

Article

Caspase-1 activation by NLRP3 inflammasome dampens IL-33-dependent house dust mite-induced allergic lung inflammation

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The cysteine protease caspase-1 (Casp-1) contributes to innate immunity through the assembly of NLRP3, NLRC4, AIM2, and NLRP6 inflammasomes. Here we ask whether caspase-1 activation plays a regulatory role in house dust mite (HDM)-induced experimental allergic airway inflammation. We report enhanced airway inflammation in *caspase-1*-deficient mice exposed to HDM with a marked eosinophil recruitment, increased expression of IL-4, IL-5, IL-13, as well as full-length and bioactive IL-33. Furthermore, mice deficient for *NLRP3* failed to control eosinophil influx in the airways and displayed augmented Th2 cytokine and chemokine levels, suggesting that the NLRP3 inflammasome complex controls HDM-induced inflammation. IL-33 neutralization by administration of soluble ST2 receptor inhibited the enhanced allergic inflammation, while administration of recombinant IL-33 during challenge phase enhanced allergic inflammation in *caspase-1*-deficient mice. Therefore, we show that caspase-1, NLRP3, and ASC, but not NLRC4, contribute to the up-regulation of allergic lung inflammation. Moreover, we cannot exclude an effect of caspase-11, because *caspase-1*-deficient mice are deficient for both caspases. Mechanistically, absence of caspase-1 is associated with increased expression of IL-33, uric acid, and spleen tyrosine kinase (Syk) production. This study highlights a critical role of caspase-1 activation and NLRP3/ASC inflammasome complex in the down-modulation of IL-33 *in vivo* and *in vitro*, thereby regulating Th2 response in HDM-induced allergic lung inflammation.

Keywords: house dust mite, allergic asthma, caspase-1, inflammasomes, IL-33, spleen tyrosine kinase (Syk), uric acid

Introduction

Allergic asthma is a chronic lung inflammation characterized by infiltration of eosinophils, type 2 innate lymphoid cells (ILC2), and lymphocytes (Th2) in the airways, increased production of IL-25, TSLP, and IL-33, mucus overproduction leading to airway obstruction, and high specific IgE production in response to environmental allergens (Finkelman and Urban, 2001; Lambrecht and Hammad, 2012).

House dust mites (HDMs) are one of the most frequent and major sources of allergens in >85% of asthmatic patients (Gregory and Lloyd, 2011). HDM contains both cysteine proteases (Der p1; papain-like protease) and serine proteases (Der p3, Der p6, and Der p9) (Gregory and Lloyd, 2011). Exposure to HDM allergens induces rapid production of IL-25, TSLP, and IL-33 by damaged epithelial cells and promotes ILC2 expansion and Th2 response (Hammad et al., 2009; Kearley et al., 2009; Neill et al., 2010).

IL-33 was primarily discovered as a chromatin-associated nuclear factor and belongs to the IL-1 family of cytokines (Haraldsen et al., 2009). IL-33 can be cleaved by caspase-1 for functional inactivation (Cayrol and Girard, 2009; Zhao and Hu, 2010). However, a 10-fold higher bioactive IL-33 can be generated

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following pro-IL-33 cleavage by other proteases such as neutrophil elastase and cathepsin G, suggesting that bioactive IL-33 may be released into the extracellular environment (Lefrancais and Cayrol, 2012; Lefrancais et al., 2012). IL-33 is mainly expressed in non-hematopoietic cells such as fibroblasts, epithelial cells, and endothelial cells (Moussion et al., 2008; Kurowska-Stolarska et al., 2009; Luthi et al., 2009). However, mucosal damage caused by allergens elicits IL-33 production from macrophages and inflammatory dendritic cells (DCs) (Prefontaine et al., 2010; Palmer and Gabay, 2011). Further, IL-33 amplifies IL-13-mediated polarization of alternatively activated macrophages with increased levels of CCL17 and CCL24, which contribute to airway inflammation (Kurowska-Stolarska et al., 2009).

Caspase-1 is an endogenous cysteine protease synthesized as inactive pro-caspase-1 and activated by dimerization and autoproteolysis within multiprotein complexes including ASC and NLRP3 called inflammasomes. Activated caspase-1 is required for IL-1 β and IL-18 release, cell death, and plays a key role in inflammation (Martinon et al., 2009; Lukens et al., 2012).

Here we address the role of caspase-1, NLRP3 inflammasome, and ASC in HDM-induced allergic airway inflammation. We report that caspase-1/11 deletion increases airway inflammation induced by HDM allergens as shown by a marked increase of eosinophil and inflammatory cell infiltration and Th2 responses, including IL-33 release. Furthermore, allergic inflammation in mice deficient for ASC and *NLRP3* was augmented, together with increased levels of IL-33. Blockade of IL-33 signaling by soluble ST2 receptor inhibited the enhanced allergic inflammation seen in *caspase-1/11*-deficient mice. These data show that caspase-1 activation regulates IL-33 *in vivo* in response to HDM allergen.

Results

Caspase-1 regulates eosinophil influx and lung inflammation in response to HDM allergen

Inflammasome complex activation recruits and activates caspase-1. We and others previously showed that NLRP3 inflammasome plays a critical role in ovalbumin-induced allergic airway inflammation either in the presence or in the absence of aluminum salt adjuvant (Eisenbarth et al., 2008; Besnard et al., 2011a). The HDM allergen causes asthma in many patients (Gregory and Lloyd, 2011; Jacquet, 2013). To investigate the role of caspase-1 and other inflammasome components in allergic lung inflammation, caspase-1-deficient mice (*Casp-1*^{-/-}) or wild-type (WT) control C57BL/6 mice were immunized with HDM (25 μ g by intranasal route on Days 0 and 7), followed by three intranasal challenges (5 μ g HDM on Days 14–16) (Figure 1A). Bronchoalveolar lavage fluid (BALF) was collected at 24 h after the last challenge and differential cell count was performed. Increased eosinophils in the BALF and lung as well as airway hyperresponsiveness (AHR) to methacholine were observed in *Casp-1*^{-/-} mice when compared with WT mice, following HDM exposure (Figure 1B–D).

IL-1 α is released by damaged cells *in vivo* and functions as alarmin to alert cells of the innate immune system upon tissue injury and to promote Th2 immunity to HDM allergens (Chen et al., 2007; Willart et al., 2012). We observed a significant increase

in IL-1 α levels in the lung homogenates of *Casp-1*^{-/-} mice compared with WT mice (Figure 1E), suggesting enhanced tissue damage upon exposure to HDM allergen.

Further, the levels of Th2 cytokines IL-4, IL-5, IL-13 and Th2 chemokines CCL11, CCL17, CCL20, CCL22, and CCL24, which are known to attract eosinophils, T lymphocytes, and dendritic cells in lung, were significantly augmented in *Casp-1*^{-/-} mice when compared with WT mice (Figure 1F–M). Moreover, the level of CCL20 increased in a non-significant manner (Figure 1K). Histological analysis of *Casp-1*^{-/-} animals showed increased inflammatory cell infiltration with goblet cell hyperplasia, mucus overproduction as assessed by PAS staining, and Muc5ac mRNA expression (Figure 2A–D).

Taken together, our data showed enhanced lung inflammation and pathology in absence of caspase-1.

NLRP3/ASC inflammasome, not NLRC4, suppresses HDM-induced allergic inflammation

Since caspase-1 is activated by different inflammasome platforms, including NLRP3, ASC, and NLRC4, we asked whether HDM-induced caspase-1-dependent regulation involved NLRP3, ASC, or NLRC4 inflammasomes.

NLRP3 inflammasome was likely to be involved in the regulation of HDM-induced allergic lung inflammation. Indeed, *NLRP3*^{-/-} mice exposed to HDM showed an augmented eosinophilia in BALF and lung (Figure 3A and B), together with increased levels of IL-1 β , IL-13, CCL5, CCL17, and CCL22 (Figure 3C–E, G, and H), while the increased levels of CCL11 and CCL24 did not reach statistical significance (Figure 3F and I). Histological analysis revealed exacerbated cell infiltration and mucus hypersecretion (Figure 3J–L).

We next hypothesized that among the NLRP3-dependent pathways, the ASC adaptor protein is essential to connect NLRP3 and caspase-1. ASC-deficient mice (*ASC*^{-/-}) sensitized and challenged with HDM showed increased eosinophilia in BALF and lung (Supplementary Figure S1A and B) together with increased levels of Th2 cytokines and chemokines including IL-1 β , IL-33 (Supplementary Figure S1C and E), CCL5, CCL11, CCL17, and CCL24 when compared with WT control mice receiving HDM (Supplementary Figure S1F–H and J), and a slight, not statistically significant increase of IL-13 and CCL22 levels (Supplementary Figure S1D and I). Histological analysis confirmed that absence of ASC exacerbated lung inflammation with increased lung pathology (Supplementary Figure S1K–M).

The NLRC4 inflammasome is important for caspase-1 activation to regulate host defense by controlling the release of IL-1 β and IL-18 (Franchi et al., 2012). To exclude a role of NLRC4 in allergic airway inflammation, we investigated the HDM lung response in *NLRC4*-deficient mice (*NLRC4*^{-/-}) (Supplementary Figure S2A). Eosinophil recruitment in BALF and lung, expression of inflammatory cytokines and chemokines, and allergic pulmonary inflammation were similar in *NLRC4*-deficient and WT mice (Supplementary Figure S2A–E).

