Allergen protease-induced Gsdmd p40 controls IL-33 secretion

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Abstract

Interleukin (IL)-33, an epithelial cell-derived cytokine that responds rapidly to environmental insult, has a critical role in initiating airway inflammation, such as that in asthma. However, the molecular mechanism underlying IL-33 secretion following allergen exposure is not clear. Here, we demonstrated that Gasdermin D (Gsdmd) functions as a conduit for IL-33 secretion following allergen protease exposure. Gsdmd was rapidly cleaved into a functional neo-form, the N-terminal p40 fragment (p40 NT-Gsdmd), in the murine airway epithelium when cells were exposed to allergen proteases from fungi, house dust mites (HDMs), or bacteria. This cleavage event that produces the p40 Gsdmd fragment was independent of inflammatory caspases-1/11, as it could not be inhibited by caspase-1 and caspase-11 deficiency in murine cells. The functional p40 NT-Gsdmd fragment directly contributed to the secretion of both the nuclear full-length form and cytosolic mature form of IL-33. Both Gsdmd deficiency and blockade of the generation of p40 by amino acid mutation or deletion of residues 308–313 (ELRQQ) in the Gsdmd sequence could efficiently prevent IL-33 release in airway epithelial cells. In mice, Gsdmd deficiency prevented IL-33 release and hindered the activation of group 2 innate lymphoid cells (ILC2s), thus alleviating airway inflammation and lung tissue damage after stimulation with HDMs or papain. Our findings uncovered a mechanism of Gsdmd-mediated IL-33 release under allergen exposure and offer insight into Gsdmd cleavage prevention as a potential approach to reduce allergic airway inflammation.

Introduction

Allergic diseases such as asthma and hay fever (allergic rhinitis) are common diseases that have been observed with increasing incidence especially in developed countries. Trapping millions of people worldwide, these diseases usually exacerbated when patients were environmentally exposed to allergens, such as asthma exacerbation induced by HDMs, moulds, bacteria, plant pollen, and animal dander[1, 2 3]. These allergens are often composed of various components, such as enzymes and complex polysaccharides. Certain enzymatically active components derived from allergens, including Der p and Der f from HDMs[4, 5], Asp f from *Aspergillus oryzae*[6], the Subtilisin protease from *Bacillus* species[7] and Per a from cockroaches[8, 9], have been reported to have the ability to induce IL-33 release and promote allergic-type 2 airway inflammation in vivo. Allergen proteases induce an allergic inflammatory response through mechanisms that involve directly damaging epithelial cell tight junctions[10, 11, 12, 13], acting on protease-activated receptors (Par2) or activating the pattern recognition receptor Toll-like receptor 4 (TLR4) on multiple cell lineages, including airway epithelial cells, macrophages and basophils[10, 14, 15]. These activated cells further boost airway inflammation by secreting damage-associated molecular patterns (DAMPs), including IL-33, IL-1, ATP, and multiple kinds of inflammatory cytokines and chemokines, such as IL-8, CCL3, and GM-CSF[16, 17, 18, 19], leading to the subsequent T helper 2 (Th2)-type inflammatory response.

IL-33 belongs to the IL-1 cytokine family, which plays an important role in maintaining barrier homeostasis. It also functions as an alarmin that is released when barriers are disrupted. The full-length

form of IL-33 (amino acids 1-270) harbors three functional domains: the N-terminal nuclear localization domain that binds with chromatin and retains IL-33 in the nucleus under healthy conditions[20]; the central protease sensor domain recognized and cleaved by various endogenous enzymes, such as calpain, cathepsin G, elastase and exogenous proteases from fungi, pollen and cockroaches, to release a potent bioactive spliced mature form of the C-terminal cytokine domain of IL-33[21, 22, 23, 24]; and the cytokine domain of IL-33, which recognizes its receptor IL-1RL1 (ST2) and activates Myd88-mediated signaling pathways in ST2-expressing cells, such as mast cells, basophils, eosinophils, ILC2s and the Th2 subset, following the production of IL-4, IL-5, IL-13, and allergen-specific IgE, contributing to systematic immune defenses and tissue repair[25, 26]. Thus, the regulation and secretion of IL-33 are critical for allergic inflammation through IL-33/ST2 signaling.

IL-33 is constitutively produced by cells in quiescent stromal tissue, such as epithelial cells and endothelial cells. It can also be produced by multiple immune cell types, including alveolar macrophages (AMs), NKT cells, and regulatory T (Treg) cells, under inflammatory conditions[27, 28]. Certain TLR and non-TLR agonists can induce IL-33 transcription in macrophages through an interferon (IFN) regulatory factor-3 (IRF-3)-dependent pathway. In addition, CREB synergizes with lipopolysaccharide (LPS) to induce IL-33 mRNA expression[29]. Deficiencies in cell death-regulating caspase 8 and apolipoprotein E also contribute to elevated IL-33 mRNA levels in mouse tissue, suggesting that multiple signaling pathways are involved in the regulation of IL-33 expression[29, 30, 31]. Similar to other members of the IL-1 family, IL-33 lacks a secretion signal and therefore cannot be released through the endoplasmic reticulum (ER)-Golgi secretory pathway and is itself unable to cross the cell membrane under physiological conditions[32]. Previous reports have suggested that death-associated damage contributes to IL-33 release, including cell lysis induced by freeze-thaw cycles or NP-40 exposure, which is accompanied by LDH release. Other damage, including damage caused by oxidative stress-induced hydrogen peroxide (H_2O_2) or NaN_3 and membrane permeabilization by the pore-forming toxin streptolysin O (SLO), was also observed to be accompanied by the release of IL-33 in both stromal cells and immune cells, such as macrophages. These stimuli contribute to IL-33 release with the occurrence of cell death features (such as LDH release or propidium iodide (PI) uptake)[20, 33, 34]. However, apoptotic stress does not lead to the secretion of IL-33. One reason is that during apoptosis, the cell membrane remains intact, and the other reason is that activated apoptotic caspases 3/7 can degrade IL-33 into inactivated forms[33]. Necrosis-associated cell membrane rupture and nuclear leakage were thought to be responsible for IL-33 release[35]. Some researchers discovered that in living cells, nuclear IL-33 can also be secreted in response to nonlethal mechanical stress[36, 37]. Other stimuli, such as cockroaches, uric acid, exogenous ATP, HDMs, and Alternaria allergens, have also been reported to induce IL-33 release from epithelial cell lineages without obvious cell death[4, 16, 17, 18, 38, 39, 40, 41]. Recently, serum amyloid A was defined as a soluble pattern recognition receptor for allergenic mite FABPs that drive the epithelial release of IL-33[42]; however, whether it participates in the recognition of other allergenic stimuli still needs to be determined. Although various natural and synthetic stimuli can induce effective IL-33 release from stromal cells, little is known about the precise mechanisms of the IL-33 secretion process.

Here, we found that Gsdmd, a gasdermin family member that functions as the executor of inflammatory pyroptosis in a variety of immune and non-immune cells, participated in the release of the inflammatory cytokine IL-1β through caspase-1/8/11-dependent cleavage[43, 44, 45] could also be cleaved into a novel functional p40 N-terminal form following simulation with various protease allergens through a caspase-independent mechanism. This p40 mNT-Gsdmd fragment (corresponding to the p35 fragment of hNT-GSDMD) responded rapidly to environmental protease stress signals and efficiently promoted the secretion of IL-33 from living cells, including airway epithelial cells and macrophages, without obvious cell death, contributing to both nuclear and cytosolic IL-33 release. Knocking out Gsdmd in mice could efficiently block early IL-33 release and subsequent ILC2s activation, thus alleviating airway inflammation and lung tissue damage after exposure to environmental allergens, including HDMs and papain. Our results revealed a central role for Gsdmd in controlling IL-33 release and the initiation of type 2 immune response in airway inflammation following allergen protease exposure.

Results

1. Gsdmd expression was elevated in airways with type 2 inflammation in both humans and mice.

The Gsdmd protein has been widely reported to function in multiple cell types, including immune cells, such as macrophages/neutrophils, and stromal cells, such as nasal epithelial cells[46, 47, 48, 49]. However, the expression of GSDMD in human lung tissue and asthma has not yet been defined. To address this question, we collected bronchial samples from asthma patients and non-asthmatic people (Supplementary Fig. 1). With activated immune responses in the lungs, the asthma patients presented elevated inflammatory cell infiltration into lung tissues, increasing amounts of smooth muscle and hypertrophy of the mucous glands compared with the non-asthmatic controls (Fig. 1a). Interestingly, lung tissue sample analysis with immunohistochemical staining indicated that GSDMD was mainly expressed in the pulmonary airway epithelium and presented an upregulated expression pattern in the asthma patient group, especially in areas with mucous gland hyperplasia (Fig. 1a, b), indicating that GSDMD may play fundamental roles in the human airway epithelium. In mice, the expression of Gsdmd presented a pattern similar to that in humans. Gsdmd was weakly expressed in the normal murine airway epithelium. With the induction of asthmatic airway inflammation by HDM exposure, Gsdmd expression was upregulated significantly (Fig. 1c, d). Together, our statistical data revealed that the expression of Gsdmd was significantly increased in human and murine lung tissues with asthma-associated inflammation, which suggested that Gsdmd might function in the pathogenesis of asthma in the lungs

2. Deletion of Gsdmd dampened ILC2s activation in both HDM-induced chronic and papain-induced acute airway inflammation

To further investigate the potential function of Gsdmd in airway inflammation, HDM extracts were administered intranasally to induce chronic asthmatic airway inflammation in both genetically Gsdmd-deficient and wild-type (WT) mice (Fig. 2a). The mice were sacrificed 17 days after the first stimulation. The deletion of Gsdmd in mice dampened the HDM-induced airway inflammatory response, with less

inflammatory infiltration into the bronchus and less mucus production in the airways (Fig. 2b). The secretion of inflammatory cytokines, such as IL-5 and IL-13, into the bronchoalveolar lavage fluid (BALF) was also significantly suppressed (Fig. 2c). The number of infiltrated eosinophils and proliferation of ILC2s in the lungs were also decreased in the Gsdmd-deficient mice (Fig. 2d, Supplementary Fig. 2a, b). In addition, Papain-induced acute airway inflammation was also evaluated in genetically Gsdmd-deficient mice (Fig. 2e). Similarly, Gsdmd deficiency alleviated acute airway inflammation in the mouse lungs, with less immune cell infiltration (Fig. 2f, g) and typically decreased alveolar eosinophil infiltration and ILC2s proliferation (Fig. 2g). The numbers of activated IL-5- or IL-13-producing ILC2s were also significantly inhibited (Fig. 2h). Secretion of the inflammatory cytokines IL-5 and IL-13 into the BALF was also obviously impaired (Fig. 2i). Together, the above data indicated that Gsdmd deficiency in mice alleviated both HDM-induced chronic and papain-induced acute airway inflammatory responses by limiting ILC2s activation and eosinophil infiltration.

