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When venom calms the storm: Stonefish venoms suppress LPS-induced Th1 cytokine expression and secretion in human PBMCs

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ABSTRACT

Venoms are known to modulate immunological processes. In this study, we investigated the immunomodulatory properties of venoms from two stonefish species, *Synanceia verrucosa* (SvV) and *Synanceia horrida* (ShV), using immunological assays including reverse-transcription quantitative polymerase chain reaction (RT-qPCR), cytometric bead array (CBA), and enzyme-linked immunosorbent assay (ELISA). Both venoms exhibited significant immunosuppressive activity, particularly in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs), with less pronounced effects on phorbol 12-myristate 13-acetate with ionomycin (P/I)-stimulated cells. The venoms primarily suppressed Th1-associated cytokines (TNF, IFN-γ, IL-6, and IL-12), as well as IL-10 (Th2) and MCP-1, indicating a stronger inhibition of the Th1 subset. SvV demonstrated greater activity compared to ShV, suppressing cytokines on which ShV had no effect, and having activity at concentrations as low as 1.25 μg/mL. Stability studies showed that both frozen and lyophilized venoms retained immunosuppressive activity comparable to fresh venom, while reversed-phase high-performance liquid chromatography (RP-HPLC) abolished this activity entirely. Size-exclusion chromatography (SEC) revealed the immunosuppressive activity was strongest in the early and late fractions of each venom. Our results highlight the selective immunosuppressive effects of *S. verrucosa* and *S. horrida* venoms on human PBMCs, particularly via modulation of Th1 cytokines in response to LPS. The stability and bioactivity of specific venom fractions underscore their potential as sources for novel immunotherapeutic agents.

1. Introduction

Animal venoms are known to modulate immune processes, which are typically associated with inflammation, and involves changes in the levels of signalling proteins called cytokines and chemokines. In general, increased cytokine levels are associated with inflammatory states, whereas decreased levels are associated with anti-inflammatory effects (Ryan et al., 2021). While many venoms induce inflammation, such as those from scorpions, snakes, toadfish, and scorpionfish by increasing levels of pro-inflammatory cytokines (Açikalin and Gökel, 2011; Fukuhara et al., 2003; Lima et al., 2003; Menezes et al., 2012), others demonstrate anti-inflammatory effects (Rainsford, 2007). Examples of the latter are seen in snake venom components from the red-bellied black snake (*Pseudechis porphyriacus*) and the Chinese cobra (*Naja atra*), which suppressed pro-inflammatory cytokine expression in activated purified human T cells (Ryan et al., 2020; Zhu et al., 2016). Similarly, fish venom components also exhibit potent anti-inflammatory properties, such as natterins characterised in the venom of the Brazilian toadfish *Thalassophryne nattereri* that reduced inflammation in a murine model (Ferreira et al., 2014). Further investigation of the toadfish

Abbreviations: RT-qPCR, reverse-transcription quantitative polymerase chain reaction; CBA, cytometric bead array; ELISA, enzyme-linked immunosorbent assay; TNF, tumour necrosis factor; IFN-γ, interferon gamma; MCP-1, monocyte chemoattractant protein-1; IL-, interleukin-; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; P/I, phorbol 12-myristate 13-acetate with ionomycin; SvV, *Synanceia verrucosa* venom; ShV, *Synanceia horrida* venom; LAL, limulus amebocyte lysate; DEX, dexamethasone; CsA, cyclosporine A; Th1, Type 1 lymphocytes; Th2, Type 2 lymphocytes.

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venom resulted in the discovery of the *Tn*P peptide, which exhibited anti-inflammatory and anti-allergic activities in mouse models (Komegae et al., 2017; Lima et al., 2022).

The discovery of the *Tn*P peptide and the immunomodulatory role of natterins prompted this investigation using stonefish venom. A transcriptomic and proteomic analysis of the estuarine stonefish (*Synanceia horrida*) venom revealed homologues to natterins and nattectin (a C-type lectin) from *T. nattereri*, as well as several unique proteins not previously described in animal venoms (Ziegman et al., 2019), raising the possibility of similar immunosuppressive effects. In this study, we investigated the immunosuppressive activity of stonefish venoms in human peripheral blood mononuclear cells stimulated with lipopolysaccharide (LPS) and phorbol 12-myristate 13-acetate with ionomycin (P/I), two of the most common and potent agents used in laboratory models of inflammation (Ai et al., 2013; Bertani and Ruiz, 2018). We assessed cytokine expression and secretion using reverse-transcription quantitative polymerase chain reaction, cytometric bead array assays, and enzyme-linked immunosorbent assays. We demonstrate that stonefish venoms suppressed both expression and secretion levels of key pro-inflammatory cytokines, suggesting the presence of anti-inflammatory agents in the venom. Venoms significantly suppressed LPS-driven inflammation, but not in P/I-stimulated cells. We further observe a bias toward the inhibition of Th1 cytokine secretion compared to Th2 cytokines.

Furthermore, given the known instability of stonefish toxins (Barnett et al., 2017; Church and Hodgson, 2000; Harris et al., 2021; Saunders and Tokes, 1961; Wiener, 1959), we assessed the impact of different storage methods (i.e. frozen, fractionated, and lyophilized) on immunosuppressive activity. We found that some fractionation techniques better preserve the anti-inflammatory effects and venoms stored at −80 °C for at least one year retain functional activity. These findings support the potential of stonefish venoms as a source of novel anti-inflammatory agents.

2. Materials and methods

2.1. Animal collection

Two species of stonefish, *Synanceia verrucosa* and *Synanceia horrida*, were used in the experiments (ethics application number A2572, James Cook University Animal Ethics Committee). Individuals were housed together and apart from other fish to allow for consecutive venom extractions from the same animal and avoid possible intra-species differences in venom activity. They were offered prawns once daily until they rejected additional food. From here on in, *S. verrucosa* venom will be referred to as SvV and *S. horrida* venom as ShV.

2.2. Venom collection

Venom was collected from either all of the dorsal spines or from only a selected number. Venom extraction was performed with at least a four-week interval between milking events to allow the venom to be replenished (Saggiomo et al., 2017). Venom collection was performed using a tube containing a membranous lid as previously reported (Wahsha et al., 2019). The tube was inverted vertically onto the spine and its lid was pressed on both venom sacs until all of the venom was excreted into the tube. Extracted venom was assessed separately to allow for direct comparison between species and the analysis of activity across time, storage forms, and fractionation methods. Venom was immediately placed in an ice bath, centrifuged at 4 °C for 10 min at 15,000 rpm to remove particulates, and used for experiments as crude fresh venom or divided into aliquots to be frozen or lyophilized. When not used as fresh venoms were subsequently stored at −80 °C.

Venom protein concentrations were assayed using a bicinchoninic acid (BCA) protein assay kit according to manufacturer's instructions (Pierce Rapid Gold, ThermoFisher). Frozen venom was allowed to thaw in an ice bath and lyophilized venom was rehydrated in Dulbecco's phosphate-buffered saline (DPBS, ThermoFisher) prior to testing. Venom was kept in an ice bath throughout the entire process.

Venom samples were tested for Gram-negative bacterial contamination using a limulus amebocyte lysate (LAL) kit (QCL-1000 kit, Lonza), according to manufacturer's instructions. Venoms were run either in duplicate or triplicate, mixed with endotoxin-free water and serially diluted to 1:10, 1:100, and 1:1000.

2.3. Human blood collection and PBMC separation

Human whole blood was obtained either from consenting local healthy donors (Ethics: H6702, James Cook University, Human Research Ethics Committee), or from the Australian Red Cross Blood Service (Ethics: H7010, QIMRB Berghofer Medical Research Ethics Committee). The study was carried out according to the rules of the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood by standard density gradient isolation (Betsou et al., 2019; Ryan et al., 2020) and were either used immediately or cryopreserved. Cells that were cryopreserved were centrifuged at 400×*g* for 5 min and were re-suspended in 1 mL freezing buffer (90 % FBS and 10 % dimethyl sulfoxide) at 10^7/mL. Cells were then cryopreserved with two-stage cooling; initially cooled to −80 °C in a controlled-rate freezing devices (Corning CoolCell FTS30 or LX), then transferred to vapor-phase liquid nitrogen within 48 h for long term storage below −150 °C.

Cryopreserved cells were thawed at 37 °C using standard procedures (Ryan et al., 2020), diluted in R10 medium [RPMI-1640 medium supplemented with penicillin (10,000 U/mL), streptomycin (10,000 μg/mL), and 10 % FBS] in a 1:10 ratio, then centrifuged at 400×*g* for 5 min. The cell pellet was re-suspended in R10 medium and DNA was digested with 20 μL of DNase-I (New England Biolabs) at 37 °C for 20 min. The cell pellet was washed twice at 500×*g* for 5 min and viable cell numbers were obtained either manually with a haemocytometer blood counting chamber (Livingstone) or with an automated cell counter (OLS CASY 2.5, OMNI Life Sciences). All cells were rested overnight (16 h) in a humidified incubator at 37 °C and 5 % CO_2 and then treated as described below.

