VENOMS AND TOXINS: AT THE CROSSROADS OF BASIC, APPLIED AND CLINICAL IMMUNOLOGY

EDITED BY: Manuela Berto Pucca, Bryan Fry, Wuelton Monteiro, Steve Peigneur and Marco Aurelio Sartim

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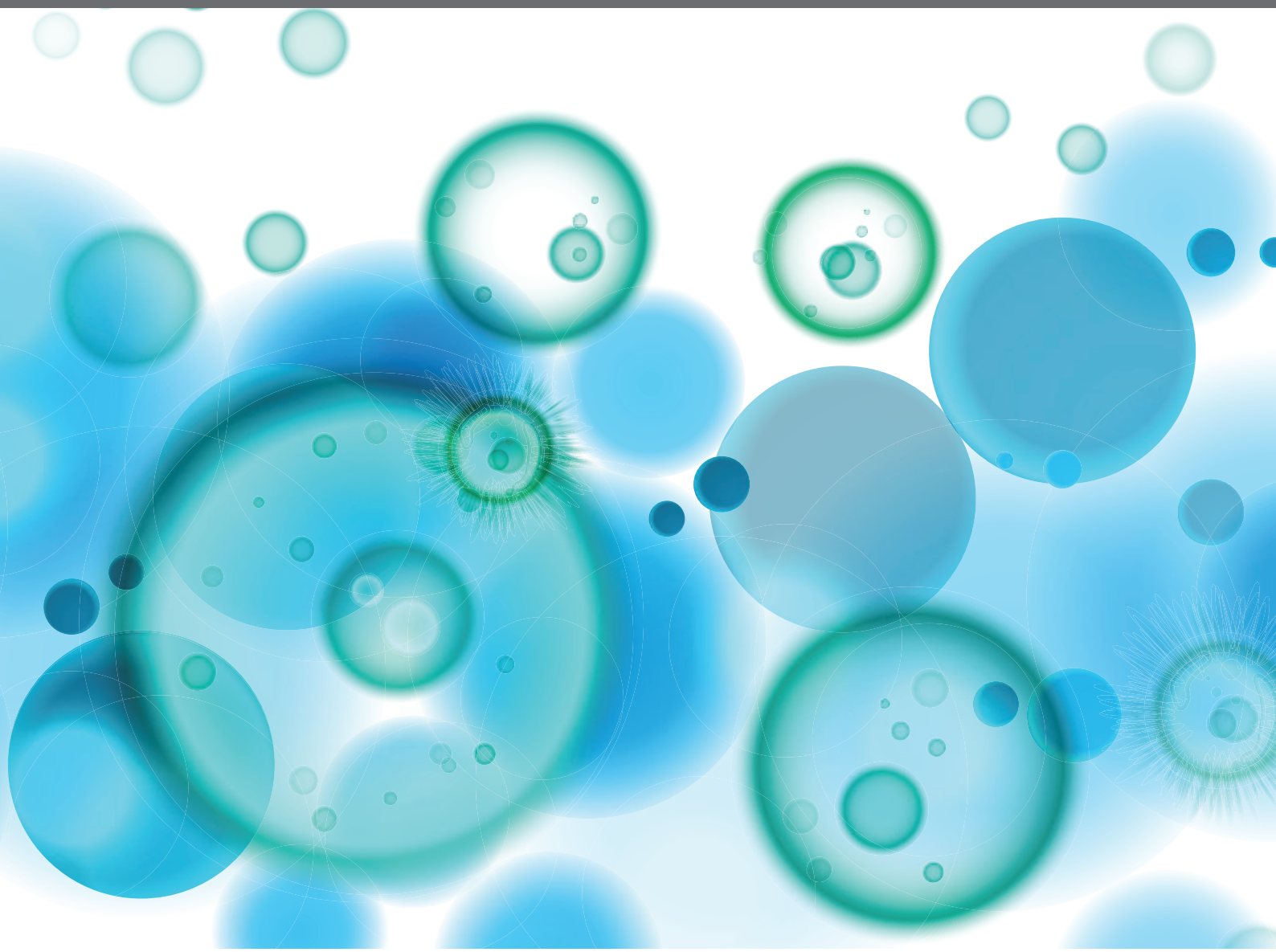

