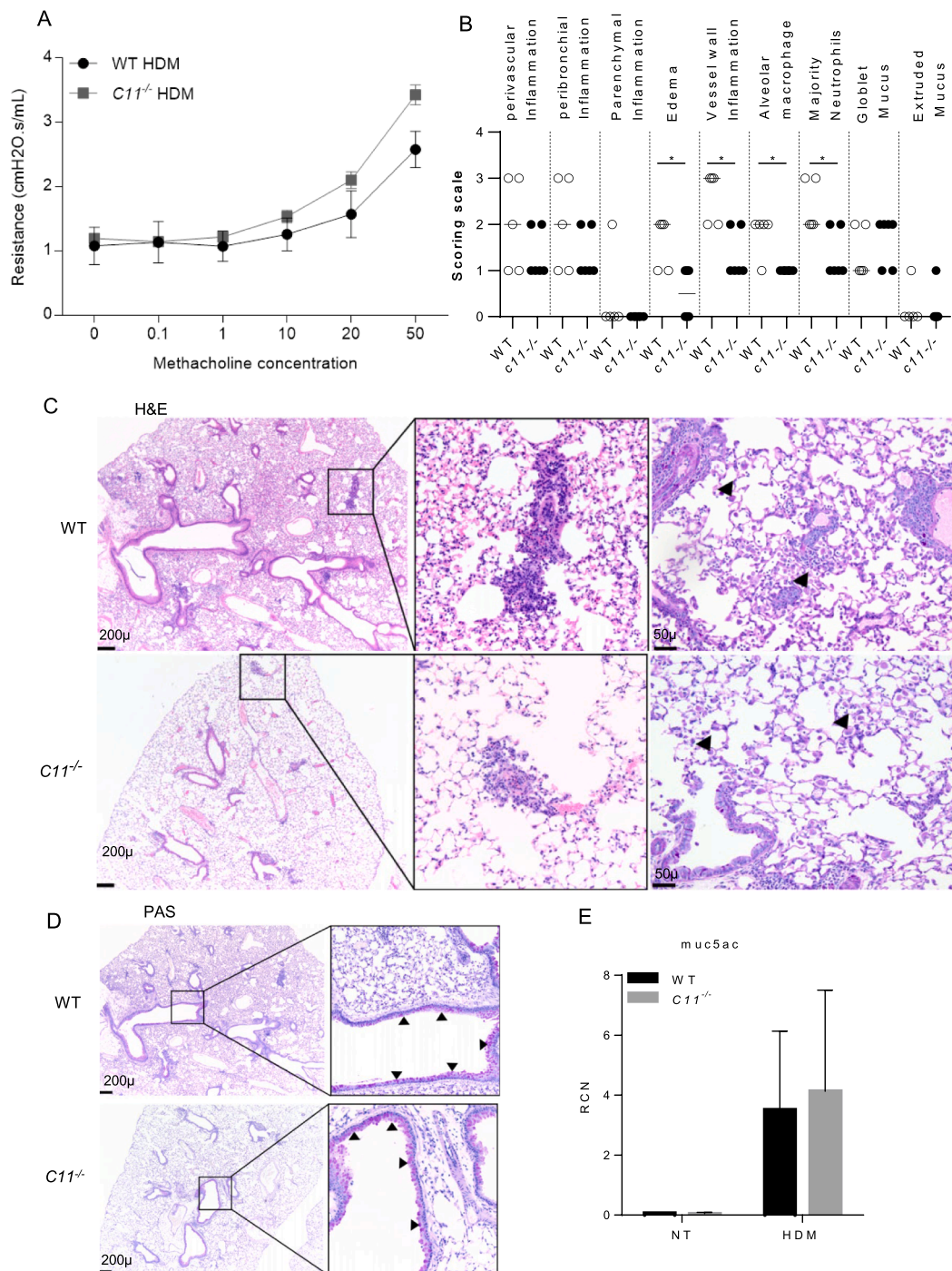


Fig. 5. *Caspase-11*^{-/-} mice show reduced lung inflammation with similar airway resistance and mucus production. Airway hyper-responsiveness to increasing concentrations of methacholine was measured 24 h after the last HDM challenge. Airway hyper-responsiveness was measured by recording changes in lung resistance of WT and *caspase-11*^{-/-} mice (A). The experiment was performed once with five mice per genotype. Data represent the mean $\pm$ SD (n = 5) per group obtained from one experiment. Two-way ANOVA performed for statistical analysis. A linear mixed model was used to take account of the correlation among observations from the same mouse within the same cage. No significant difference was seen between *caspase-11*^{-/-} and WT on the lung contraction averaged across the drug concentrations. Semi-quantitative scoring of inflammation and cellular infiltration in the lungs scored was performed in a blinded fashion by a board-certified veterinary comparative pathologist (B). Representative H&E sections showing reduced lung inflammation in *caspase-11*^{-/-} mice exposed to HDM compared to WT. WT mice had more significant perivascular and peribronchiolar inflammation, while there were no statistical differences in alveolar macrophages (arrows). Inset shows a blood vessel with perivascular and vascular wall inflammation at higher magnification (C). Total magnifications are 40x for lung overviews and 200x for other images including insets. Scale bar for 40x photomicrographs = 200 μ m, scale bar for 200x photomicrographs = 50 μ m. Data represent the mean $\pm$ SD (n = 5) per group obtained from the second *in vivo* experiment. Mann Whitney test performed for statistical analysis, * P < 0.05. The experiment was done twice with seven mice per group in the first *in vivo* experiment and 5 mice per group in the second *in vivo* experiment. Representative lung images derived from WT and *caspase-11*^{-/-} mice are shown from the second *in vivo* experiment. Lung sections derived from WT or *caspase-11*^{-/-} stained with periodic acid schiff (PAS). Similar quantities of PAS-positive mucosubstances (arrows) are visible in bronchiolar epithelium in both WT and *caspase-11*^{-/-} mice (D). Representative (n = 2) expression of *muc5ac* mRNA was measured by quantitative PCR obtained from the second *in vivo* experiment (E).