2.4. Cytotoxicity assays of stonefish venoms

Venom cytotoxic effects were quantified using CellTox™ Green Cytotoxicity Assay (CellTox Green, Promega) following the manufacturer's instructions. Briefly, PBMCs (10^5 cells/well) were incubated with venom at concentrations of 1.25–100 μg/mL from 20 min to 24 h, depending on the experiment. Treatment conditions were performed in triplicate, plated in a white flat-bottom opaque 96-well plate (BMG Labtech), with fluorescence measured in a FLUOStar Omega (BMG Labtech) plate reader. Lysis buffer (positive control), DPBS (negative control), and venom treatments were run in parallel. Assays were independently repeated a minimum of three times. Each venom batch was tested separately to ensure the concentrations used were not cytotoxic.

2.5. Immunomodulation assessment of stonefish venoms

Levels of cytokine expression and secretion were measured using three different assays, namely reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) to measure mRNA transcription levels, and cytometric bead array (CBA) assay and enzyme-linked immunosorbent assay (ELISA) to measure protein translation levels. To contrast the pro-inflammatory action of stonefish venoms, we used positive controls of the well-characterised pro-inflammatory endotoxin lipopolysaccharide (LPS) and cell stimulatory cocktail phorbol 12-myristate 13-acetate and ionomycin (P/I), as previously described (Ai et al., 2013; Bertani and Ruiz, 2018). To contrast the anti-inflammatory action of

stonefish venoms, we used the well-characterised drugs dexamethasone (DEX) and cyclosporine A (CsA) to strongly inhibit LPS- and P/I-driven inflammation, respectively, as previously described (Arbabian et al., 2011; Castano et al., 2002). All assays were independently repeated a minimum of three times.

2.5.1. Human PBMC stimulation for CBA, ELISA, and RT-qPCR assays

All immunoassays were seeded at 10^5 PBMCs/well and incubated overnight for 16 h in 96-well U-bottom plates (Falcon). Cells were incubated with media only, inflammation controls LPS (10 ng/mL, Sigma-Aldrich) or P/I (0.5 X, Sigma-Aldrich), immunosuppression controls DEX (10 μg/mL, Sigma-Aldrich) with LPS or CsA (10 μg/mL, Sigma-Aldrich) with P/I, venom alone (20, 5 or 1.25 μg/mL), and venom with LPS or P/I. All treatments were plated in triplicate in filtered R10 medium, except for the immunosuppression controls, which were plated in duplicate. After overnight stimulation (16 h) plates were removed from the incubator, checked under the microscope and pelleted by centrifugation. Cell lysate was used for RT-qPCR, or culture supernatant was used for CBA or ELISA assays (detailed below).

2.5.2. Cytometric bead array (CBA) assay using S. verrucosa fresh venom

CBA was used to screen the secreted concentration of various cytokines and chemokines upon incubation with fresh SvV. This assay was only performed on SvV due to sample availability. The CBA kit was run as per manufacturer's instructions (CBA Human Soluble Protein Master Buffer Kit, BD Biosciences) and measured cytokines produced by Type 1 T helper lymphocytes (Th1): TNF, IFN-γ, IL-1β, IL-2, IL-12; Type 2 lymphocytes (Th2): IL-4, IL-6, IL-10, IL-13; and the chemokines MCP-1 and IL-8 (Austin et al., 1999; Lucey et al., 1996). This assay was performed with fresh SvV at 20, 5, and 1.25 μg/mL kept on ice until the end of the experiment. Human PBMCs (n = 4 biological replicates) were plated as described above. Data were acquired on a BD Fortessa X-20 (BD Biosciences) using BD FACSdiva Software (BD Biosciences; Ver Diva 8.0.1), analysed using the BD FCAP Array v3 (Ver 3.0.19.2091) and GraphPad Prism Ver 9.

2.5.3. Enzyme linked immunosorbent assay (ELISA)

Multiple ELISAs were performed to assess human TNF secretion levels using both SvV and ShV, and were used fresh, frozen, fractionated, or lyophilized depending on the experiment. Incubation and stimulation of PBMCs was conducted in 96-well half-area flat bottom microplates (Corning Costar) following manufacturer's protocol (human TNF-alpha DuoSet, R&D Systems). The optical density of the plate was immediately determined using a plate reader (FLUOStar Omega Ver 5.11R4, BMG Labtech). The final output was transferred to an online data analysis website designed for ELISAs (www.elisaanalysis.com) and the export from the website was multiplied by the dilution factor and the final TNF concentration was used for statistical analysis in GraphPad Prism Ver 9. Specific details of biological replicates and treatments are provided for each experiment below.

2.5.4. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

This assay was performed with both SvV and ShV at only 20 μg/mL. PBMCs (n = 4 biological replicates) were plated as stated above, however, cells were supplemented with 10 % human serum (Sigma Alderich; Batch: SLBN8825V) rather than FBS and received additional 10 mM HEPES (ThermoFisher Scientific) and 1 % non-essential amino acids (Gibco). RNA was extracted and converted to cDNA as previously described (Browne et al., 2022). Briefly, RNA was isolated using a MagMAX™ mirVana™ Total RNA Isolation Kit (Applied Biosystems) and converted to cDNA with SuperScript™ IV First-Strand Synthesis System (ThermoFisher) following the manufacturer's instructions, except that all reagents were used at 25 % of the volume recommended by the manufacturer, and the Superscript™ IV reverse transcriptase was used at 5 U/μL RNA. PrimerBank Primers (Spandidos et al., 2009) were used to quantify the mRNA expression of TNF (PrimerBank ID: 25952110c1), IFN-γ (PrimerBank ID 56786137c1), and IL-10 (PrimerBank ID 24430216c1) following stimulation relative to the negative control (media) background [Log2 (stimulation/background)], using 500 nM desalt-grade primers (Sigma-Aldrich) with ssoAdvanced™ Universal SYBR® Green Master-Mix (Bio-Rad), as previously described (Browne et al., 2020). All reactions were run in technical triplicate in accordance with MIQE guidelines (Bustin et al., 2009) at 5 μL total volume with 1 μL of reverse transcription eluent diluted 1:4 in Ultra-Pure™ H_2O (Invitrogen). Data was acquired using a QuantStudio5 Real-Time PCR system running QuantStudio Design and Analysis Software (v1.4.3, Applied Biosystems).

2.6. Assessment of stonefish venom activity through ELISA

It has been long known that stonefish venom activity is particularly labile with changes in pH, temperature, lyophilization, and fractionation (Church and Hodgson, 2000, 2002; Harris et al., 2021). The following experiments were designed to assess whether there was a change in immunological activity in human TNF suppression comparing fresh venom, frozen venom, lyophilized venom and fractionated venom through reversed-phase high performance liquid chromatography (RP-HPLC). All aliquots came from the same venom batch to ensure they could be directly comparable.

2.6.1. Comparison of fresh, frozen, lyophilized, and RP-HPLC fractions from S. verrucosa venom

To investigate whether physical or chemical changes can negatively affect immunological activity, SvV was extracted as described above, and aliquoted so that samples were used fresh (kept on ice), frozen (for 1 h in −80 °C), lyophilized, or fractionated through RP-HPLC [Agilent 1260 Infinity HPLC (Agilent Technologies, Hanover) and Phenomenex Kinetex 5 μm, C8, 100 Å, 50 × 2.1 mm (Phenomenex, Torrence, CA, USA) column]. SvV (20–30 μL) was mixed with 20 μL of solvent A [H_2O/0.05 % trifluoroacetic acid (TFA, Auspep)] and centrifuged at 4 °C for 10 min at 15,000 rpm (Mikro 200R, Hettich Zentrifugen, LabGear). The supernatant (~60 μL) was injected through an autosampler and run with a 0.5 % gradient solvent B [90 % acetonitrile (ACN; Sigma-Aldrich)/10 % H_2O/0.045 % TFA] (0–60 % B, 60 min; 60–90 % B, 5 min; 90 % B, 10 min; 90-0 % B, 5 min) at a flow rate of 1 mL/min. The column oven was set to 35 °C and the absorbance monitored at 214 and 280 nm. All fractions were manually collected and pooled into a single to investigate whether immunosuppressive activity was lost after the venom was subjected to RP-HPLC. The pooled fractions were subsequently aliquoted, labelled, freeze-dried and kept in −80 °C until required. PBMCs (n = 3 biological replicates) were seeded as described above and an ELISA was performed as described above using both LPS and P/I agents. Due to the loss of activity after venom was fractionated with RP-HPLC, only SvV was tested.