3. Gsdmd deficiency impaired early IL-33 release in vivo.

Multiple inflammatory cytokines are important in the initiation and development of airway inflammation, among which epithelial cell-derived IL-33, IL-25, and TSLP are believed to play fundamental roles in the initiation of type 2 inflammation[50, 51, 52]. To identify the factors that dominate airway inflammation in this process, we evaluated the secretion of inflammatory cytokines into the BALF of mice exposed to papain. With an MSD multiple cytokine detection systems, we analyzed the levels of different inflammatory cytokines, including IFNγ, IL-13, IL-33, IL-25, and IL-1β, in the BALF 3 hours after papain stimulation. Among these cytokines, IL-33 displayed an extremely high abundance (Fig. 3a), which suggested that the IL-33 release event is fundamental in boosting inflammation at this early stage. To expand our knowledge on IL-33 secretion into the BALF, we monitored the secretion of IL-33 over time. The results showed that the secretion of IL-33 into the BALF reached a peak at 3 hours after papain exposure (Fig. 3b). In contrast, both IL-25 and TSLP displayed no significant change after papain exposure (Fig. 3c). Our observations suggest that in the early stage, IL-33 is responsible for the enhancement of lung inflammation after allergen protease stimulation in vivo.

The airway epithelium functions as a physical barrier, initiating multiple innate and adaptive immune responses by secreting alarmins, including IL-33, which respond rapidly to allergens, viruses, and environmental pollutant insults. To clarify whether Gsdmd is involved in the early IL-33 release event, under the same experimental conditions, we collected BALF from WT and Gsdmd-deficient mice 3 hours after papain exposure. The data showed that Gsdmd deficiency inhibited IL-33 release into the BALF in the early stage (Fig. 3d) but did not interfere with the IL-33 mRNA expression level in lung tissues (Fig. 3e). These results indicated that Gsdmd might function by controlling the secretion of IL-33 and imply a crucial function for Gsdmd in airway epithelial cells during airway inflammation.

4. Exogenous recombinant IL-33 supplementation blunted the alleviation of inflammation in Gsdmd-/- mice.

As Gsdmd deficiency impairs IL-33 release in the early stage, we speculated that Gsdmd deficiency constrained IL-33 within cells. To identify whether the functions of Gsdmd in blocking ILC2s activation and eosinophil infiltration in mice were dependent on IL-33 signaling, we intranasally challenged Gsdmd-deficient mice with recombinant murine IL-33 (rIL-33) (Supplementary Fig. 3a). As expected, activating airway inflammation through IL-33/ST2 axis directly with rIL-33 stimulation resulted in no obvious inflammatory immune responses differences in inflammatory cell infiltration, ILC2s activation, or cytokine secretion between Gsdmd-deficient and WT mice (Supplementary Fig. 3b-g). Indicating that Gsdmd might function upstream of IL-33 within cells before its secretion, thus blocking the activation of ST2-expressing effector cells, such as ILC2s

5. The protease activity of papain is needed for IL-33 release and Gsdmd cleavage in vitro.

As the pulmonary epithelium has been reported to be an important source of IL-33[16, 53, 54], we further analyzed the expression of IL-33 in murine lungs with immunofluorescence staining. We observed a typical expression pattern of IL-33 in lung surfactant protein C (SPC)-positive airway epithelial type 2 (AT2) cells (Supplementary Fig. 4a). Both IL-33 and Gsdmd are preferentially expressed in murine airway epithelial cells. Considering that Gsdmd has been proved with the pore-forming ability on the cell membrane in regulating pyroptosis and mediating IL-1β release, we hypothesized that Gsdmd may function similarly in controlling IL-33 release in the airway epithelium. Endogenous IL-33-expressing murine AT2 mle12 cells were used for an in vitro functional study. A549 airway epithelial cells constitutively expressing C-terminal-eGFP-fusional HA-tagged human IL-33 (A549-IL-33-eGFP) and the smallest nuclear localization sequence (NLS) linked with eGFP (A549-NLS-eGFP) were also used for functional analysis (Supplementary Fig. 5a).

Epithelial cells were exposed to papain, and IL-33 secretion from the nucleus was recorded for 30 min with a live cell imaging recording system (Fig. 4a; Supplementary Videos 1 and 2). IL-33 responded rapidly to papain protease stimulation in epithelial cells, while NLS-eGFP did not show any decrease in the GFP fluorescence signal in the nucleus (Fig. 4a, b), suggesting that papain was able to induce IL-33 secretion without extensive nuclear leakage. Cells and cell culture supernatants were collected and analyzed by western blotting, and we found that papain-stimulated IL-33 release occurred with the generation of a p40 mNT-Gsdmd fragment in murine alveolar epithelial cells (Fig. 4c) and a p35 hNT-GSDMD fragment in human epithelial cells (Supplementary Fig. 5b-d). In addition, papain-stimulated IL-33 release and Gsdmd cleavage presented a dose- and time-dependent manner (Fig. 4c, d). Moreover, cells did not present obvious death morphology during this time series (Supplementary Videos 1 and 2). Even with a relatively high dose of papain stimulation, LDH release from cells into the supernatant was nearly undetectable, and neither apoptosis-associated caspase-3 cleavage nor necroptosis-associated Mlkl phosphorylation was detected in cells (Fig. 4e). These observations suggested that papain-induced Gsdmd cleavage and IL-33 release did not accompany by cell death. Taken together, our results revealed that papain stimulation did not activate classic cell death pathways, such as apoptosis or necroptosis, whereas the activation of a neo-form of the p40 mNT-Gsdmd fragment was found to be involved in this papain-stimulated IL-33 release event. In addition, directly activating p-Mlkl-mediated necroptosis or

caspase-mediated apoptosis did not contribute to IL-33 release in mle12 airway epithelial cells, which implied that the p-Mlkl-mediated necroptosis pathway did not dominate nuclear IL-33 release.

Papain is a proteolytic enzyme with cysteine protease activity. To determine whether Gsdmd cleavage and the IL-33 secretion process are dependent on the protease activity of papain, the irreversible cysteine protease inhibitor E64 was incubated with papain before papain was added to cell culture supernatants. With the inhibition of cysteine protease activity, the activation of Gsdmd in cells and the release of IL-33 into the supernatant were suppressed (Fig. 4f). Similarly, after heat inactivation at 100 °C for 10 min, papain no longer possessed dose-dependent activating effects on Gsdmd cleavage and IL-33 secretion (Fig. 4g). These observations suggested that the protease activity of papain was a fundamental element for Gsdmd cleavage and IL-33 secretion in epithelial cells.

Constituted with active enzymatic components is a common feature of allergens, and different enzymatic components from various allergens have been proven to possess the ability to induce IL-33 release both in vitro and in vivo. Next, we wanted to address whether Gsdmd activation is a common phenomenon after cells were exposed to allergen protease stimuli. Allergen-derived active enzymatic components, including the fungus *A. oryzae*, the bacteria *Bacillus licheniformis*, HDM extracts and the purified natural HDM proteases Der p and Der f 1, were cultured with mle12 murine airway epithelial cells at different concentrations. After exposure for 30 min, we collected the cell pellets and detected Gsdmd cleavage with immunoblotting. The results showed that all these tested allergen proteases were able to activate the Gsdmd-p40 N-terminal fragment in a dose-dependent manner, which was similar to papain stimulation (Fig. 4j). This raised the possibility that allergen proteases share a common cellular protease stress-sensing pathway that leads to Gsdmd activation by producing one p40 fragment, which might be associated with IL-33 release from the nucleus at an early stage.

