ARQUIVOS DE ASMA, ALERGIA E IMUNOLOGIA

Original Article

Distinctive inflammasome activation in individuals allergic to Hymenoptera venoms

Resposta distintiva do inflamassoma aos venenos de himenópteros em indivíduos alérgicos

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ABSTRACT

Introduction: Previous studies have shown that different types of venoms, such as those from snakes, arachnids, and Hymenoptera insects, can active the inflammasome complex, particularly through the NLRP3 receptor, as seen with the honeybee (*Apis mellifera*) venom component melittin. Furthermore, inflammasome activation has been linked to increased susceptibility to hypersensitivity. This study aimed to investigate inflammasome activation in individuals allergic to Hymenoptera venoms based on the hypothesis that allergic individuals may exhibit enhanced inflammasome activity in response to Hymenoptera venoms. **Methods:** Ten allergic individuals and 17 nonallergic healthy adults were recruited. Inflammasome activation was determined by immunofluorescence detection of activated caspase-1 using the FAM-FLICA kit and pyroptosis. Cytokine levels were measured by ELISA. **Results:** Allergic individuals exhibited constitutive inflammasome-derived cytokine release and an exaggerated inflammasome response to honeybee, wasp, and fire ant venoms in monocyte-derived macrophages, as evidenced by elevated interleukin-1ß levels and pyroptosis. **Conclusion:** These findings suggest that, in addition to the immunoglobulin E-mediated response, a proinflammatory profile may also contribute to hypersensitivity to insect venom.

Keywords: Inflammasome allergy, Hymenoptera venom, IL-1ß, pyroptosis, pyrin domain-containing 3 protein NLR family.

RESUMO

Introdução: Estudos anteriores mostraram que diferentes tipos de veneno, como os de cobras, aracnídeos e insetos *Hymenoptera*, podem induzir a ativação do complexo inflamatório, e especificamente do seu receptor NLRP3, como o componente melitina do veneno da abelha (*Apis mellifera*). Além disso, o complexo inflamatório pode aumentar a suscetibilidade à hipersensibilidade. Este estudo teve como objetivo caracterizar a ativação do inflamassoma em indivíduos alérgicos ao veneno de himenópteros a partir da hipótese de que a ativação do inflamassoma pode estar desregulada em indivíduos alérgicos e, portanto, contribuir para o traço alérgico. **Métodos:** Dez indivíduos alérgicos e 17 adultos saudáveis não alérgicos foram recrutados. A ativação do inflamassoma foi medida por imunofluorescência da caspase-1 ativada com o kit FAM-FLICA e pela piroptose. As citocinas foram medidas por ELISA. **Resultados:** Indivíduos alérgicos apresentam uma liberação constitutiva de citocinas derivadas do inflamassoma e uma hiper-resposta do inflamassoma aos venenos de abelha, vespa ou formiga "fire" em macrófagos derivados de monócitos, tanto em termos de liberação de IL-1ß quanto de piroptose. **Conclusão:** Os achados do estudo sugerem que um contexto pró-inflamatório pode influenciar a reação alérgica ao veneno de inseto além da resposta relacionada à IgE.

Descritores: Alergia ao inflamassoma, veneno de Hymenoptera, IL-1ß, piroptose, proteína 3 que contém domínio de pirina da família NLR.

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Funding statement: This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under grant number 2021/05420-4.

Submitted April 22 2024, accepted July 1 2024.

Arq Asma Alerg Imunol. 2024;8(2):139-45.

http://dx.doi.org/10.5935/2526-5393.20230086-en

Introduction

Stings from Hymenoptera insects, such as honeybees, wasps, and fire ants, can trigger allergic reactions or even cause fatal anaphylaxis.[1] Although immunoglobulin E (IgE)-mediated type I hypersensitivity is recognized as a primary immediate reaction, the precise molecular mechanisms triggered by injected venom, as well as the subsequent innate immune response, remain poorly understood.