Taken together, the data demonstrate that the NLRP3 inflammasome complex with ASC and caspase-1, but not NLRC4, contributes to the regulation of airway eosinophilia and Th2 cytokine and chemokine production in the allergic lung inflammation induced by HDM allergens.

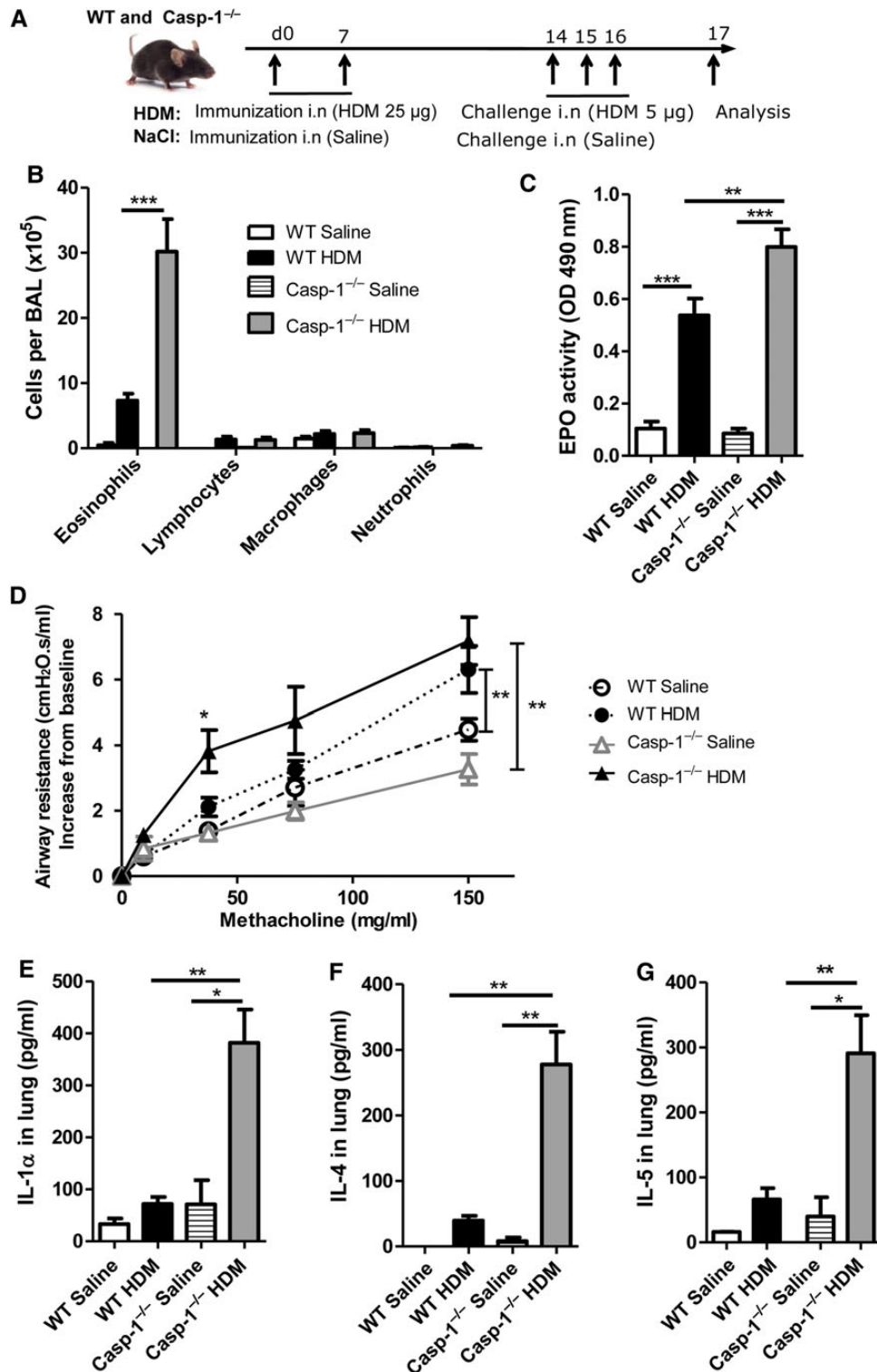


Figure 1 *Caspase-1*-deficient mice show increased airway inflammation in experimental HDM model. **(A)** HDM-sensitized WT and *Casp-1*^{-/-} mice were challenged three times with HDM extract as indicated. **(B)** The numbers of eosinophils, lymphocytes, macrophages, and neutrophils were determined in bronchoalveolar lavage (BAL) at 24 h after the last HDM exposure. **(C)** The eosinophil peroxidase activity (EPO) was measured in lung homogenates. **(D)** Airway hyper-responsiveness to increasing doses of methacholine (Mch; 9.375–150 mg/ml) was measured at 24 h after the last HDM challenge on WT and *Casp-1*^{-/-} mice by recording changes in lung resistance. **(E–M)** The concentrations of IL-1α, IL-4, IL-5, IL-13, CCL11, CCL17, CCL20, CCL22, and CCL24 were measured in lung homogenates by ELISA and Multiplex assay. Results are expressed as mean ± SEM of eight mice per group and are from one representative of two similar independent experiments. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001; ns, not significant.

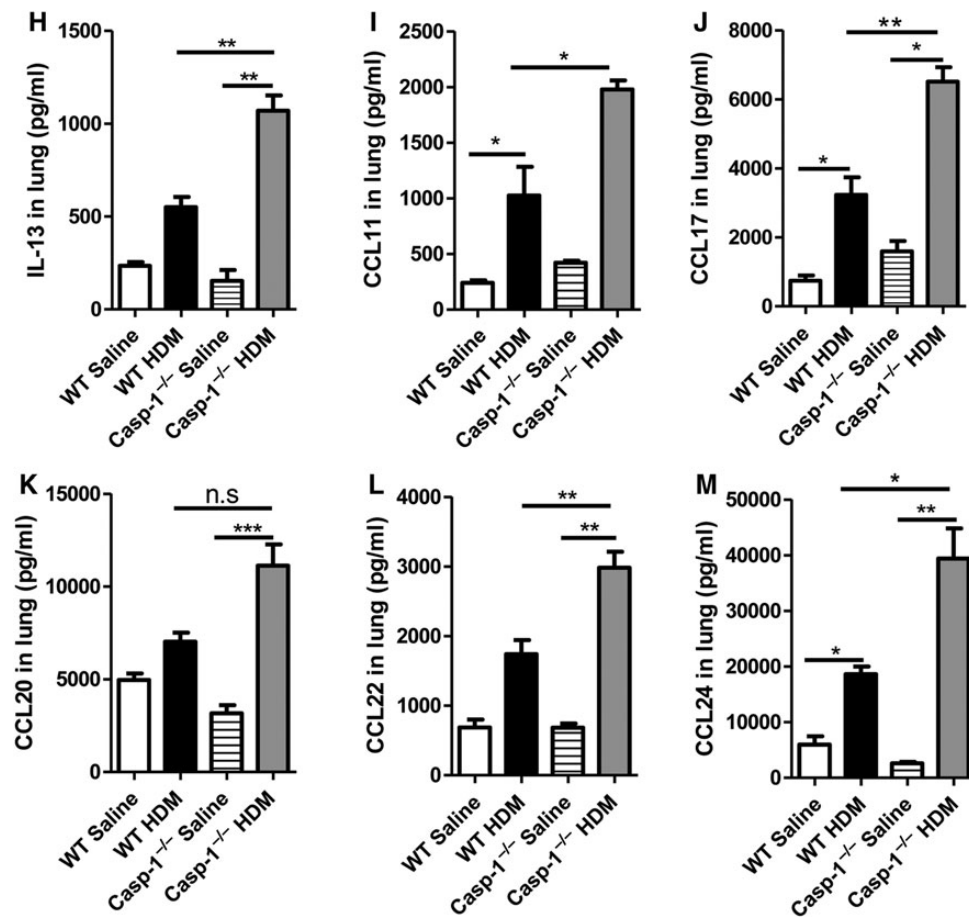


Figure 1 Continued.

Enhanced Th2 response in caspase-1-deficient mice depends on pulmonary IL-33 expression upon HDM allergen exposure

IL-33, a member of the IL-1 family is a strong inducer for a Th2-driven airway inflammation and is a substrate for caspase-1 (Liew et al., 2010). IL-33 is cleaved by caspase-1 after ASP₁₇₈ residue into an inactive form, while by other proteases including cathepsin G and neutrophil elastase into bioactive forms (IL-33_{95–270}, IL-33_{99–270}, and IL-33_{109–270}), which have 10-fold higher biological activity than the full-length IL-33 (Cayrol and Girard, 2009; Lefrancais et al., 2012). To determine whether enhanced pulmonary eosinophil recruitment observed in the airways of Casp-1^{-/-} mice exposed to HDM is linked to IL-33, we analyzed the expression of IL-33 in the lung of HDM-treated WT and Casp-1^{-/-} mice.