2.6.2. Comparison of fresh and frozen venoms stored up to 15 months from both S. verrucosa and S. horrida

This assay was designed to understand whether there is a reduction in immunomodulation activity in venom stored for long-term at −80 °C. To reduce potential confounding factors, the same batch of venom was used across the assays. ELISAs were performed with fresh venoms, and aliquots stored at −80 °C for 12 months for ShV and 15 months for SvV. Fresh venoms were tested on PBMCs (n = 4 biological replicates for SvV; n = 3 for ShV) for both LPS- and P/I-induced cells but the follow-up assay using frozen venom (n = 3 biological replicates) was only performed using LPS-induced cells.

2.7. Fractionation and partial purification of stonefish venoms to further assess venom immunosuppression activity

To provide insight into the fractions contributing to the bioactivity of

stonefish venom, crude SvV and ShV were fractionated using a range of chromatography techniques and the immunosuppression activity of the fractions were analysed on human TNF.

2.7.1. Size-exclusion chromatography (SEC)

As previously described (Saggiomo et al., 2024), SEC was performed with both SvV and ShV using standard procedures. In summary, crude venom samples were fractionated using an Agilent 1260 Infinity High Performance Liquid Chromatography (HPLC) instrument (Agilent Technologies) and a Phenomenex Yarra 3 μm SEC-2000, 300 × 7.8 mm column (Phenomenex). Venoms were diluted (1:1) with DPBS, centrifuged, and loaded with an isocratic gradient of DPBS at a flow rate of 0.8 mL/min for 60 min. Individual fractions were collected manually. SEC fractions were pooled from repeat experiments, freeze-dried and kept in −80 °C until they were required. SEC fractions were also pooled, where equal volumes of each fraction were pooled to test bioactivity (referred to as "pooled fractions" in the assays) following this particular chromatography technique.

2.7.2. Solid-phase extraction (SPE) of S. verrucosa venom

To partially purify the small molecules found in SvV, a solid-phase extraction column (Phenomenex Strata C18-U 55 μm 70 Å) was used as previously described (Saggiomo et al., 2024). The column was first equilibrated twice with 1 mL 100 % methanol and washed twice with 1 mL 100 % H_2O prior to loading, then washed with 1 mL 5 % methanol/H_2O, and the partially purified fraction was eluted with 100 % methanol, aliquoted, freeze-dried, and kept in −80 °C until required. ShV was not used due to limited venom availability.

2.7.3. RT-qPCR and ELISA assays of fractionated and partially purified stonefish venoms

We tested SvV and ShV fractionated and partially purified venom components. Assays were performed as described above using PBMCs (n = 3 biological replicates). For these assays, given the non-significant results with P/I and limited venom sample availability, only the effects against LPS-stimulated PBMCs were tested.

3. Results

A range of assays were carried out to analyse the cellular effects of stonefish venoms. All *S. verrucosa* and *S. horrida* venom samples were tested for the presence of endotoxin using LAL kits. The results established the venom samples were not contaminated by endotoxin and were appropriate for use in the cellular assays.

3.1. Cytotoxicity of stonefish venoms

The cellular toxicity of fresh SvV and ShV was analysed on PBMCs using fluorescence-based cytotoxicity kits (Fig. 1). Treatment of PBMCs with 80 μg/mL of SvV was significantly increased from untreated control (maroon vs black; Fig. 1A), indicating some level of cytotoxicity at this concentration. In contrast, there was no difference in ShV cytotoxicity up to 100 μg/mL (Fig. 1B), suggesting a potential difference in venom composition or mode of action compared to SvV. Due to these results, we used venom concentrations of up to a maximum of 20 μg/mL for further experiments for both species.

3.2. Assessment of the immunosuppressive activity of fresh stonefish venom on cytokine secretion

To assess whether stonefish venom showed immunosuppressive activity on cytokine secretion from stimulated PBMCs, a cytometric bead array (CBA) assay was conducted using fresh SvV. ShV was not tested due to limited venom availability. Three concentrations of SvV (up to 20 μg/mL) were tested. A total of 12 cytokines were measured: TNF, IFN-γ, MCP-1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and IL-13. Some cytokines had expression levels of zero or close to zero following stimulation with LPS (IL-2, IL-4, IL-5, and IL-13) or P/I (IL-10 and IL-12) (Figs. S1 and S2, respectively).

Venom treatment had a dose-dependent effect on cytokine secretion after stimulation with LPS or P/I (Fig. 2). TNF secretion was significantly suppressed by SvV at concentrations of 20, 5, and 1.25 μg/mL (83.2 %, 61.2 %, and 40.1 %, respectively), as well as by DEX (87.9 %). A similar suppression pattern was observed for IL-12, with reductions of 100 %, 94.6 %, and 90.2 %, at SvV concentrations of 20, 5, and 1.25 μg/mL, respectively, and 99.5 % for DEX. Secretion levels of IFN-γ and IL-6 were significantly reduced by SvV only at 20 μg/mL (88 % and 73.5 %, respectively), with DEX or CsA showing reductions of 99.5 % and 77.5 %, respectively. MCP-1 levels were suppressed by SvV at every concentration tested (79.3 %, 75.7 %, and 62.5 % for 20, 5, and 1.25 μg/mL, respectively), and were suppressed to a greater extent than with DEX, which showed no effect on MCP-1 levels (Fig. 2). These results indicate that SvV exhibits anti-inflammatory activity comparable to the pharmacological drugs used. However, SvV did not suppress IL-1β, IL-6, IL-8 or IL-10 levels in LPS-stimulated PBMCs, nor did it significantly reduce MCP-1, IFN-γ, TNF, IL-1β, IL-2, IL-4, IL-5, IL-8, or IL-13 secretion in P/I-stimulated cells (Figs. S1 and S2, respectively).

In conclusion, venom from *S. verrucosa* exhibits potent immunosuppressive activity highlighted by the significant inhibition of cytokine secretion from PBMCs. This activity seems to be more potent towards LPS-stimulated cells compared with P/I stimulation, with a preferential effect on Th1 cytokines rather than Th2 cytokines.

Cells were incubated alone, with 20μg/mL *of SvV, LPS or P/I, LPS or P/I + 20, 5 or 1.*25μg/mL *of SvV. Twelve cytokines, representing a range of Type 1, Type 2, and regulatory cytokines, were measured and the graphs show only those with significant suppression levels: TNF, IL-12, IFN-γ, MCP-1, and IL-6.*

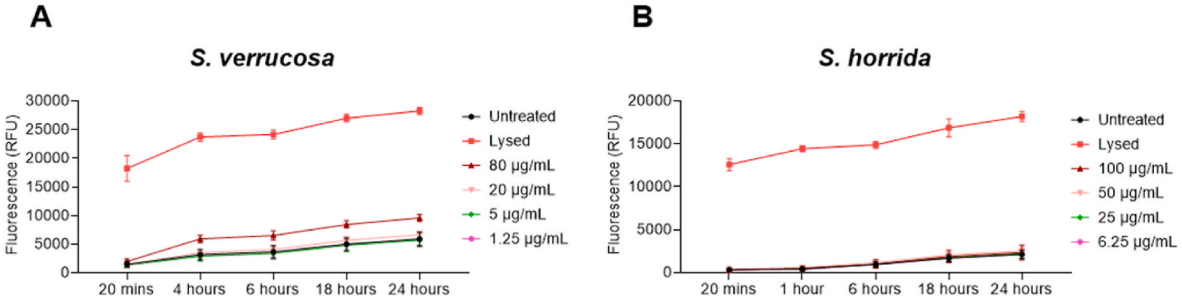


Fig. 1. Stonefish venom is not cytotoxic towards PBMCs. (A) Fresh SvV (n = 4 biological replicates), (B) Fresh ShV (n = 3 biological replicates). Results represent the cellular response of PBMCs to crude fresh venom treatments. Data are shown as mean fluorescence ± SD. Untreated cells are negative controls (black); lysed cells are positive controls (red); venom dilution treatments are shown in different colors. A two-way ANOVA with multiple comparisons was performed for each venom species separately and showed a significant difference between untreated and venom-treated cells at 80 μg/mL for SvV ($p < 0.0001$), but no significant differences for ShV ($p = 0.9990$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