Previous studies have shown that different types of venom, such as those from snakes, arachnids, and Hymenoptera insects, can activate the inflammasome complex,[2] particularly through the NLRP3 receptor, as seen with the honeybee (*Apis mellifera*) venom component melittin.[3] Moreover, activation of the inflammasome complex may increase susceptibility to hypersensitivity reactions.[4-5] The inflammasome-dependent cytokine interleukin (IL)-18 has been associated with the development of allergic disorders,[6] airway inflammation in asthma,[7] eosinophilic activity,[8] and the activation of Th2 cytokines.[9]

Considering these findings and the increasing body of evidence regarding the role of the NLRP3 inflammasome in allergic conditions,[4-5,10] we hypothesized that allergic individuals may exhibit enhanced inflammasome activity in response to Hymenoptera venom.

Patients and methods

Study participants

All participants, including patients and healthy donors (HDs), provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committees (CAEE no. 55881222.0.0000.0068 and no. 52647921.8.0000.5467). We recruited 10 allergic individuals from the Allergy and Clinical Immunology Clinic at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (FMUSP), Brazil, and 17 healthy nonallergic adults (HD) from the general community at the Institute of Biomedical Sciences of Universidade de São Paulo, Brazil (Table 1).

In vitro assays and inflammasome analysis

Peripheral blood monocyte-derived macrophages (MDMs) were obtained from volunteers as described elsewhere.[11] They were treated with venom extracts from *Apis mellifera*, *Polybia paulista*, and *Solenopsis invicta*, with or without priming with lipopolysaccharide (*E. coli* O111:B4 strain; Sigma-Aldrich/Merck) for 3 hours. Dose- and time-response are shown in Figure 1. ATP (Sigma-Aldrich/Merck) was added at the end of the stimulation period. MDMs were pretreated with the NLRP3 inhibitor MCC-950 (InvivoGen) when applicable. Inflammasome activation was determined by immunofluorescence staining of activated caspase-1 using the FAM-FLICA kit (Immunochemistry Technologies), alongside measurements of IL-1ß release and pyroptosis.

Cytokine quantification

Cytokine levels were determined using commercial ELISA kits (R&D Systems; Biolegend).

Pyroptosis analysis

Propidium iodide (PI; Thermo Fisher Scientific) uptake was measured using real-time fluorometric analysis (Synergy instrument; BioTek Instruments) or immunofluorescence staining. Lactate dehydrogenase (LDH) release was quantified using a commercial kit (ThermoFisher Scientific). Cleavage of gasdermin D (GSDMD) was assessed via western blot analysis.

Data analysis

The public dataset GSE92866 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE92866) was reanalyzed using the GEO2R software (https://www.ncbi.nlm.nih.gov/geo/) and the EnrichR platform (https://maayanlab.cloud/Enrichr/).

Student's *t*-test or ANOVA was employed to compare two or more groups (or variables). Categorical variables were evaluated using Fisher's exact test, and Spearman's correlation analysis was performed.

Results

Allergic individuals (n=12) from the public database GSE92866 exhibited enrichment of proinflammatory genes and pathways compared with HD (n=12) (Figure 2A-B), suggesting that a proinflammatory profile may contribute to hypersensitivity. Accordingly, allergic patients exhibited significantly elevated plasma levels of IL-6 and IL-18 compared with HD (Figure 2C-D), strongly suggesting constitutive and systemic inflammasome activation in allergic donors.

Table 1
Clinical data from Hymenoptera allergic patients

ID	Sex (F/M)	Age (years)	Insect	Atopy	Degree of anaphylaxis	Total serum IgE (UI/mL)	Allergic crysis at sampling
1	M	42	*Apis mellifera*	None	–	9	No
2	M	33	*Apis mellifera*	Allergic rhinitis	III	387	No
3	M	46	*Apis mellifera*	Allergic rhinitis	IV	N/A	No
4	F	40	*Apis mellifera*	Food allergy	III	N/A	Yes
5	M	30	*Polybia paulista*	None	IV	N/A	No
6	M	54	*Polybia paulista*	Asthma	III	116	No
7	M	10	*Solenopsis invicta*	Allergic rhinitis, asthma, and atopic dermatitis	III	1563	Yes
8	F	23	*Solenopsis invicta*	Allergic rhinitis	III	87.7	Yes
9	M	5	*Solenopsis invicta*	Allergic rhinitis, asthma, and food allergy	III	519	No
10	M	13	*Solenopsis invicta*	Allergic rhinitis, asthma, and atopic dermatitis	III	1832	No

IgE = immunoglobulin E; M = male; F = female; N/A = not available.
Clinical data obtained at the Ambulatório de Alergia e Imunologia Clínica from Hospital das Clínicas e Faculdade de Medicina, Universidade de São Paulo (HC-FMUSP).