We found a significant increase of IL-33 mRNA expression and IL-33 protein levels in the lungs of Casp-1^{-/-} mice compared with WT mice (Figure 4A and B). Western blot analysis revealed increased full-length IL-33 (34 kDa) and highly bioactive IL-33 (21 and 18 kDa), corresponding to Cathepsin G and neutrophil elastase cleavage products (Figure 4C).

The processing of pro-caspase-1 into its p20 and p10 subunits is a hallmark of caspase-1 activation. Consistent with this, we found an increased caspase-1 p20 subunit in the lung homogenates of WT mice, but not Casp-1^{-/-} mice, in response to HDM exposure

(Figure 4D), which may explain the increased expression of full-length IL-33 in the absence of caspase-1.

IL-1β is cleaved into its mature form by caspase-1. Total IL-1β levels in lung homogenates from Casp-1^{-/-} mice were increased when compared with WT mice (Figure 4E), which was mainly due to the pro-IL-1β as determined by western blot analysis (Figure 4F).

Therefore, full-length and processed IL-33 expressions are augmented in the absence of caspase-1. These data suggest that endogenous caspase-1 restrains IL-33 production and function, acting as a suppressor of the allergic response. ASC-deficient mice (ASC^{-/-}) sensitized and challenged with HDM showed significant increased level of IL-1β and IL-33 (Supplementary Figure S1C and E).

Uric acid released in the airways upon HDM exposure has been identified as an essential initiator and amplifier of HDM allergen-induced type 2 immunity through NLRP3-independent mechanisms (Kool et al., 2011; Kuroda et al., 2011). Moreover, uric acid induces IL-33 production in lungs of mice treated with MSU crystals (Hara et al., 2014). We found increased levels of uric acid in the BALF of Casp-1^{-/-} mice compared with WT mice treated with HDM allergen (Figure 4G), suggesting that uric acid might indeed amplify the inflammatory response.

Moreover, uric acid induces Th2 cell immunity by triggering DC activation in a spleen tyrosine kinase (Syk)-dependent manner

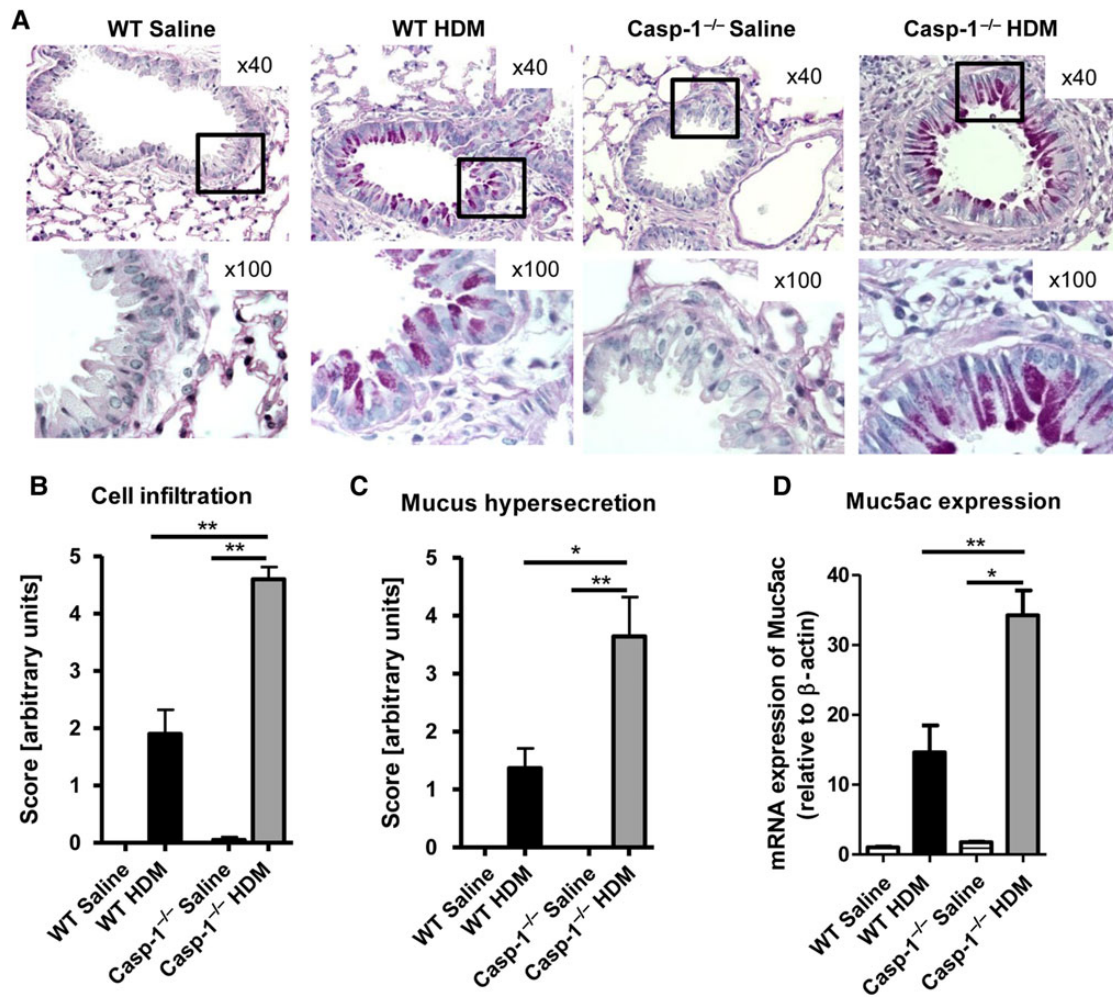


Figure 2 Exacerbated lung inflammation and mucus hypersecretion in *caspase-1*-deficient mice exposed to HDM. (A) Formalin-fixed lung sections were stained with periodic acid Schiff reagent (PAS) to visualize mucus. Representative sections from saline control and HDM-treated WT and *Casp-1*^{-/-} mice are shown. Magnifications are 40 $\times$ and 100 $\times$ as indicated. (B and C) A semi-quantitative histological assessment of inflammatory cell infiltration (B) and mucus hypersecretion (C) was performed by two independent observers. A scale from 0 to 5 is given on the axis. (D) Muc5ac mRNA expression was measured by quantitative PCR. Results are expressed as mean $\pm$ SEM of eight mice per group and are from one representative of two similar independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$.

(Kool et al., 2011; Kuroda et al., 2011). Total Syk protein was indeed increased in the lung of *Casp-1*^{-/-} mice, suggesting caspase-1-dependent Syk kinase regulation as well as GM-CSF production (Figure 4H and I).

Therefore, the data suggest that absence of caspase-1 is associated with increased expression of IL-33, uric acid, and spleen tyrosine kinase (Syk) production, which is critical for the amplification of lung inflammation in response to HDM.

IL-33 blockade with muST2-Fc fusion protein reduces the development of Th2 immunity to HDM in caspase-1-deficient mice

IL-33 is a specific ligand for ST2, the IL-33 receptor (Schmitz et al., 2005), and drives Th2 responses (Townsend et al., 2000; Oboki et al., 2010). To examine whether the increased IL-33 production upon HDM exposure contributes to the exacerbation of airway inflammation in *caspase-1*-deficient mice, we administered

a murine ST2-Fc fusion protein (muST2-Fc) to *Casp-1*^{-/-} mice during the immunization phase only (Figure 5A). Soluble muST2 protein has previously been shown to abrogate IL-33 activity *in vitro* and *in vivo* (Palmer et al., 2008; Smithgall et al., 2008). Murine ST2-Fc fusion protein treatment significantly reduced the exacerbated eosinophil recruitment in BAL and lung tissue seen in *Casp-1*^{-/-} mice (Figure 5B and C). Furthermore, muST2-Fc fusion protein significantly inhibited the production of IL-1 α , IL-1 β , IL-4, IL-5, IL-13, IL-33, CCL11, CCL17, CCL20, CCL22, and CCL24 in lung, as well as uric acid in BALF after HDM exposure (Figure 5D–O). Moreover, cell infiltration, mucus overproduction, and Muc5ac mRNA expression in lung were significantly reduced by muST2-Fc fusion protein treatment in HDM-exposed *Casp-1*^{-/-} mice (Figure 5P–S).

Overall, these data emphasize a critical role played by IL-33 in the exacerbation of lung eosinophilia in *Casp-1*^{-/-} mice, and IL-33 neutralization attenuates lung inflammation.