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VENOMS AND TOXINS: AT THE CROSSROADS OF BASIC, APPLIED AND CLINICAL IMMUNOLOGY

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EDITORIAL
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Editorial: Venoms and Toxins: At the Crossroads of Basic, Applied and Clinical Immunology

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Editorial on the Research Topic

Venoms and Toxins: At the Crossroads of Basic, Applied and Clinical Immunology

Animal venoms are a rich source of biologically active molecules, and represent an important field in toxinology given its medical importance and bioprospecting potential of novel drugs. The studies explore a wide range of areas of knowledge, from animal biology to biomedical and chemical aspects of venom toxins and their biological effects including biochemistry, pathology, molecular biology, pharmacology, and others. In particular, immunology represents an essential field in toxinology, in which basic and clinical research covers four main pillars: pathophysiology, diagnosis, treatment, and drug discovery. In this Research Topic, authors from all over the world present new data or reviews regarding the effects triggered by toxins from a variety of venoms in the immune system, besides presenting new approaches for antivenom development and diagnosis.

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PATHOPHYSIOLOGY

It is well established that immunological bases of envenomation pathophysiology are possibly one of the most studied topics in toxinology. Ryan et al. presented a review article on the major immunopathological aspects induced by animal venoms. The work consists of a detailed review of the latest efforts in the literature, covering innate and adaptative responses to envenomation as well as the toxin-induced effects on tissue cellular components and leukocyte populations. Among the topics, the authors approached mechanistic aspects of toxins recognition directly or indirectly (via tissue-damaged products) by the immune system and the pathological and protective responses towards venom compounds in order to neutralize the aggressor and symptom resolution.

The inflammatory response corresponds to a major issue considering its relevance to local and systemic damage caused by animal venoms, which the investigation of the mechanisms involved corresponds an essential aspect to better understand pathophysiological aspects of envenomation. Caterpillar envenoming is responsible for cutaneous reactions, and among its manifestations, the development of osteochondritis is associated with severe cases of envenoming. In order to better understand the inflammatory mechanism on joints, Villas-Boas et al. evaluated the *Premolis*

semirufa (found in the Amazon forest region) hair extract on human chondrocytes culture. The authors demonstrated that venom extracts were capable of increasing the production of inflammatory mediators such as cytokines, chemokines, eicosanoids, and complement components, as well as matrix metalloproteinases and HMGB1, and reduced the expression of cartilage components aggrecan and type II collagen. Moreover, using transcriptome analysis, the study identified pathways associated with the inflammatory process of joint diseases, such as the inflammatory response, chemotaxis of immune cells, and extracellular matrix remodeling, evidencing an osteoarthritis-like signature.

Scorpion stings are responsible for the most cases of envenoming by venomous animals worldwide. Among the main pathological aspects, pain represents an important clinical manifestation in scorpion envenoming, which is associated with the direct action of neurotoxins on ion channels as well as by the inflammatory process induced by the venom. Abreu et al. carried out a pioneering study with the venom of *Rhopalurus crassicauda*, an endemic species that inhabits exclusively the northernmost state of Brazil. The authors demonstrated that the venom and its isolated β-neurotoxin, Rc1, were capable to modulate the production of inflammatory cytokines and NFκ-B activation by macrophages, besides inducing a nociceptive behavior in mice. Rc1 was also shown to activate the voltage-dependent ion channels Nav1.4, Nav1.6, and BgNav1. The authors also demonstrated that scorpion antivenom used in Brazilian health units were unable to recognize *Rhopalurus crassicauda* venom and Rc1, indicating the need for different approaches for the treatment of envenomings regarding *Rhopalurus crassicauda* accidents.

Complement activation induced by snake venoms has been widely reported. Silva-de-França et al. investigated the effects of *Naja annulifera* venom on the complement system and explored its contribution to envenomation pathological aspects. Using *in vitro* and *in vivo* assays, the authors found that the venom was capable to induce an inflammatory response, characterized by local and systemic reactions (leukocyte influx, oedema, acute lung injury, and production of inflammatory mediators) in a complement-dependent manner. The research group also indicated the C5a-C5aR1 axis as a possible therapeutical target for *Naja annulifera* snakebite treatment.