In vitro assays showed that insect venoms induced rapid and pronounced release of IL-1ß (Figure 2E-G), but not IL18 (undetectable), and tumor necrosis factor (TNF) (Figure 2H-J) in MDMs from HD and allergic donors. IL-1ß, but not TNF, was significantly elevated in allergic individuals compared with HD (Figure 2H-J). Honeybee venom caused the most severe membrane damage in MDMs, occurring more rapidly in allergic donors than HD (Figure 2K-P). Wasp and fire ant venoms caused moderate damage in MDMs, which was also more pronounced in allergic donors than HD.

These findings suggest venom-associated inflammasome activation, as supported by the correlation between IL-1ß levels and PI uptake (Figure 3A). The venoms rapidly activated caspase-1,

leading to loss of membrane permeability (Figure 3B-C), indicative of pyroptosis. Western blot analysis revealed that ant and wasp venoms, but not honeybee venom, enhanced cleavage of GSDMD (Figure 3D-E). LDH release was elevated in all venom-treated MDM cultures (Figure 3F). The NLRP3 inhibitor MCC-950 significantly reduced IL-1ß levels and PI uptake induced by all venoms, underscoring the central role of NLRP3 inflammasome activation in response to Hymenoptera venoms (Figure 3G-H).

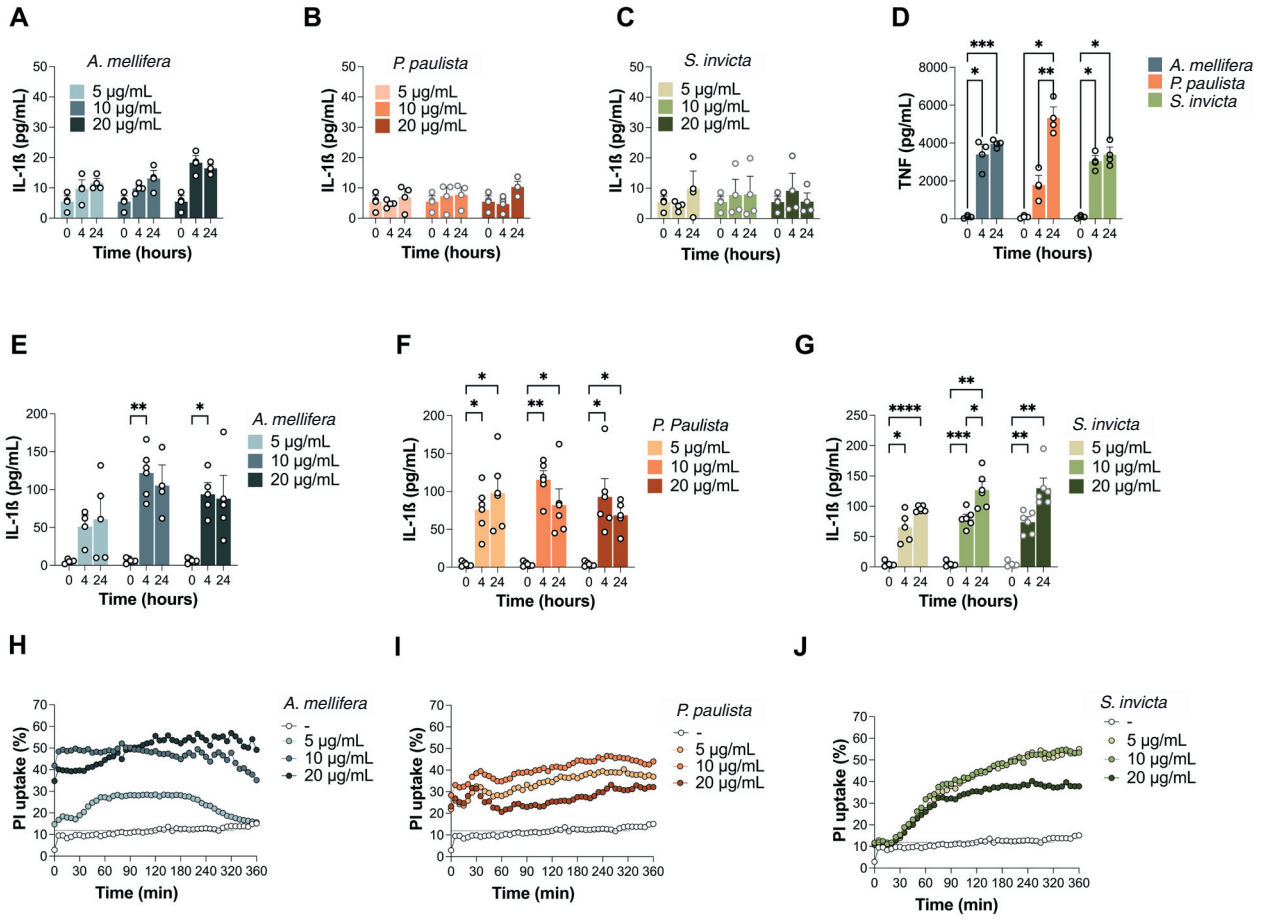

Figure 1
Standardization of stimulation time and concentration of Hymenoptera venoms

Monocyte-derived macrophages (MDMs) from healthy donors (8×10^4 cells) were stimulated for 4 or 24 hours with 5, 10, and 20 µg/mL of venom extracts from (A) *Apis mellifera* (n=6), (B) *Polybia paulista* (n=5), and (C) *Solenopsis invicta* (n=6) without lipopolysaccharide priming. Interleukin (IL)-1ß