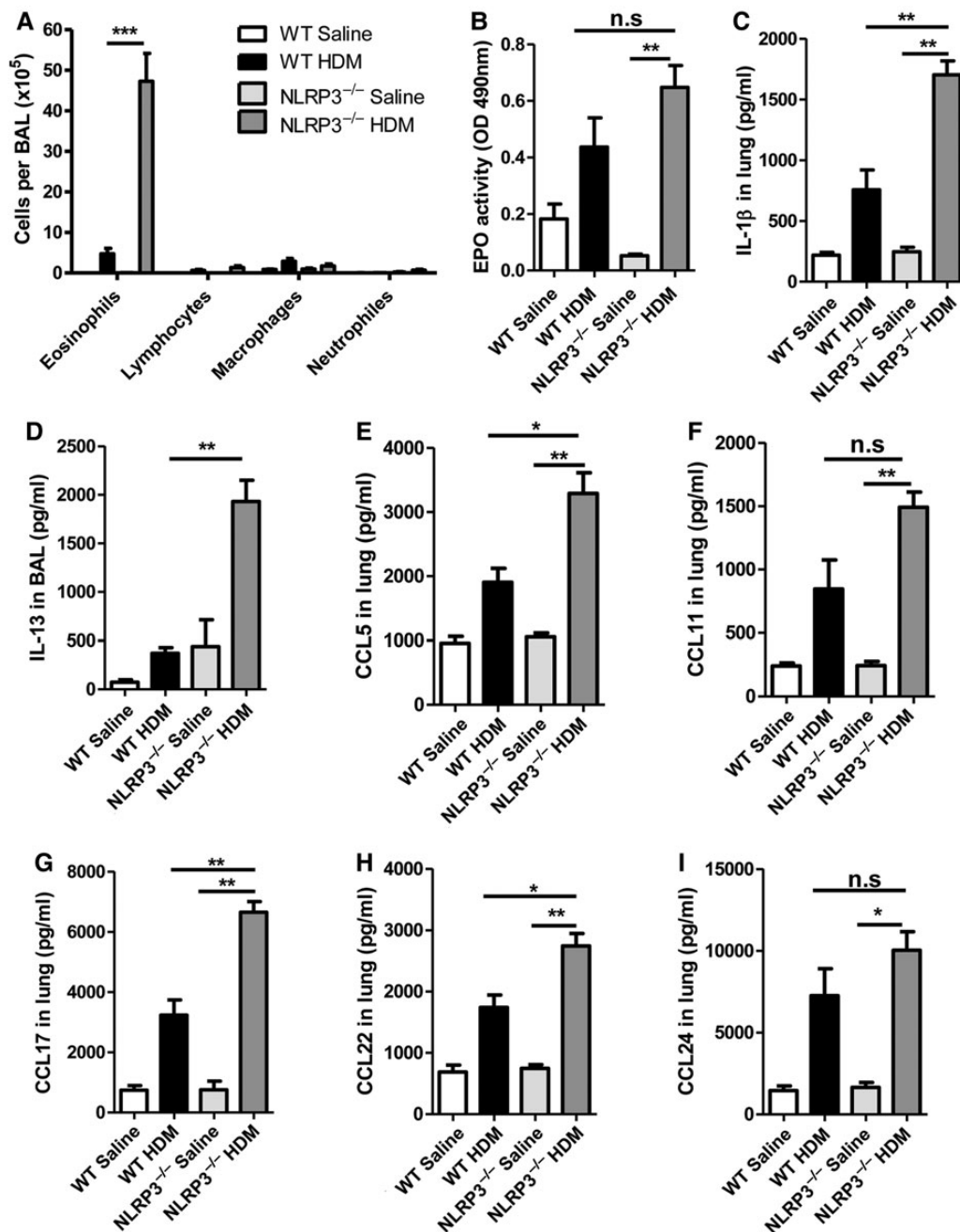


Figure 3 Increased allergic airway inflammation in *NLRP3*^{-/-} mice exposed to HDM. HDM-sensitized WT and *NLRP3*^{-/-} mice were challenged three times with HDM extracts as shown in Figure 1A. (A) Twenty-four hours after the third challenge, eosinophil, lymphocyte, neutrophil, and macrophage counts were determined in BAL fluid. (B–I) EPO activity was evaluated, and IL-1β, IL-13, CCL5, CCL11, CCL17, CCL22, and CCL24 were measured in lung homogenates by ELISA. (J) Histological sections of lungs from WT and *NLRP3*^{-/-} mice were stained for PAS. Magnifications are 40× and 100× as indicated. (K and L) A semi-quantitative histological scoring of cell infiltration (K) and mucus hypersecretion (L) was performed by two independent observers. A scale from 0 to 5 is given on the axis. Data are presented as mean ± SEM and are representative of three independent experiments (*n* = 8 mice per group). **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

IL-33 increases airway inflammation and induces uric acid in the absence of caspase-1/11

To investigate whether recombinant (rm) IL-33 alone can induce exacerbated airway inflammation and the production of uric acid in the absence of caspase-1, we treated WT and *Casp-1*^{-/-} mice with

IL-33 recombinant protein (three times by intranasal route) (Figure 6A). Increased eosinophils in the BALF were observed in *Casp-1*^{-/-} mice when compared with WT mice, following rmIL-33 exposure (Figure 6B). Furthermore, uric acid was increased in the lung of *Casp-1*^{-/-} mice treated with IL-33 (Figure 6C). Therefore,

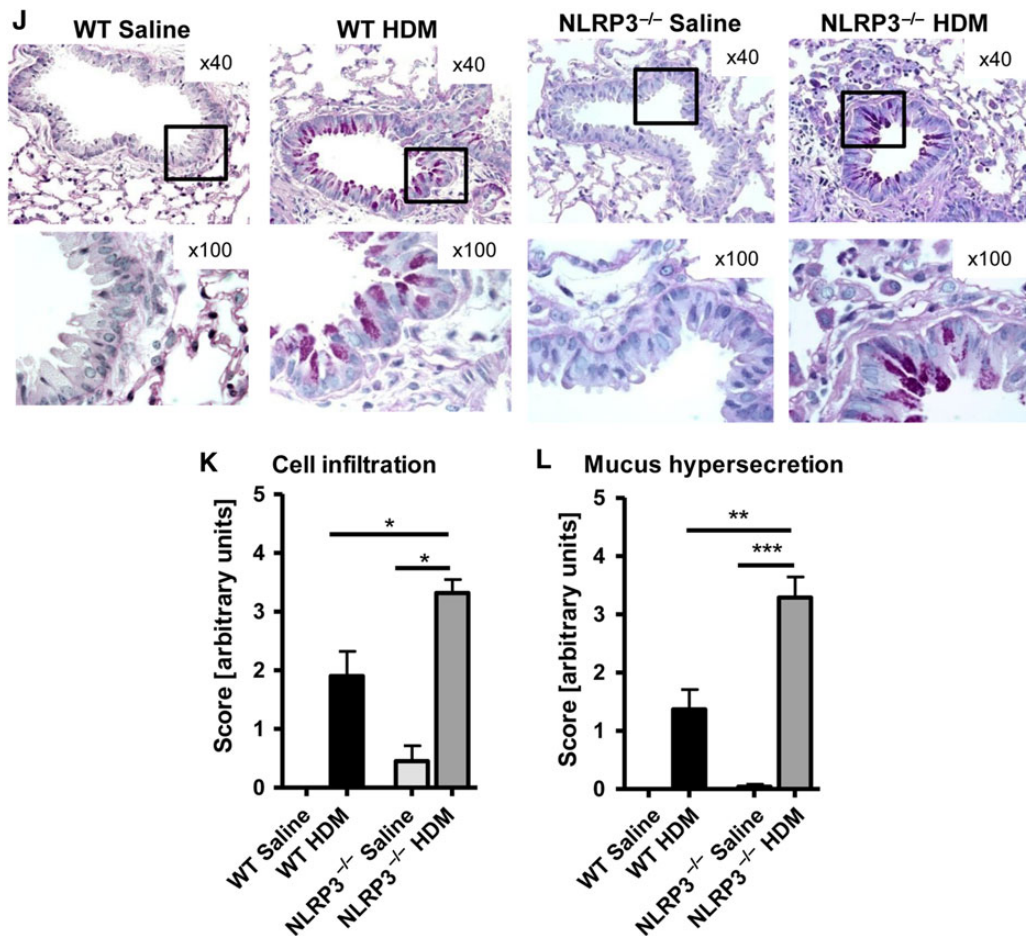


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IL-33 augments uric acid release that is NLRP3- and caspase-1-independent, amplifying the inflammatory response via Syk kinase downstream of IL-33/ST2.

Macrophages are critical producers of IL-33 in response to HDM stimulation

Since the population of lung macrophages was augmented after HDM sensitization, we next investigated whether macrophages express IL-33 during HDM-induced lung inflammation. We cultured *in vitro* bone marrow-derived macrophages (BMDMs) and primary fibroblasts from WT or *Casp-1*^{-/-} mice, and stimulated them with HDM (Figure 7). HDM increased IL-33 protein in macrophages, and IL-33 levels were significantly higher in cell lysate from *Casp-1*^{-/-} BMDM when compared with WT BMDM, while IL-1 α levels were similar (Figure 7A). We obtained similar results using alveolar macrophages from *Casp-1*^{-/-}, *ASC*^{-/-}, and *NLRP3*^{-/-} mice (data not shown).

However, IL-33 was undetectable in the cell lysates or supernatants from fibroblasts stimulated with HDM (data not shown). To verify that fibroblasts can produce other cytokines in response to HDM stimulation, we measured TNF α , IL-6, and KC (CXCL1) protein levels in fibroblast supernatants. Interestingly, TNF α , IL-6, and KC levels were significantly increased in fibroblasts from

Casp-1^{-/-} mice compared with WT (Figure 7B), which suggest that upon HDM exposure, other inflammatory mediators are regulated by caspase-1 and might contribute to the enhanced inflammatory response.