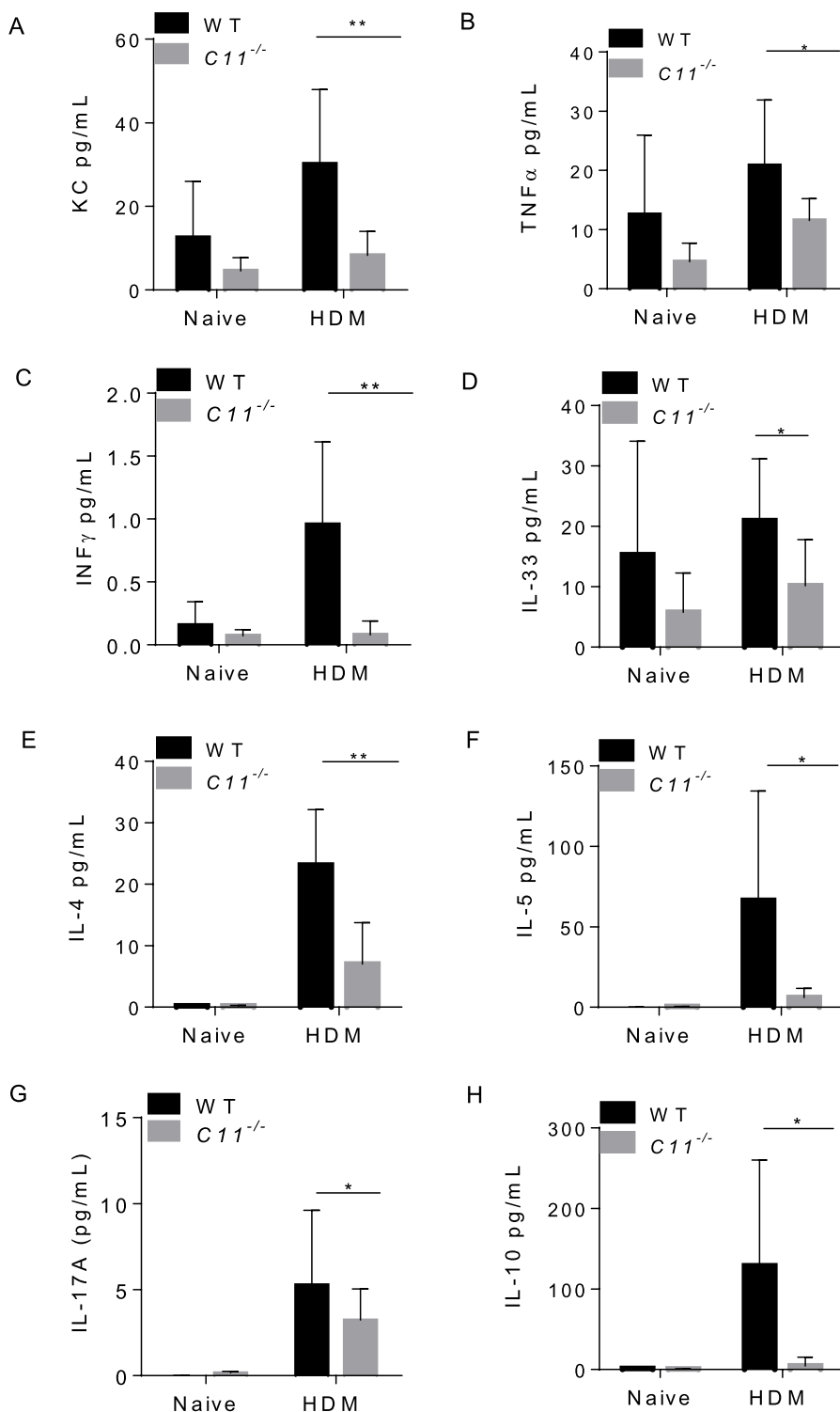


Fig. 6. BALF from *caspase-11*^{-/-} mice show differential levels of Th1 and Th2 cytokines compared to WT. BALF samples were obtained from mice on day 35 post HDM challenge. Levels of BALF cytokines were quantified by multiplex MSD electrochemiluminescence (OSU core facility). Day 0 represents serum obtained from naïve animals, while day 35 represents serum obtained from animals exposed to HDM for 35 days. KC (A), TNFα (B), IFNγ (C), IL-33 (D), IL-4 level (E), IL-5 (F), IL-17A (G), IL-10 level (H). Data represent the mean ± SD (n = 4) per group obtained from the second *in vivo* experiment. Multiple t tests performed for statistical analysis, * P < 0.05, ** p < 0.01.

chemokine that is associated with the recruitment of eosinophils into sites of inflammation) can promote tissue eosinophilia independent of IL-5 [81]. We have tested the expression of CCL11 in lungs derived from *caspase-11*^{-/-} mice challenged with HDM. Expression of CCL11 is higher in *caspase-11*^{-/-} compared to WT lungs (Supplementary Fig. 1I). This finding could explain the increase in eosinophils in *caspase-11*^{-/-} derived BALF while IL-5 is reduced.

Additionally, *caspase-11*^{-/-} mice exhibit reduced airway inflammation and reduced cellular infiltration including neutrophils,

macrophages and lymphocytes in their lungs. The reduced infiltration of cells could be due to an inherent defect in their ability to migrate to sites of inflammation due to defects in the cytoskeleton [24,70]. We have shown that *caspase-11*^{-/-} neutrophils are defective in migration toward chemokines such as KC *in vivo* [24]. Furthermore, caspase-11 promotes neutrophil directional trafficking in an acute model of gout [24]. Others have shown that splenocytes and macrophages derived from *caspase-11*^{-/-} mice are defective in migration toward different chemokines *in vitro* and *in vivo* [23,70]. It was found that *caspase-11*^{-/-} T cells migrate

less efficiently into lymphoid tissues [14]. Modulation of actin polymerization by caspase-11 could regulate additional aspects of T cell biology, including T cell receptor (TCR) signalling [88] and hence affect their ability to migrate.