levels were measured by ELISA. MDMs (8×10^4 cells) were also stimulated for 1, 4, or 24 hours with 5, 10, and 20 µg/mL of venom extracts from (E) *Apis mellifera* (n=6), (F) *Polybia paulista* (n=5), and (G) *Solenopsis invicta* (n = 6) after priming with lipopolysaccharide (1 µg/mL) for 3 hours. IL-1ß levels were measured by ELISA. (D) Tumor necrosis factor (TNF) concentrations were determined by ELISA in the supernatants of MDMs stimulated with 10 µg/mL of each venom for 1 and 4 hours. For propidium iodide (PI) uptake, approximately 2.5 × 104 MDMs were stimulated with 5, 10, and 20 µg/mL of venom extracts from (H) *Apis mellifera* (n=4), (I) *Polybia paulista* (n=4), and (J) *Solenopsis invicta* (n=4) for 4 hours. 2.5 µg/mL of PI per well was added before the start of real-time fluorometric readings. The kinetics of PI incorporation are reported. Two-way ANOVA, followed by multiple post-test comparisons, was applied to compare cytokine production between the 3 experiments. Differences were considered statistically significant when $p < 0.05$.

ns= nonsignificant; * = $p < 0.05$; ** = $p < 0.01$.

Discussion

Understanding and managing the immune response to Hymenoptera venom allergy is crucial for at-risk individuals. While Th2 response and IgE production typically characterize type-1 hypersensitivity reactions, our findings suggest that atopic individuals also exhibit enhanced inflammasome activation, even during asymptomatic periods. This aligns with other medical hypothesis[6-8] and previous genetic studies that have associated NLRP3 activation with type-1 hypersensitivity, such as aspirin and food allergies and asthma.[4-5,10]