6. Papain-activated Gsdmd is independent of the inflammasome caspase 1/11 pathway

Gsdmd serves as an executor of pyroptosis and can be cleaved by inflammatory caspases-1/11 through the activation of canonical and non-canonical inflammasome pathways promoting IL-1β release in multiple cell types, typically macrophages[55]. Macrophages can also be an efficient source of IL-33 upon inflammatory stimulation[27, 56]. To compare Gsdmd cleavage events between inflammatory caspase and papain stimulation, we conducted papain stimulation in murine bone marrow-derived macrophages (BMMs). After pre-stimulation with bacterial LPS, papain was added as a second stimulatory signal, similar to ATP. LPS activated TLR4 signaling, which led to the production of pro-IL-1β in BMMs. After the second stimulation with ATP or nigericin (Nig), the NLRP3 inflammasome was activated, which promoted the generation of p20 caspase 1 and the 35-kDa fragment of mNT-Gsdmd. This p35 fragment of mNT-Gsdmd displayed a high binding affinity to cell membrane-associated lipids with pore-forming capability, resulting in the release of IL-1β and LDH (Fig. 5a-d). In contrast, papain stimulation did not promote the activation of p20 caspase 1; thus, no mature p18 IL-1β was generated and secreted into the cell supernatant (Fig. 5a, b). However, papain promoted the generation of the p40 mNT-Gsdmd fragment and IL-33 release in BMMs, which kept the same pattern as airway epithelial cells. Additionally, this process

did not result in obvious LDH release (Fig. 5a). Indeed, BMMs with Gsdmd deficiency presented both impaired caspase 1-dependent Gsdmd cleavage and IL-1β secretion upon pyroptotic stimulation, as well as impaired IL-33 release with papain stimulation (Fig. 5a-c), which further supported the conclusion that Gsdmd processing is needed for sufficient IL-33 secretion. These results indicate that the inflammatory caspase 1-generated 1-276 aa mNT-Gsdmd fragment does not contribute to the release of IL-33 and that the generation of the p40 mNT-Gsdmd fragment is independent of the active p20 caspase 1 signaling pathway.

Since BMMs can respond to papain stimulation by generating the p40 mNT-Gsdmd fragment and secreting IL-33, we isolated caspase 1 and caspase 11 double-deficient BMMs and conducted the same stimulation to exclude the involvement of inflammatory caspases 1/11. As reported[55], caspase 1/11 double deficiency blocked the generation of the pyroptotic mNT-Gsdmd fragment and the secretion of IL-1β (Fig. 5d, e). However, caspase 1/11 double deficiency in macrophages did not prevent the generation of p40 mNT-Gsdmd or secretion of IL-33 when stimulated with papain (Fig. 5d, f), which revealed that inflammatory caspase 1/11 did not participate in p40 mNT-Gsdmd cleavage. To further exclude the potential function of inflammatory caspases in Gsdmd cleavage and IL-33 release in airway epithelial cells, the pan-caspase inhibitor Z-VAD-FMK (Z-VAD) was pre-cultured with mle12 murine airway epithelial cells before papain stimulation (Fig. 5g). The results showed that neither Gsdmd cleavage nor IL-33 release could be limited by the inhibition of caspase activity, which implied that the caspase family does not contribute to Gsdmd cleavage upon papain stimulation. Thus, there may be other enzymes involved in this protease stress-sensing pathway that mediate Gsdmd cleavage. Taken together, these results indicated that activating the pyroptotic residue 1-276 mNT-Gsdmd fragment with activated inflammatory caspase 1 did not contribute to the release of IL-33. Instead, the papain-induced p40 mNT-Gsdmd fragment promoted IL-33 release via a caspase 1/11-independent pathway.

7. The truncated p40 mNT-Gsdmd fragment contributed to the release of IL-33.

As described above, we observed an association between Gsdmd cleavage and IL-33 secretion in both mle12 airway epithelial cells and BMMs. However, whether this generation of the p40 N-terminal fragment of Gsdmd directly contributes to IL-33 release is still unknown. As we have no specific information on what kind of enzyme contributes to the generation of the p40 Gsdmd fragment, the online pro-protein conversion prediction tool ProP-1.0 was used to predict the possible cleavage sites of functional Gsdmd[57]. Excluding those amino acid sites within the 1-276 aa fragment generated by caspase 1/8/11 cleavage, we obtained a list of six possible arginine(R) or lysine(K) cleavage sites, among which sites R311, K394, and K409 were chosen for a broad screen to narrow the range of the functional p40 cleavage site (Supplementary Fig. 6).

We constructed predicted possible functional murine Gsdmd fragments (aa 1-311, 1-394 and 1-409) with a Flag-tagged N terminus, and the full-length (FL) form of Gsdmd was used as a non-functional control (Fig. 6a). To further confirm their function in IL-33 release, these truncated fragments were co-transfected into HEK293T cells with C-terminal HA-tagged IL-33 expression vectors. Cell supernatants were collected

for immunoblotting and ELISA, and cells were collected for immunoblotting. We observed that compared to full-length Gsdmd, the truncated 1-311aa mNT-Gsdmd fragment presented an extremely high efficiency in promoting nuclear IL-33 release, whereas the other predicted fragments did not exhibit this capability (Fig. 6b, c). Next, we evaluated whether this 1-311aa mNT-Gsdmd fragment also functions in promoting the secretion of the cytosolic mature form of IL-33 (without the N-terminal nuclear localization signal peptide). As predicted, the truncated 1-311aa mGsdmd fragment also worked robustly in promoting cytosolic mature IL-33 release from cells into the culture supernatant, making this fragment unique among these selected truncated forms of mGsdmd (Fig. 6d, e). Differential point mutations and deletions near 311aa were constructed and stably expressed in epithelial mle12 cells via lentiviral infection. Mle12 cells with differentially mutated Gsdmd overexpression were stimulated with papain as described. We found that the amino acid mutation or deletion of residues 308–313 (ELRQQ) in the Gsdmd sequence could efficiently block the generation of the p40 mNT-Gsdmd fragment and prevent IL-33 release in airway epithelial cells, which suggested that the amino acid 308–313 (ELRQQ) sequence is important for the recognition and generation of the functional p40 fragment.

To determine the specific cleavage site of the p35 hNT-GSDMD fragment in human cells, we carried out a similar prediction as described in murine Gsdmd. Three possible truncations, 1-290 aa, 1-320 aa, and 1-327 aa, were cloned into tetracycline-responsive expression vectors and infused with eGFP (Supplementary Fig. 7a). With a HEK293T expression system, we analyzed the functions of these truncated fragments in mediating both nuclear and cytosolic IL-33 release. The results revealed that the corresponding 35-kDa fragment (1-290 aa hNT-GSDMD) presented the unique ability to promote IL-33 release from both the nucleus and the cytosol after tetracycline-induced expression (Supplementary Fig. 7b, c). To confirm the specific cleavage site in hGSDMD, we constructed Flag-tagged hGSDMD expression vectors with several mutations around aa 290. These vectors were transfected into and expressed in hGSDMD-deficient HeLa cells following papain stimulation, and Flag-GSDMD cleavage was analyzed with immunoblotting. We found that the substitution of 5 amino acids around aa 290, i.e., mutating GLRAE into AAAAA, could efficiently block the generation of the p35 hNT-GSDMD fragment after papain exposure, which suggested that the functional p35 fragment cleavage site is located in this region. The site was further narrowed to L289-R290, as deletion of both arginine 290 and leucine 289 could also effectively prevent the generation of p35 hGSDMD (Fig. 7b, c). Together, the results revealed that the human 1-290 aa fragment could sufficiently promote the secretion of IL-33 into the supernatant and that the endogenous cleavage of hGSDMD following papain treatment could be blocked by deletion of L289-R290.

8. GSDMD expression is positively correlated with IL-33 in patients with asthma.

It has been reported that patients with asthma presented higher IL-33 protein expression in the lungs[58]. As we observed elevated expression of GSDMD in patients, we analyzed both GSDMD and IL-33 protein expression in lung tissues with immunohistochemically staining of serial bronchial sections from a total of 11 patients with asthma. Statistical analysis results showed that the expression of GSDMD and IL-33 was positively correlated in the asthma patients (Fig. 7a, b), which suggested that IL-33 and GSDMD

might function through the same axis during type 2 inflammation and work closely together to regulate the pathogenesis and development of asthma.

Discussion

IL-33/ST2 signaling plays fundamental roles in mediating the development of type 2 inflammation in the lungs. The extracellular function of IL-33 has been explored extensively in multiple cell types both in vivo and in vitro[59, 60, 61]. However, little is known about how cells sense various exogenous and endogenous stimulators, thus initiating IL-33 secretion. Although IL-33 is observed to be released during cell death (LDH release, PI uptake, and death-associated morphology) with certain stimulations, including H_2O_2 or lytic treatment with Triton or freeze-thaw cycles, these treatments can also activate many cellular stress-associated signaling pathways, which may involve IL-33 release. Whether cell death is necessary for the release of IL-33 is still controversial.

In this study, we uncovered that various allergen-derived proteases share a common downstream event in generating the p40-mGsdmd fragment in airway epithelial cells, which could directly mediate the release of IL-33 without obvious cell death. In addition, activating certain types of cell death signaling pathways, including caspase-mediated apoptosis (stimulated with TNF-α + SM-164) and p-Mlkl-dependent necroptosis (stimulated with TNF-α, SM-164, and Z-VAD), was not sufficient to induce IL-33 release in airway epithelial cells. Furthermore, our results also revealed that in BMMs, activating inflammatory caspase 1-mediated lytic cell death with membrane pores formed by murine p35 mNT-Gsdmd did not contribute to IL-33 release, suggesting that IL-33 secretion is independent of those cell death pathways. Thus, cells exhibited an intrinsically effective exogenous allergen sensing signaling pathway that responded rapidly (in our case, within 30 min) by generating a p40 fragment of NT-Gsdmd and thus helped to deliver nuclear danger signals to neighboring cells via secretion of the alarmin IL-33 without cell death in an early stage. If the danger signal remains uncleared, cell death-associated pores in the plasma membrane formed by p35 NT-mGsdmd may be generated and help to promote exaggerated processed cytosolic IL-33 release, thus boosting downstream immune cell-activating events.