The evaluation of circulatory inflammatory mediators in patients has brought important information on the inflammatory behavior of envenomation, guiding the search for novel biomarkers. Gerardo et al. introduce a novel prognostic model by associating inflammatory mediators (obtained pre and post antivenom therapy) and clinical features to functional recovery up to 28 days of patients from Crotalinae snakebite in the United States. The authors discovered biomarkers networks, as well as clinical variables, to predict patient recovery and increase patient prognosis as a strategy to be applied in the clinical management of snakebite. During animal envenomation, much is discussed around the direct effects of inflammation and its association to local tissue damage. However, the inflammatory response is also responsible for interfering in other physiological systems, such as hemostasis known as the inflammation-coagulation crosstalk. The study performed by Wellmann et al. investigated the correlation of circulating inflammatory cytokines and chemokines to fibrinogen levels observed in victims of the lancehead *Bothrops atrox* snakebite in North Brazil. The authors have found distinguished cytokines/chemokines profile between patients presenting normal and decreased levels of fibrinogen, highlighting that patients with hypofibrinogenemia showed increased levels of CCL-5 and decreased INF-γ, as well as a negative correlation between CXCL-8 and IL-2 to fibrinogen levels. These data support the relationship of the immune response and hemostatic disturbances observed in the envenomation pathophysiology, and the use of inflammatory mediators as possible biomarkers of coagulopathy prognosis.

DIAGNOSIS

Despite the great advance in the studies of the pathophysiological aspects of snakebite, and the established protocols for clinical management and treatment, an important issue that is neglected is the snakebite diagnosis. The snakebite diagnosis is based majorly on the patient's clinical aspects and the accident history to identify the aggressor. Knudsen et al. performed a review article exploring clinical and epidemiological aspects of snakebite worldwide and focused on research efforts within snakebite diagnosis. The study brings important data on different technological strategies for the diagnosis of snakebite and discuss new trends in the development of novel and rapid methods for snakebite diagnosis in order to promote a faster recovery and shorter time to hospital discharge.

TREATMENT

The unique specific treatment available for treating envenomings caused by venomous animals is the use of heterologous antivenoms. These antivenoms are composed of preparations containing IgGs or derived-fragments from the plasma of hyperimmunized animals. Although antivenoms have been produced using a century technique, conventional antivenoms are currently being used to save countless lives worldwide.

Most commercial antivenoms are polyspecific, meaning that they are obtained from animals immunized with a mixture of venoms from more than one species. However, based on similarities of venom toxins, antivenoms can cross-neutralize toxins from species that were not used in the immunization protocol (paraspecific neutralization). Thus, although there are known only regional and national antivenom preparations (depending on the composition of the venomous animal fauna), the development of universal antivenoms would be of immediate benefit. Ratanabanangkoon reviewed the possibility of manufacturing a plasma-derived antivenom (PDAV) against neurotoxic snake venoms. The review discusses that with the correct diverse toxin repertoire composed of a careful selection of

elapid toxins, a universal PDAV against elapid neurotoxic venoms can be produced and used for the treatment of envenomation by elapid snakes at a global level.

On the other hand, it is well-explored that heterologous antivenoms can trigger both acute (anaphylactic or pyrogenic) and delayed (serum sickness) reactions, evidencing the urgent need for improving the specific treatment for envenomings caused by venomous animals. Fortunately, with the advent of novel antibody discovery technologies and improved methods for protein engineering, there are now several advances in the field. Føns et al. reported the first recombinant human monoclonal immunoglobulin G antibody that binds α-latrotoxin (from the Mediterranean black widow spider *Latrodectus tredecimguttatus*) and shows its *ex vivo* neutralization efficacy.

