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Interleukin (IL)-33 is an IL-1 family nuclear cytokine, constitutively expressed in epithelial cells of environmentally exposed tissues (skin, gastrointestinal tract and lungs) and endothelial cells. It is involved in type-2 innate immunity via activation of eosinophils, basophils, mast cells, group 2 innate lymphoid cells and macrophages through its receptor ST2 (also called IL1RL1).[1] Due to the absence of a secretory signal sequence, IL-33 was thought to be passively released during cell necrosis, physical stress or tissue damage, and was accordingly considered as an alarmin.[2] However, Chen et al. recently demonstrated that, upon exposure to allergen proteases, IL-33 is transported from the nucleus to the cytosol via stress granules (SG) assembly with subsequent release of the active cytokine through membrane pores formed by the unusual p40 N-terminal fragment of gasdermin D (Gsdmd).[3] Importantly, these events were not associated with any signs of cell death, thus uncovering a new pathway in IL-33 release distinct from the "alarmin" prototype (Fig. 1).

The role of Gsdmd is well described in pyroptosis, which is a form of cell death associated with tissue damage. During canonical inflammasome-induced pyroptosis, Gsdmd is proteolytically cleaved by activated caspase-1 to generate an active fragment with membrane pore-forming ability, allowing the release of IL-1β and IL-18 by 'unconventional protein secretion.[4] In contrast, following stimulation with an allergen protease papain, Chen et al. observed the formation of a novel p40 or p35 N-terminal (NT) Gsdmd fragment in murine MLE-12 or human A549 epithelial cell lines respectively.[3] The appearance of these neo-fragments was concomitant with the delocalization of IL-33 from cell nucleus and its release in the supernatant without apparent cell death or caspase activation. This effect was reversible after the removal of papain from the medium. In order to differentiate this observation from the conventional inflammasome-dependent pyroptosis effect of Gsdmd, Chen and colleagues compared the effect of inflammasome activation and papain stimulation in murine bone marrow-derived

macrophages (BMDMs). While activation of inflammasome with lipopolysaccharide (LPS) and ATP or Nigericin led to the cleavage of caspase-1 and the generation of conventional pyroptotic fragment (35-kDa) of Gsdmd together with lactate dehydrogenase (LDH) and IL-1β release in these cells, stimulation with papain promoted the appearance of the p40-NT Gsdmd and the secretion of IL-33. The non-involvement of caspases in papain-induced IL-33 secretion was confirmed with casp-1/casp-11-deficient BMDM and by the use of pan-caspase inhibitor Z-VAD-FMK. On the other hand, the protease activity of papain and other allergen proteases was indispensable for IL-33 secretion. However, proteases from *Alternaria alternata* failed to induce the p40-NT Gsdmd but induced IL-33 release *in vitro*, suggesting an alternative pathway for IL-33 secretion with this protease.

The essential role of SGs in this newly described mechanism of IL-33 secretion was detected by the formation of G3BP1-positive SG puncta in response to papain stimulation, which permitted the nucleus-cytosol translocation of IL-33. SGs are described as dynamic compartments assembled in the cytoplasm for the transport of RNA, ribosomal subunits and various proteins following translational arrest in response to stress.[5] Though SG assembly induced by arsenite as well as papain resulted in the nucleo-cytoplasmic translocation of IL-33, papain stimulation exclusively triggered the secretion of IL-33 to the supernatant, implying that SG assembly is an independent prerequisite event in IL-33 secretion.

Chen et al. then explored the potential cleavage sites that generate the p40-NT fragment responsible for IL-33 secretion. Among the *in silico* predicted fragments and the caspase-induced pyroptotic fragments they have generated, only the $Gsdmd^{1-311}$ and the pyroptotic $Gsdmd^{1-276}$ fragments induced efficient IL-33 secretion when co-transfected with mature IL-33 lacking the nuclear localization signal peptide to bypass the need for nucleocytoplasmic translocation. However, the $Gsdmd^{1-311}$ fragment triggered less LDH release compared to the

latter. The introduction of site-specific mutations further identified the residues 309-313 and 288-292 as putative cleavage sites to obtain the murine p40 NT and human p35 NT Gsdmd fragments respectively.