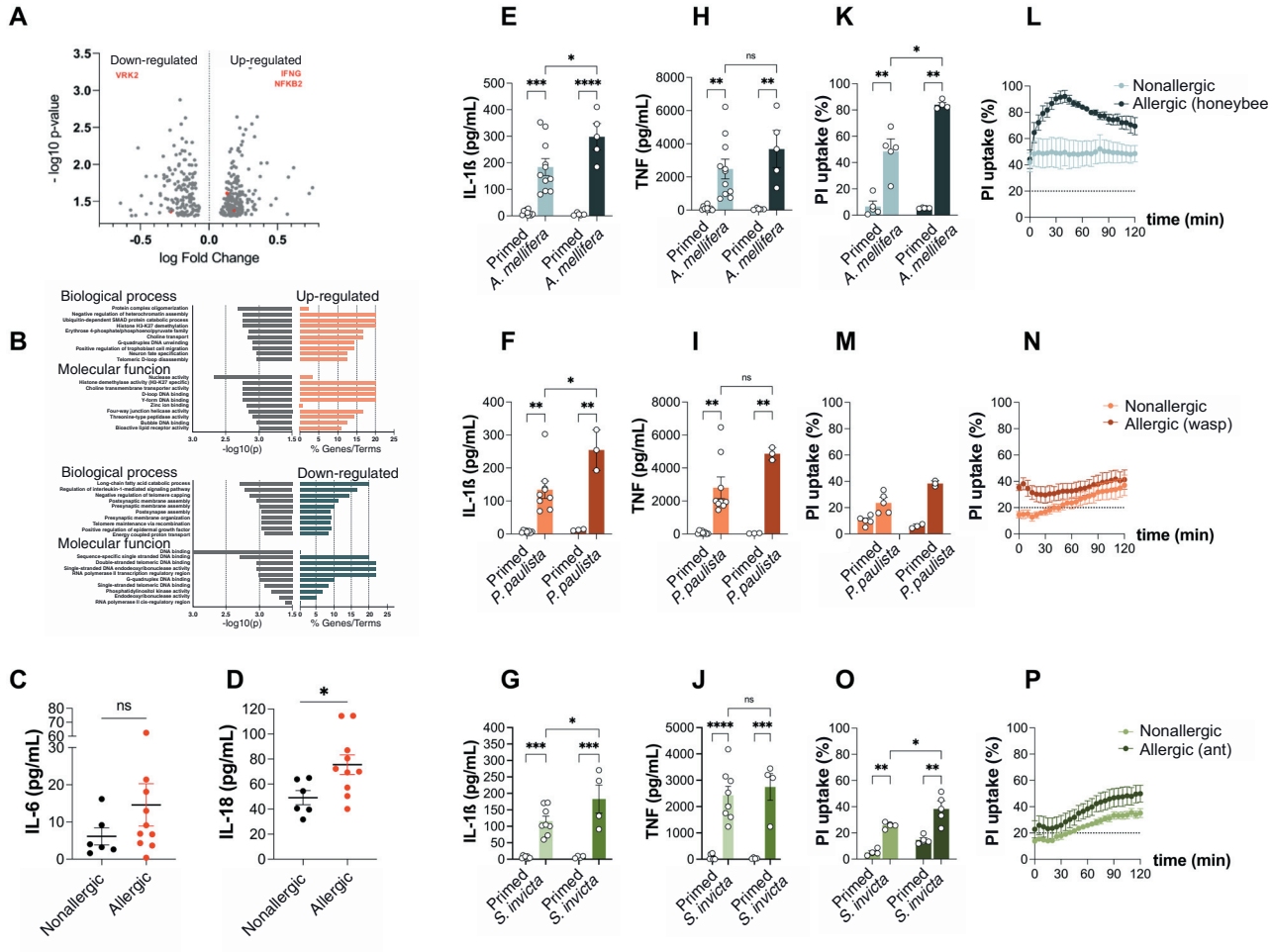

Figure 2

Inflammasome activation by Hymenoptera venoms in allergic and nonallergic donors

(A-B) Reanalysis of the GSE92866 database. (A) Differentially expressed genes (DEGs) between allergic patients and nonallergic individuals are shown in a volcano plot. Genes with fold change (FC) > 1.5 and p < 0.05 were considered DEGs. DEGs of interest are depicted in red. (B) Gene ontology enrichment analysis identified gene sets characteristic of allergic patients. Up-regulated pathways are shown in the upper graph, while down-regulated pathways are shown in the bottom graph. (C-D) Plasma concentrations of interleukin (IL)-6 and IL-18 were measured by ELISA in allergic (n=10) and nonallergic (n=17) donors. Monocyte-derived macrophages (MDMs) from allergic (n=10) and nonallergic (n = 17) donors were primed with lipopolysaccharide (1 µg/mL) for 3 hours and then treated with *Apis mellifera*, *Polybia paulista*, and *Solenopsis invicta* venoms (10 µg/mL) for 4 hours. IL-1ß (E-G) and tumor necrosis factor (TNF) (H-J) release was measured in MDM supernatants from both groups by ELISA. Propidium iodate (PI) uptake was measured over 120 minutes after venom treatment, with the percentage of PI-positive cells reported at 60 minutes (K, M, O) and throughout 120 minutes in real-time (L, N, P).