Another possibility for the reduced inflammation in *caspase-11*^{-/-} mice could be associated with the increased level of total IgA in these mice. In addition to inhibition of bacterial colonization and neutralizing viruses at mucosal surfaces [19,107], IgA plays a protective role at mucosal surfaces by mediating tolerance and preventing hyperinflammation toward allergens that can induce allergic inflammation, such as asthma [9,38]. Therefore, it is plausible that the reduced lung inflammation in *caspase-11*^{-/-} is associated with the increased level of IgA, which could potentially limit binding of HDM to host cells in our experimental model.

The reduced inflammation in *caspase-11*^{-/-} lungs was evident by an overall reduction in KC, Th1 (TNF α and IFN γ), Th2 (IL-33, IL-4, IL-5 and IL-10), and Th17 (IL-17A) levels in BALF. *Caspase-11*^{-/-} mice also show reduced level of IL-17A, which is secreted by a distinct subset of CD4⁺ T helper cells and innate lymphoid cells [110]. By secreting IL-17, Th17 cells orchestrate the recruitment of neutrophil granulocytes in the lungs and their activation directly through CXCL8 production [84]. Several reports have linked IL-17A production with asthma severity [10,97]. Therefore, it is possible that the reduced number of neutrophils we found in the *caspase-11*^{-/-} mice is due to lower IL-17A release.

The level of Th2 cytokines is reduced in the BALF of *caspase 11*^{-/-} mice. IL-33 is of particular importance since it is a chromatin-associated nuclear cytokine from the IL-1 family [94]. IL-33 is involved in polarization of T cells towards a Th2 phenotype and must be released extracellularly in order to bind to the ST2 receptor. Full length (FL) IL-33 is released passively during cell necrosis or when tissues are damaged, and functions as an alarmin [25,26,49,69,72]. While proteolysis of IL-33 by caspase-1 suppresses IL-33 bioactivity [25,72,99] inflammatory proteases including cathepsin G and neutrophil elastase also cleave IL-33 full length (FL) into a shorter mature form with higher activity than IL-33 FL [69,80].

