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Natterin an aerolysin-like fish toxin drives IL-1β-dependent neutrophilic inflammation mediated by caspase-1 and caspase-11 activated by the inflammasome sensor NLRP6

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ABSTRACT

Natterin is an aerolysin-like pore-forming toxin responsible for the toxic effects of the venom of the medically significant fish *Thalassophryne nattereri*. Using a combination of pharmacologic and genetic loss-of-function approaches we conduct a systematic investigation of the regulatory mechanisms that control Natterin-induced neutrophilic inflammation in the peritonitis model. Our data confirmed the capacity of Natterin to induce a strong and sustained neutrophilic inflammation leading to systemic inflammatory lung infiltration and revealed overlapping regulatory paths in its control. We found that Natterin induced the extracellular release of mature IL-1β and the sustained production of IL-33 by bronchial epithelial cells. We confirmed the dependence of both ST2/IL-33 and IL-17A/IL-17RA signaling on the local and systemic neutrophils migration, as well as the crucial role of IL-1α, caspase-1 and caspase-11 for neutrophilic inflammation. The inflammation triggered by Natterin was a gasdermin-D-dependent inflammasome process, despite the cells did not die by pyroptosis. Finally, neutrophilic inflammation was mediated by non-canonical NLRP6 and NLRC4 adaptors through ASC interaction, independent of NLRP3. Our data highlight that the inflammatory process dependent on non-canonical inflammasome activation can be a target for pharmacological intervention in accidents by *T. nattereri*, which does not have adequate specific therapy.

1. Introduction

In the North and Northeast of Brazil, Thalassophryne nattereri is responsible for several accidents amongst fishermen and bathers [1,2]. According to Fonseca and Lopes-Ferreira [3], the palm of the hands or soles of the feet are the most commonly areas affected. The main symptoms of envenomation including local edema and excruciating pain that develops immediately after the accident and can remain for several days were reproduced in mice [4].

These symptoms are followed by intense necrosis [5] characterized by vascular alterations as stasis and presence of thrombi in venules, focal transient constrictions in arterioles and increased vascular permeability with consequent ischemia. The *Thalassophryne nattereri* venom (V*Tn*) lacks direct pro-coagulant activity, but exerts a strong cytolytic action on platelets and endothelial cells *in vitro* [6]. The effects of V*Tn* on the delayed recruitment of neutrophils [7] and its action decreasing collagen fiber content during the healing phase by the activation of matrix metalloproteinases (MMP-2 and MMP-9) [8] are added to the mechanisms responsible for ischemic and necrotic injury characterized by the lack of anti-inflammatory drug therapy. Indomethacin (a cyclo-oxygenase inhibitor), dexamethasone (a steroid anti-inflammatory agent), cyproheptadine (an antagonist of serotonin receptors), fentanyl

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(an opioid analgesic), or L-NAME (an inhibitor of nitric oxide synthase) did not affect the venom-induced nociceptive and edematogenic responses [9].

Based on the fact that V*Tn* induces pain, edema and necrosis dependent on its kininogenase activity [9], using combined pharmacological, proteomic, and transcriptomic approaches we isolated into purified fractions of the venom by chromatography and identified in these fractions as well as in the cDNA library of the venom-producing gland the Natterin family composed by five orthologs 1–4 and -P [10,11], which are responsible for the main toxic effects of the V*Tn* [9,10,12].

Natterins 1 and 2 are very close related, presenting 84% of identity;

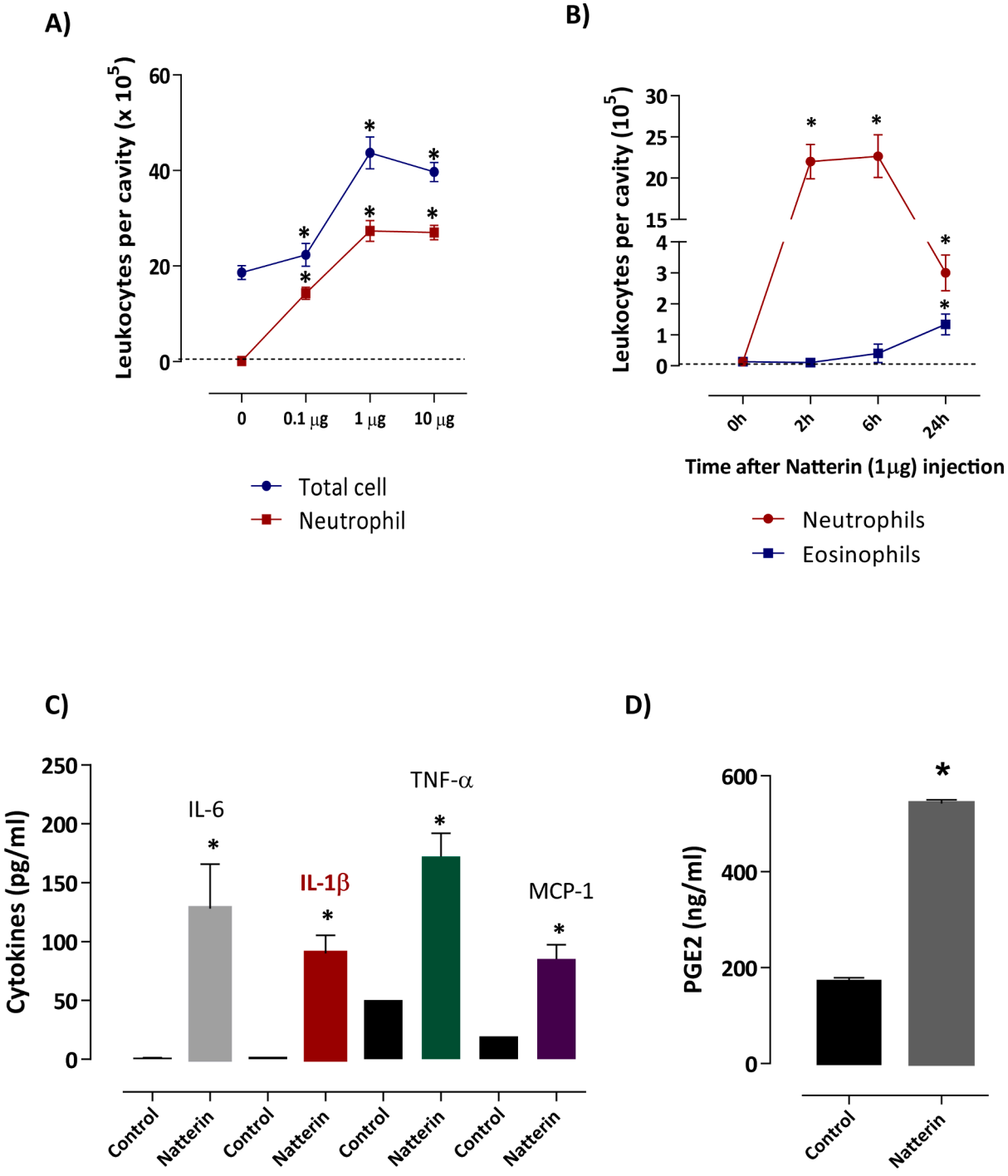


Fig. 1. Natterin induces a strong and sustained neutrophilic inflammation in murine model of peritonitis. Natterin (0.1, 1 and 10 µg) in 500 µL of PBS was injected i.p. into BL/6 wt mice and as a negative-control mice were injected i.p. with PBS. Two hours later, peritoneal cells were harvested and aliquots were applied on glass slides, subjected to centrifugation and stained with Diff-Quick Stain kit and leukocytes were imaged with a microscope Axio Imager A1 (Carl Zeiss, Germany) for count (*A*). Natterin (1 µg) in 500 µL of PBS was injected i.p. into BL/6 wt mice and as a negative-control mice were injected i.p. with PBS. After 2, 6, and 24 h the peritoneum exudates were harvested and aliquots were applied on glass slides, subjected to centrifugation and stained with Diff-Quick Stain kit and leukocytes were imaged for count (*B*). Two hours after Natterin (1 µg) injection, the peritoneum exudates were harvested for cytokine determination (*C*) by cytokine-specific ELISA (IL-1β and MCP-1 or cytometric bead array (IL-6 and TNF-α); and PGE2 (*D*) levels determination by cytokine-specific ELISA. The results represent the mean ± SEM of 3–5 animals/group. *$p < 0.001$ compared with negative-control group.