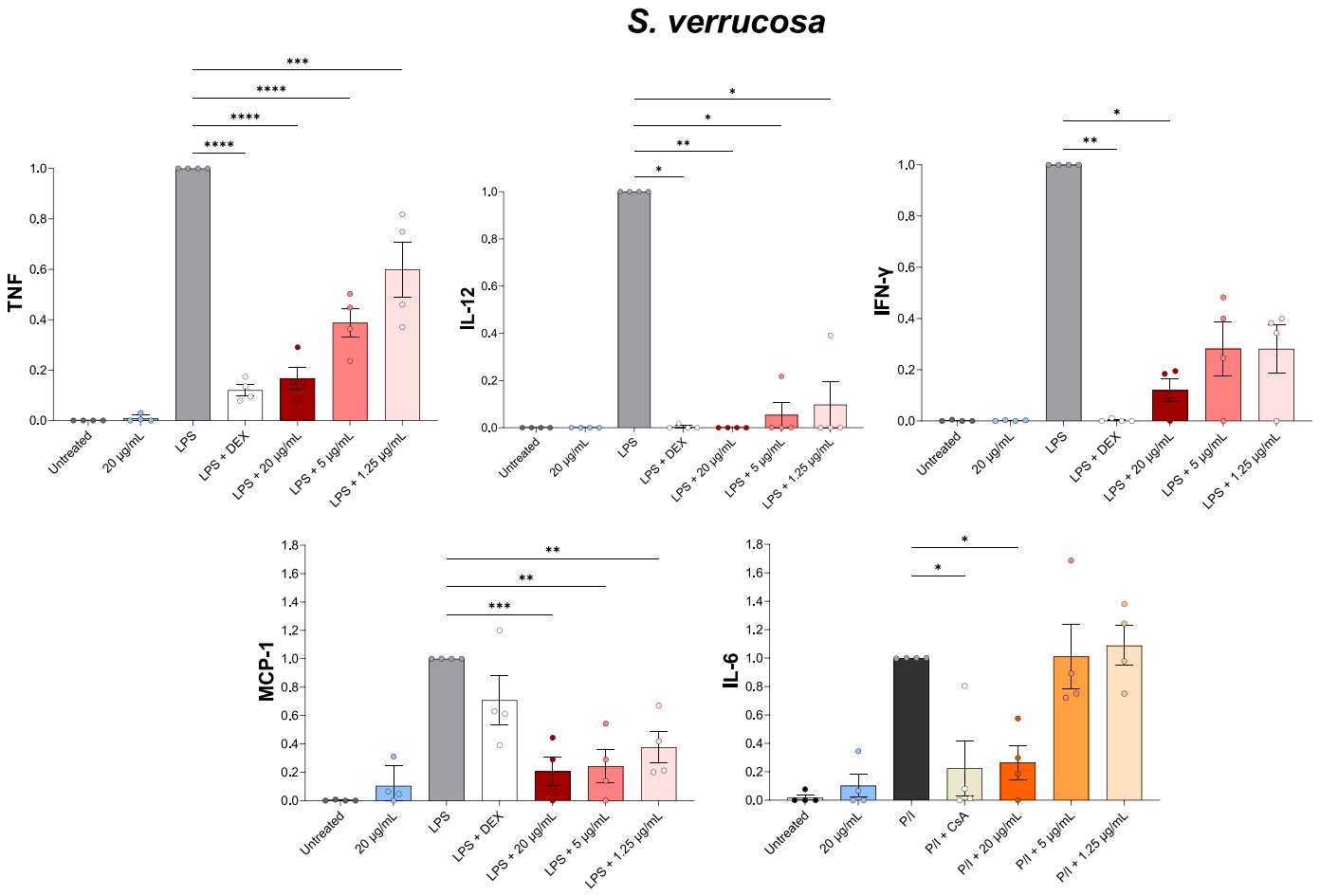


Fig. 2. Stonefish venom treatment (fresh) significantly reduces cytokine secretion levels from PBMCs stimulated with LPS (grey) or P/I (black).

All measurements are shown relative to LPS or P/I. Histograms illustrate soluble cytokine levels released from PBMCs, where error bars are mean ± SEM (n = 4 biological replicates). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). *p < *0.05,* **p < *0.006,* ***p < *0.009,* ****p < *0.0001.*

3.3. Assessment of stonefish venom activity on immunosuppressive activity

To investigate the effects of venom activity on immunosuppressive activity, a series of assays were designed and performed using ELISAs, with TNF serving as the marker of interest due to its central role in immune response (Akdis et al., 2016; Cruz et al., 2008; Jaffer et al., 2010).

3.3.1. Comparison of fresh, frozen, lyophilized, and RP-HPLC fractions from S. verrucosa venom

Fresh SvV was aliquoted into four different forms: fresh, frozen (stored at −80 °C for 1 h), lyophilized, and pooled RP-HPLC fractions. These forms were tested to determine whether physical state or chemical fractionation affected the venom's suppression activity. The RP-HPLC profile revealed seven distinct fractions between 33 and 43.5 min, which were subsequently pooled to represent a RP-HPLC-treated venom sample (Fig. 3A).

Immunological testing of all venom forms through ELISA showed that SvV completely lost its immunosuppressive activity after being passed through a RP-HPLC column, regardless of concentration, for both LPS- and P/I-driven TNF secretion in PBMCs (Fig. 3B and C, respectively). In LPS-stimulated cells, specifically, SvV maintained its immunosuppressive activity when tested fresh, frozen, or lyophilized at a concentration of 20 µg/mL, achieving near complete suppression of TNF secretion (100 %, 95 %, 100 %, respectively). SvV also significantly reduced TNF secretion at the lower concentration of 5 µg/mL when used frozen or lyophilized (54.4 % and 48.9 %, respectively) (Fig. 3B). A similar trend was observed with P/I-stimulated cells, where frozen and lyophilized forms significantly suppressed TNF secretion levels (44.8 % and 44.2 %, respectively), whereas fresh venom showed no significant suppression at 20 µg/mL. Additionally, a significant suppression was observed with 1.25 µg/mL of lyophilized venom (40.7 %) (Fig. 3C).

3.3.2. Effect of fresh venoms and long-term frozen storage at −80 °C on TNF secretion levels in PBMCs

ShV and SvV that was stored at −80 °C for at least 12 months was tested on LPS- or P/I-stimulated PBMCs to determine whether long-term storage at −80 °C negatively affects the suppression of TNF secretion. Fresh SvV significantly reduced TNF secretion in LPS-stimulated PBMCs by 61.5 % and 30.3 % at 20 and 5 µg/mL, respectively, whereas DEX reduced TNF secretion by 70.3 % (Fig. 4A). For P/I-stimulated cells, fresh SvV at 20 µg/mL caused a significant reduction in TNF levels (36.1 %) (Fig. 4B), suggesting that SvV may have some activity against P/I-stimulated cells, though this effect was not consistent across all assays. In contrast, ShV only suppressed TNF secretion in LPS-stimulated PBMCs, with a 29.4 % reduction at 20 µg/mL, whereas DEX reduced TNF by 67.3 % (Fig. 4B). Following P/I stimulation, TNF secretion increased, but this increase was not statistically significant (Fig. 4B).

For venoms stored at −80 °C for at least 12 months, the same trend seen in Fig. 3 for frozen venom was noted. Both venoms showed further