Nuclear alarmins function as danger signals to help deliver cell damage information to initiate immune rescue and anti-infectious pathogen defense in living organisms. Non-inflammatory apoptotic stress-inducing cell death was proven to be irrelevant to the secretion of DAMPs, including IL-33, IL-1 and HMGB1[33, 62, 63]. All three of these nucleus-derived cytokines are secretory proteins without signal peptides. Proteins belonging to this type are released through unconventional protein secretion (UPS) pathways, including direct protein penetration across the plasma membrane and vesicular trafficking that bypasses the ER-Golgi trafficking process[64]. Multiple kinds of stimulators have been reported to induce the release of HMGB1 and IL-1α[65, 66, 67,] and both were observed to be released through the activation of the inflammatory caspase-1-activated pyroptosis pathway in macrophages or dendritic cells in conjunction with LDH release when cells were stimulated with LPS plus Nig/ATP[68, 69, 70]. Inflammasome-associated HMGB1 and IL-1α release can be inhibited efficiently by blocking the activation of both

inflammatory caspases and Gsdmd[71, 72, 73], suggesting that the caspase-dependent inflammasome signalling pathway can transmit nuclear stress signals and that the generation of the pyroptotic p35 mGsdmd fragment is associated with the release of both cytokines. However, we determined that IL-33 was not released through the activation of inflammatory caspase 1, which generates the pyroptotic p35 mGsdmd fragment. Thus, IL-33 works differently from IL-1 and HMGB1, suggesting that compact regulatory machinery exists in cells that works strictly in controlling the secretion of different nuclear cytokines, responds to various stress signals and contributes to different downstream immune activation events.

Previous studies have reported that both GSDMA and GSDMB are preferentially expressed in the human airway epithelium. GSDMB promotes asthma pathogenesis in the epithelium of patients through classic functions by promoting IL-1β release through inflammatory caspase cleavage[74, 75]. Our investigation noted that GSDMD also presented a relatively high expression pattern in the human airway epithelium, it responded rapidly to environmental allergen proteases by generating new GSDMD cleavage forms of 1-290 aa hNT-GSDMD and 1-311 aa mNT-Gsdmd fragments, which control the release of both nuclear full-length IL-33 and cytosolic mature IL-33 and determines the fate of airway inflammation. Additionally, traditional caspase-cleaved p35 mNT-Gsdmd did not contribute to nuclear IL-33 release from cells (Fig. 8). Although studies have shown that active caspases can cleave IL-33 into inactive forms[33, 76], we did not detect any forms of IL-33 released into the supernatant after caspase-mediated murine p35 mNT-Gsdmd activation. This suggested that the p35 mNT-Gsdmd fragment might not be sufficient to deliver the stress signal for nuclear IL-33 processing, suggesting that the amino acid sequence in Gsdmd between residues 275–290 in humans and residues 275–311 in mice might determine the cellular organelle targeting orientation, which allows Gsdmd to target nuclear IL-33. In addition, introducing mutations in the amino acid sequence of positions 308–313 (ELRQQ) in mGsdmd could efficiently prevent the cleavage of Gsdmd into the functional p40 fragment and block IL-33 release in airway epithelial cells, suggesting that sites located in positions 308–313 (ELRQQ) are vital for the recognition and generation of functional p40 Gsdmd. Similarly, the human residues L289-R290 are needed for the generation of the functional hNT-GSDMD fragment, thus the cleavage of lysine and arginine might provide clues for enzymes associated with this process. Although we did observe a neo-function of this p40 fragment of mGsdmd, we did not define which kind of enzyme is involved in this protease stress-sensing pathway and contributes to the generation of Gsdmd p40. Further research on this question is needed.

Our data highlight a unique role for Gsdmd in controlling cytokine release by switching between two different cleaved forms: the pyroptotic p35 mNT-Gsdmd fragment reacts to classic caspase-1/8/11 cleavage that contributes to typical IL-1β release, and the p40 fragment responds to different protease stress signals, resulting in IL-33 secretion without obvious cell death and amplifying different downstream immune cell activation events that further contribute to disease pathogenesis(Fig. 8). Thus, Gsdmd works in the center as a gatekeeper in limiting both type 1 and type 2 inflammatory immune responses by varying the generation of different cleaved forms. As GSDMA and GSDMB also presented

the same expression pattern as Gsdmd, there is a great possibility that other gasdermin proteins can also be involved in the process. Overall, our study uncovered a novel activation pathway for Gsdmd that responded to protease allergen stimulation, functioning in controlling IL-33 release and ILC2s activation during airway inflammation.

Methods

Mice. C57BL/6 WT mice were purchased from the Shanghai Laboratory Animal Center. Gsdmd-/- mice on the C57BL/6J background were from Dr. Feng Shao, Caspase1/11 double deficient mice were kindly provided by Dr. Guangxun Meng (Institut Pasteur of Shanghai, CAS). Six- to ten-week-old mice were used for all experiments; all animals were age- and sex-matched and then randomized into different groups. All mice were maintained in specific pathogen-free animal facilities at the Animal Care Facility of the Chinese Academy of Sciences and used according to protocols approved by the Institutional Animal Care and Use Committee.

Induction of allergic airway inflammation. For the induction of acute type 2 airway inflammation, C57BL/6 WT and Gsdmd-/- mice were anaesthetized by isoflurane inhalation, treated intranasally with papain (5 μg) in 40 μl PBS every day for 5 days, and euthanized for analysis on day 6. For the induction of chronic type 2 airway inflammation, HDMs (50 μg) were applied intranasally for 3 continuous days; the mice were allowed to rest for 11 days and then treated with HDMs (10 μg) for 4 continuous days. The mice were sacrificed for analysis 24 hours after the final intranasal treatment. Recombinant IL-33 (25 μg) was administered intranasally for 4 continuous days to induce acute airway inflammation.

Primary cells isolation

BMMs cells were prepared as follows: bone marrow were flushed from the femurs and tibias of C57BL/6 mice following removed of red blood cells using ammonium chloride. Cells were seeded at a density of 1×10^6 cells per well in 24-well plates in DMEM supplemented with 20 ng/ml murine M-CSF. Cell culture medium was freshed every 2 days and cells were harvested on day 6.

Allergen extracts and protease allergens. HDM extracts were obtained from Greer Laboratories. The purified natural HDM proteases Natural Der p 1 (NA-DP1-1) and Natural Der f 1 (NA-DF1-1) were obtained from Indoor Biotechnologies. A protease from *A. oryzae* (Sigma-Aldrich #P6110), papain from papaya latex (Sigma #P3125) and subtilisin A protease from *B. licheniformis* (Sigma-Aldrich #P5380) were used as sources of purified protease allergens. All allergen extracts and protease allergens were reconstituted in PBS (1 mg/ml) and stored at -80 °C, except for the *A. oryzae* protease (500 U/g; 4 °C).

Cleavage of recombinant GSDMD with allergen extracts and allergen proteases. The plasmids pcDNA3-flag-hGSDMD and pcDNA3-flag-mGsdmd were transfected into 293T cells with Lipofectamine 2000 (Invitrogen 11668019). After 24 hours, the cells were collected and lysed in RIPA buffer supplemented with a protease inhibitor cocktail (Roche). Flag-tagged GSDMD protein was enriched with anti-Flag M2 affinity gel (Millipore A2220). The full-length GSDMD protein in the gel was incubated with increasing

amounts of allergen extracts (up to 1 µg for all allergens) or allergen proteases (125 ng to 1 µg for Der p 1 and Der f 1, 8-125 ng for papain) in 30 µl PBS for 10–30 min at 37 °C. Cleavage products were analysed by SDS-PAGE and immunoblotting.

Generation of constitutive IL-33/GSDMD-expressing cells. To generate cells with stable constitutive overexpression of IL-33/GSDMD, a lentivirus was generated by transiently transfecting HEK293T cells with either the pLVX-IL-33-HA-eGFP/Flag-GSDMD-Puro plasmid or the empty vector with an improved packaging plasmid and the VSdVG plasmid. Then, A549/mle-12 cells were infected with lentiviral supernatants in the presence of polybrene (5 µg/mL) for 12 hours. The following day, puromycin selection was applied to generate pools of cells with stable overexpression. Following at least 1 week of puromycin treatment, single-cell clones were generated using limiting dilution.

Cleavage of endogenous GSDMD with allergen extracts and protease allergens. GSDMD deficient HeLa cells were kindly provided by Dr. Feng Shao. Cells seeded in 24-well plates were grown to form monolayers and then exposed to increasing amounts of allergen extracts and proteases (up to 50 µg for papain and *B. licheniformis*, up to 10 µg for Der p and Der f, up to 200 µg for HDMs, and up to 5 µl for *A. oryzae*; ~1×10^6 cells) in 300 µl/well DMEM supplemented with 1% FBS and cultured for 30 min at 37 °C. The cells were collected for analysis by SDS-PAGE and immunoblotting, and the supernatants were collected for ELISA detection or analysed with SDS-PAGE after precipitation with FreeZone Freeze Dryers.

Antibodies, plasmids and reagents.