and both orthologs present identity around 40% with Natterins 3 and 4. Natterin P is the shortest among the Natterin family (71 amino acids long), and shows high homology (84% of identity) mainly to the Natterin 4 at the first 55 amino acids residues [10]. Structurally, Natterins 1–4 contain in the N-terminal region two DM9 domains [13] followed in C-terminal by an aerolysin domain into a so-called domain 2 similar to the conserved common core of aerolysin produced by *Aeromonas* species [14], important for toxicity [10], membrane penetration [15], and induction from cell death to cell survival, being able to activate the NLRP3 inflammasome-dependent caspase-1 maturation leading to IL-1β-dependent neutrophilic inflammation [16,17].

After the first identification of Natterin family by our group, several protein sequences assigned as members of this family, for presenting identity with the aerolysin domain in the C-terminal region of Natterin proteins from the venom of the *Thalassophryne nattereri*, have been found in another venomous fish such as *Plotosus lineatus* [18], as well as in non-venomous fish species including *Gadus morhua*, *Lampetra japonica* and *Lampetra morii, Salvelinus alpinus*, and *Danio rerio* [15,19–22] and aquatic species as Oyster *Crassostrea gigas* [23] and Cnidaria Acropora digitifera [24].

Due to their capacity to form pore resulting in the activation of inflammation, a tissue specific manner expression (gills, followed by expression in head kidney, skin and spleen), and also differently expression during the development and after infections, these natterin-like proteins have been described as defense molecules for the host, promoting protection against pathogens and microorganisms [25].

Given that Natterin is a family of aerolysin-like pore-forming toxin responsible for the toxic effects of the venom of the medically significant fish *T. nattereri* and the envenomation is characterized by the lack of anti-inflammatory drug therapy, we hypothesized that the inflammatory response triggered by Natterin may be related to its aerolysin domain involved in the activation of the inflammasome complex helping to promote neutrophilic inflammation into peripheral tissues. To test this hypothesis a mouse model of peritonitis was used with a combination of genetic loss-of-function and pharmacologic approaches to investigate the regulatory mechanisms controlling acute neutrophilic inflammation induce by Natterin.

2. Results

2.1. Natterin induces a robust neutrophilic inflammation in peritonitis model

To gain insights into the capacity of Natterin to induce neutrophilic inflammatory response, we undertook *in vivo* investigations using this isolated family of fish venom toxins in a murine model of peritonitis. First, the dose-response of neutrophil recruitment to peritoneal cavity of wild type (*WT*) C57BL/6J (BL/6) mice induced by Natterin was shown in Fig. 1A. In control-mice (0 h), neutrophils (red line) were barely detectable, and resident macrophages were the major myeloid cells in the peritoneal cavity (data not shown). The high amount ($\times 10^5$) of neutrophils induced 2 h post-injection was dose-dependent for Natterin in the range of 0.1–1 μg (i.p. injection in 500 μL), which remained high at 10 μg. The infiltration of neutrophils triggered by all doses of Natterin 2 h into peritoneal cavity was not accompanied by a significant increase in the amount of eosinophils at his time point (2 h, blue line, Fig. 1B).

In addition, the kinetics of inflammation induced by Natterin was shown in Fig. 1B. Natterin at 1 μg promoted a high and sustained infiltration of leukocytes until 6 h, decreasing at 24 h. At this time (24 h) when neutrophilia decreased the infiltration of eosinophils increased.

These results confirm the capacity of Natterin to induce a strong and sustained neutrophilic inflammation in murine model of peritonitis.

2.2. Natterin induces the release of mature IL-1β in exudate of peritoneal cavity

We start exploring the mechanisms involved in Natterin-induced neutrophilic inflammation by measuring different mediators in supernatants of peritoneal exudates. IL-1α works similarly to IL-1β [26], but it also plays a key role in promoting cellular senescence, promoting the senescence-associated secretory phenotype, SASP, a response characterized by secretion of IL-6 and IL-8 [27]. Active IL-1β is inducible and requires posttranslational proteolysis by inflammatory caspases to signal via IL-1R to promote neutrophil recruitment. As shown in Fig. 1C, peritonitis induced by Natterin was accompanied by elevated levels of cytokines as IL-1β, IL-6, and TNF-α which jointly promotes neutrophil recruitment, as well as the chemokine MCP-1 that regulate the migration and infiltration of macrophages. In contrast, Natterin did not promote the release of KC (CXCL1), chemokine controlling neutrophils recruitment (data not shown). Finally, we observed a production of PGE2, a pro-inflammatory mediator [28] produced in response to Natterin (Fig. 1D).

Our results show that the acute neutrophil recruitment to the site of inflammation induced by Natterin was associated with extracellular release of mature IL-1β, IL-6, TNF-α and PGE2, independent of KC.

2.3. Natterin induces IL-33-expressing bronchial epithelial cells

In the search for the mediators involved in neutrophilic infiltration, we also investigated if Natterin was able to produce IL-33, an alarmin resident constitutively in the nucleus, primarily of epithelial cells, endothelial cells, and fibroblasts that is released when barriers are breached and binds to a heterodimeric receptor comprising ST2 and IL-1 receptor accessory protein - IL-1RAcP [29].

For this, we chose to use of an elegant strain of mice that constitutively express the *il-33* gene built with a fluorescent report, allowing us to localize the IL-33-producing cell subtypes in different tissues after Natterin i.p. injection. We demonstrated the production of IL-33 by bronchial epithelial cells at 2 h post-Natterin i.p. injection in IL-33/citrine reporter (citR) (right, Fig. 2A). Twenty-four hours later Natterin induced several-fold higher IL-33 positive cells compared to 2 h (Fig. 2B). The histological examination of lung sections of both, *WT* or citR mice receiving i.p. injection of sterile PBS (control-mice) revealed absence of IL-33-expressing cells (left, Fig. 2A). Also, no detection of IL-33 was associated with vascular structures of the lung sections from control- or Natterin-injected citR mice (data not shown). No detection of IL-33-expressing cells was observed in peritoneal membrane of control- or Natterin-injected citR mice (Fig. 2C).

2.4. Natterin drives lung neutrophilic inflammation mediated by IL-33/ST2 engagement

To better define the mechanism by which cytokines are involved in neutrophilic inflammation induced by Natterin, we employed knockout mice (*KO*) in the genes of IL-17A, IL-17RA, TNF-α, and *WT* BL/6 mice treated with anti-ST2 receptor neutralizing antibody via i.p. injection, a classical route used for the control of pulmonary inflammation in murine models of asthma, chronic obstructive pulmonary disease (COPD) or bronchopulmonary dysplasia [30–32].