Caspase-1 activation is accompanied by an efficient secretion of mature IL-1 β and cell death (Broz et al., 2010). *Caspase-1*-deficient macrophages or dendritic cells stimulated with HDM extracts showed drastically reduced IL-1 β release, while similar TNF α levels were seen in WT cells (Supplementary Figure S3A–D). Importantly, HDM stimulation caused higher cell death than LPS stimulation in WT macrophages, while *Casp1*^{-/-} macrophages showed less cell death after HDM stimulation (Supplementary Figure S4A), indicating that both IL-1 β release by macrophages and cell death were dependent on caspase-1 *in vitro*.

Thus, macrophages and epithelial cells, but not fibroblasts, produced IL-33 in response to HDM, which was enhanced in the absence of caspase-1 or NLRP3, supporting a regulatory role of the inflammasome for IL-33 production.

Discussion

A regulatory role of caspase-1 on IL-33 release and regulation of HDM-induced lung inflammation has so far not been reported, while several studies investigated the role of caspase-1 on cell

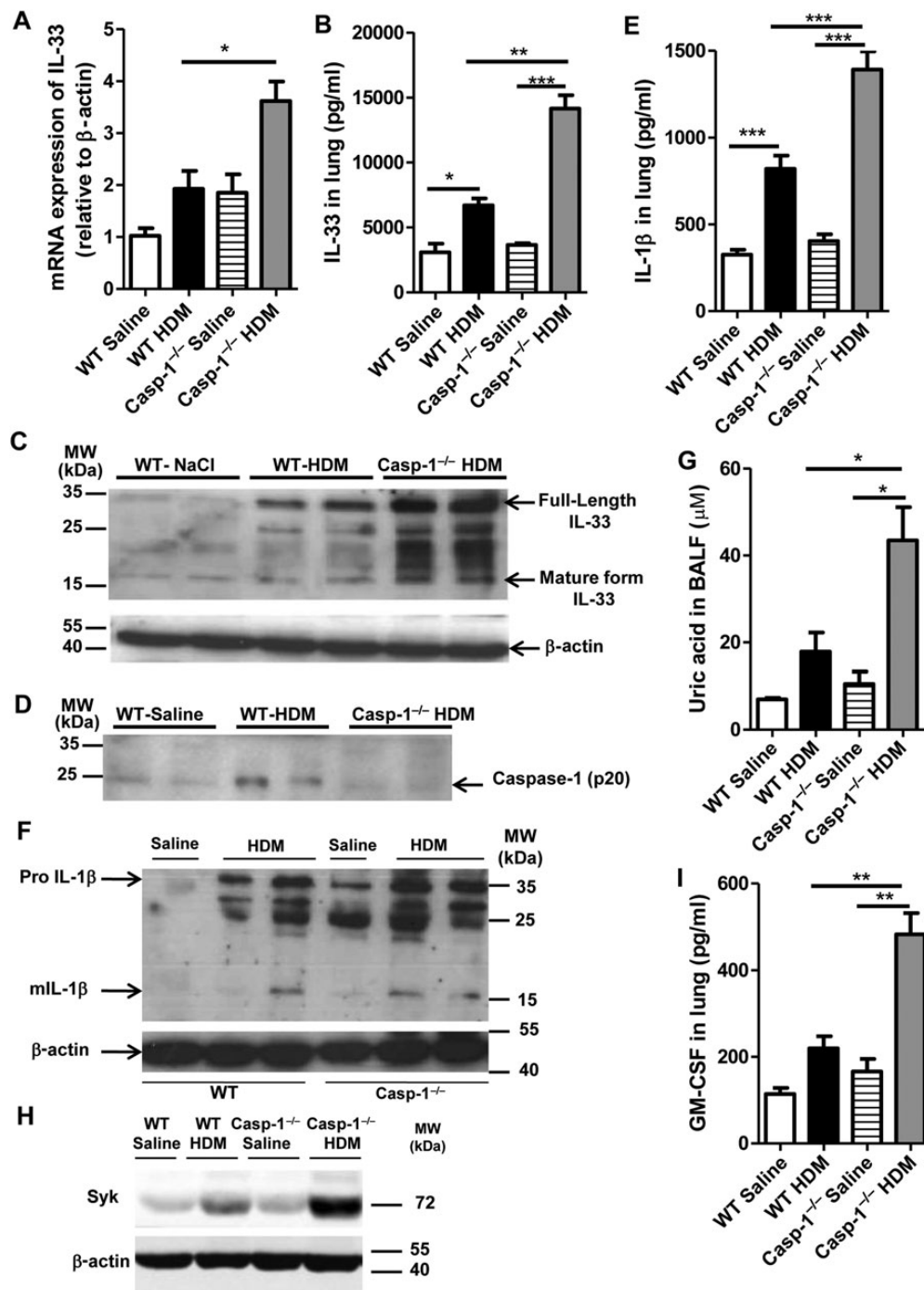


Figure 4 Increased pulmonary IL-33 levels upon HDM exposure in *caspase-1*-deficient mice. (A) IL-33 mRNA expression was measured by Q-PCR. (B–F) The total concentration and molecular length of IL-33 (B and C), IL-1 β (E and F), and caspase-1 p20 subunit (D) were determined in lung supernatant at 24 h after the last HDM exposure by ELISA (B and E) or western blot analysis (C and F). Full-length IL-33 (34 kDa) and mature form of IL-33 (18 kDa) are differentiated by western blot analysis in lung supernatant of HDM-treated WT and *Casp-1*^{-/-} mice or saline control mice (C). Caspase-1 p20 subunit is determined in lung supernatant of HDM-treated WT and *Casp-1*^{-/-} mice or saline control mice (D). Pro-IL-1 β (35 kDa) and mature form (17 kDa) are differentiated by western blot analysis in lung supernatant of HDM-treated WT and *Casp-1*^{-/-} mice or saline control mice (F). (G) Uric acid was measured in BALF. (H) Total Syk protein was determined by western blot analysis in lung supernatant of HDM-treated WT and *Casp-1*^{-/-} mice or saline control mice. (I) GM-CSF cytokine production was measured by ELISA in the lung of mice. β -actin is quantified in all western blot experiments to determine the quantity of sample per well. Results are expressed as mean $\pm$ SEM of eight mice per group and are from one representative of two similar independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