It has been shown that *caspase-1*^{-/-}/*caspase 11*^{-/-} mice exposed to HDM exhibit enhanced lung inflammation associated with a marked eosinophil recruitment, increased expression of IL-4, IL-5, IL-13, as well as FL and cleaved bioactive IL-33 [73]. In our *caspase-11*^{-/-} mouse model, we detected decreased IL-33 in BALF and decreased Th2 cytokines associated with reduced inflammation. We demonstrated reduced IL-4, IL-5 and IL-10 in *caspase-11*^{-/-} mice. The reduction of Th2 cytokines could be a consequence of the reduced IL-33 in the BALF, given the role of IL-33 in enhancing Th2 cytokine production.

On the inflammasome side, it is possible that caspase-1 is activated *in vivo* in the absence of caspase-11. We have seen a reduction in IL-1 β level in *caspase-11*^{-/-} macrophages treated with HDM *in vitro*. However, IL-1 β level in WT and *caspase-11*^{-/-} BALF derived from mice challenged with HDM on day 35 were below the detection of the multiplex. As a pro-inflammatory cytokine, it is possible that IL-1 β level is more important at earlier time points in our model.

Even though we have not identified a direct mechanism by which caspase-11 regulates airway inflammation in response to HDM, we speculate that *caspase-11*^{-/-} lungs exhibit global reduction in inflammation as evidenced by reduced chemokines and Th1, and Th2 cytokines. This reduction could impact cell recruitment into the lungs of these mice in response to HDM. Intriguingly, a new paper was published by Zaslona et al. on the role of *caspase-11*^{-/-} in response to OVA and alum in which the authors demonstrated that *caspase-11*^{-/-} mice show reduction in Th1, Th2 and Th17 cytokines [111]. The findings reported by Zaslona et al. are in line with our data despite the use of a different allergen, exposure times and routes of administration. Macrophages and dendritic cells experience the allergen early on and present processed fragments to naïve T cells. Following activation, T cells release cytokines that polarize immune cells and skew the environment toward asthma. It is possible that macrophages and dendritic cells show reduced ability to present the

allergen to naïve T cells *in vivo*, which results in an overall reduction of cytokines and chemokines involved in cells polarization as well as the disease progression.

In conclusion, caspase-11 regulates lung inflammation in response to HDM during the priming and the effector phases of asthma. Our study offers several intriguing scenarios for the diverse functions of caspase-11. Expression of caspase-11 in innate immune cells following HDM exposure may be required for the antigen presentation by macrophages and dendritic cells to naïve T cells. Subsequently, insufficient antigen presentation leads to reduction in cytokines and chemokines released in the BAL fluids. We have shown that caspase-11 regulates autophagy, which is an essential process for antigen presentation by immune cells [65]. Our published work and that of others also support the notion for inherent defect in migration of *caspase-11*^{-/-} cells due to defect in the actin cytoskeleton [24,70]. Furthermore, [73] showed that lack of caspase-1 is associated with increased Th2 inflammation. As a major player in IL-1 β release, we speculate that that caspase-1 activation plays a role at earlier stages of the disease. However, our data suggest decreased Th2 cytokine in a longer and more chronic model of inflammation. These data suggest that caspase-11 might play a role in later stages of asthma progression which is associated with increased Th2 response. Future studies will explore these possibilities.

4. Materials and methods

4.1. Mice

C57BL/6 wt mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The *caspase-11*^{-/-} mice were generously provided by Dr. Junying Yuan. (Harvard Medical School, Boston, MA, USA) [104]. All mice are in the C57BL/6 background. All mice were housed in a pathogen-free facility and experiments were conducted with approval from the Animal Care and Use Committee at The Ohio State University (Columbus, OH, USA) in accordance with NIH and OSU IACUC guidelines. Mice were maintained in a temperature-controlled facility with a strict 12h light/ dark cycle and were given free access to food and water.

4.2. Cell culture and treatment of BMDMs with HDM and inflammatory stimuli

BMDMs from WT or *caspase-11*^{-/-} were derived from the femurs of five to eight-week-old mice as previously described [1,63], and grown in IMDM (Iscove's modified Dulbecco's medium) supplemented with L929 fibroblast cell line (ATCC)-cultured supernatant and 10% heat inactivated fetal bovine serum (HIFBS) (Gibco). Approximately 2x10⁶ cultivated macrophages were washed twice with PBS and incubated in IMDM media during treatments. Caspase-11 induction was measured by treating WT macrophages with various inflammatory mediators including IL-1 α (R&D Systems, 400-ML-005). (20 ng/mL), IL-1 β (20 ng/mL) (R&D Systems, 401-ML-005), LPS (100 ng/mL) (tlrl-eklps) for 4 h. WT macrophages were stimulated with IL-4 (5 ng/mL) or IL-13 (20 ng/mL) (PeproTech, 214-14, 210-13) for 1, 4, 6 and 24 h in IMDM media. In some instances, macrophages were pre-treated with IL-4 overnight, and then macrophages were un-treated or treated with HDM, HDM + IL-4 for 4 or 24 h (Stallergenes Greer, XPB82D3A2.5). The vial was suspended with phosphate buffer saline to obtain 10 mg/ml stock, then 10 μ l of the stock was used for each mouse which included ~ 7.85 ng of endotoxin.