In Fig. 3A, we confirmed that the recruitment of neutrophils promoted by Natterin stimulation was completed dependent on ST2 receptor, since a drastic reduction of 97 ± 0.1% was observed in the number of neutrophils recruited to peritoneal cavities of mice that had blocked ST2 receptors by treatment with anti-ST2 neutralizing Abs 30 min before Natterin injection, confirming the importance of ST2/IL-33 signaling on the neutrophils migration. IL-17A/IL-17RA signaling was also decisive for the recruitment of neutrophils (Fig. 3B), since the deficiency of both genes in BL/6 mice injected with Natterin resulted in a strongly reduction in the number of neutrophils into peritoneal

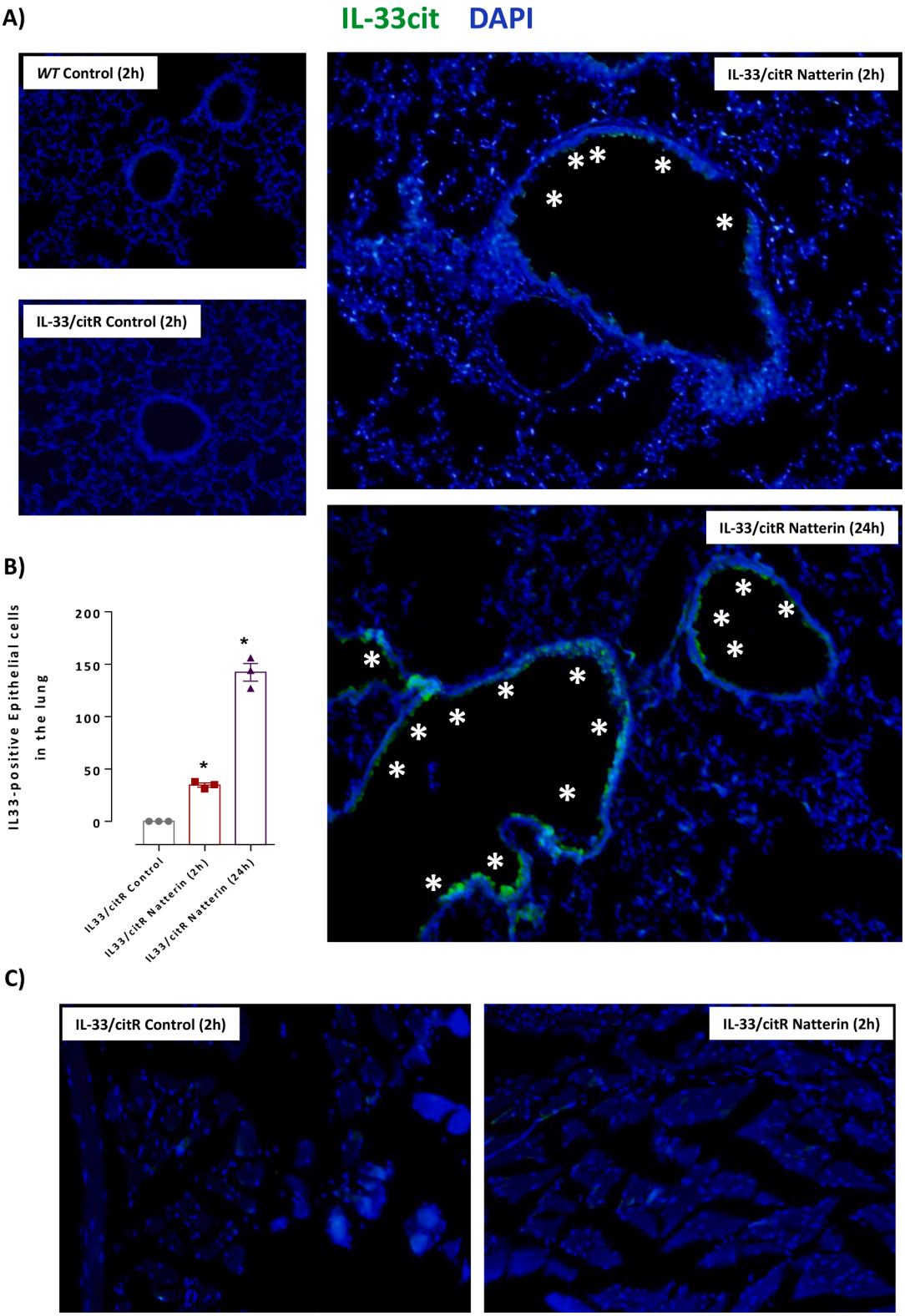


Fig. 2. Natterin induces IL-33-expressing bronchial epithelial cells. Natterin (1 μg) in 500 μL of PBS or PBS alone were injected i.p. into BL/6 wt or IL-33/citrine reporter (citR) mice. After 2 or 24 h, mice were anesthetized, killed and perfused through the ascending aorta with saline followed by 4% formaldehyde. After perfusion, lung (*A*) and peritoneal membrane (*C*) were removed, fixed and kept in 40% sucrose in phosphate buffer, mounted in OCT compound, sectioned at 15 μm on a cryotome and processed for immunofluorescence. All sections were stained with DAPI. IL-33-producing cells (green cells indicated by white asterisks) were imaged with a fluorescence microscope OLYMPUS BX51, objective 10×, magnification bar 200 μm and counted (*B*). For each group of mice, four stained lung sections from each mouse were analyzed and IL-33-positive cells were counting using an image processing package FIJI Image J. *$p < 0.001$ compared with negative-control IL-33 citR mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