Independent of the type and nature (heterologous or homologous) of the antivenoms, their efficacy is evaluated at the preclinical level by assessing its capacity to neutralize the lethal action of venoms in animal models (e.g. mice). Therefore, there is a growing awareness of the need to significantly reduce the number of mice used based on the 3Rs (Replacement, Reduction and Refinement) philosophy. Gutiérrez et al. performed a systematic review about the development of *in vitro* tests for antivenom preclinical efficacy assessment, focusing mainly on studies in which the correlation between *in vitro* and *in vivo* tests was evaluated.

The variability in the venom composition among snakes from the same genus or species is directly associated with possible limitations on antivenoms effectiveness. Seneci et al. have demonstrated that geographical and ontogenic variations in venom composition of Mexican rattlesnakes implicate different coagulotoxicity. The authors demonstrated that Antivipmyn®, the major antivenom used in Mexico for rattlesnake envenoming, presented drawbacks in neutralizing the true procoagulant activity (mediated by factor X activation), differently from prinomastat (a matrix metalloprotease inhibitor), which was capable of inhibiting stable clot formation induced by factor X activating metalloproteases.

Pucca et al. have shown that in South America, there are some differences in relation to the venom composition and clinical pictures resulting from different subspecies of *C. durissus.* For instance, envenoming caused by *C. d. ruruima*, an important public health issue in the northernmost state of Brazil, Roraima, evolve with a higher frequency of unclottable blood and bleeding compared to other *C. durissus* subspecies, thus deserving special attention in the therapeutic management using the available antivenoms. Authors discuss the efficacy of the Brazilian horse-derived antivenoms to treat *C. d. ruruima* envenomings, focusing on the discovery and development of monoclonal antibodies against *Crotalus* toxins to design recombinant antivenoms based on oligoclonal mixtures of antibodies targeting all medically important venom toxins. Likewise, Chowdhury et al. have demonstrated that clinically significant variations in snakebite effects and antivenom neutralization efficacy are underpinned by the dynamic evolution of the Palearctic vipers' venoms (Daboia, Montivipera, and Vipera genera). Testing of antivenom neutralization by three commercial antivenoms (Inoserp Europe, VIPERFAV, and ViperaTAb) revealed differential intra-species and inter-species ability to neutralize the lethal FX activating effects, reflective of variations in the composition of the venoms used in the immunizing processes.

Several efforts have been performed in order to improve the treatment of snakebite, such as the search for novel drugs or antivenoms improvement in order to neutralize venom toxins. However, the strategy for the treatment of clinical manifestations has gained a spotlight with the advent of biological techniques. Sanchez-Castro et al. performed a review article on the use of mesenchymal stromal cells (MSCs) as a promising treatment for muscle regeneration following a snakebite, detailing the local damage pathophysiology during snakebite and the benefits of MSCs due to its anti-inflammatory and pro-regenerative properties. Moreover, the authors also presented an experimental section with original data on the applicability of a MSCs protocol developed by the research group on *Bothrops atrox* experimental envenoming in mice, showing a potential beneficial effect on muscle damage.

DRUG DISCOVERY

Aside from the role of venom components on envenomation pathogenesis, toxins are important candidates in the prospection of novel biomolecules with biotechnological and therapeutical relevance. Among them, crotoxin, the major toxic component of the venom of South American rattlesnake *Crotalus durissus*, has been in the spotlight due to its immunotherapeutic properties concerning its anti-inflammatory and immunosuppressive behavior. Sant'anna et al. conducted a study on the potential of a nanoformulation complexed with crotoxin to treat multiple sclerosis in an experimental autoimmune encephalomyelitis model. The nanoformulation was more efficient in reducing pain, ameliorate motor impairment and mitigate peripheral Th17 response and cell infiltration to the central nervous system, compared to animals treated with crotoxin alone.

Another applicability of venom toxins in medicine comprises the use of toxins as coadjuvant molecules, without a direct pharmacological-target purpose to treat the patients. Abbade et al. conducted a phase I/II non-randomized, single-arm clinical trial using heterologous fibrin sealant for the treatment of chronic venous ulcers. Fibrin sealants are widely used in dermatological treatments, due to their tissue repair properties, which the authors used a formulation containing heterologous fibrinogen-rich cryoprecipitate and a thrombin-like toxin from the venom o *Crotalus durissus* as the inducer of the fibrin formation. The study showed promising results, which the bioproduct proved to be safe and non-immunogenic with good preliminary efficacy for the treatment of chronic venous ulcers.