4.3. Induction of HDM-mediated allergic airway inflammation

To induce HDM-driven airway inflammation, mice were injected with HDM (100 μ g) adsorbed with alum (ImjectTM Alum, Thermo Scientific 77161) intraperitoneally on day 0 and day 7. Isoflurane anesthetized mice were challenged with (25 μ g) HDM intranasally for 15 challenges from day 14 until day 35. Twenty-four hours after the last challenge, mice were sacrificed for bronchoalveolar lavage (BAL),

bronchial hyperresponsiveness, cytokines and chemokines concentration, histology and quantitative real time PCR.

4.4. Assessment of surface body temperature

Body temperature was assessed by measuring surface body temperature with the aid of infrared thermometers [Heat spy infrared thermal imaging camera (Wahl, Culver City, CA, USA)]. Mice were incubated for 10 min at room temperature, and then their body temperatures were measured. Subsequently, mice were challenged with HDM and incubated for 30 min, and their body temperatures were measured again. Then, the body temperature after the challenge was subtracted from that before the challenge for each mouse. This is shown as drop in body temperature as indicated in the figures.

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

Bronchoalveolar lavage was performed by washing the lungs once with 0.8 ml of saline solution at room temperature. After centrifugation at 1500 RPM for 5 min at 4° C, the supernatant (cell-free BALF) was stored at -20° C for cytokine analysis. To determine the percentages of inflammatory cells infiltration in BALF, cells were suspended in FACS buffer and either unstained or single/ multiple stained with the following antibodies: anti CD3 (T-cells), anti CD8 (cytotoxic T cells), anti CD19 (B cells), anti C-kit (mast cells), anti SiglecF (eosinophils), anti CD49b (NK-cells) and anti CD11c/F480 (alveolar macrophages). Stained cells were analyzed with an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA).

4.6. Assessment of HDM-specific IgG1 and IgA Ab responses and total IgA levels

Total and HDM-specific Ab responses were measured in sera and BALF by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated with HDM (25 µg/ml). For detection of HDM-specific IgG1 and IgA Abs, serial dilutions of serum or BALFs were added to the plates and the binding antibodies were detected with HRP-conjugated antisera (Southern Biotech Associates Inc., Birmingham, AL, USA). Biotin-conjugated rat anti-mouse IgG1 monoclonal Abs and HRP-conjugated streptavidin (BD Bioscience, San Jose, CA, USA) were used to measure IgG1. The Ab titers were determined as the last dilutions of samples that with an absorbance of > 0.1 above that of control samples. Total IgA levels were determined by ELISA using extrapolation against IgA standards [33].

5. Total IgE quantification

Total IgE Ab levels were determined by a mouse IgE ELISA kit (ThermoFischer scientific). Sample dilution and IgE detection were done according to instructions from the manufacturer.

5.1. Real-time PCR

Tissues were collected; snap frozen, and reduced to powder before adding TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was isolated and cDNA was synthesized by using Superscript III (Invitrogen) and real-time PCR was performed as previously described [2,37] with the aid of specific primers. The gene of interest Ct was normalized to β -actin Ct and expressed as relative copy number (RCN) of mRNA expression ($RCN = 2^{-\Delta Ct}$).

5.2. Lung histopathology

The right lungs from each group (n = 5 per group) were fixed with 4 % buffered formalin solution. Sections were stained with hematoxylin

and eosin (H&E) for quantifying inflammation and Periodic Acid Schiff (PAS, Sigma-Aldrich) for visualizing mucosubstances and bronchiolar mucus goblet cells. Histology services were performed in the Histology Laboratory of the Comparative Pathology and Digital Imaging Shared Resource (CPDISR) of the Ohio State University Comprehensive Cancer Center. Tissues were routinely processed for histopathology on a Leica Peloris 3 Tissue Processor (Leica Biosystems, Buffalo Grove, IL), embedded in paraffin, sectioned at an approximate thickness of 4–5 µm, and batch stained with hematoxylin and eosin (H&E) on a Leica ST5020 autostainer (Leica Biosystems, Buffalo Grove, IL) using a routine and quality controlled protocol. Slides were then evaluated by a board-certified veterinary comparative pathologist (Dr. Corps) using a Nikon Eclipse Ci-L Upright Microscope (Nikon Instruments, Inc., Melville, NY). Representative photomicrographs were taken using an 18 megapixel Olympus SC180 microscope-mounted digital camera and cellSens imaging software (Olympus Life Science, Center Valley, PA). For evaluation of mucins and mucosubstances, serial histology sections taken immediately following the sections utilized for H&E were routinely stained with PAS and counterstained with hematoxylin. Semi-quantitative scoring was performed by board-certified comparative veterinary pathologist Dr. Kara N. Corps, DVM, PhD, DACVP, of the CPDISR of The Ohio State University Comprehensive Cancer Center. Dr. Corps developed the standardized scoring system based on the requirements of our project and employing standard semi-quantitative methods for the assessment of pulmonary inflammation and mucus production, with modifications of the published methods as necessary [4,29]. Several parameters were evaluated, including perivesicular, peribronchiolar and parenchymal inflammation; inflammatory cell infiltration in vascular walls; perivascular and peribronchiolar edema; alveolar macrophages distant to affected airways/blood vessels; predominance of neutrophils; and goblet cell mucus in bronchioles. Scoring scale was 0–5 with 0, 1, 2, 3, 4 and 5 assigned for no increase/within normal limits, mild (<25%), moderate (26–50%), marked (51–75%), severe (>75%), and maximal inflammation, respectively.