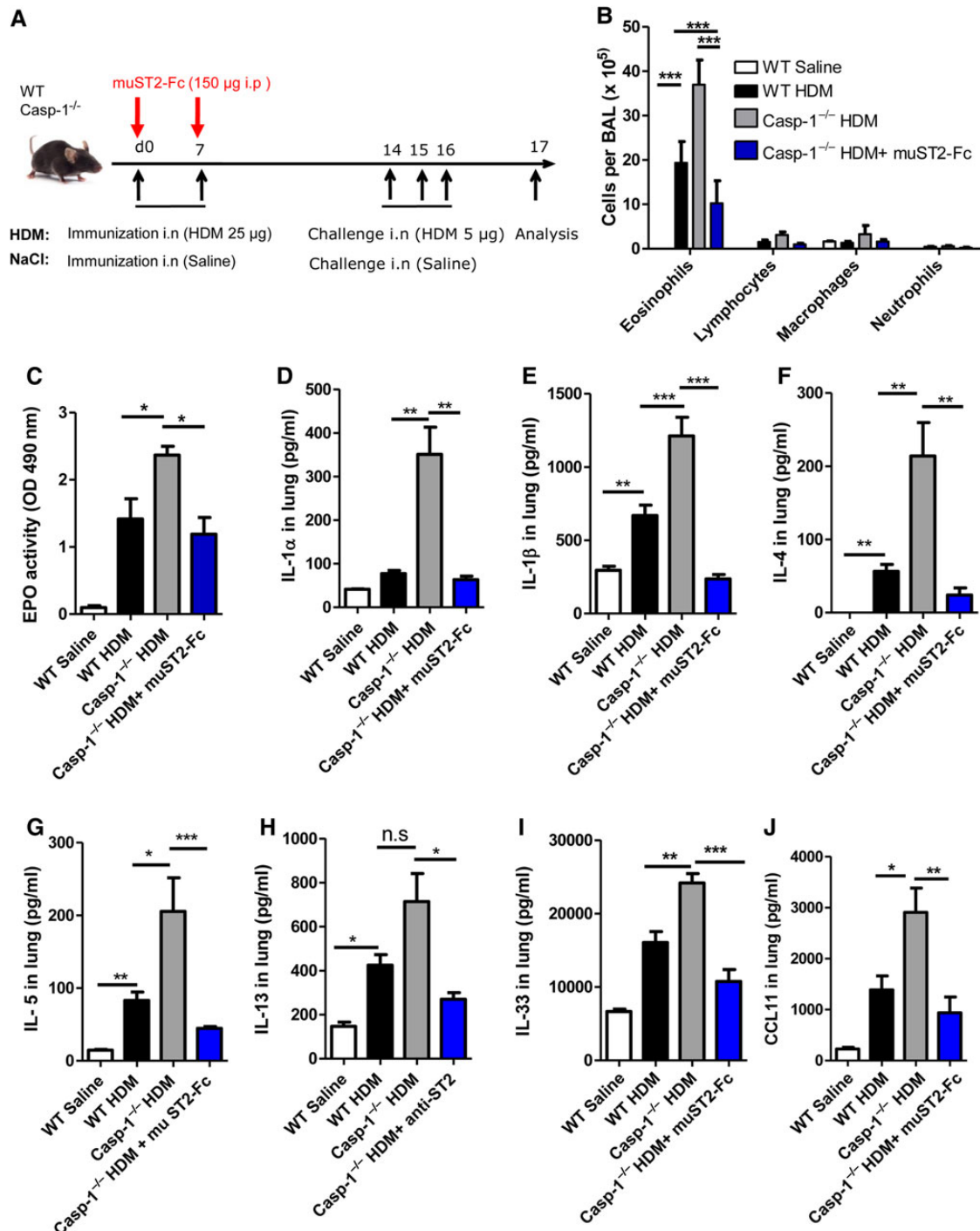


Figure 5 Blockade of IL-33 with murine ST2-Fc fusion protein during sensitization phase reduces airways eosinophilia and inflammation in *caspase-1*-deficient mice. **(A)** WT or *Casp-1*^{-/-} mice were sensitized with HDM (25 µg on Days 0 and 7) with or without ST2-Fc fusion protein (150 µg by i.p. injection), and were challenged three times with HDM instillation (5 µg on Days 14–16). **(B and C)** Twenty-four hours after the third challenge, eosinophil numbers in the BAL fluid **(B)** and EPO activity in the lung **(C)** were determined. **(D–N)** IL-1α, IL-1β, IL-4, IL-5, IL-13, IL-33, CCL11, CCL17, CCL20, CCL22, and CCL24 were measured by ELISA in the lung homogenates. **(O)** Uric acid production was measured in BALF. **(P)** Histological sections of lungs from WT and *Casp-1*^{-/-} mice were stained for PAS. Magnifications are 40× as indicated. **(Q and R)** A semi-quantitative histological score of cell infiltration **(Q)** and mucus hypersecretion **(R)** was performed by two independent observers. A scale from 0 to 5 is given on the axis. **(S)** Muc5ac mRNA expression was measured by Q-PCR. Results are expressed as mean ± SEM of eight mice per group and are from one representative of two similar independent experiments. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

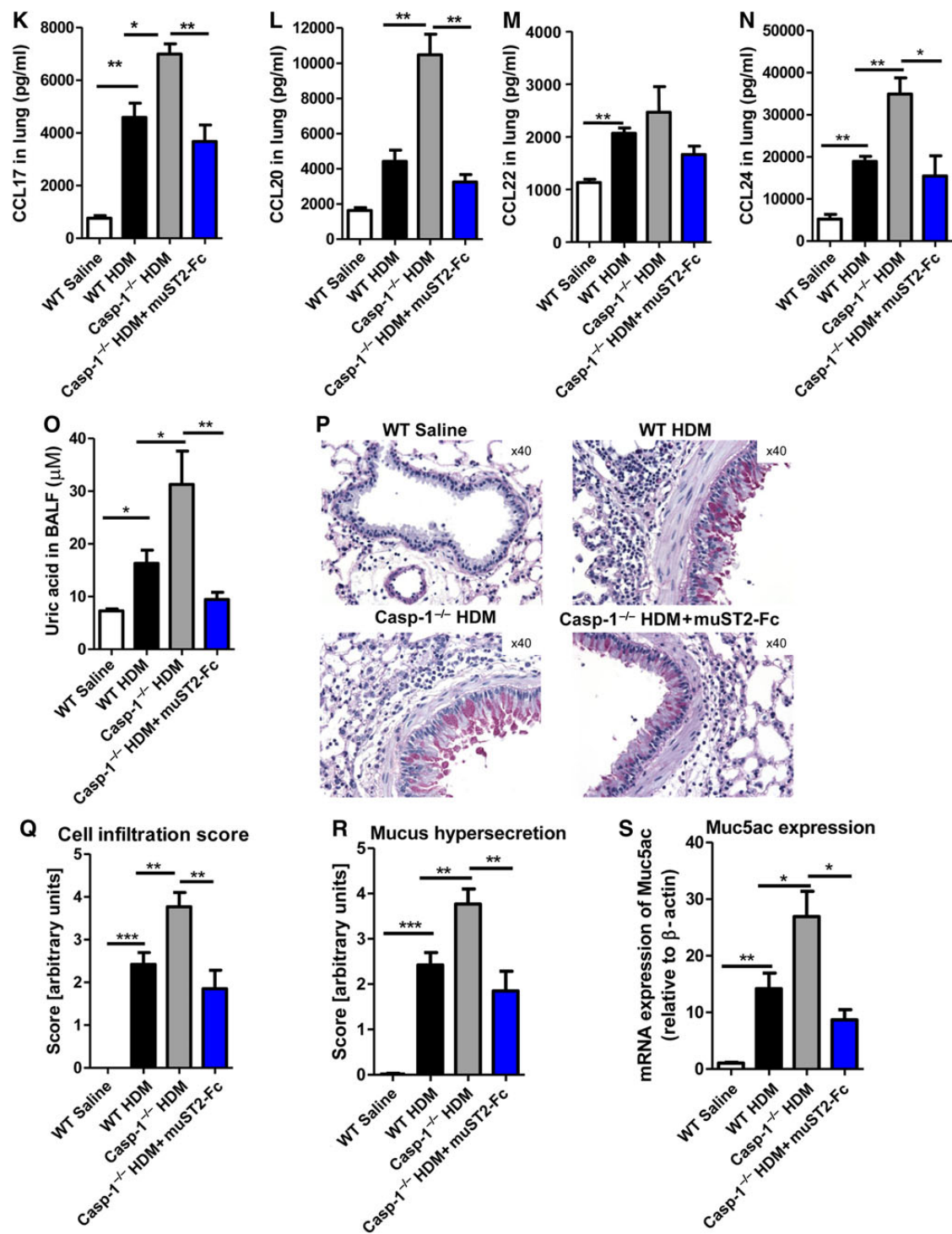


Figure 5 Continued.

death and IL-1 β release *in vitro* and *in vivo* (Kuida et al., 1995; Gurcel et al., 2006; Yu and Finlay, 2008). Here, we have uncovered a novel critical regulatory role of caspase-1 on the Th2-dependent allergic response to HDM allergen. HDM-induced Th2 responses result from the damaging effects of protease-containing allergens on airway epithelia. We

demonstrate here that caspase-1 and NLRP3 activation attenuates HDM-induced allergic lung inflammation with reduced eosinophil recruitment and IL-33 production. Upregulation of HDM-induced airway inflammation in *caspase-1*-deficient mice revealed increased Th2 cytokine production. The increased Th2 response correlated with higher IL-33 production, which promotes airway

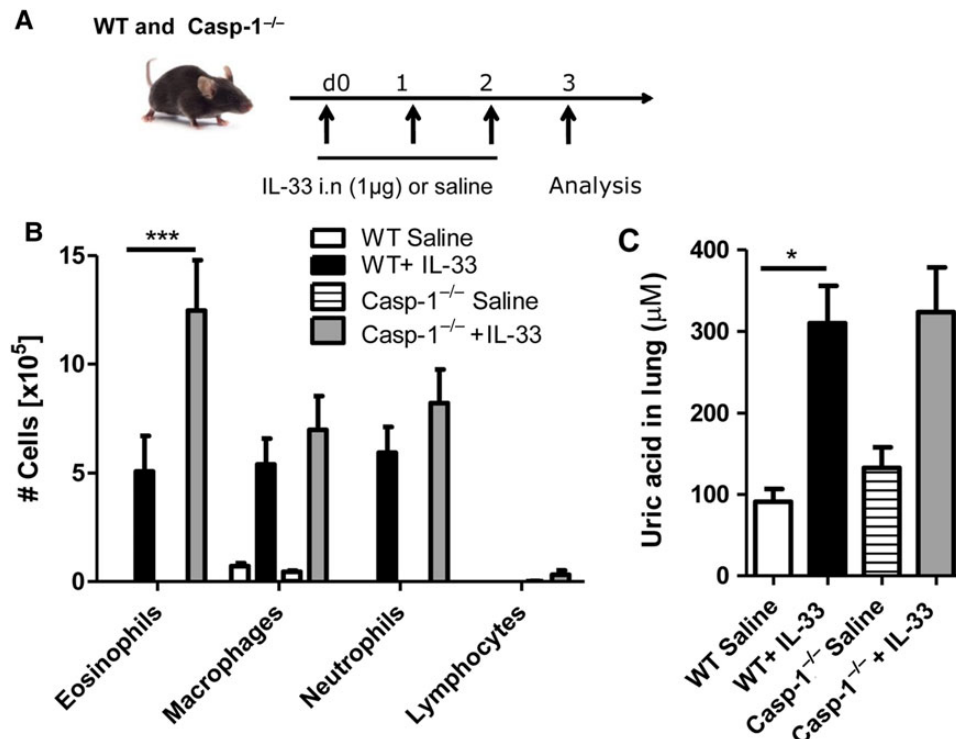


Figure 6 Intranasal administration of recombinant IL-33 increases airway inflammation in *Casp-1*^{-/-} mice. (A) WT or *Casp-1*^{-/-} mice were treated with three intranasal instillations of 1 μg of IL-33. (B) Twenty-four hours after the last treatment, eosinophil, macrophage, neutrophil, and lymphocyte were determined in BALF. (C) Uric acid concentration was determined in lung homogenate. Results are expressed as mean ± SEM of eight mice per group and are from one representative of two similar independent experiments. **P* ≤ 0.05, ****P* ≤ 0.001; ns, not significant.

inflammation with increased eosinophil influx in the lung, consistent with the notion that IL-33/ST2 signaling is critical for Th2 development (Townsend et al., 2000).