Anti-haemagglutinin (HA.11 Covance #CO-MMS-101R), anti-GSDMDC1 (Novus Biologicals #NBP2-33422), and mouse anti-GSDMD (Abcam #ab219800) antibodies were used. A mouse anti-IL-33 pro-peptide antibody (R&D #AF5010-SP), an anti-IL-33 antibody (mouse clone Nessy-1, Alexis Biochemicals #ALX-804-840), an anti-IL-33-Cter antibody (Alexis Biochemicals #AT-110), an anti-IL-33 (human) monoclonal antibody (Cayman #10809), a donkey anti-sheep IgG NorthernLights™ NL557-conjugated antibody (R&D #NL010), a sheep IgG HRP-conjugated antibody (R&D #HAF016), an anti-HA tag antibody (#ab130275), an anti-Caspase-1 mouse antibody (Adipogen #AG-20B-0042-C100), an anti-mouse IL-1β/IL-1F2 antibody (#AF-401-NA), an anti-Cleaved Caspase-3 (Asp175) antibody (CST #9661S), an anti-MLKL (phospho S345) antibody (Abcam #ab196436), an anti-LDH rabbit monoclonal antibody (Beyotime Biotechnology #AF1660), a goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 555 (Invitrogen #A-21428), a goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor plus 488 (Invitrogen #A32723), an anti-Flag M2 antibody (Sigma-Aldrich #F4049), and an anti-actin antibody (Sigma-Aldrich #A2066) were purchased.

DAPI for nucleic acid staining (Sigma-Aldrich #D9542-5MG), Z-VAD (Selleck #S7023), the endocytosis inhibitor chlorpromazine HCl (Selleck #S2456), (E)-necrosulfonamide (MCE #HY-100573), puromycin (Invitrogen #A1113803), LPS (Sigma #L2630) the Pierce™ BCA Protein Assay Kit for protein quantification (Thermo Scientific #23227), the cysteine protease inhibitor E64 (Sigma #E3132) were obtained. The following ELISA kits were used for cytokine detection: Human IL-33 Quantikine ELISA Kit

(R&D #D3300B), Mouse IL-33 DuoSet ELISA (R&D #DY3626), Mouse IL-1β/IL-1F2 DuoSet ELISA (R&D #Y401-05), and Mouse IL-6 DuoSet (R&D #DY406). The KOD-Plus-Mutagenesis Kit (TOYOBO #SMK-101) was used to construct GSDMD constructs with different point mutations. The Necroptosis Inducer Kit with TSZ and the Apoptosis Inducer Kit (TNF-α + SM-164) (Beyotime Biotechnology #C1058S and #C0006S) were used.

Complementary DNA (cDNA) for human GSDMD (#HG25207-UT), human IL-33 (#HG10368-CY) and mouse IL-33 (#MG50118-CY) were purchased from Sino Biological Inc. Mouse Gsdmd was amplified from reverse-transcribed cDNA derived from BMMs. A lentiviral vector encoding a doxycycline-inducible eGFP-fusion Flag-tagged GSDMD construct was modified in a Lenti-iCas9-neo vector (Addgene plasmid #85400). The improved lentiviral packaging plasmid was a gift from Dr. Jing Zhong (Institut Pasteur of Shanghai, CAS).

Quantitative real-time PCR. Total RNA was extracted from tissues and cells using TRIzol (Invitrogen). The purified RNA was quantified and reverse transcribed using the ReverTraAce qPCR RT Kit (TOYOBO). The expression levels of mRNA transcripts were calculated relative to the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the 2^{-dCT} method.

Flow cytometry. Lung cell suspensions were obtained by digesting the lungs with 0.5 mg/ml collagenase I for 60 min at 37 °C and mechanical dissociation through a 70-μm cell strainer. Red blood cells were lysed after digestion by applying ammonium chloride potassium (ACK) lysis buffer for 2 min. Leukocytes were obtained by density gradient centrifugation with a 40/80% Percoll (GE Healthcare) gradient. Cells in the lungs and BALF were counted with a haemocytometer. Single-cell suspensions were stained with combinations of the following antibodies: anti-CD4 (clone GK1.5, eBioscience), anti-SiglecF (clone E50-2440, BD Biosciences), anti-CD45R (clone RA3-6B2, BD Biosciences), anti-NK1.1 (clone PK136, eBioscience), anti-CD3 (clone 2C11, eBioscience), anti-CD11b (clone M1/70, eBioscience), anti-Ter119 (clone Ter119, eBioscience), anti-Ly-6G (clone RB6-8C5, eBioscience), anti-FcεR1 (clone MAR-1, eBioscience), anti-CD11c (clone N418, eBioscience), anti-CD90.2 (clone 53–2.1, BD Biosciences), anti-CD127 (clone A7R34, eBioscience) and anti-IL-33R (T1/ST2, clone DJ8, MD Biosciences). Eosinophils were characterized with the markers SiglecF and CD11c using the antibodies identified above. ILC2s were identified as lineage marker$^-$ (Lin^-: TCRγ/δ$^-$CD45R$^-$NK1.1$^-$CD3$^-$CD11b$^-$CD11c$^-$Ter119$^-$Ly6G$^-$FcεR1$^-$Gr1$^-$) CD90.2$^+$ST2$^+$ cells. Single-cell suspensions were incubated with unlabelled purified anti-Fc receptor blocking antibodies (anti-CD16/CD32) before staining with fluorochrome-conjugated antibodies. Dead cells were excluded by using the fixable viability dye eFluor780 (eBioscience). Samples were acquired on a CytoFLEX flow cytometer (Beckman Coulter), and the data were analysed with FlowJo V10 (FlowJo).

Live-cell imaging. A549-IL-33-HA epithelial cells were seeded in 4-chamber dishes (Cellvis #D35C4-20-1.5-N), and live-cell imaging experiments were performed 12 hours later. For Hoechst co-localization experiments, cells were pre-treated with Hoechst 33342 DNA dye (1 μg/ml) for 10 min prior to imaging in

DMEM (Gibco™ 31053028) supplemented with 10% FBS with a Nikon A1R LUN-V inverted confocal microscope with a 60×/1.27NA water objective.

Human samples. Clinical information is summarized in Supplementary Table 1 (accession number: PJ2019-015).

Tissue collection.

All tissues were collected after subjects provided informed consent, with the approval of tissue-specific protocols by the Medical Ethical Committee of the Academic Medical Centre, Affiliated Hospital, Institute of Respiratory Diseases, Guangdong Medical College, Zhanjiang, China. Inflamed lung tissue samples were obtained from patients with asthma. Uninflamed control lung tissue samples were obtained from adult patients undergoing lung tumour surgery or lung transplantation surgery; tissue samples were obtained at an appropriate distance from the tumour. BALF samples were obtained from patients undergoing bronchoscopy for diagnostic purposes.

Statistical analysis

Statistical significance was analysed as described in the figure legends, and values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism.

Declarations

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Figures

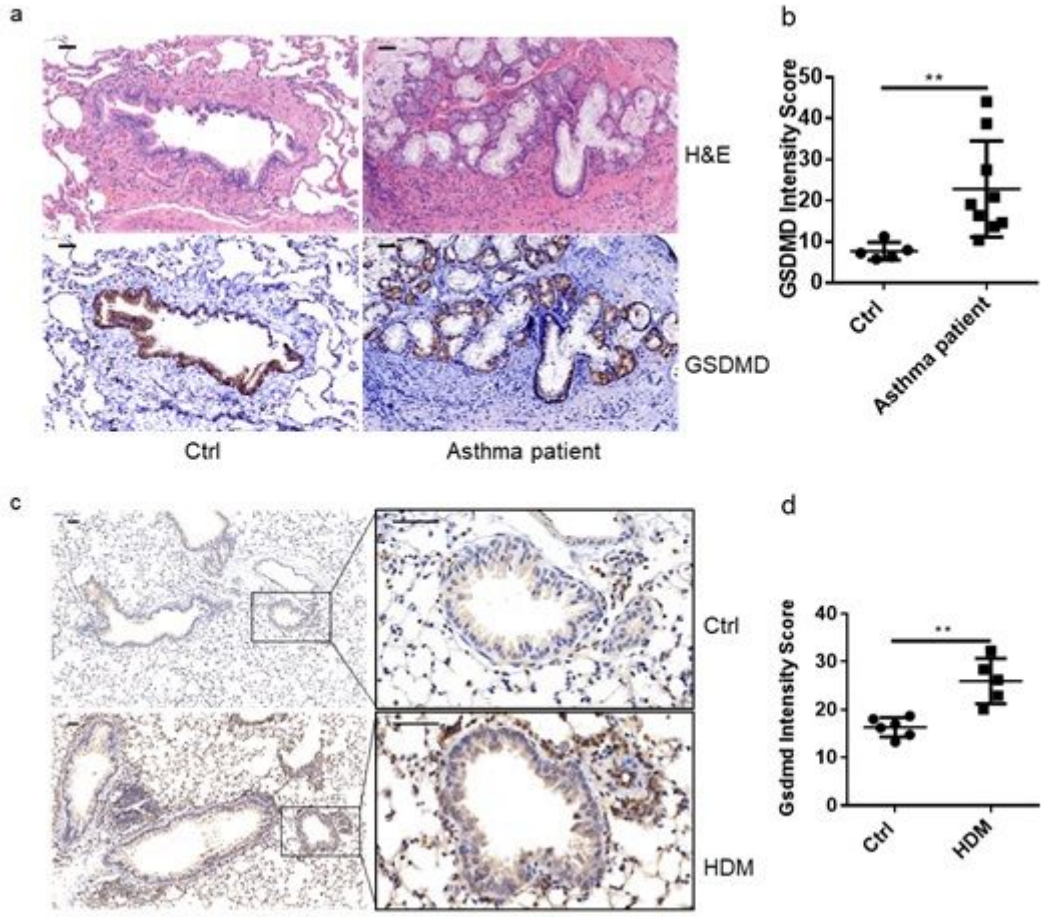


Figure 1

Gsdmd expression is elevated in type 2 inflammation in both humans and mice. a. Microscopy images of haematoxylin-eosin staining (H&E) and immunohistochemical (IHC) staining of human GSDMD (brown) in bronchial tissue from asthma patients and non-asthmatic controls. The GSDMD protein intensity score in (a) was statistically analyzed. Each point represents one person. b. Visual quantification of the GSDMD protein intensity score in (a). Each point represents one person. c. Microscopy imaging of immunohistochemically stained mouse Gsdmd protein in lung tissues. Balb/c mice were administered HDM or PBS intranasally for up to 3 weeks to induce asthma symptoms. d. Statistical analysis of the Gsdmd protein intensity scores corresponding to (c). Each point represents one mouse. Scale bars (a, c), 50 μm. Immunohistochemical signals were analyzed with ImageJ software, and the average IHC intensity score of each tissue sample was obtained by evaluating 5-8 different fields that were selected blindly. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using the Mann-Whitney test.