Two-way ANOVA, followed by multiple post-test comparisons, was applied to compare cytokine production and PI uptake between the 3 experiments. Differences were considered statistically significant when p < 0.05.

ns = nonsignificant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001.

Venoms from several Hymenoptera insects, including wasps and ants in addition to honeybees,[3] lead to inflammasome activation and may serve as initial triggers for the development of type-1 hypersensitivity. Ant and wasp venoms, but not honeybee venom, significantly enhanced cleavage of GSDMD, which is in agreement with earlier studies on honeybee venom.[3] This suggests that distinct molecular mechanisms underlie venom-induced inflammasome activation.

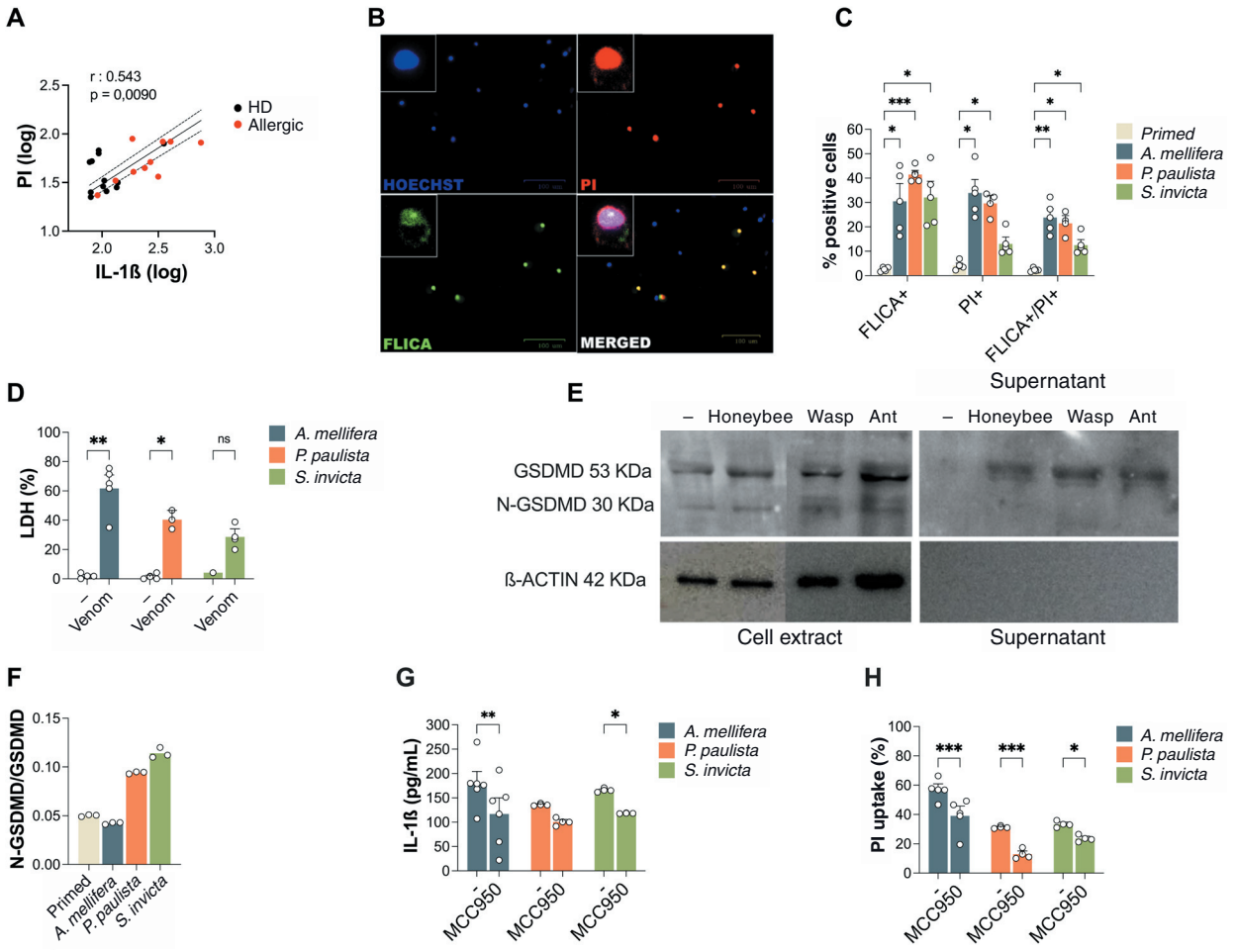

Figure 3

Hymenoptera venoms activate the inflammasome complex and promote pyroptosis

Monocyte-derived macrophages (MDMs) from healthy donors (n=10) were primed with lipopolysaccharide (1 µg/mL) for 3 hours and then treated with *Apis mellifera*, *Polybia paulista*, and *Solenopsis invicta* venoms (10 µg/mL) for 4 hours. Activation of caspase-1 was assessed using the FAM-FLICA kit. Interleukin (IL)-1ß and lactate dehydrogenase (LDH) release was measured in the supernatants, and propidium iodate (PI) uptake was measured after 60 minutes of venom treatment. Cells were lysed for western blot analysis. (A) Correlation analysis was performed between IL-1ß levels and PI uptake in venom-treated MDMs, with Spearman's r and p-values reported. (B) Immunofluorescence images (100× magnification) show active caspase-1-positive (green) and PI-positive (red) cells, with nuclei stained using Hoechst (blue) in untreated and wasp venom-treated MDMs. (C) Total counts of MDMs positive for activated caspase-1 and PI in 3 fields/experiments for each venom are reported. (D) LDH release was measured using the LDH Activity Assay Kit (ThermoFisher Scientific) and expressed as the percentage of Triton-X100 lysed cells. (E) A representative western blot shows GSDMD cleavage (55 kDa) into N-GSDMD (33 KDa), with ß-actin as the loading control. (F) Data from 3 western blots are reported as mean