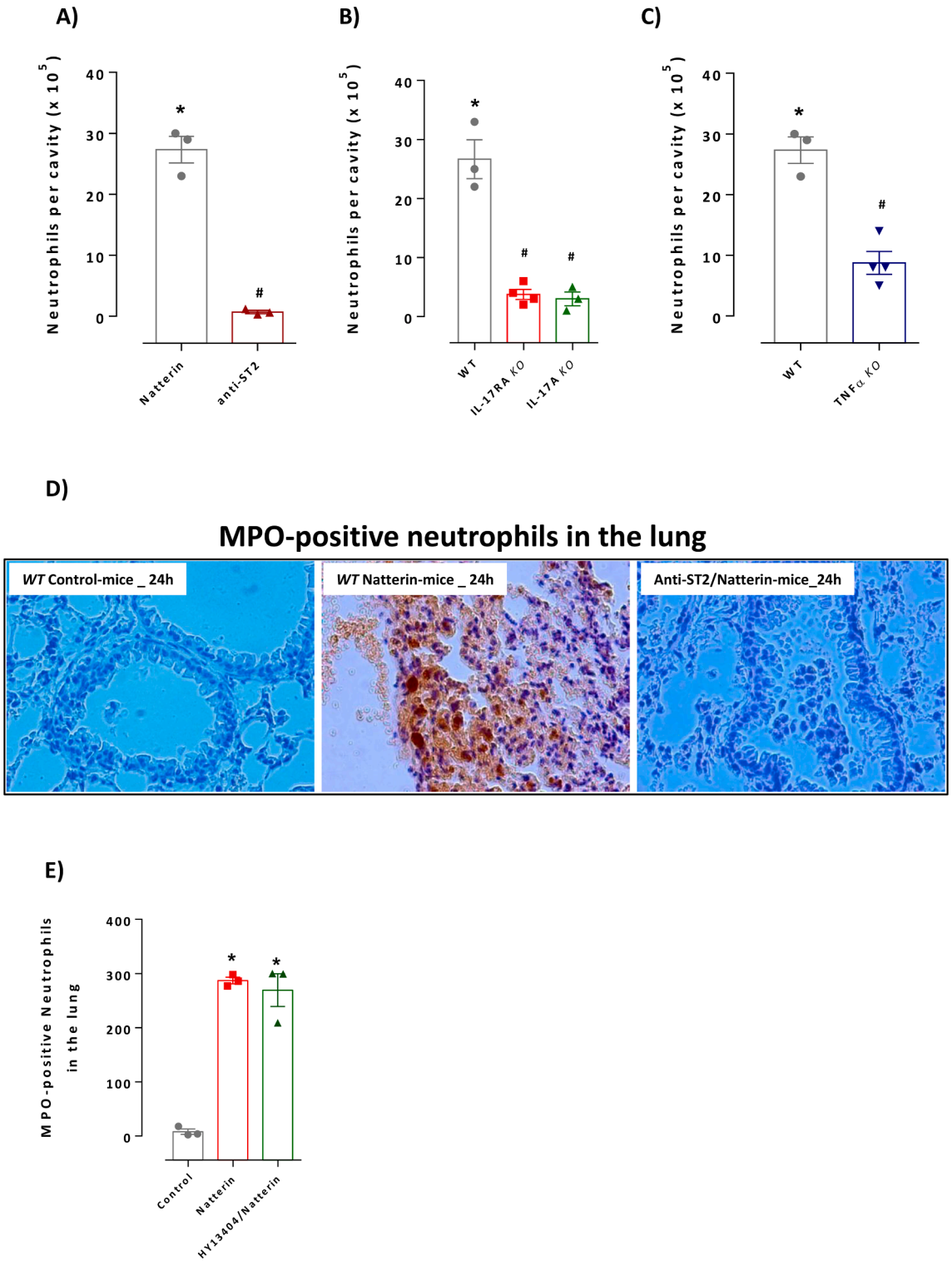


Fig. 3. Natterin drives lung neutrophilic inflammation mediated by IL-33/ST2 engagement. Natterin (1 μg) in 500 μL of PBS was i.p. injected into BL/6 wt mice or deficient in IL-17RA and IL-17A (*B*) or TNF-α (*C*). *WT* mice previously treated with anti-ST2 neutralizing antibody before Natterin injection were also used (*A*). Two hours after Natterin injection, peritoneal cells were harvested and neutrophils were count in cytospin preparation subjected to centrifugation and stained with Diff-Quick Stain kit. An independent group of *WT* mice was also treated 2 times a day 24 h before Natterin injection with HY13404 (*E*). Sections harvested from lungs 24 h post Natterin injection were incubated with anti-MPO. The brown reaction product was detected after incubation with anti-IgM HRP followed by DAB chromogenic substrate. Sections were counterstained with hematoxylin and MPO-positive cells were counted (*D* and *E*). The results represent the mean ± SEM of 3–5 animals/group. *$p < 0.001$ compared with negative-control group and # $p < 0.001$ compared with Natterin-group.

cavities (reduction of 86 ± 0.1% and 89 ± 2%, respectively). Moreover in Fig. 3C we also observed that TNF-α *KO* mice exhibit a partial decrease of 68 ± 2% in neutrophil recruitment to peritoneal cavity 2 h post-Natterin injection compared to Natterin-injected *WT* mice.

The demonstrated production of IL-33 by bronchial epithelial cells at 2 h and mainly at 24 h post-Natterin i.p. injection (Fig. 2A and Fig. 2B) and the complete dependence of ST2 on neutrophil recruitment (Fig. 3A) were accompanied by intense and late infiltration of MPO-positive neutrophils into airways of mice at 24 h (brown color, Fig. 3D) compared to PBS-injected control mice, characterizing a systemic inflammatory syndrome. In contrast, no infiltration of neutrophils was observed into the lungs of mice treated with anti-ST2 receptor neutralizing antibody before Natterin injection (Fig. 3D), implying IL-33/ST2 as a key driver of local (peritoneal cavity) and remote (lung) systemic inflammation induced by Natterin.

For discharged the role of reverse transendothelial migration (rTEM) [33] in the recruitment of neutrophils into the lungs directly from the peritoneal cavity, we treated mice before Natterin injection with selective pharmacological intervention of c-MET by capmatinib [34]. As shown in Fig. 3E the previous treatment of Natterin-injected *WT* mice with a potent and selective c-Met tyrosine kinase inhibitor (HY13404) did not reverse the neutrophilic infiltration into the lungs and mice presented the same amount of MPO-positive neutrophils in the lungs after Natterin injection compared to Natterin-injected *WT* mice, demonstrating that pulmonary infiltration was derived from bone marrow-mature neutrophils and migration through the bloodstream to the inflamed lung tissues. However, we cannot rule out that pulmonary neutrophils came from the pulmonary marginal reservoir. Within the lung alone, this pool constitutes the most prominent reservoir of neutrophils in the systemic circulation (~40% of total body neutrophils), enabling rapid neutrophil recruitment inside of the tissue following injury and/or infection.

2.5. IL-1/IL-1R-dependent neutrophilia requires caspase-1 and caspase-11

To determine the relative contributions of IL-1α and IL-1β to neutrophil recruitment induced by Natterin, we utilize IL-1α/β *KO* or IL-1β *KO* mice and deficient mice in type I IL-1 receptor (IL-1R *KO*). We observed that IL-1R *KO* (73 ± 2%) and IL-1α/β *KO* (82 ± 4%) mice exhibit a significant decrease in neutrophil recruitment to the peritoneal cavity 2 h post-injection. IL-1β *KO* presented an inhibition of 74 ± 2% in the recruitment of neutrophils compared to BL/6 wt Natterin injected-mice, suggesting that another ligand of IL-1R receptor as IL-1α can control the cell infiltration at the site of injury as confirmed in mice that had blocked IL-1α by treatment with anti-IL-1α neutralizing specific monoclonal Abs 30 min before Natterin injection (Fig. 4A).

Next we confirmed the role of caspase-1 and caspase-11 in Natterin-neutrophil mobilization (Fig. 4B). First, we found that ICE *KO* (Casp1$^{-/-}$Casp11$^{129mt/129mt}$) mice deficient in both caspase-1 and caspase-11 were not susceptible to neutrophil recruitment induced by Natterin (decrease of 82 ± 0.4%). Moreover, neutrophilic infiltration in peritoneal cavities of mice was completely prevented when caspase-1 was blocked by the inhibitor YVAD-CMK Ac or caspase-11 by wedelolactone (99 ± 0.1%).

We further investigated the dependence of IL-1α and caspase-1 and caspase-11 on the modulation of Natterin-induced lung neutrophilia. We noticed in Fig. 4D that neutralization of IL-1-α by specific antibody or inhibition of caspase-1 and caspase-11 by treatment with YVAD-CMK and wedelolactone respectively completely blocked neutrophil influx into the lungs 24 h after Natterin injection into peritoneum.

Our results demonstrate that caspase-1 and caspase-11 were required for processing of pro-IL-1β, and together with IL-1α both cytokines control the systemic neutrophilic response.