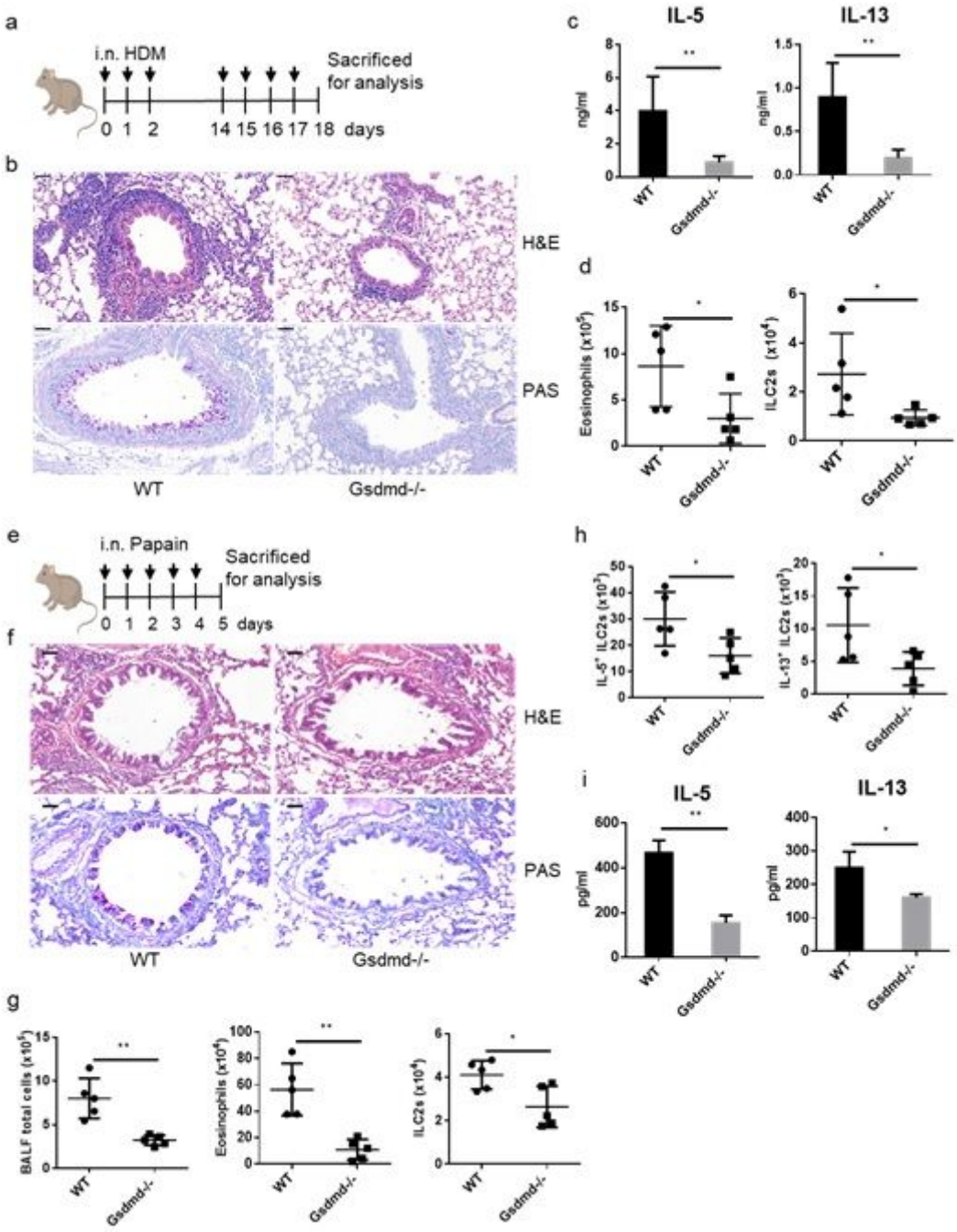


Figure 2

Deletion of Gsdmd dampened ILC2s activation in both HDM-induced chronic and papain-induced acute airway inflammation. a. Scheme of asthmatic inflammation induction in Balb/c mice intranasally administered HDM. Eighteen days after the first stimulation, the BALF and lung tissues were collected. b. Representative H&E staining and periodic acid-Schiff (PAS) staining of lung tissue sections from HDM-treated WT and Gsdmd-/- mice. c. Concentrations of IL-5 and IL-13 in the BALF of HDM-treated WT and Gsdmd-/- mice detected by ELISA. d. The numbers of SiglecF+CD11c+ eosinophils in the BALF and Lin–CD90.2+ST2+ ILC2s in the lungs after HDM exposure, as determined by flow cytometry. e. Scheme of acute airway inflammation induction in Balb/c mice with papain. Mice were sacrificed for analysis 5 days after the first exposure. f. Representative H&E staining and periodic acid-Schiff (PAS) staining of lung

sections from papain-treated WT and Gsdmd-/- mice. Scale bars, 50 μm. g. The numbers of total cells SiglecF+CD11c+ eosinophils in the BALF and Lin−CD90.2+ST2+ ILC2s determined with FACS analysis. h. The numbers of IL-5- and IL-13-expressing ILC2s (Lin−CD90.2+ST2+) in the lungs after papain exposure, as determined by flow cytometry. i. The concentrations of IL-5 and IL-13 in the BALF of were papain-treated WT and Gsdmd-/- mice detected by ELISA. Each point represents one mouse, and 5-6 mice were used per group. Scale bars (b), 50 μm. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using the Mann-Whitney test.

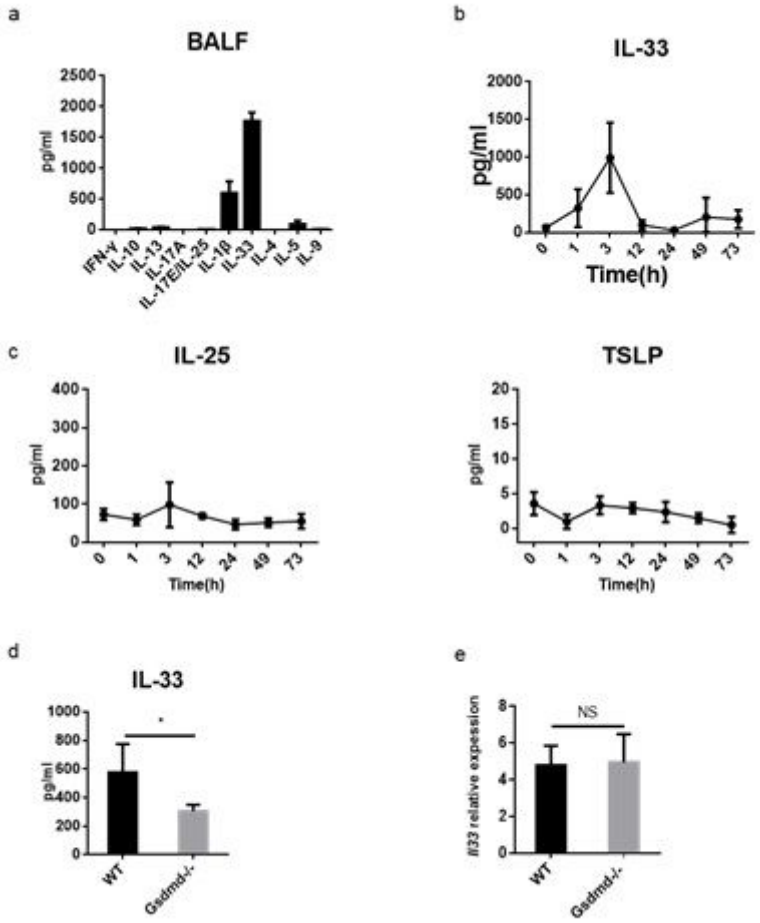


Figure 3

Gsdmd deficiency impairs IL-33 release in vivo. a.WT Balb/c mice were exposed to 5 μg papain in 40 μl PBS intranasally and sacrificed for BALF collection 3 hours after exposure. Ten cytokines, including IFN-r, IL-10, and IL-33, were detected with an MSD multi-cytokine detection system. The data represent the mean ± SEM of pooled data from 3 different mice. b.ELISA was used to analyze the IL-33 content in the BALF, which was collected at the indicated time points after exposure to a single dose of papain (5 μg). At least three individual mice were used at each time point. c.ELISA was used to analyze IL-25 and TSLP levels in the BALF of WT and Gsdmd-/- mice 3 hours after papain exposure. d.ELISA was used to analyze the IL-33 content in the BALF of WT and Gsdmd-/- mice 3 hours after papain exposure. e.IL-33 in lung homogenates was analyzed with immunoblotting after quantification by a BCA protein assay. Lung

tissues from WT and Gsdmd-/- mice were collected at the indicated time points and suspended in PBS with a protease inhibitor for homogenization. The mRNA expression levels of IL-33 were analyzed in the remaining mouse lungs, and 5-6 mice were used per group. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using the Mann-Whitney test.