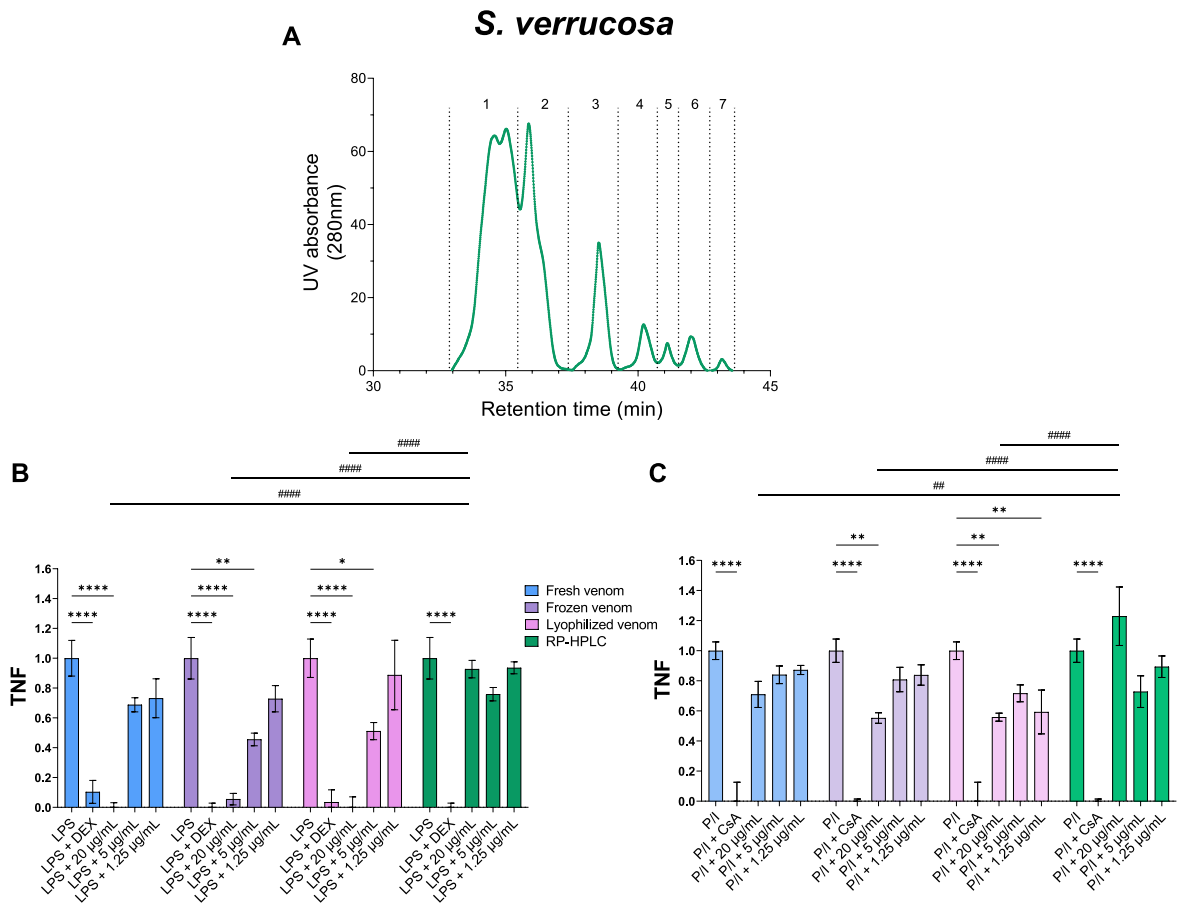


Fig. 3. Stonefish venom maintains immunosuppressive activity when frozen or lyophilized, but not when it is fractionated through a RP-HPLC column. *(A) Profile of RP-HP*LC fractionation of SvV indicating retention times of each fraction, which were pooled prior to testing. (B) ELISA of fresh, frozen, lyophilized, and pooled RP-HPLC fractions on TNF secretion levels from PBMCs post LPS stimulation. Cells were incubated alone, with 20 µg/mL of SvV, LPS, or LPS +20, 5 or 1.25 µg/mL of SvV. All measurements are shown relative to LPS. (C) ELISA of fresh, frozen, lyophilized, and pooled RP-HPLC fractions on TNF secretion levels from PBMCs post P/I stimulation. Cells were incubated alone, with 20 µg/mL of SvV, P/I, or P/I + 20, 5 or 1.25 µg/mL of SvV. All measurements are shown relative to P/I. Histograms illustrate levels of soluble cytokine levels released from PBMCs, where error bars are mean ± SEM (n = 3 biological replicates). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). *p < 0.05, **p < 0.006, ***p < 0.009, ****p < 0.0001. To calculate statistical differences between groups, a two-way ANOVA was performed followed by Tukey's multiple comparison test. ##p < 0.006, ####p < 0.0001.

reduction of TNF secretion after long-term storage compared to fresh venom (Fig. 4C). Specifically, SvV reduced TNF secretion by 88 % after long-term storage, compared to 61.5 % with fresh venom, and ShV reduced TNF secretion by 59 % when frozen, compared to 29.4 % when used fresh. These differences were statistically significant. As expected, DEX treatment of LPS-stimulated PBMCs produced consistent effects between assays, reducing TNF levels by 70.3 % in the fresh SvV assay, 67.3 % in the frozen SvV assay, and an identical reduction (67.3 %) in both fresh and frozen ShV assays. These consistent results suggest that the statistically significant differences observed between fresh and frozen venoms were due to intrinsic properties of the venoms themselves, rather than assay variability. Unfortunately, due to limited venom availability, P/I- stimulated cells were not tested, so the long-term effect on P/I-driven TNF secretion remains unknown.

3.4. Assessment of the immunosuppressive activity of stonefish venom on cytokine expression

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to assess the gene regulation of three cytokine markers, Th1: TNF, IFN-γ, and Th2: IL-10, following stimulation with LPS or P/I. These markers were selected based on the CBA results or their well-established roles in immunomodulation: TNF is generally one of the first pro-inflammatory cytokines released by immune cells; IFN-γ may inhibit viral replication and activate macrophages to kill intracellular pathogens; and IL-10 typically acts as an anti-inflammatory cytokine (Cohen and Cohen, 1996). Both SvV and ShV were included in the analysis, tested at 20 µg/mL (Fig. 5).

For LPS-induced cells, SvV treatment significantly down-regulated the mRNA levels of all three cytokines tested (Fig. 5A). The most dramatic reduction in expression levels were seen with *IFN-γ* levels, reduced by 91.6 % following SvV treatment and by 96.9 % by DEX. SvV also reduced *TNF* mRNA expression levels by 48.2 % and *IL-10* levels by 61.1 %, while DEX treatment showed no significant reduction in either experiment. In the case of ShV, there was significant down-regulation of mRNA levels of *TNF* and *IL-10* genes (34.9 % and 54.9 %, respectively; Fig. 5B). DEX also only showed a significant reduction in the levels of *IFN-γ* (84.6 %) and, although ShV showed a reduction in *IFN-γ* levels, these did not reach significance, contrasting with the results seen with SvV (Fig. 5B).

For P/I-induced cells, neither SvV (Fig. 5C) nor ShV (Fig. 5D) showed a tendency to decrease the mRNA levels of any of the genes tested, remaining at a similar level as P/I. These data align with the protein secretion results observed in our previous experiments.