2.6. Natterin promotes neutrophilic infiltration via gasdermin-D-dependent process

Physiological roles for gasdermin-D in both pyroptosis and IL-1β/ IL-1α release during inflammasome signaling have been extensively characterized in macrophages and other mononuclear leukocytes [35]. Next, we investigated the role of gasdermin-D in the recruitment of neutrophils induced by Natterin and found that like IL-1R1 *KO*, IL-1α neutralizing-mice, and caspase-1/11 *KO* mice, *gasdermin-D* gene deficiency mice also displayed an intense decrease in the number of neutrophils (99% ± 0.1%) recruitment to the peritoneal cavity 2 h post-injection, compared to Natterin-injected *WT* mice (Fig. 4C).

We tested whether pyroptosis drives the influx of neutrophils induced by Natterin in pre-treated mice with glycine, a potent inhibitor of pyroptosis [36]. In Fig. 4C we demonstrated that *WT* mice treated with the cyto-protective agent glycine demonstrated intense neutrophil infiltration following Natterin injection compared to control-mice.

Together, these data demonstrate that the neutrophilic inflammation triggered by Natterin was a gasdermin-D-dependent inflammasome process, despite the cells did not die by pyroptosis and remained viable and continue to participate in the inflammatory response.

2.7. NLRP6/NLRC4, but not NLRP3, drives the neutrophilic infiltration induced by Natterin

NLRP3, the most extensively studied member of inflammasome family has been implicated in the sensing of a multiplicity of pathogens as bacteria, virus, fungus, parasites, and several aerolysin-like pore-forming toxins from bacteria or viruses [37,38]. In response to cellular stress the NLRP3 inflammasome activates multimerization of the adaptor molecule ASC (apoptosis-associated speck-like protein with a caspase recruitment domain) and pro-caspase-1, resulting in the processing and secretion of the pro-inflammatory cytokine IL-1β [39].

Next we tested the functional relevance of NLRP3 and ASC to Natterin-induced neutrophilia. In Fig. 5A, we observed that the recruitment of neutrophils in ASC deficient mice was drastically decreased (80 ± 0.4%) compared to Natterin-injected *WT* mice. However in NLRP3 *KO* or mice treated with the inhibitor of NLRP3-dependent ASC oligomerization, MCC950 [40], the recruitment of neutrophils remained elevated compared with BL/6 wt Natterin-mice.

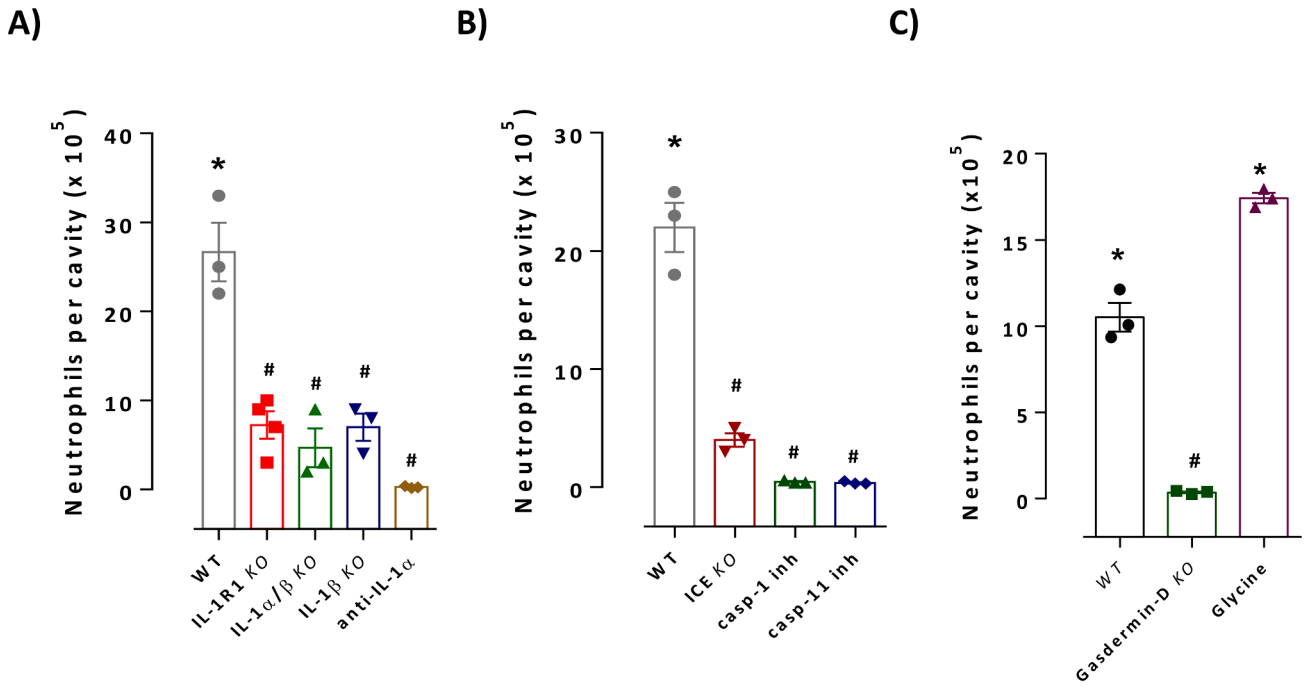
In the search for the requirement for another member besides NLRP3 to activate the ASC/caspases complex assembly, we investigated the participation of sensors such as NLRP6 and NLRC4 adaptor, both expressed and active in epithelial cells [41].

Our results in Fig. 5B demonstrated that the absence of NLRP6 virtually abolished (98 ± 0.5%) the recruitment of neutrophils into the peritoneal cavities of mice injected with Natterin. In Fig. 5C we further found that NLRC4 deficiency in BL/6 mice led to significantly decrease of neutrophil infiltration (62 ± 3%) after injection of Natterin. However, we also confirmed that mitochondrial anti-viral signaling protein (MAVS) a sensor required for recruitment of NLRP3 to the mitochondria and IL-1β production [42] was not involved in neutrophilic inflammation induced by Natterin (Fig. 5D).

These results are indicative that IL-1β/caspase-1/caspase-11-dependent neutrophilic inflammation was mediated by non-canonical sensors through ASC interaction.

3. Discussion

Here in this work, our objective was to investigate the regulatory mechanisms controlling acute neutrophilic inflammation induce by Natterin a family of toxins responsible for the toxic effects of the V*Tn*, specifically its ability to induce IL-1β-dependent neutrophilic inflammation mediated by inflammasome activation. For this, using a combination of genetic loss-of-function and pharmacologic approaches we conduct a systematic investigation in mice of the regulatory mechanisms