Neutralization of IL-33 by muST2-Fc fusion protein during the immunization phase of HDM-induced lung inflammation diminished allergic airway inflammation, and was associated with a reduced expression of IL-1α, IL-1β, IL-5, and IL-33. Our data are consistent with earlier reports that ST2 deletion or blockade impairs type 2 responses *in vivo* in other models (Coyle et al., 1999; Townsend et al., 2000).

The molecular and cellular mechanisms that initiate IL-33 release *in vivo* remain a topic of considerable debate and controversy. In mouse and human tissues, IL-33 is constitutively expressed in endothelial cells and in some epithelial cells (Carriere et al., 2007; Prefontaine et al., 2009, 2010; Pastorelli et al., 2010). Regulated expression of IL-33 has been reported in cultured mouse and human myeloid cells, such as monocytes/macrophages and DCs (Ohno et al., 2009; Nile et al., 2010; Shimamoto et al., 2010).

We detected a significant increase of intracellular IL-33 protein expression in murine macrophages. Our data support the notion that IL-33 plays an important role in innate rather than acquired immunity. This finding is also in agreement with observations in IL-33-deficient mice (Oboki et al., 2010). Consistent with our findings, expression of IL-33 has been demonstrated in the lungs of

patients with severe asthma (Prefontaine et al., 2010), and IL-33 has also been identified as a susceptibility gene for human asthma in genome-wide association studies (Gudbjartsson et al., 2009; Moffatt et al., 2010).

A key finding from our studies is that caspase-1 restrains the expression of full-length and bioactive, cleaved IL-33, which in turn attenuates inflammation, since the expression of full-length and cleaved IL-33 is drastically increased in *Casp-1*^{-/-} mice. This is an unexpected and novel finding that has not been reported hitherto. We confirmed the absence of caspase-1 mRNA and protein in *Casp-1*^{-/-} mice. A contribution by caspase-11 cannot be excluded, as the mice used in this study were recently shown to be also deficient for *caspase-11*, which is engaged by non-canonical inflammasome activation (Kayagaki et al., 2011). Therefore, the inactivation of caspase-11 (known also as caspase-4) could be involved in the unslashed inflammation seen in the double *caspase-1/11*-deficient mice exposed to the HDM allergens. The role of a non-canonical inflammasome, which might be controlled by caspase-11, needs to be further investigated.

Further, caspase-1, caspase-8, and calpain appeared not to be required for IL-33 production by macrophages (Ohno et al., 2009). Interestingly, other proteases such as neutrophil elastase and cathepsin G were recently shown to cleave IL-33 at different sites, resulting in bioactive IL-33_{95–270}, IL-33_{99–270}, and IL-33_{109–270} (Lefrançois et al., 2012).

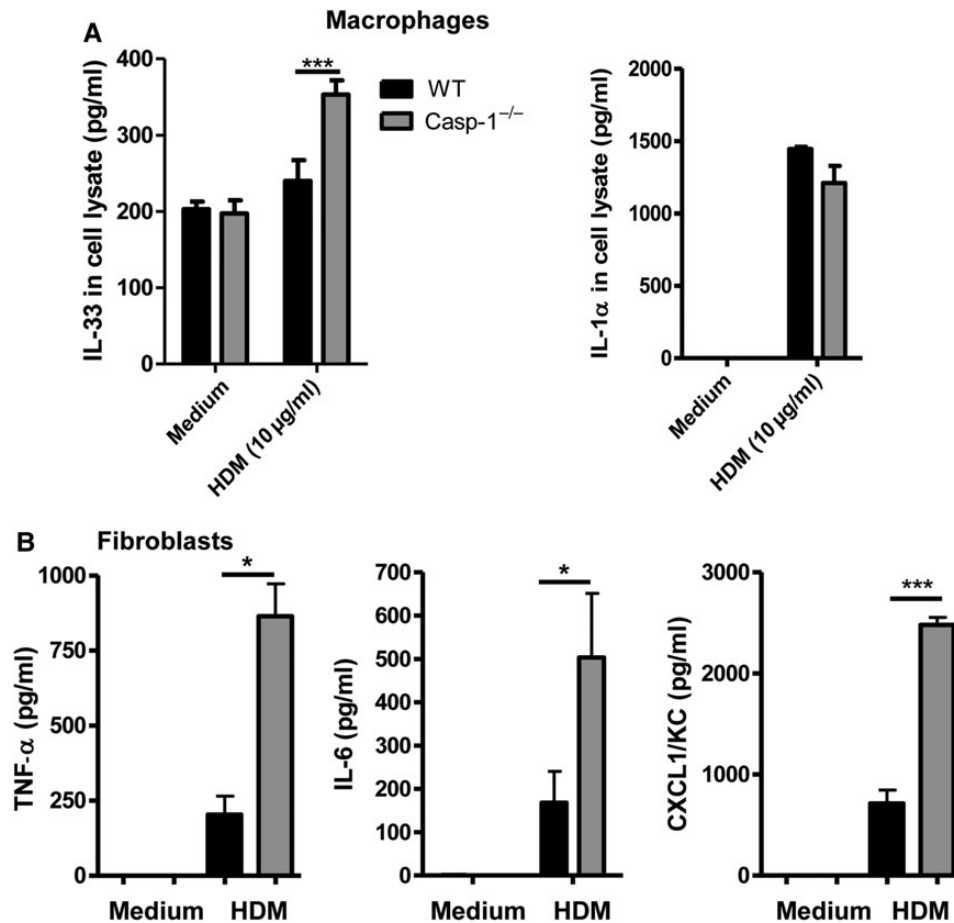


Figure 7 Increased IL-33 production in *caspase-1*-deficient macrophages *in vitro*. (A) Macrophages from WT and *Casp-1*^{-/-} mice were stimulated for 6 h with HDM (10 µg/ml), and the cell lysate was analyzed for IL-33 and IL-1α by ELISA. (B) Fibroblasts from WT and *Casp-1*^{-/-} mice were stimulated for 24 h with HDM (10 µg/ml), and TNF-α, IL-6, and KC (CXCL1) levels in the supernatant were quantified by ELISA. Results are expressed as mean ± SEM of four mice per group and are from one representative of two similar independent experiments. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001; unpaired *t*-test.

Exposure to HDM leads to the release of uric acid activating the NLRP3 inflammasome, but our data and recent report suggest an NLRP3-independent activation with increased bioactive IL-33 (Kool et al., 2011). Furthermore, our data showed that recombinant IL-33 administration to naïve mice induces uric acid production in the absence of caspase-1/11.

Furthermore, IL-33 augments the release of uric acid, which activates Syk kinase in DC. Most strikingly, we found increased pulmonary Syk expression upon HDM exposure in *caspase-1*-deficient mice, suggesting that caspase-1 modulates the development of allergic asthma via enhanced IL-33 production with increased uric acid production signaling through Syk kinase.

We demonstrate here that the double absence of caspase-1/11 allows a massive inflammatory response with release of abundant IL-1α, IL-1β, and other inflammatory mediators that augment the HDM-driven Th2 response, with increased production of full-length, but also more bioactive, cleaved IL-33 forms. The NLRP3/ASC inflammasome complex is involved, as HDM-induced Th2

inflammation and IL-33 activation are increased in *NLRP3*^{-/-} mice, similar to that in *Casp-1*^{-/-} mice.

Our previous findings using OVA-induced allergic asthma model demonstrate that the NLRP3 inflammasome is an integral part of OVA-induced allergic lung inflammation, absence of NLRP3 reducing OVA-induced response (Besnard et al., 2011a). It is important to note that there are major differences concerning the antigenicity between OVA and HDM allergens. While OVA does not cause allergy without prior immunization, HDM is allergenic by itself upon direct exposure. HDM is a mixture of serine/cysteine proteases (Der p1, Der p9) and proteins without enzymatic activity (Der p2 and Der p7) that are highly allergenic (Gregory and Lloyd, 2011). HDM antigens stimulate bronchial epithelial cells to produce TSLP, IL-25, IL-33, and GM-CSF that are known to prime allergic inflammation (Hammad et al., 2009, 2010). Here we show increased HDM-induced response in the absence of NLRP3. This apparent discrepancy can be explained by the fact that HDM, but not OVA, induces IL-33 expression in macrophages, bronchial epithelial, and possible other cells.

The amplifying role of IL-33 in the inflammatory response in the absence of NLRP3/ASC/caspase-1 is supported by the fact that IL-33 neutralization abrogates the exacerbated HDM-induced allergic response seen in the absence of caspase-1/11.