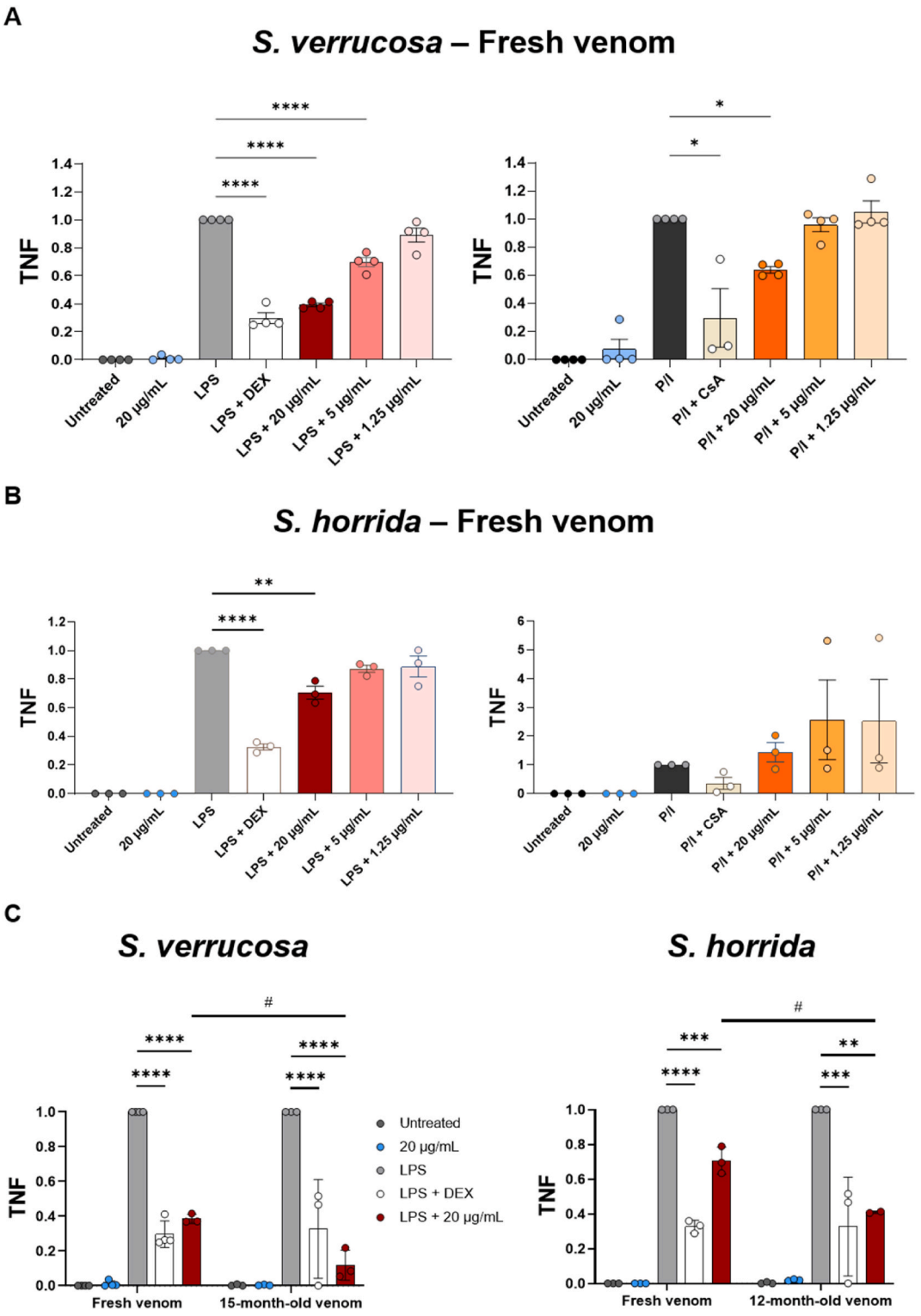


Fig. 4. Stonefish venoms maintain immunosuppressive activity after long-term storage in −80 °C. (A) Fresh SvV treatment effects on PBMCs (n = 4 biological replicates) stimulated with LPS (grey) or P/I (black). (B) Fresh ShV treatment effects on PBMCs (n = 3 biological replicates) stimulated with LPS (grey) or P/I (black); (C) Comparison between fresh SvV (n = 4 biological replicates) and SvV kept at −80 °C for 15 months (n = 3 biological replicates); and comparison between fresh ShV and ShV kept at −80 °C for 12 months (n = 3 biological replicates) for LPS-stimulated PBMCs. Histograms display untreated cells, cells treated with 20, 5 or 1.25 µg/mL of venom, with LPS or P/I, with LPS + DEX or P/I + CsA, and cells incubated with LPS or P/I + 20, 5 or 1.25 µg/mL of venom. All measurements are shown relative to LPS or P/I. Error bars indicate mean ± SEM. Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). *$p < 0.05$, **$p < 0.006$, ***$p < 0.009$, ****$p < 0.0001$. To calculate statistical differences between groups, a mixed-effects analysis was performed followed by Šídák's multiple comparisons test. #$p < 0.05$.

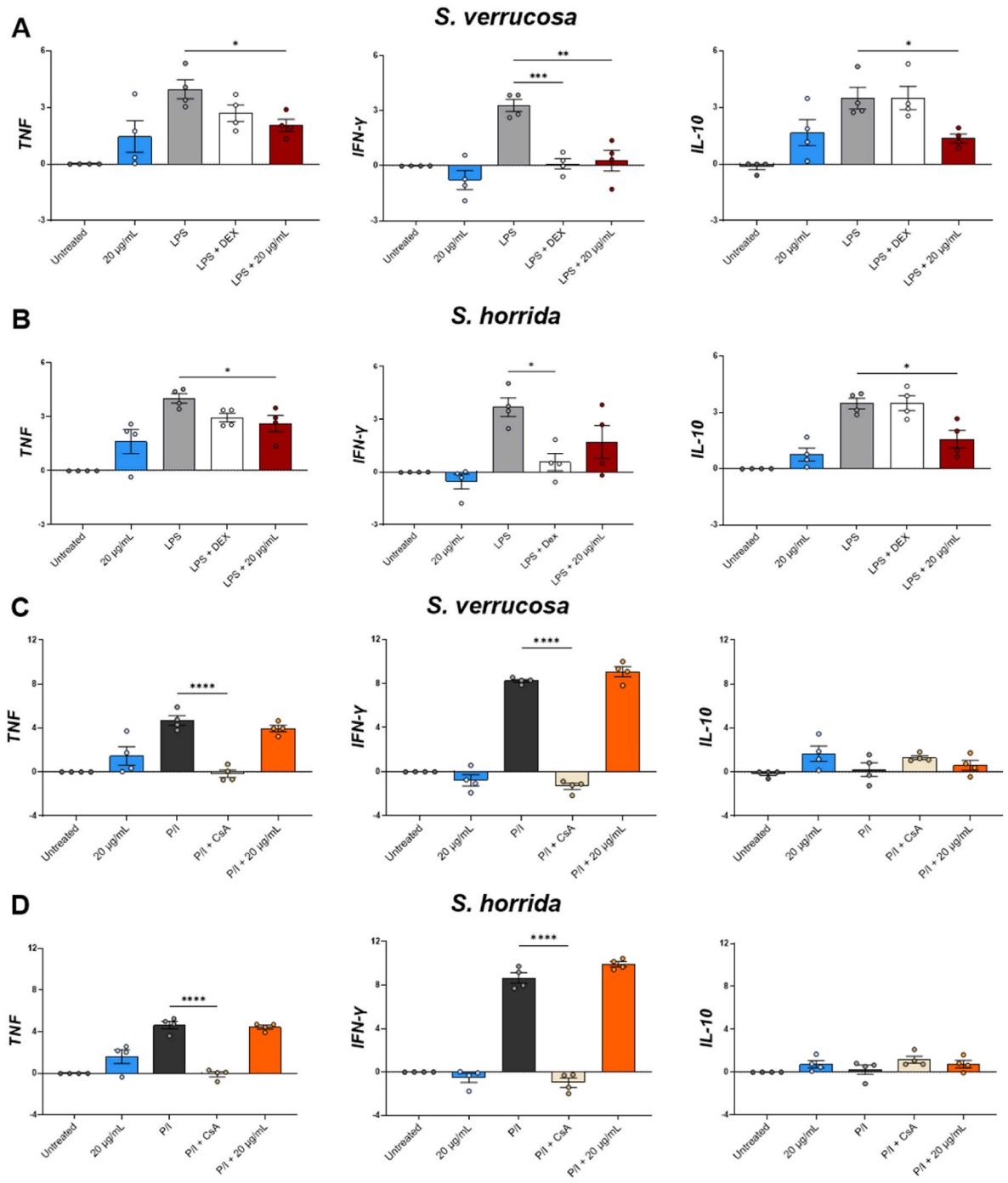
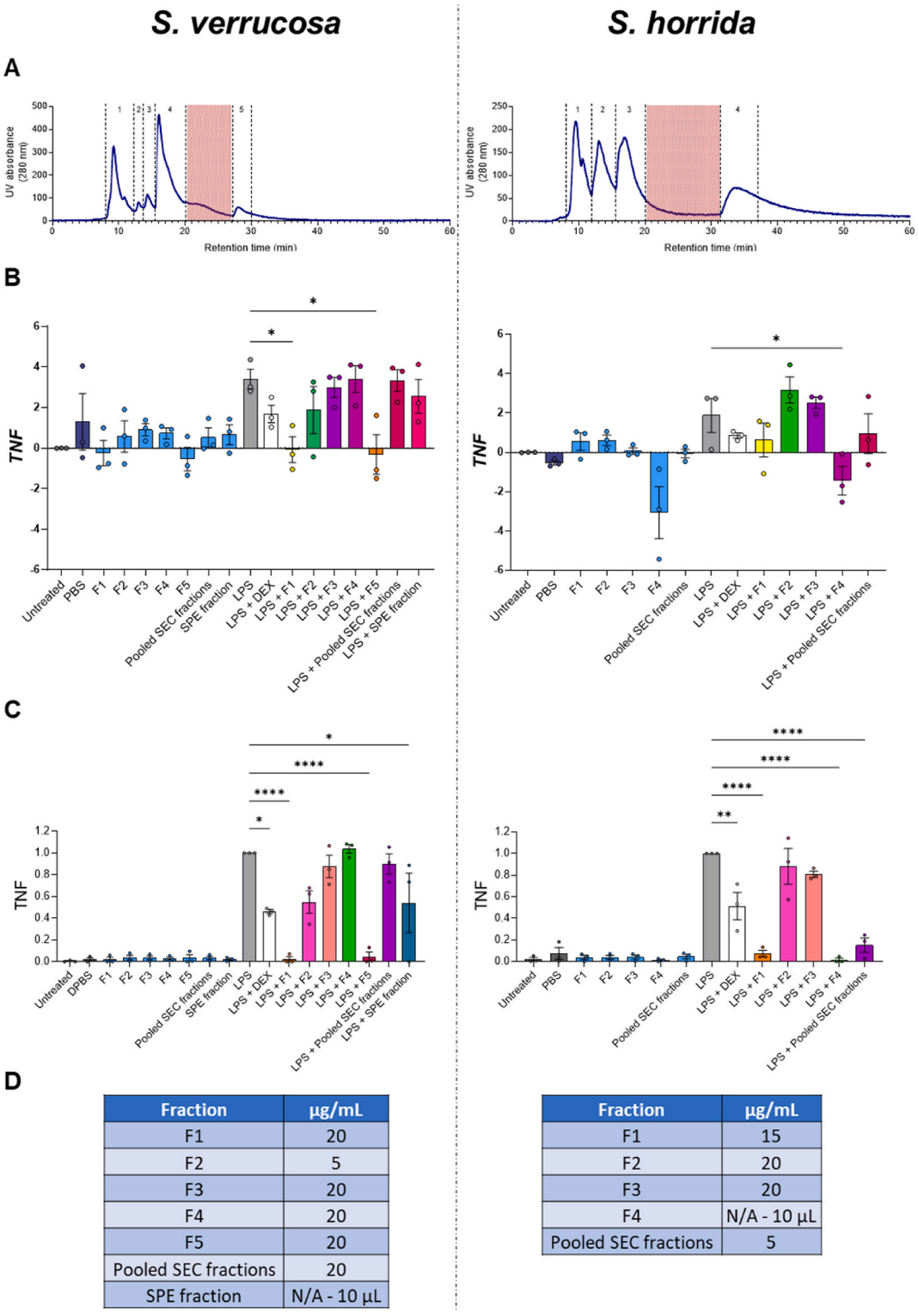