The contribution of Gsdmd to the development of type 2 inflammation was confirmed in asthmatic patients and in a mouse model of asthma induced with house dust mite (HDM). In asthmatic patients, Gsdmd expression in lung airway epithelium correlated with IL-33 secretion in the bronchoalveolar lavage (BAL) and elevated serum IgE levels. Similarly, in mice intranasally challenged with HDM, inflammatory infiltration in the lungs was associated with a pulmonary raise in Gsdmd level. When mice were challenged with papain, IL-33 was significantly upregulated while the levels of other type 2 inflammatory cytokines IL-25 and thymic stromal lymphopoietin remain unchanged. However, a slight increase of IL-1β was also detected in papain-challenged mice suggesting a residual activation of the canonical inflammasome pathway in these mice. Analysis of the BAL from papain-exposed Gsdmd-deficient mice confirmed the requirement of Gsdmd in the secretion, but not in the neo-synthesis of IL-33 as the RNA levels were unaffected in the transgenic mice. Furthermore, when $Gsdmd^{-/-}$ and WT mice were exposed repetitively to HDM to mimic chronic asthma or acutely with papain for 5 days, a decrease in the IL-5 and IL-13 levels was associated with less lung infiltration in $Gsdmd^{-/-}$ mice, confirming the involvement of Gsdmd in the development of type 2 inflammation.

The data described here explain a plausible alternative mechanism for the secretion of IL-33 in response to allergen proteases. However, several molecular players involved in this pathway remain to be elucidated such as direct sensing receptor of the allergen protease in this context and the pathway(s) that leads to the p40 NT-Gsdmd fragmentation. Although it has been well established that the pyroptotic fragment is generated by the cleavage of Gsdmd by caspase 1,

the enzyme which generates the p40 NT-Gsdmd is not identified. The ability of Gsdmd to create membrane pores that can have divergent physiological responses in distinct cell types or in response to diverse stimuli has been proposed.[6] From this work, we could postulate that the pores formed by the pyroptosis fragment or the p40 NT-Gsdmd generated in response to different environmental cues could be structurally dissimilar. Gsdmd seems like a molecular switch to orientate the immune response depending on the trigger or stimuli. Moreover, the current data might help in identifying the mechanism(s) by which therapy with intravenous immunoglobulin, one of the commonly used immunotherapeutic drugs, leads to enhanced IL-33 in the circulation.[7]

Chen et al. identified the SG as a major player in IL-33 transport in the cytosol after allergen exposure. However, the trigger and the regulatory mechanisms for SG assembly are not known. IL-1β has been shown to induce SG assembly in human osteoarthritis chondrocytes.[8] IL-1β released from necroptosis cells could also regulate the formation of SG and have an indirect effect on the IL-33 secretion in epithelial cells and macrophages. Involvement of SG in the production of IL-1β in macrophages has also been suggested to modulate the Th1/Th17 balance,[9] showing the importance of these dynamic compartments in the regulation of immune responses.

The allergen protease papain has also been shown to activate basophils leading to the production of the Th2 cytokine IL-4. Despite previous attempts to identify the signalling pathways activated by papain in basophils, the specific molecular mechanisms are not yet clear. Studies by using various knockout mice have revealed that the activation mechanism in basophils is independent of many common cell signalling pathways including the caspase-1

inflammasome pathway.[10] In this setting, a pathway similar to p40 NT-Gsdmd could be envisioned in the case of basophils or other innate cells and release of cytokines.

In line with the findings of Chen and colleagues, could GSDMD targeting be useful to curb type 2 inflammation and airway inflammatory responses? Efforts in targeting the Cys191/Cys192 site of Gsdmd, which is indispensable for Gsdmd oligomerization and pore formation, have shown success with the discovery of necrosulfonamide (NSA)[2] and the FDA-approved drug disulfiram[11] as an inhibitor of pyroptosis by blocking gasdermin pore formation (Fig. 1). In a recent study, the pharmacological inhibition of Gsdmd with disulfiram prevented neutrophil extracellular traps (NET) formation, and reduced inflammation and lung tissue damage in an experimental model of COVID-19 highlighting the importance of targeting this molecule in multiple inflammatory pathologies.[12] Of note, SARS-CoV-2 infection has been shown to induce IL-33 in epithelial cells.[13] Similar small-molecule drug approaches to target the cleavage site of the newly discovered p40-NT Gsdmd fragment could be proposed as a viable therapeutic option for the benefit of patients with chronic airway inflammation. As another example, the necroptosis inhibitor GW80 attenuated lung inflammation *in vivo* in an IL-33-dependent *Aspergillus fumigatus* extract-induced asthma model.[2] These drugs have been described in the context of pro-inflammatory forms of cell death that results in cell lysis. However, with the novel discovery of the involvement of Gsdmd in IL-33 secretion, it would be worth studying them in the context of allergen protease sensitization and type 2 inflammation.

Altogether, Chen et al. convincingly shed light on two uncoupled mechanisms that could explain the transport of IL-33 in the cytosol via SG assembly and its active secretion in the extracellular milieu through the generation of pores by a newly described fragment of Gsdmd. These results bring many attractive possibilities to tackle type 2 inflammation.