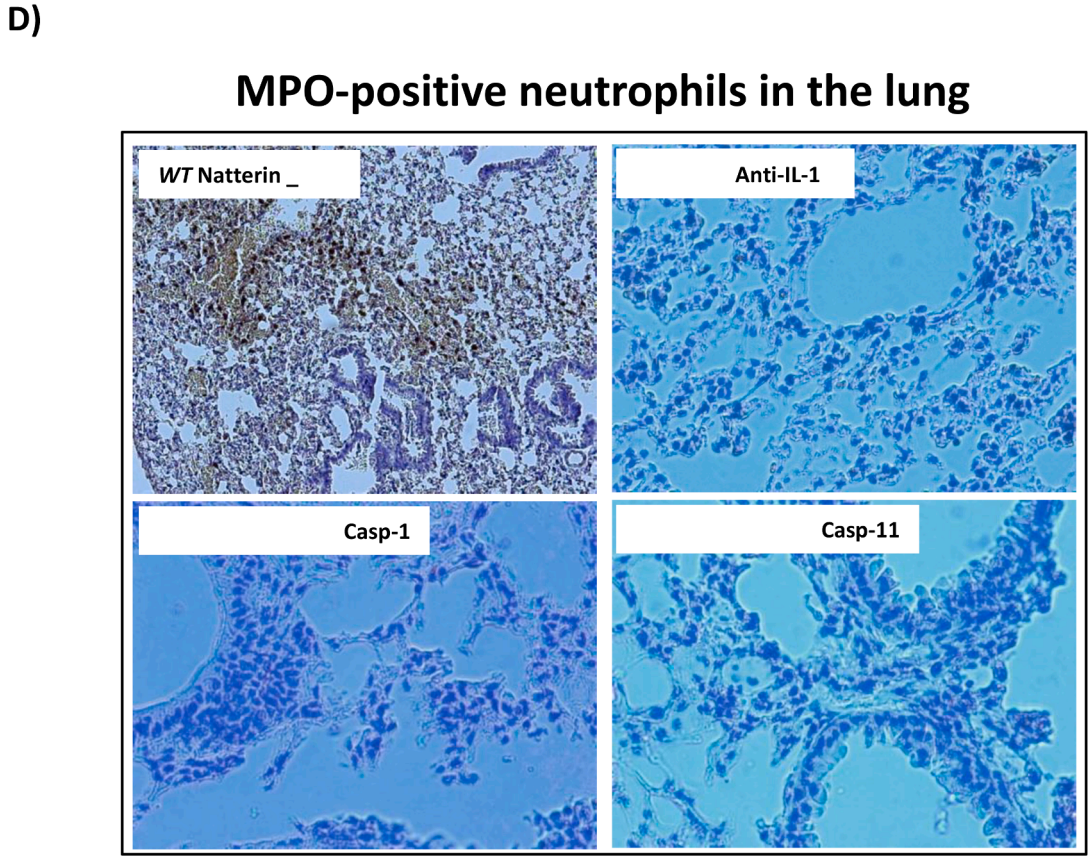


Fig. 4. IL-1/IL-1R-dependent neutrophilia requires caspase-11/caspase-1 and Gasdermin-D. Natterin (1 μg) in 500 μL of PBS was i.p. injected into BL/6 wt or deficient in IL-1R1, IL-1α/β, IL-1β (*A*) or ICE (*B*) or Gasdermin-D (*C*). *WT* mice previously treated with anti-IL-1α neutralizing antibody (*A*); YVAD-CMK Ac and wedelolactone (*B*) or glycine (*C*) before Natterin injection were also used. Two hours after Natterin injection, peritoneal cells were harvested and neutrophils were count in cytospin preparation subjected to centrifugation and stained with Diff-Quick Stain kit. Sections harvested from lungs 24 h post Natterin injection were incubated with anti-MPO. The brown reaction product was detected after incubation with anti-IgM HRP followed by DAB chromogenic substrate. Sections were counterstained with hematoxylin (*D*). The results represent the mean ± SEM of 3–5 animals/group. *$p < 0.001$ compared with negative-control group and # $p < 0.001$ compared with Natterin-group.

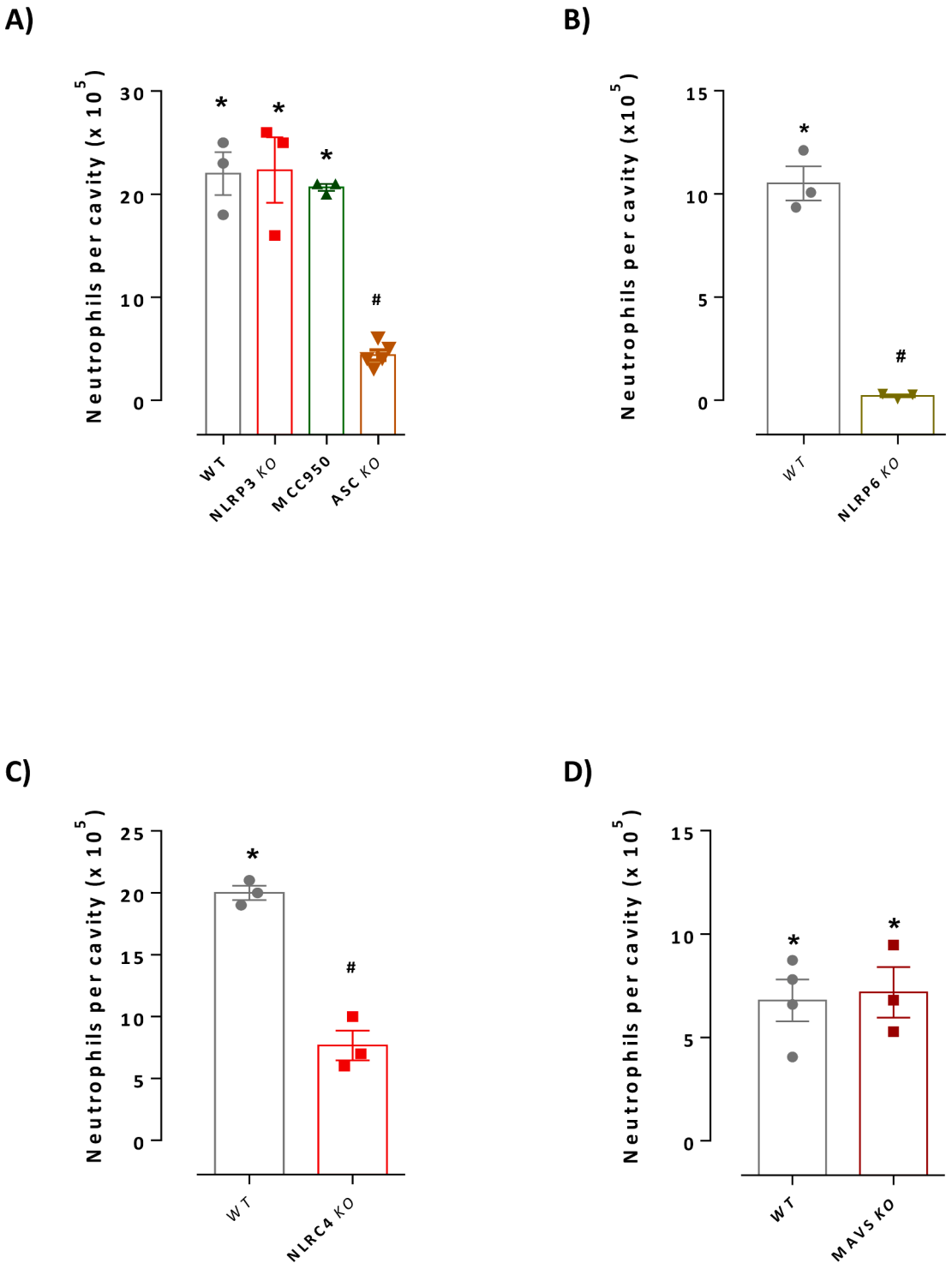


Fig. 5. NLRP6/NLRC4, but not NLRP3 drives the neutrophilic infiltration induced by Natterin. Natterin (1 μg) in 500 μL of PBS was i.p. injected into BL/6 wt or deficient in NLRP3 and ASC (*A*), NLRP6 (*B*), or NLRC4 (*C*) or deficient in MAVS (*D*). *WT* mice previously treated with MCC950 (*A*) before Natterin injection were also used. Two hours after Natterin injection, peritoneal cells were harvested and neutrophils were count in cytospin preparation subjected to centrifugation and stained with Diff-Quick Stain kit. The results represent the mean ± SEM of 3–5 animals/group. *$p < 0.001$ compared with negative-control group and # $p < 0.001$ compared with Natterin-group.

that control Natterin-induced neutrophilic inflammation in the peritonitis model.