6. Western blotting

Protein extraction from macrophages was performed using TRIzol™ reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Briefly, after phase separation using chloroform, 100% ethanol was added to the interphase/phenol-chloroform layer to precipitate genomic DNA. Subsequently, the phenol-ethanol supernatant was mixed with isopropanol in order to precipitate out proteins. Isolated protein was then denatured in a urea-based lysis buffer. The Bradford method was used to determine protein concentrations. Equal amounts of protein were separated by 12% SDS- polyacrylamide gel (Biorad, 161–0158) and transferred to a polyvinylidene fluoride (PVDF) membrane (Biorad, 162–0177). Membranes were immunoblotted with antibodies that recognize caspase-11 (Sigma Aldrich, C1354), GAPDH (Cell Signaling Technology, 2118), Phospho-Stat6 (Tyr641) (D8S9Y) (Cell Signaling Technology, 56554), total Stat6 D3H4 (Cell Signaling Technology, 5394). Corresponding secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, 7074; Santa Cruz Biotechnology, sc-2006) and in combination with enhanced chemiluminescence reagent (Amersham, RPN2209,) were used to visualize protein bands

7. Cytokine measurements.

Cytokines including IL-1 β , KC, TNF α , IL-4, IL-5, IL-13, IL-10, IFN γ and IL-33, were measured in serum and BALF samples using multiplex ELISA and Meso Scale Diagnostics (MSD) electro-chemiluminescence according to recommendations of the manufacturer. The level of IL-1 β , IL-6, IL-12 and KC in the supernatant of treated macrophages was determined by specific sandwich ELISA following the manufacturer's protocol (R&D) system Inc.

7.1. Statistical analysis

Figures display the mean and standard deviation (SD) as indicated in the figure legends. Comparisons between groups were conducted by multiple t-test, two-way ANOVA or linear mixed effects models were used for analysis to take account of the correlation among observations from the same animal within the same cage. Holm's procedure was used to adjust for multiple comparisons or multiple outcomes to control for type I error at 0.05. Mice were age and sex matched, however no difference was seen in the gender. We did two independent *in vivo* experiments with seven and five mice per group, respectively which lead to similar results. The numbers of animal/group are indicated for each statistical analysis and this is indicated in the figure legends. Statistically significant differences were defined as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

8. Availability of data and materials

All the obtained data are available in the laboratory of Dr. Amal Amer and Dr. Prosper N Boyaka.

9. Ethics statement

The animal study was reviewed and approved by OSU IACUC.

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CRediT authorship contribution statement

Arwa Abu Khweek: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Marisa R. Joldrichsen:** Investigation, Methodology, Data curation, Formal analysis. **Eunsoo Kim:** Investigation, Methodology, Visualization, Supervision. **Zayed Attia:** Investigation, Methodology, Formal analysis. **Kathrin Krause:** Investigation, Methodology, Formal analysis. **Kylene Daily:** Investigation, Methodology, Formal analysis. **Shady Estfanous:** Investigation, Methodology, Formal analysis. **Kaitlin Hamilton:** Investigation, Methodology, Formal analysis. **Asmaa Badr:** Investigation, Methodology, Formal analysis. **Midhun N.K. Anne:** Investigation, Methodology. **Mostafa Eltooby:** Investigation, Methodology. **Kara N Corps:** Validation, review & editing, Data curation. **Cierra Carafice:** Investigation, Methodology. **Xiaoli Zhang:** Data curation, Formal analysis. **Mikhail A. Gavrilin:** Supervision, Resources, Data curation. **Prosper N. Boyaka:** Conceptualization, Writing – original draft, Project administration, Writing – review & editing, Resources. **Amal O. Amer:** Conceptualization, Writing – review & editing, Project administration, Resources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2021.104425>.