fluorescence intensity (MFI) for GSDMD and N-GSDMD, normalized to ß-actin MFI. MDMs from healthy donors (n=10) were primed with lipopolysaccharide (1 µg/mL) for 3 hours, then treated with the NLRP3 inhibitor MCC-950 (10 µM) before exposure to the venoms (10 µg/mL) for 4 hours. (G) IL-1ß release was measured in cell supernatants, while (H) propidium iodate (PI) uptake was measured after 60 minutes of venom treatment. (F) Two-way ANOVA, followed by multiple post-test comparisons, was applied to compare cytokine production and PI uptake. Differences were considered statistically significant when $p < 0.05$.

ns = nonsignificant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Honeybee and wasp venoms contain at least 2 compounds known to trigger NLRP3 activation, phospholipase A2[2] and hyaluronidases,[12] along with bee melittin.[3] Ant venom consists of water-soluble hyaluronidases and phospholipases, as well as water-insoluble alkaloids that have cytotoxic and hemolytic properties,[2,12] which may activate NLRP3 via cell membrane damage.

Conclusions

Although further mechanistic studies are necessary, our findings provide novel insights into the role of inflammasome activation in atopic individuals. In addition to the classical Th2-related cytokines, our results suggest that proinflammatory markers may help in the characterization of type-1 hypersensitivity. These results strongly support the involvement of the NLRP3 inflammasome in allergic disease and indicate that specific NLRP3 inflammasome inhibitors may be potential therapeutic targets for allergic diseases.

Acknowledgments

We would like to thank the Allergy and Clinical Immunology Clinic at Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo for assisting with patient blood collection and our colleagues at the Laboratory of Transplant Immunology and the Laboratory of Experimental Immunology of the Department of Immunology at Universidade de São Paulo (ICB/USP) for providing equipment and technical support.

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No conflicts of interest declared concerning the publication of this article.

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