Neutrophils are typically the first responders to infection and injury where they help to contain and destroy antigens through a number of mechanisms [43]. Our data confirm the capacity of Natterin to induce a strong and sustained neutrophilic inflammation in murine model of peritonitis leading to systemic inflammatory lung infiltration and reveal overlapping regulatory paths in the control of neutrophilia, including the co-participation of the IL-33/ST2, and IL-17A/IL-17RA signals dependent on IL-1α/IL-β/IL-1R1.

Biologically active cytokine IL-1β is generated by the cleavage of the precursor pro-IL-1β through active caspase-1, a cysteine protease [44].

Recent reports show a role for caspase-11 in regulating the activation of a non-canonical inflammasome that promotes cell death as well as IL-1α and IL-1β secretion and neutrophil recruitment during infection response [45].

Through multiple systemic and local effects, IL-1β is an essential actor of the inflammatory and immune responses. IL-1β induces the expression of multiple pro-inflammatory genes such as IL-6, IL-8, MCP-1 (or CCL2), IL-1α, platelet activating factor, and eicosanoids [46]. By enhancing the production of the alarmin IL-33 and subsequent release by damaged cells, IL-1β promotes neutrophil recruitment and activation to the site of inflammation [47,48]. IL-33/ST2 axis seems to be involved in Th17 [49] response and IL-17A seems to up-regulate IL-33 expression in NHEK, maybe by induction of ERK, p38/MAPK, and JAK/STAT pathways [29].

Further, the participation of IL-33 in neutrophil infiltration was demonstrated be dependent on IL-33R expression on peritoneal mast cells and on dependent- or independent mast cell release of TNF-α [50,51]. Our data here highlight the view that neutrophils and Th17 cells establish a communication network in chronic inflammatory diseases, once we confirmed that Natterin-induced systemic neutrophilia was totally dependent on the binding of IL-33 to its ST2 receptor with the entire participation of IL-17A/IL-17RA signaling, as well as partially the cytokine TNF-α.

An increasing body of evidence supports the role of pro-inflammatory cytokines like IL-1β, IL-17 and TNF-α in the pathophysiology of neutrophilic diseases similarly to classic monogenic auto-inflammatory diseases, suggesting common physiopathological mechanisms [52]. Neutrophilic inflammation, Th1 and Th17 cells establish a communication network in the airways of some severe asthmatics, refractory to treatment by inhaled or oral corticosteroids. Also, the association between IL-17 and neutrophilic inflammation is well-documented in other diseases such as psoriasis, where targeting the IL-17R with Brodalumab reduces neutrophil infiltration of the skin with improvement in disease.

Our data confirming the role of IL-33/ST2 as a mechanism underlying the systemic effect of Natterin inducing the recruitment of neutrophils to the inflamed cavity and later to the lung highlight that Natterin is a potent pro-inflammatory molecule and are evidence that a large number of cells may sense and respond to Natterin. In addition, we can infer that the envenomation caused by *T. nattereri* can trigger a systemic inflammatory response in human patients requiring more intense care.

Altogether our data adds another level of complexity to the Natterin-induced neutrophilic inflammatory process. First, we demonstrated that the IL-1/IL-1R-dependent neutrophilic inflammation induced by Natterin was mediated by caspase-1 and caspase-11 activated by the inflammasome sensor NLRP6 in an ASC dependent-manner. And second, we found that the neutrophilic inflammation triggered by Natterin was gasdermin-D-dependent, but was not affected by glycine treatment. In murine macrophages, glycine markedly delays pyroptosis (via an unknown mechanism), but does not inhibit IL-1β release or assembly of N-gasdermin-D pores [53,54], which led us to think that the neutrophilia induced by IL-1-dependent Natterin is mediated by its excretion in the gasdermin-D-dependent process without the requirement of cell lysis by pyroptosis, as recently described by others [55–60].

Unlike several studies showing that different bacterial [61,38,17,37] aerolysin-like pore-forming toxins open pores in the membrane bilayer and trigger NLRP3 inflammasome-dependent caspase-1 activation leading to IL-1β-dependent neutrophilic inflammation, the activation of NLRP6/NLRC4 inflammasome by Natterin seems to be determinant for the for neutrophil recruitment, independently of NLRP3.

NLRP6 has a wide range of functions in innate immune signaling suppressing inflammation [62,63] or playing a role as an inflammasome [64–70]. Recently, Leng et al [71] provided biochemical and cellular evidences of an alternative intracellular LPS recognition mechanism through NLRP6. NLRC4 inflammasome acts as an adaptor to recruit and activate caspase-1 and requires NAIP for binding to ligands [72–74]. We proposed that cytosolic Natterin or DAMPs (*host-derived danger associated molecular patterns) generated in response to Natterin* could be recognized by NLRP6 facilitating their interaction with NLRC4 allowing inflammasome assembly through ASC interaction and resulting in caspase-11 activation, corroborating the involvement of NLRP6 in inflammation.

Interestingly, our data extend those previously published by others showing NLRP3 as the most extensively member of inflammasome family implicated in the sensing of several aerolysin-like pore-forming toxins from bacteria or other such as fungal trichothecene mycotoxin [75], viral viraporins [76], bee venom melittin [77], and the frog aerolysin-like [78] and emphasized that the NLRP6/NLRC4-dependent neutrophil-mediated response may be part of an innate immune mechanism underlying aerolysin from fish venoms.

In conclusion, we were able to clarify that the IL-1β-dependent neutrophilic inflammation induced by Natterin is the result of non-canonical activation of the inflammasome complex with the participation of cytosolic NLRP6/NLRC4 sensors. Our data highlight that this inflammatory process dependent on non-canonical inflammasome activation can be a target for pharmacological intervention in accidents by *T. nattereri*, which does not have adequate specific therapy.

4. Methods

4.1. Mice

Male or female 7–8 weeks old C57BL/6J wild types (*WT*) or IL-1α/β-, IL-1β-, IL-1R-, ICE- ($Casp1^{-/-}Casp11^{129mt/129mt}$), NLRP3-, ASC-, IL-17RA-, IL-17A-, NLRP6-, NLRC4-, MAVS-, Gasdermin-D-, and TNF-α-deficient mice – *KO*, and IL-33/citrine reporter (citR) mice (all on a BL/6 background) were obtained from a colony at Transgenose Institute (Orleans, France). Mice were maintained in sterile microisolators with sterile rodent feed and acidified water, and were housed in positive-pressure air-conditioned units (25 °C, 50% relative humidity) on a 12 h light/dark cycle. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian and French College of Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Butantan Institute (Permit Number: 9381060819) and of Transgenose Institute (Permit Numbers: 1085 and 1088).

4.2. Natterin preparation

The purified 30–45 kDa Natterin proteins from *T. nattereri* fish venom were prepared with a pool of venom collected in different months of the year in the state of Alagoas. The venom was fractionated by cation exchange chromatography, using the fast protein liquid chromatography system (FPLC - Pharmacia, Uppsala, Sweden) according to Lopes-Ferreira et al [9]. Endotoxin content was evaluated (resulting in a total dose < 0.8 pg LPS) with QCL-1000 chromogenic Limulus amoebocyte lysate assay (Bio-Whittaker) according to the manufacturer's instructions.