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SUPPLEMENTAL MATERIALS

Caspase-11 regulates lung inflammation in response to house dust mites

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Legend supplemental figures

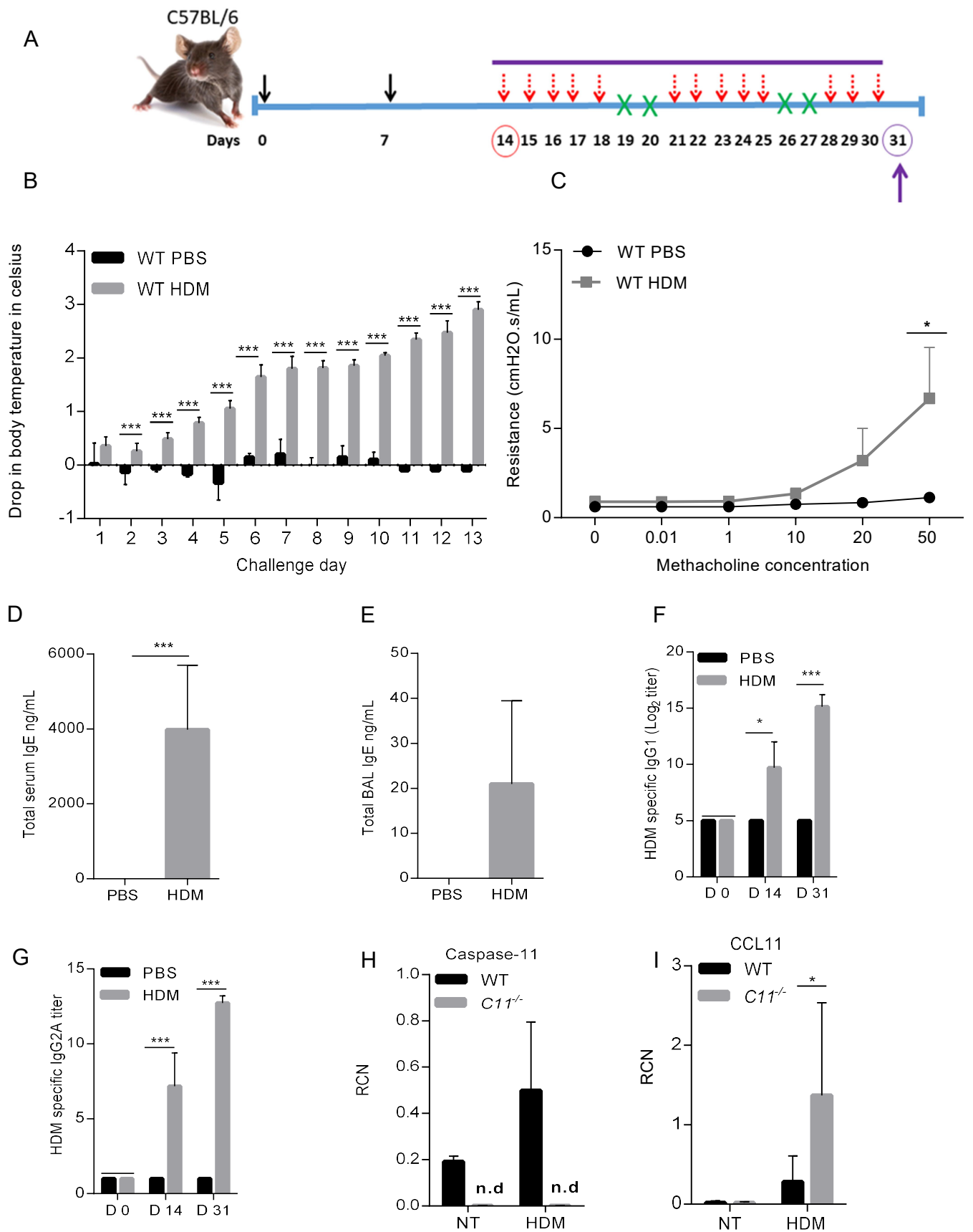
Supplementary figure 1. HDM sensitization and challenge promote allergic inflammation in WT mice.

Experimental HDM model (A). Seven WT mice were sensitized with alum+ 100µg HDM on day 0 and 7 intra-peritoneally (solid arrows). Mice were challenged with 13 doses of HDM intranasally (25µg) as indicated (dashed arrows). Days without a challenge are indicated by (X). Six WT mice were challenged with PBS and used as control. Drop in surface body temperature after intra-nasal challenges with HDM was measured before and thirty minutes after each challenge (B). Data indicates the difference in body temperature before and after each challenge. Data represent the mean $\pm$ SD (n=5) obtained from one experiment. The experiment was performed once for the purpose of optimization. Two-way ANOVA performed for statistical analysis, * $p < 0.05$, ** $p < 0.01$. The experiment was performed once for the purpose of optimization. Two-way ANOVA performed for statistical analysis. Airway hyper-responsiveness was measured by recording changes in lung resistance of WT mice challenged with HDM or PBS on day 31 (C). Data represent the mean $\pm$ SD (n=5) per group obtained from one experiment. The experiment was performed once for the purpose of optimization. Two-way ANOVA performed for statistical analysis. Inflammation is shown by measuring the total sera (D) and BALF IgE level (E). Level of IgG1 (F) and IgG2A (G) were quantified in the serum of HDM-treated mice. Titer on day 0 is below the detection level. Day 0, 14 and 31 represent naïve, sensitized, and sensitized + challenged mice. Data represent the mean $\pm$ SD (n=7) for WT mice challenged with HDM, (n=6) for WT mice challenged with PBS. The experiment was performed once for the purpose of optimization. Multiple t-tests performed for statistical analysis. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Representative (n=2) caspase-11 expression by RT-PCR from