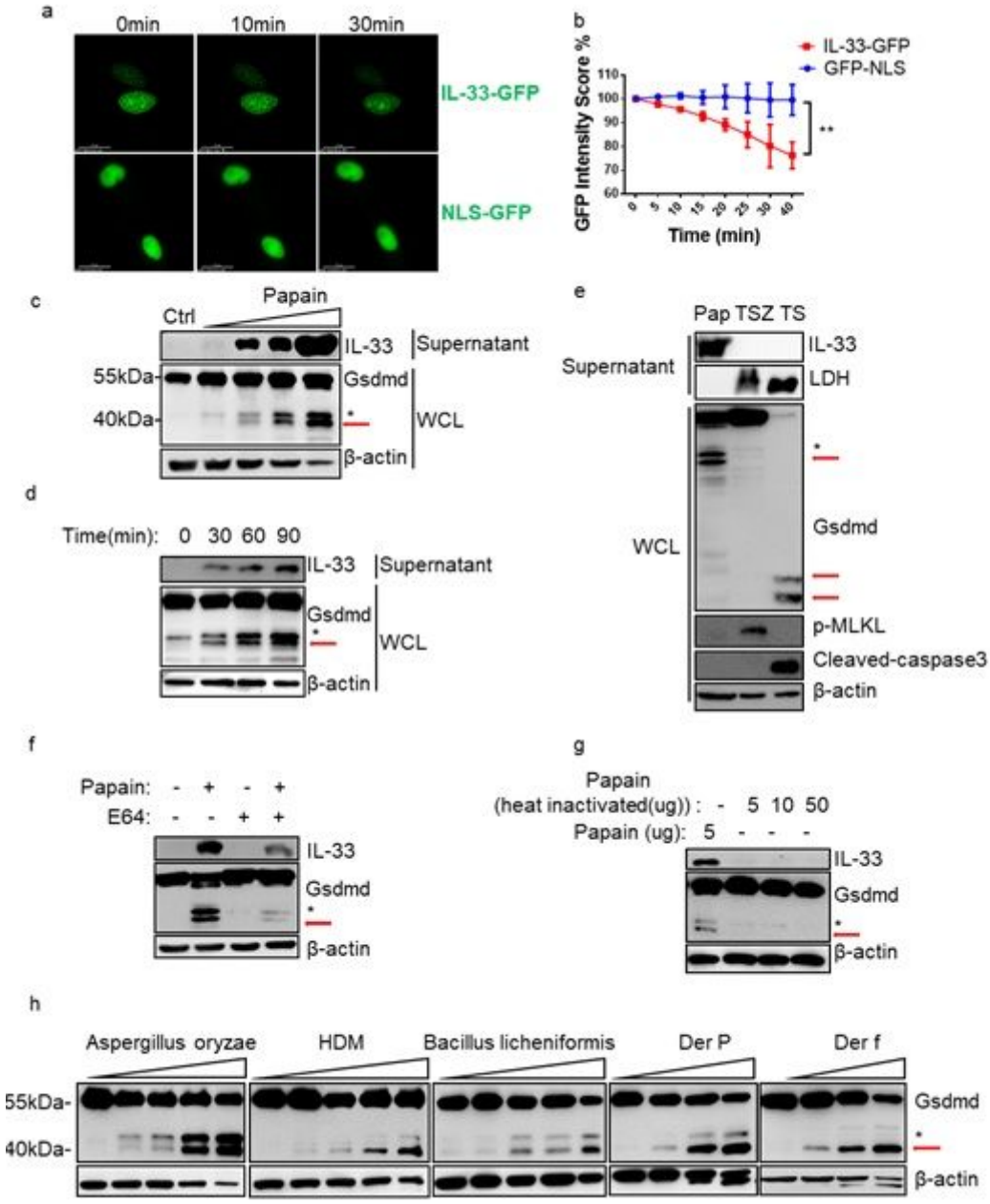


Figure 4

The protease activity of papain is needed for IL-33 release and GSDMD cleavage in airway epithelial cells. a.Time-lapse images of IL-33-GFP- and NLS-GFP-expressing A549 cells after exposure to 5 μg papain in a 3.5-cm-diameter cell culture dish for 30 min. b.The GFP intensity of cells at the indicated times after papain exposure was analyzed with ImageJ software. c.Mle12 murine airway type 2 epithelial cells were seeded in 24-well plates at a density of 80% and stimulated with different amounts of papain (1 μg, 5 μg, 10 μg and 50 μg per well) for 30 min. The cells and culture supernatants were analyzed with

immunoblotting to evaluate IL-33 secretion and Gsdmd processing. d.Mle12 cells were exposed to 5 μg/well papain for the indicated time. The cells and culture supernatants were collected for immunoblotting. e.Mle12 cells were exposed to a relatively high dose of papain (100 μg/well) for 30 min to induce IL-33 release. Mle12 cells were individually induced to undergo necroptosis with a TNF-α, SM-164 and Z-VAD-FMK (TSZ) mixture or undergo apoptosis with TNF-α and SM-164 (TS) stimulation for 12 hours. f. Immunoblot analysis of collected cells and culture supernatants treated with papain (5 μg) in the presence of the cysteine protease inhibitor E64. g. Papain inactivated with heat treatment at 100°C for 10 min was added to the cell culture supernatant at the indicated concentration per 24 wells, and the cells and culture supernatants were collected for immunoblot analysis. h.Different amounts of allergen proteases were added to cell cultures, and following an exposure time of 30 min, the cells were collected to analyze Gsdmd processing. WCL: whole-cell lysates. The red arrow indicates the neo-form of p40 NT-Gsdmd. Asterisk-marked nonspecific fragments were not discussed in our work. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using the Mann-Whitney test.