In conclusion, IL-33 regulates HDM-induced allergic airway inflammation via NLRP3 inflammasome complex consisting of ASC and caspase-1 downregulating IL-33 production and the Th2 response. Importantly, HDM activation of *caspase-1/11*-deficient macrophages produced more IL-33, establishing a direct link between caspase-1 activation and IL-33 release. Alternative caspase-1 activation pathway coupled with cell death, uric acid, and syk kinase activation may be critical for the amplification of lung inflammation in response to HDM.

Materials and methods

Mice

C57BL/6 wild-type, *Casp-1*^{-/-} (Kuida et al., 1995), and *NLRP3*^{-/-} (generated directly on C57BL/6 background) (Martinon et al., 2006) mice were bred in our specific pathogen-free animal facility at CNRS, Orleans, France. Experiments were performed with gender-matched mice aged 8–10 weeks. All protocols were approved by the local ethics committee (under the number CLE CCO 2013-1004), and complied with the French Government's Ethical and Animal Experiment Regulations.

Induction of HDM-induced allergic airway inflammation

To induce HDM-driven eosinophilic airway inflammation, isoflurane-anesthetized mice were administered intranasally twice on Days 0 and 7 with 25 µg HDM extracts (ALK-Abello) without adjuvant and were challenged on Days 14–16 with 5 µg of HDM extracts. Control mice were challenged with saline alone. Twenty-four hours after the last challenge, mice were sacrificed for bronchoalveolar lavage (BAL), EPO enzyme, uric acid, cytokines, chemokines, histology, qPCR, and bronchial hyperreactivity.

Determination of bronchial hyperresponsiveness

For invasive measurement of dynamic resistance, mice were anesthetized with intra-peritoneal injection of solution containing ketamine (100 mg/kg, Merial) and xylazine (10 mg/kg, Bayer), paralyzed using D-tubocurarine (0.125%, Sigma), and intubated with an 18-gauge catheter. Respiratory frequency was set at 140 breaths per min with a tidal volume of 0.2 ml and a positive end-expiratory pressure of 2 ml H₂O. Increasing concentrations of aerosolized methacholine (9.375, 18.75, 37.5, 75, and 150 mg/ml) were administered. Resistance was recorded with a plethysmograph (Buxco). Baseline resistance was restored before administering the subsequent doses of methacholine.

Intranasal IL-33 administration

Mice were administered intranasally three times with 1 µg of IL-33 recombinant protein (gift of J.P. Girard, Institute of Pharmacology and structural biology, Toulouse, France). Control mice received saline alone. Twenty-four hours after the last challenge, mice were sacrificed for bronchoalveolar lavage (BAL), cytokines, chemokines, and uric acid examinations.

Administration of neutralizing murine ST2-Fc fusion protein

HDM-treated mice were injected intraperitoneally with 150 µg of murine ST2-Fc fusion protein (mu ST2-Fc, Amgen) at 30 min before HDM challenge. Twenty-four hours after the last challenge and mu ST2-Fc fusion protein treatment, mice were sacrificed and different parameters were analyzed.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by washing the lungs four times with 0.5 ml of saline solution at room temperature. After centrifugation at 400 × *g* for 10 min at 4°C, the supernatant (cell-free BAL fluid) was stored at –20°C for further analyses.

Pulmonary eosinophil peroxidase activity

Eosinophil peroxidase (EPO) activity was determined in order to estimate the recruitment of eosinophil counts in lung parenchyma as described (Besnard et al., 2011a).

Lung histology

Lungs were fixed in 4% buffered formaldehyde, and 3 µm sections were stained with periodic acid Schiff reagent (PAS) and examined with a Leica DM2500 microscope (40× or 100× magnification). Peribronchial infiltrates and mucus hypersecretion were assessed by a semi-quantitative scoring (0–5) by two observers independently as described before (Besnard et al., 2011b).

Uric acid measurement

Uric acid concentration was determined in lung samples using an Amplex Red Uric Acid/Uricase Assay Kit (Molecular Probes). Briefly, uricase catalyzes the conversion of uric acid to allantoin, hydrogen peroxide (H₂O₂), and carbon dioxide. In the presence of horseradish peroxidase, H₂O₂ reacts stoichiometrically with Amplex Red reagent to generate the red fluorescent oxidation product resorufin, which is measured spectrophotometrically.

Cytokine measurement

IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-13, mIL-33, GM-CSF, TNFα, CCL5 (Rantes), CCL11 (Eotaxin), CCL17 (TARC), CCL20 (MIP-3α), CCL22 (MDC), and CCL24 (Eotaxin-2) concentrations in cell culture supernatant from mediastinal lymph nodes or lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA, R&D) or by Multiplex (BioRad) according to the manufacturer's recommendations.

Bone marrow-derived macrophages

Murine bone marrow cells were isolated from femurs of WT and *Casp-1*^{-/-} mice and differentiated into macrophages. Cells (1 × 10⁶/ml) were cultured for 10 days in DMEM (Sigma) supplemented with 20% horse serum (Gibco) and 30% L929 cell-conditioned medium as a source of M-CSF. After 7 days, cells were washed and re-cultured in fresh medium for another 3 days. The cell preparation contained a homogenous population of macrophages. For the experiment, the cells were resuspended in a medium containing 0.2% FCS and plated in 48-well microtiter plates (1 × 10⁶ cells/well), and stimulated upon adherence with HDM (10 µg/ml, Stallergenes).

Cell supernatants or cell lysates were harvested after 6 or 24 h and analyzed directly for cytokine quantification or stored frozen at -80°C .

Lung fibroblasts

Mice were sacrificed by CO_2 exposure and lungs were perfused with 10 ml of Isoton (Beckman Coulter). Lungs were excised and cut in small pieces and digested for 45 min with DNase from bovine pancreas (0.5 mg/ml, Sigma) and collagenase type IV (1 mg/ml, Gibco). Digested lungs were crushed and passed through 100 μm pore size filters (BD Bioscience). The cell suspension was washed and cultured in DMEM medium (Sigma) supplemented with 10% FCS (Perbio), 1% penicillin-streptomycin (Gibco), and 1% L-glutamine (Gibco) in a T25 flask until confluent. Passage of adherent cells for four times allowed removal of macrophage and monocyte contaminants. The resulting fibroblasts were digested for 5 min with Trypsin–EDTA (Gibco) and plated in 24 well plates (1×10^4 cells/well) until they reached 90% confluence.

Muc5ac and IL-33 mRNA expression

Total RNA was isolated from homogenized mouse lung using Tri Reagent (Sigma) and quantified by NanoDrop (Nd-1000). Reverse transcription was performed with SuperScript III Kit according to the manufacturer's instructions (Invitrogen). cDNA was subjected to quantitative PCR using primers for Muc5ac (sense 5'-CAGCCGAGAGGAGGGTTTGATCT-3' and anti-sense 5'-AGTCTCTCTCCGCTCTCTCA-3'; Sigma) or IL-33 (Qiagen QT00135170). Relative transcript expression of a gene is given as $2^{-\Delta\text{C}_t}$ ($\Delta\text{C}_t = \text{C}_{t\text{target}} - \text{C}_{t\text{reference}}$), and relative changes compared with control are $2^{-\Delta\Delta\text{C}_t}$ values ($\Delta\Delta\text{C}_t = \Delta\text{C}_{t\text{treated}} - \Delta\text{C}_{t\text{control}}$) (John et al., 2014).

Immunoblotting

Lung tissues were homogenated in Tris–HCl (0.05 M, pH8) with Polytron PT 2100 (Kinematica AG). Protein concentration was determined using the Bradford method (Bio-Rad). Equal amounts of protein (30 μg /sample) were denatured by boiling (95°C , 5 min), separated by SDS/PAGE and transferred to nitrocellulose membranes (GE Healthcare). The membrane was blocked in Tris-buffer saline (TBS) with Tween-20 (TBST), containing 10% lipid free-dry milk. Antibodies used for detection were diluted in TBST, containing 5% lipid free-dry milk, and were from Sigma unless otherwise stated.

The control β -actin was detected with mouse anti β -actin antibody followed by biotinylated goat anti-mouse IgG1 and extravidin peroxidase. IL-33 was detected with goat anti-IL-33 antibody (R&D Systems) followed by HRP-conjugated rabbit anti-goat IgG. Caspase-1 (p20) was detected with mouse anti-caspase-1 (p20) monoclonal antibody (Adipogen) followed by biotinylated goat anti-mouse IgG1 and extravidin peroxidase. IL-1 β and Syk were detected with goat anti-mouse IL-1 β (Sigma) or rabbit anti-syk (Cell Signaling) followed by HRP-conjugated anti-goat IgG (Sigma) or HRP-conjugated anti-rabbit IgG (Cell Signaling), respectively. Immuno-reactive bands were visualized by enhanced chemiluminescence kit (GE Healthcare).

Statistical analysis

To determine whether the data come from Gaussian distribution, column statistic using D'Agostino and Pearson test is performed before running the statistical analysis. Statistical evaluation of differences between the experimental groups was made by using parametric (one-way ANOVA followed by a Bonferroni post-test) and nonparametric (Kruskal Wallis followed by Dunns multiple comparison post-test) tests according to the results of column statistic. All tests were performed with Graphpad Prism, Version 4.03 for Windows (GraphPad Software, Inc., www.graphpad.com). Data are expressed as mean $\pm$ SEM. Statistically significant differences were defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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