Fig. 5. Stonefish venom treatment significantly down-regulates cytokine mRNA levels from PBMCs stimulated with LPS (A and B), but not with P/I (C and D). Histograms illustrate levels of mRNA expression relative to media background Log2 (stimulation/background) of LPS- or P/I-induced PBMCs: TNF, IFN-γ and IL-10. (A and B) Cells were incubated alone, with 20 µg/mL of either SvV (A) or ShV (B), with LPS only, LPS + DEX or LPS + 20 µg/mL of SvV or ShV. (C and D) Cells were incubated alone, with 20 µg/mL of either SvV (C) or ShV (D), with P/I only, P/I + CsA or P/I + 20 µg/mL of SvV or ShV. Error bars are mean ± SEM (n = 4 biological replicates). Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). *$p < 0.05$, **$p < 0.006$, ***$p < 0.009$, ****$p < 0.0001$.

3.5. Assessment of the immunosuppressive activity of fractionated stonefish venom on cytokine expression and secretion

To further explore the immunological activity of the venoms, crude venom samples were fractionated using size-exclusion chromatography (SEC) to separate components based on molecular size. Five fractions were collected from SvV and four from ShV (Fig. 6A). These fractions were subsequently tested on PBMCs to assess cytotoxicity, as previously reported (Saggiomo et al., 2024). Additionally, solid-phase extraction (SPE) was used to crudely separate the small molecules from the small proteins and salts in the crude venom samples, and subsequently tested.

The results from the RT-qPCR showed a similar profile for both SvV and ShV, where the first and last fractions had a marked reduction in *TNF* mRNA levels (Fig. 6B). For SvV, this reduction was statistically significant for both SEC fractions 1 and 5, whereas only the effect from fraction 4 of ShV was statistically significant. In contrast, DEX did not



Fraction	µg/mL
F1	20
F2	5
F3	20
F4	20
F5	20
Pooled SEC fractions	20
SPE fraction	N/A - 10 µL

Fraction	µg/mL
F1	15
F2	20
F3	20
F4	N/A - 10 µL
Pooled SEC fractions	5

Fig. 6. Identification of immunosuppressive components in stonefish venom fractions. (A) Profile of SEC fractions of SvV and ShV indicating retention times of each fraction collected. (B) RT-qPCR assay of SEC and SPE fractions on TNF mRNA expression levels [relative to media background Log2 (stimulation/background)] from PBMCs post LPS stimulation. (C) ELISA of SEC and SPE fractions on TNF secretion levels from PBMCs post LPS stimulation. Cells were incubated alone, with DPBS, with various fractions from SvV or ShV at different concentrations, with LPS only, LPS + DEX, or LPS + fractions. ELISA measurements are shown relative to LPS or P/I. Error bars are mean ± SEM (n = 3 biological replicates). (D) Tables specifying the protein concentration in µg/mL or volume in µL used for each fraction from either SvV or ShV. Statistical differences were quantified with a one-way ANOVA followed by Dunnett's multiple comparison tests or their non-parametric equivalent (Kruskal-Wallis followed by Dunn's tests). *$p < 0.05$, **$p < 0.006$, ***$p < 0.009$, ****$p < 0.0001$.

cause a significant reduction in *TNF* mRNA levels in either case, although a reduction in *TNF* levels is seen. We note that, although fraction 2 of SvV showed some reduction in *TNF* mRNA levels, this result did not reach statistical significance; however, fraction 2 was only tested at 5 μg/mL, and it is possible it would have resulted in a significant reduction in *TNF* levels if tested at 20 μg/mL, as it was the case with the other fractions.

To assess protein secretion following gene translation, ELISAs were performed on LPS-stimulated PBMCs (Fig. 6C). For SvV, fractions 1, 5, and the SPE fraction significantly suppressed TNF secretion levels by 97.5 %, 85.3 %, and 45.9 %, respectively, while DEX significantly reduced TNF secretion by 53.7 %. Fraction 2 reduced levels by 45.2 %, a reduction comparable to DEX, and could have shown a significant reduction in TNF secretion levels had it been tested at 20 μg/mL. The SPE fraction had a statistically significant reduction in TNF protein expression, while this reduction was not significant at the transcription level.

For ShV, fractions 1, 4, and pooled SEC fractions significantly reduced TNF secretion by 92.6 %, 98.4 % and 84.6 %, respectively, while DEX caused a significant reduction of 48.9 % (Fig. 6C). These results were consistent with the RT-qPCR data (Fig. 6B), showing that although some fractions did not reach statistical significance for the down regulation of *TNF* gene transcription, this reduction was sufficient to cause significant reduction in protein levels.

Overall, both SvV and ShV demonstrated stronger suppression of TNF secretion in LPS-stimulated PBMCs than in P/I-stimulated cells. This trend was observed for both gene and protein levels of multiple cytokines. SvV showed a greater effect than ShV, reducing both cytokine expression and secretion, with its activity comparable to that of the anti-inflammatory drug DEX. SEC fractionation revealed that the suppressive activity was localized to specific venom fractions. We hypothesize that both high-molecular-weight components, found in early SEC fractions (e.g., SvV and ShV fractions 1), and low-molecular-weight compounds, indicated by the activity in SvV SEC fraction 5, SvV SPE fraction, and ShV fraction 4, contribute to the observed immunosuppressive effects.

4. Discussion

Animal venoms are typically associated with painful and potentially life-threatening effects following envenomation; however, they also represent a valuable source of pharmacologically active compounds with therapeutic potential. In this study, we investigated the immunomodulatory properties of venoms from two species of stonefish, *Synanceia verrucosa* and *Synanceia horrida*. Using a range of complementary immunological assays such as RT-qPCR, CBA, and ELISAs, we assessed both transcriptional and translational responses, gaining a more comprehensive understanding of venom-induced immune modulation. PBMCs were simulated with LPS, a bacterial endotoxin known to induce strong cytokine responses in humans (Bertani and Ruiz, 2018) and strongly inhibited by dexamethasone (DEX) (Castano et al., 2002), and P/I, known to activate T cells and induce cytokine production (Ai et al., 2013), being suppressed by cyclosporin A (CsA) (Arbabian et al., 2011).

4.1. Stability of immunosuppressive activity in stonefish venoms

The storage of venom is an essential safeguard to ensure its consistent availability for research, as various factors, such as limited venom production or unexpected animal mortality may affect venom supply. Stonefish venom toxins are extremely labile, with significant reductions in activity due to temperature fluctuation, storage conditions, lyophilisation, or repeated freeze-thaw cycles (Barnett et al., 2017; Church and Hodgson, 2000; Harris et al., 2021; Saunders and Tokes, 1961; Wiener, 1959). Therefore, assessing whether long-term storage or chemical processing negatively impacts the immunosuppressive activity of stonefish venom is essential. We focused on TNF for these assays due to the important roles it plays in immune modulation, being one of the first cytokines to be expressed upon pathogen detection, starting the cascading events that lead to the recruitment of other cells and expression of further cytokines to protect the host (Akdis et al., 2016; Cruz et al., 2008; Jaffer et al., 2010).

The initial assays with SvV showed that frozen and lyophilized venoms not only maintained the immunosuppressive activity when compared to fresh venom when tested at 20 μg/mL after LPS stimulation, but also showed a statistically significant reduction in TNF protein levels at 5 μg/mL, which was not seen with fresh venom. When cells were stimulated with P/I, frozen and lyophilized forms were able to significantly reduce TNF levels at 20 μg/mL, which was not seen with fresh venom tested at the same concentration. Similar results were observed with both venoms after long-term storage at −80 °C (12 months for ShV and 15 months for SvV), exhibiing a significant enhancement in their activity. Given the known lability of toxins (Garnier et al., 1995; Gwee et al., 1994; Kreger, 1991), we hypothesize that there is a decrease in the relative proportion of toxic compounds in the venom samples and an increase in the relative proportion of the compounds responsible for the immunosuppressive activity, as noted by Garnier and colleagues (Garnier et al., 1996), ultimately leading to a stronger suppression activity.