In summary, studies of venom components reveal more and more examples of natural toxins with actions on the immune system, which can be used both as pharmacological tools and as a useful starting point for drug development. Moreover, understanding the effects triggered by venoms and their toxins is particularly important to elucidate the mechanism of clinical

envenomings and help in the discovery of better antivenoms and ancillary treatments.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Correlating Fibrinogen Consumption and Profiles of Inflammatory Molecules in Human Envenomation's by *Bothrops atrox* in the Brazilian Amazon

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Snakebites are considered a major public health problem worldwide. In the Amazon region of Brazil, the snake *Bothrops atrox* (*B. atrox*) is responsible for 90% of the bites. These bites may cause local and systemic signs from acute inflammatory reaction and hemostatic changes, and present common hemorrhagic disorders. These alterations occur due the action of hemostatically active and immunogenic toxins which are capable of triggering a wide range of hemostatic and inflammatory events. However, the crosstalk between coagulation disorders and inflammatory reaction still has gaps in snakebites. Thus, the goal of this study was to describe the relationship between the consumption of fibrinogen and the profile of inflammatory molecules (chemokines and cytokines) in evenomations by *B. atrox* snakebites. A prospective study was carried out with individuals who had suffered *B. atrox* snakebites and presented different levels of fibrinogen consumption (normal fibrinogen [NF] and hypofibrinogenemia [HF]). Seventeen patients with NF and 55 patients with HF were eligible for the study, in addition to 50 healthy controls (CG). The molecules CXCL-8, CCL-5, CXCL-9, CCL-2, CXCL-10, IL-6, TNF, IL-2, IL-10, IFN-γ, IL-4, and IL-17A were quantified in plasma using the CBA technique at three different times (pre-antivenom therapy [T0], 24 h [T1], and 48 h [T2] after antivenom therapy). The profile of the circulating inflammatory response is different between the groups studied, with HF patients having higher concentrations of CCL-5 and lower IFN-γ. In addition, antivenom therapy seems to have a positive effect, leading to a profile of circulating inflammatory response similar in quantification of T1 and T2 on both groups. Furthermore, these results suggest that a number of interactions of CXCL-8, CXCL-9, CCL-2, IL-6, and IFN-γ in HF patients are directly affected

by fibrinogen levels, which may be related to the inflammatory response and coagulation mutual relationship induced by *B. atrox* venom. The present study is the first report on inflammation-coagulation crosstalk involving snakebite patients and supports the better understanding of envenomation's pathophysiology mechanisms and guides in the search for novel biomarkers and prospective therapies.

Keywords: hemostasis, immune response, *Bothrops* snakebites, inflammation-coagulation, crosstalk

INTRODUCTION

Snakebite is a neglected tropical disease and a major public health problem in developing countries worldwide. It is an important cause of morbidity and mortality, especially in areas of extreme poverty in the tropics and subtropics, such as sub-Saharan Africa, South, and Southeast Asia, Papua New Guinea, Central and Latin America (1). Approximately 421,000 cases of snakebites and 20,000 deaths occur worldwide each year (2, 3). In Brazil, specifically in the Amazon region, 57,374 notifications of snakebites were reported, resulting in incidence rate of 37.2 cases per 100,000 inhabitants/year, during the period from 2010 to 2015 (4). The *B. atrox* snake is widely distributed in the northern region of Brazil (5, 6), and is the species that causes 90% of envenomings in the region (7).

Clinical manifestations observed in victims of *Bothrops* snakebites are characterized by varied local and systemic effects, as a result of the action of biologically active toxins in the venom (2, 8). Among these effects, inflammatory and hemostatic disorders are frequently observed in patients. Envenomation's by viperid snakes often cause local and systemic bleeding (9, 10), such as gingivorrhagia, epistaxis, hematemesis, hematuria, bleeding in the uteris, and placenta (in pregnant women) (11, 12) and bleeding in the central nervous system (13–16).