WT and *caspase-11*^{-/-} derived lungs treated with HDM, day 35 (H). Results are obtained from the first *in vivo* experiment. Representative (n=2) CCL11 expression by RT-PCR from WT and *caspase-11*^{-/-} derived lungs treated with HDM, day 35 (I). Results are obtained from the first *in vivo* experiment.

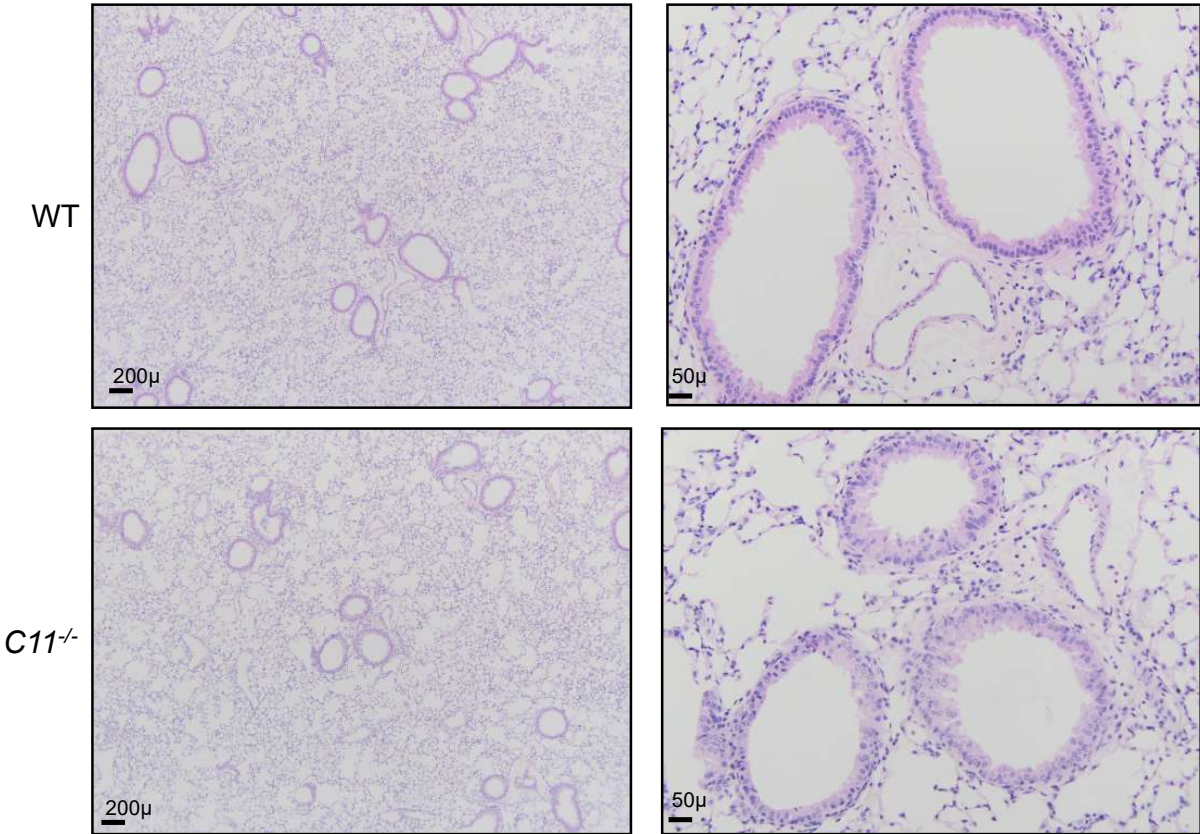
Supplementary figure 2. Representative H&E sections showing no difference in lung inflammation in *caspase-11*^{-/-} mice exposed to PBS compared to WT. Total magnifications are 40x for lung overviews and 200x for other images including insets. Scale bar for 40x photomicrographs = 200 micrometers, scale bar for 200x photomicrographs = 50 micrometers (A). Images scoring (B), data represent the mean $\pm$ SD (n=3) per group obtained from the second *in vivo* experiment. Mann Whitney test performed for statistical analysis, * P < 0.05.

Supplementary Figure 1.



Supplementary Figure 2.

A



B