4.3. Acute inflammation induced by Natterin and pharmacological treatments

WT or *KO* mice ($n = 3$–5/group) were intraperitoneally (i.p.) injected with Natterin at 0.1, 1 or 10 μg in 500 μL. As negative-control mice were injected i.p. with PBS. After 2, 6, and 24 h the peritoneum exudates were harvested for total and differential cell count and for processed proteins determination. Independent group of *WT* mice was pre-treated 30 min before with MCC950 at 100 mg/Kg; anti-ST2 neutralizing antibody at 25 μg/mL (16–9333-82, eBioscience™); anti-IL-1α neutralizing antibody at 2 μg/mL (IgG mabg-mIL-1α-5, Invivogen); YVAD-CMK Ac at 1.8 mg/mL, (17–8603-78–6, Invivogen); wedelolactone at 80 μM (SC-

200648, Santa Cruz); and glycine at 5 mM (00163, Chem-Impex). An independent group of mice was also treated 2 times a day 24 h before Natterin injection with capmatinib at 10 mg/Kg (HY13404, INCB28060, MedChenExpress). Groups of mice were injected with an equivalent volume of NaCl, PBS or isotype control (rat IgG, or rat IgG1 or rat IgG2b). Mice were killed after 2 or 24 h for analysis.

4.4. Peritoneal cell suspension collection

At time points indicated after Natterin injection, mice were killed, and peritoneum exudates were harvested with 2 × 2.5 mL of cold PBS + 10 mM EDTA for cell suspensions that were centrifuged at 1500 rpm for 10 min at 4 °C. The supernatants were stored at −20 °C; and cell pellets were resuspended in 1 mL of PBS + 0.1% BSA. The total leukocyte count was performed in a 10 squares of Malassez chamber with Turk solution. For differential counts, aliquots containing 100 μL of cell suspension were applied on glass slides, subjected to centrifugation at 1000 rpm for 10 min and stained Cytospin with kit Diff-Quick Stain Set and analyzed in an optical microscope with a 40× objective. For differential cell counts, 300 leukocytes were enumerated and identified as mononuclear cells, eosinophils or polymorphonuclear neutrophils, based on staining and morphologic characteristics using a light microscope Axio Imager A1 (Carl Zeiss, Germany) with an AxioCam ICc1 digital camera (Carl Zeiss).

4.5. Cytometric bead array for determination the level of cytokines

Concentrations of peritoneal exudates of IL-6 and TNF-α were determined by cytometric bead array (CBA), according to the manufacturer's protocol (560485, BD Biosciences) with minor modification. Briefly, 50 μL samples were subjected to analysis in duplicate using the cytometric bead array kit on a FACSCalibur cytometer. Cytokine concentrations were quantified using CellQuestPro and CBA software (Becton Dickinson). The detection limits for IL-6 and for TNF-α were 1.4 pg/mL and 0.9 pg/mL, respectively.

4.6. Quantification of IL-1β and MCP-1

IL-1β, KC and MCP-1 levels were measured in exudates of peritoneal cavity using a specific two-site sandwich ELISA with OpEIA Kits (BD-Pharmingen, San Diego, CA, USA). Biotinylated monoclonal antibodies binding has been detected using streptavidin-horseradish peroxidase complex and TMB (3, 3′, 5, 5′-tetramethylbenzidine) substrate solution containing hydrogen peroxide. Detection limit was 7.8 pg/mL for both IL-1β and MCP-1 and 25 pg/mL for KC.

4.7. Eicosanoid assay

Concentrations of PGE2 were measured in the peritoneal exudate lavage fluid collected 2 h after Natterin or PBS injection, by a specific enzymatic immunoassay, using a commercial kit (Cayman Chemicals, MI, USA). In brief, 100 μL aliquots of each sample were incubated with the eicosanoid conjugated with acetyl cholinesterase and the specific rabbit antiserum in 96-well microtitration plates, coated with anti-rabbit IgG mouse monoclonal antibody. After addition of the substrate, the absorbances of the samples were recorded at 412 nm in a microplate reader, and concentration of the eicosanoid was estimated from standard curve.

4.8. Immunofluorescence for detection of IL-33

Natterin (1 μg) or sterile saline in 500 μL were injected i.p. into BL/6 wt or IL-33/citrine reporter (citR) mice. After 2 or 24 h mice were anesthetized with a ketamine (50 mg/mL)/xylasine (25 mg/mL) and perfused through the ascending aorta with saline followed by 4% formaldehyde. After perfusion, lung and peritoneal membrane were removed and post fixed. For cryosectioning, fixed organs were transferred to 40% sucrose in phosphate buffer, mounted in OCT compound (Neg-50, Richard Allan), and sectioned at 15 μm on a cryotome (Cryostat HM 505E) and processed for immunofluorescence. All of the sections were stained with DAPI (sc-300415) and fluoromount-G (00–4958, eBioscience) was added to the slides prior to mounting with cover slips and imaged with a fluorescence microscope OLYMPUS BX51, objective 10×, magnification bar 200 μm. For each group of mice, four stained lung sections from each mouse were analyzed and IL-33-positive cells were counting using an image processing package FIJI Image J.

4.9. Immunohistochemical for detection of MPO in the lungs

Sections harvested from lungs 24 h post Natterin injection were deparaffinized, rehydrated, and incubated in 3% H_2O_2 in PBS for 10 min at room temperature to block endogenous peroxidase activity. The sections were then washed with PBS three times, incubated in 0.1% trypsin (Sigma Chemical) in 0.1% $CaCl_2$ at 37 °C for 10 min, and rinsed with 4 M HCl for 15 min. After nonspecific binding blocking the lung sections were incubated for 1 h with anti-MPO (sc-271881, Santa Cruz, at 1/500). After incubation, anti-IgM HRP (A8786, Sigma, at 1/5000) was detected by incubating the sections for 5 min with chromogenic substrate for the peroxidase 3, 3′-diaminobenzidine (DAB) (Sigma), providing a brown reaction product. Sections were counterstained with hematoxylin and lungs were imaged with an upright microscope (Axiolab, Carl Zeiss, Oberkochen, Germany) coupled to a photographic camera (AxioCam Icc1, Carl Zeiss, Oberkochen, Germany) using a 10/0.3 longitudinal distance objective/numeric operture and 1.6 optovar (Carl Zeiss, Oberkochen, Germany). For each group of mice, four stained lung sections from each mouse were analyzed and MPO-positive cells were counting using an image processing package FIJI Image J.

4.10. Statistical analysis

All values were expressed as mean ± SEM. Experiments using 3 to 5 mice per group were performed independently two times. Parametric data were evaluated using analysis of variance, followed by the Bonferroni test for multiple comparisons. Non-parametric data were assessed using the Mann-Whitney test. Differences were considered statistically significant at $p < 0.001$ using GraphPad Prism (Graph Pad Software, v6.02, 2013, La Jolla, CA, USA).

CRediT authorship contribution statement

Maria Alice Pimentel Falcao, Aline Ingrid Andrade-Barros, Ana Carolina Seni-Silva, Lidiane Zito Grund, Eniko Balogh, and Katia Conceiçao: Formal analysis, Investigation, Methodology, Writing - original draft. **Valerie F. Queniaux and Bernhard Ryffel:** Formal analysis, Writing - original draft. **Monica Lopes-Ferreira and Carla Lima:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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