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AUTHOR CONTRIBUTIONS

C.C., S.V.R. and J.B. performed the literature search and analyses and drafted the manuscript

COMPETING INTERESTS

Authors declare no competing interests

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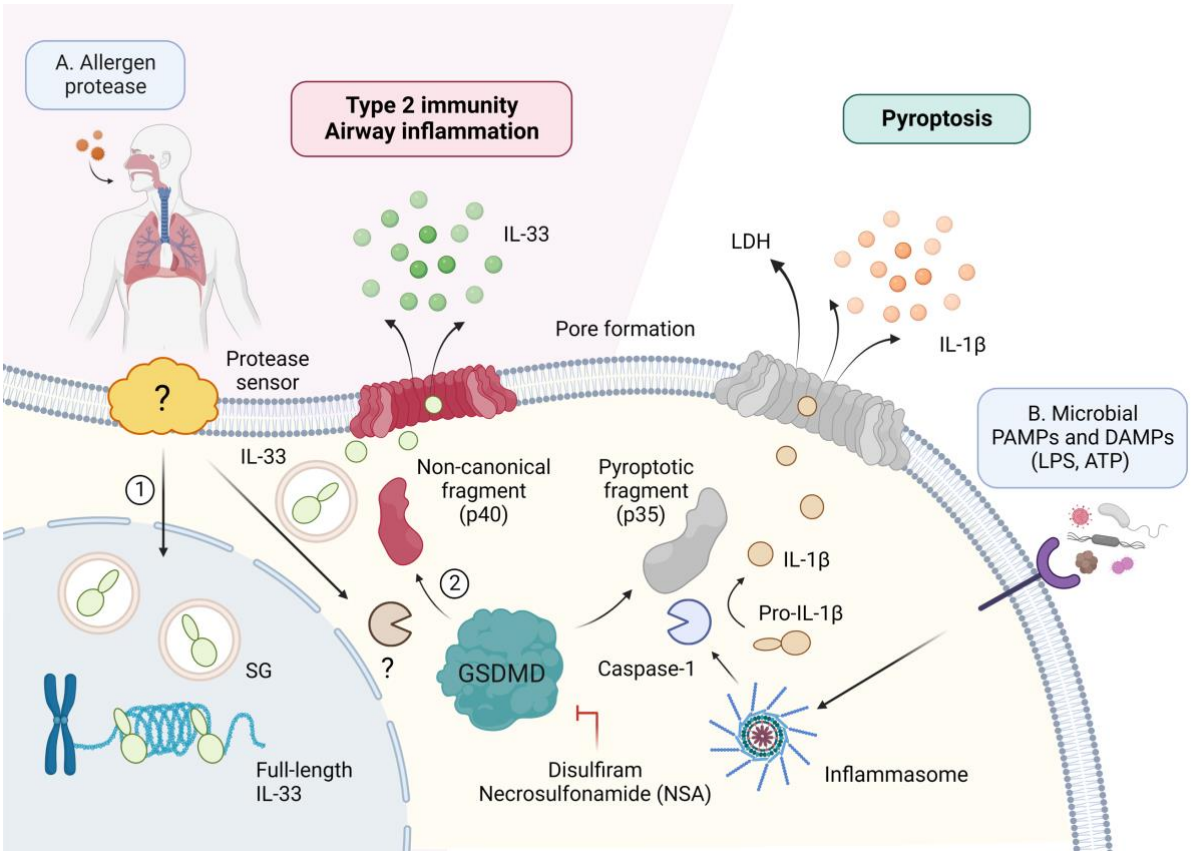
Figure Legend

Fig 1. **Context-dependent role of Gasdermin D (Gsdmd) in the release of IL-33 and IL-1β.** In response to allergen protease stimulation (A), nuclear IL-33 is transported into the cytosol via dynamic SG assembly (1). The subsequent generation of pores by the newly described fragment of Gsdmd (p40-NT in mouse/p35-NT in human) (2) leads to the active release of IL-33 into the extracellular space, independent of canonical inflammasome pathway activated in response to microbial pathogen-associated molecular patterns (PAMPS) like LPS and molecules associated with endogenous danger-associated molecular pattern (DAMP) ATP (B). Drugs targeting Gsdmd could be used as therapeutic agents to curb type-2 inflammation. Nevertheless, the nature of the sensing receptor of the allergen protease and the identity of the enzyme, which generates the p40 NT-Gsdmd need to be investigated. Figure created in BioRender.com.


A. Allergen protease
Type 2 immunity Airway inflammation
Pyroptosis
IL-33
LDH
IL-1β
Pore formation
Protease sensor
?
IL-33
B. Microbial PAMPs and DAMPs (LPS, ATP)
Non-canonical fragment (p40)
Pyroptotic fragment (p35)
IL-1β
Pro-IL-1β
SG
?
GSDMD
Caspase-1
Full-length IL-33
Disulfiram Necrosulfonamide (NSA)
Inflammasome