When SvV was subjected to the RP-HPLC, it completely lost its ability to suppress TNF secretion, independently of the stimuli. This suggests the key immunosuppressive constituents of the venom are likely proteins, as small molecules and peptides are typically stable under the conditions used during fractionation (Bill, 1996), but this remains to be determined. Loss of activity may also result from exposure to the acidic environment (pH ~2.1) of the RP-HPLC buffers, the presence of organic solvents (i.e. acetonitrile), or protein precipitation prior to sample loading (Bill, 1996; Mant and Hodge, 1991).

4.2. Immunomodulatory effects of stonefish venoms

A cytokine screening assay was conducted to assess the immunomodulatory activity of *S. verrucosa* venom on key cytokines and chemokines associated with viral immune response (Cohen and Cohen, 1996). SvV significantly suppressed all Th1 cytokines tested (TNF, IFN-γ, and IL-12) in LPS-stimulated cells, except IL-2, without a significant reduction in Th2 cytokines. In P/I-stimulated cells, only IL-6 was suppressed. RT-qPCR and ELISA assays confirmed that SvV downregulated mRNA levels of *TNF*, *IFN-γ*, and *IL-10*, while *S. horrida* venom suppressed *TNF* and *IL-10* in LPS-stimulated PBMCs. Both venoms showed limited cytokine modulation for gene transcription and protein secretion in P/I-stimulated cells.

This inhibition of Th1 subset relative to Th2 coupled with the stronger inhibition of LPS-driven inflammation relative to P/I may suggest that the mechanism of action for immunosuppression may happen through specific T cell regulatory pathways. LPS-induced inflammation involves the activation of immune cell surface receptors, such as the toll-like receptor 4 (TLR4), leading to the recruitment of transcription factors such as NF-κB and subsequent secretion of pro-inflammatory cytokines and chemokines (Heinbockel et al., 2018). Natterins, a protein from the Brazilian toadfish *T. nattereri* venom, have been shown to inhibit inflammation via TLR2-TLR4/MyD88-mediated signalling cascade(Ferreira et al., 2014), suggesting a possible similar mechanism for stonefish venoms. Since various diseases are mediated by Th1 cell subsets, such as multiple sclerosis and autoimmune thyroiditis (Del Prete, 1998), stonefish venoms may offer potential for targeted immunosuppression treatments, warranting further study.

4.3. Immunomodulation of venom fractions

Given the diversity of components in stonefish venom, it was of interest to fractionate the venom to identify bioactive components with potential immunosuppressive activity.

4.3.1. S. verrucosa venom

Fractions 1 and 5 exhibited the most potent immunosuppressive effects, significantly reducing TNF expression and secretion in LPS-induced inflammation. Using liquid chromatography mass spectrometry (LC-MS), our group previously identified the main proteins in these fractions (Saggiomo et al., 2024). Fraction 1 contained a protein of around 13,960 Da, which was also found in fractions 2, 3, and 4. Fraction 5 primarily contained a 12,365 Da protein, which was also present in fraction 4. Fraction 2 also showed a reduction in TNF secretion, albeit not statistically significant given the low concentration used (5 μg/mL instead of 20 μg/mL). Fraction 2 contained the 13,960 Da molecule, along with gamma-aminobutyric acid (GABA) and a 17,375 Da protein (Saggiomo et al., 2024). The SPE fraction also demonstrated a significant reduction in TNF secretion, albeit weaker compared to fractions 1 and 5. The variability in activity observed may be due to the unknown concentration of the SPE fraction. Our previous analysis (Saggiomo et al., 2024) identified GABA and norepinephrine within the SPE fraction, which might contribute to the strong immunosuppressive effects observed, warranting further investigation.

The absence of a decrease in TNF levels observed with the pooled SEC fractions was unexpected given the potent TNF suppression observed with fractions 1, 2, and 5. We hypothesize this may be due to the removal of bioactive molecules during fraction collection (i.e. discarded with the area marked in red; Fig. 6A), or due to a different ratio of bioactive molecules found in the pooled sample compared to the ratio found in the crude venom, as the pooled fraction was reconstituted using equal volumes of each fraction rather than replicating the relative ratio each fraction occurs in crude venom. This may have diluted key components, disrupting natural molecular ratios. Future studies should consider maintaining the natural proportion of each fraction to avoid dilution effects.

4.3.2. S. horrida venom

Fractions 1, 4, and pooled SEC fractions demonstrated significant immunosuppression activity in LPS-induced inflammation. Our previous LC-MS analysis revealed that the main constituent of fraction 1 was a 14,007 Da molecule, which was also found in all four fractions (Saggiomo et al., 2024). Fraction 1 additionally contained two other molecules shared with fractions 2 and 3, but a unique 11,920 Da molecule was exclusive to this fraction and may be responsible for the immunosuppressive activity seen. In fraction 4, the main constituent was a 12,068 Da molecule, with a molecule of 13,776 Da, both of which were unique to this fraction, in addition to detectable levels of the 14,007 Da molecule (Saggiomo et al., 2024). Despite the overlap of certain molecules among fractions, it is possible that they might play a role in the suppression of TNF levels, depending on their relative abundance and synergistic interactions. We note that fraction 1 was tested at 15 μg/mL, so it is possible that if used at 20 μg/mL, suppression would be further improved. Similarly, the concentration of fraction 4 was not assessed, so it is possible that fraction 4 was used at a higher concentration than 20 μg/mL. Standardising concentrations and molecular compositions will be essential in future studies.

Importantly, ShV fraction 4 and SvV SPE fraction were previously assessed for cytotoxicity in PBMCs at the same volumes used in this study, and neither was cytotoxic towards PBMCs (Saggiomo et al., 2024), so the results observed are not due to cellular damage or death. Significant suppression of TNF levels was observed with the ShV pooled SEC fractions at a concentration as low as 5 μg/mL. This suggests that the pooling process did not compromise the immunosuppressive activity, unlike what was observed with the SvV pooled fractions. The likely explanation is that the starting volumes of individual fractions were similar, allowing equal-volume pooling without significantly diluting the active components.

We also hypothesize that the major proteins observed in fractions 1 from both SvV (13,690 Da) and ShV (14,007 Da) are possibly homologues or isoforms, based on the similarity in their molecular weights and immunosuppressive activity. This is also likely true for the main proteins in SvV fraction 5 (12,365 Da) and ShV fraction 4 (12,068 Da). Further molecular characterisation of these components is needed to elucidate their specific roles in immune modulation. Finally, when comparing across species, *S. verrucosa* venom seemed to have a stronger and more consistent immunosuppressive effect compared to *S. horrida* venom, highlighting interspecies differences in venom bioactivity.

5. Conclusion

This study reveals that the venoms of *Synanceia verrucosa* and *S. horrida* have immunosuppressive effects on PBMCs. Both venoms significantly downregulated mRNA levels of *TNF* and *IL-10*. Additionally, SvV significantly suppressed protein levels of TNF, IL-12, MCP-1, IFN-γ and IL-6, which shows a selective modulation of Th1 cells, particularly in response to LPS stimulation. These findings suggest a selective and potentially targeted modulation of Th1 cell responses, indicating the presence of specific regulatory pathways within the venom components, which may lead to the identification of immunotherapeutic agents. Another key contribution of this study is the identification of stable bioactive components with consistent TNF-suppressing activity, even after long-term storage at −80 °C, highlighting their potential for future immunotherapeutic drug development. Lastly, but of equal importance, this study demonstrates that the bioactive components can be successfully separated using SEC and SPE fractionation, underscoring the value of these techniques in venom-based bioactive compound discovery.

CRediT authorship contribution statement

Silvia Luiza Saggiomo: Writing – review & editing, Writing – original draft, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Daniel Browne:** Writing – review & editing, Investigation. **Yide Wong:** Writing – review & editing, Investigation. **John J. Miles:** Writing – review & editing, Supervision, Conceptualization. **Denise Doolan:** Writing – review & editing, Supervision, Resources. **Norelle L. Daly:** Writing – review & editing, Supervision, Data curation. **David Thomas Wilson:** Writing – review & editing, Supervision, Data curation.

Data accessibility

The data that support the findings of this study are available from the corresponding author (silvia.saggiomo@qimrb.edu.au) upon reasonable request.

Ethical statement

Human whole blood was obtained either from consenting local healthy donors (Ethics: H6702, James Cook University, Human Research Ethics Committee), or from the Australian Red Cross Blood Service (Ethics: H7010, QIMRB Berghofer Medical Research Ethics Committee). The study was carried out according to the rules of the Declaration of Helsinki.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2025.108579.

Data availability

Data will be made available on request.

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