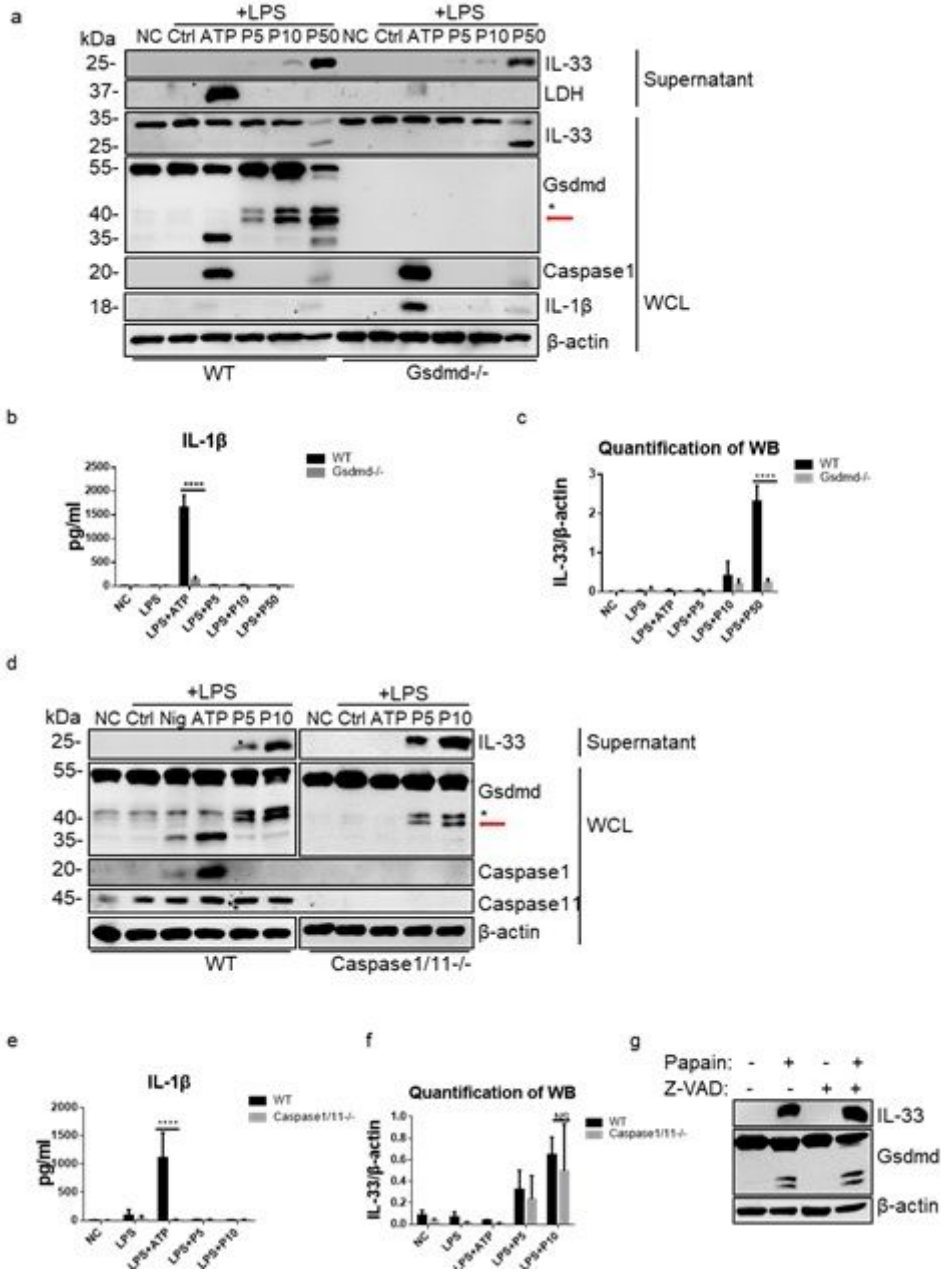


Figure 5

Papain-activated Gsdmd is independent of the inflammatory caspase pathway. Bone marrow-derived macrophages were treated with the indicated stimuli. NC: cells not treated with an exogenous stimulus; Ctrl: cells treated with only 200 ng/ml LPS for 4 hours; Nig/ATP: cells treated with LPS for 4 hours plus 5 mM Nig/ATP treatment for 30 min; and P5/P10/P50: cells treated with LPS for 4 hours plus 5, 10 or 50 μg/well papain treatment for 30 min. a.Immunoblot analysis of the indicated proteins in whole-cell lysates (WCL) generated from cells given different treatments and supernatants from WT and Gsdmd-/- BMMs. The red arrow indicates the cleaved functional Gsdmd N-terminal fragment and mature form of IL-1beta. Asterisk-marked nonspecific fragments were not discussed in our work. b.BMM cell culture supernatants corresponding to (a) were evaluated with an IL-1beta ELISA. c.Quantification of the IL-33 content (relative to the β-actin content) in cell culture supernatants from cells treated as indicated in (a) with ImageJ. d.Immunoblot analysis of BMMs and culture supernatants derived from WT and Caspase1/11-/- mice given certain stimulations. f.Quantification of the IL-33 content (relative to the β-actin content) in cell culture supernatants from cells treated as indicated (d) with ImageJ. g.Immunoblot analysis of Mle12 cells and cell culture supernatants that were pretreated with the pan-caspase inhibitor Z-VAD-FMK (Z-VAD) for 30 min before papain stimulation. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using Sidak's multiple comparisons test.

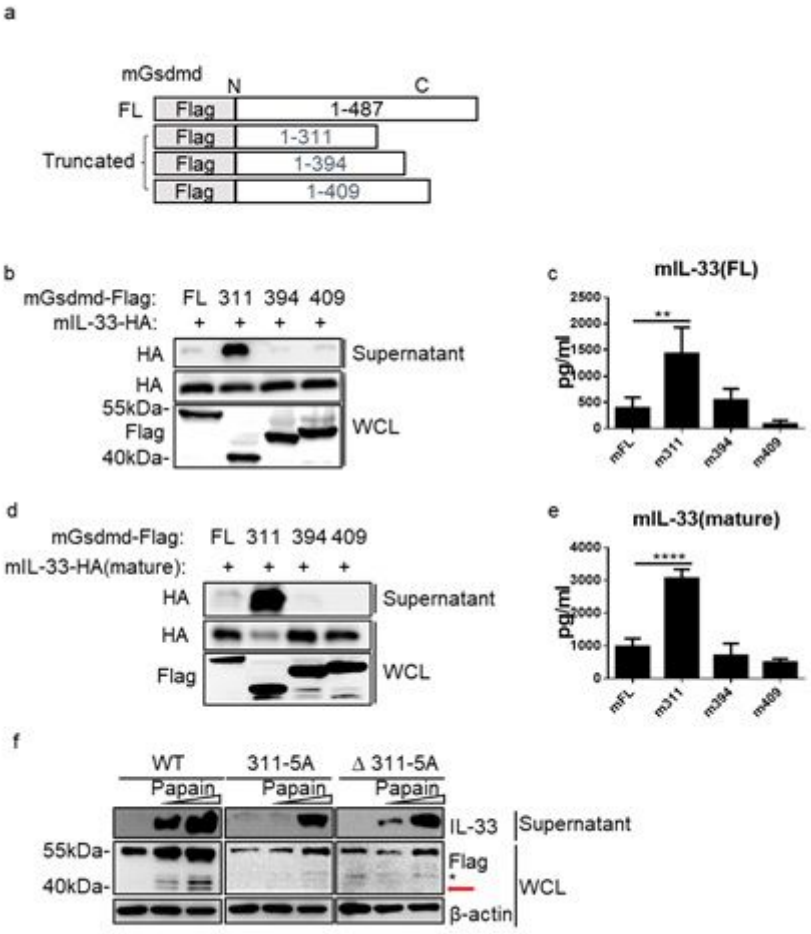


Figure 6

Truncated 1-311 aa mNT-Gsdmd contributes to IL-33 release. a.Schematic showing truncation mutants of mGsdmd with an N-terminal Flag tag. b.Different truncated Gsdmd fragments were co-expressed with murine C-terminal HA-tagged full-length IL-33 in HEK293T cells, and whole-cell lysates and cell culture supernatants were collected for immunoblot analysis after the cells were transfected for 18 hours. c.Cell culture supernatants from cells treated as indicated in (b) were evaluated with an IL-33 ELISA. d.Different truncated Gsdmd fragments were co-expressed with the murine C-terminal HA-tagged mature form of IL-33 in HEK293T cells, and whole-cell lysates and cell culture supernatants were collected for immunoblot analysis after the cells were transfected for 18 hours. e.Cell culture supernatants from cells treated as indicated in (d) were evaluated with an IL-33 ELISA. f.Mle12 cells were engineered to express the indicated mutations in a full-length mGsdmd construct with a Flag tag, and whole-cell lysates and cell culture supernatants were collected for immunoblot analysis after papain exposure for 30 min. 311-5A represents mutation of residues 308-313 (ELRQQ) to AAAAA; Δ 311-5A represents deletion of residues 308-313 (ELRQQ) in the full-length Gsdmd sequence. Results are depicted as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 using Dunnett's multiple comparisons test.

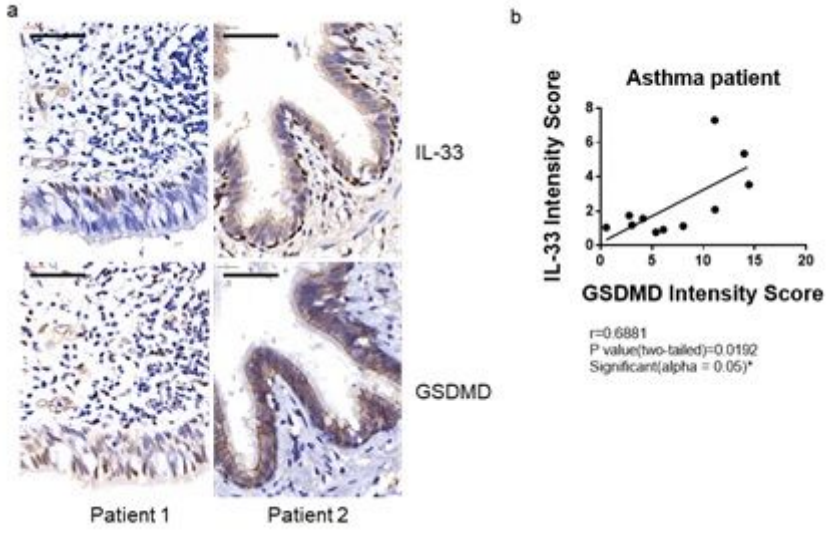
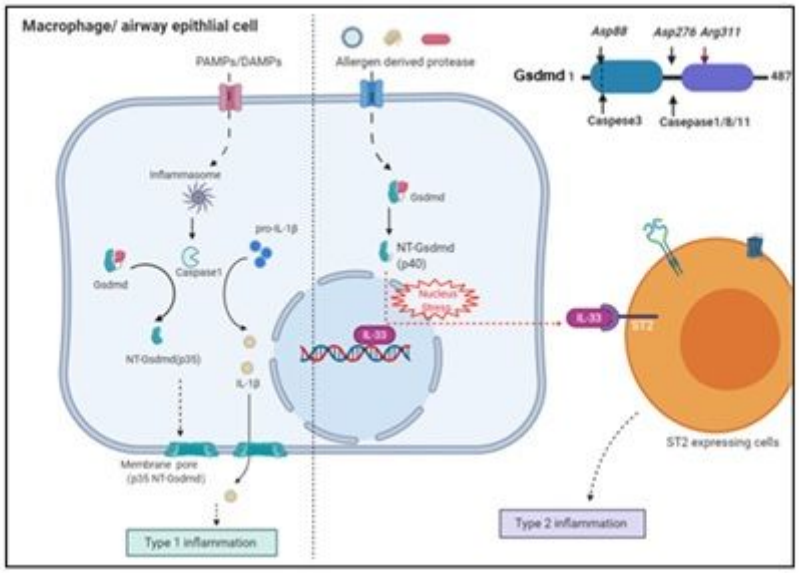


Figure 7

GSDMD expression is positively correlated with IL-33 in patients with asthma. a.Representative images of immunohistochemical (IHC) staining of GSDMD and IL-33 in serial sections of bronchial tissues from asthma patients. b.Statistical analysis of GSDMD protein intensity scores related to the data in (a). Each point represents one person. Scale bars (a), 50 μm. Immunohistochemical signals were analysed with ImageJ software, and the average IHC intensity score of each tissue sample was obtained by evaluating 6-8 different fields that were selected blindly.



Highlights

1. Discover a new cleavage form of Gsdmd which is independent on inflammatory caspase and function as an conduits for IL-33 secretion;

2. Discover several exogenous allergen protease effectors which could inducing a common functional p40 mGsdmd fragment;

3. Discover a pro-inflammatory function of Gsdmd protein in regulation of type 2 immunity;

4. Gsdmd presented an elevated expression pattern in asthma patient which might be a potent drug target for therapy.

Figure 8

Working model of protease allergen induced Gsdmd cleavage in epithelial cells

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- IL33eGFPpapain.mp4
- NLSeGFPpapain.mp4
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