Coagulopathy as a result of a snakebite is characterized by the action of hemostatically active toxins, which are capable of interfering with coagulation factors, by activating, inhibiting, or modulating platelet function, and inducing fibrinolysis. These effects can lead to blood incoagulability, which is characterized by the depletion of coagulation factors, and this depletion can be accompanied by thrombocitopenia, as well as intravascular thrombotic events. Consumption coagulopathy, thrombocytopenia and the effect of hemorraghins (toxins that act in the degradation of components of the basement membrane of vessels and with direct cytotoxic action on endothelial cells) are involved in the development of hemorrhagic events. The results consist of local and systemic bleeding disorders, hypovolemic shock, and thrombotic microangiopathy (17).

Regarding the inflammatory process, the *Bothrops* snakebite is characterized by the ability of venom toxins to directly activate inflammatory cells that are circulating or tissue located (18). It can also indirectly activate them as leukocytes recognize products of tissue damage caused by the action of venom components, known as venom-associated molecular patterns (VAMPs), and damage-associated molecular patterns (DAMPs) (19–21). In both cases, leukocyte stimulation is responsible for the production of inflammatory mediators, such as cytokines, chemokines, lipid mediators, and components of the complement system, resulting in a leukocyte infiltrate. The exacerbated inflammatory reaction enhances the tissue damage and reduces repair mechanisms, contributing to local complications from snakebites such as edema formation, necrosis, compartment syndrome, functional deficit, and amputation, as well as alterations in organs such as the kidneys and lungs (16, 20, 22, 23).

Coagulation and inflammation are highly integrated and well-balanced biological systems since systemic inflammation can lead to activation of coagulation and the components of these can modulate the inflammatory response. Deregulation components of these systems can affect this balance, resulting in a large number of diseases with different levels of severity associated with excessive inflammation and thrombosis (24). However, the crosstalk between coagulation disorders and inflammatory reaction still has gaps in snakebites. Current knowledge demonstrates that isolated *Bothrops* venom and toxins can be associated with acute inflammatory processes and coagulation disorders (25).

Patients bitten by *B. atrox* snakes in the Amazonas State, Brazil, presented the installation of a consumption coagulopathy, in which 85% of the patients showed hypofibrinogenemia (26). In addition, increased levels of soluble inflammatory mediators in patients have been demonstrated, and are associated with increased severity of the accident (27).

The aims of this study were to describe the relationship between the consumption of fibrinogen and the profile of inflammatory molecules (cytokines and chemokines) in *B. atrox* snakebite patients in the Brazilian Amazon. Our results show that inflammatory molecules showed different interactions associated with the levels of fibrinogen, which suggests crosstalk between coagulation disorders and inflammatory reactions.

MATERIALS AND METHODS

Study Design

A prospective study was carried out with individuals who had suffered *B. atrox* snakebites and who had sought medical assistance at the *Fundação de Medicina Tropical Doutor Heitor Vieira Dourado* (FMT-HVD).

Patients and Sampling

The study population consisted of 72 individuals with clinical and laboratory diagnosis of *Bothrops* snakebite and who had sought medical assistance at FMT-HVD. Pregnant women or individuals who reported a history of chronic inflammatory disease, autoimmune diseases, or immunodeficiency were not

included. *Bothrops* identification was performed by a zoologist from the research group. Patients were included, and then followed-up for 48 h, with clinical and laboratory evaluations carried out on three occasions: before antivenom therapy (T0), at 24 h (T1), and at 48 h (T2) after the administration of antivenom. Patients were classified into two subgroups (normal fibrinogen [NF], values ≥200 mg/dL; and hypofibrinogenemia [HF] <200 mg/dL) according to their results of fibrinogen consumption (**Figure 1**). Furthermore, blood samples from patients were subsequently sent to the Instituto Butantan (IBu) to confirm snakebite by *Bothrops* sp. using a gender-specific ELISA (28). In addition, 50 healthy individuals, of either gender and no history of snakebite, were included in the control group (CG) as a comparison parameter considering the baseline levels of cytokines and chemokines obtained at the *Fundação Hospitalar de Hematologia e Hemoterapia do Amazonas* (HEMOAM).

Ethical Issues

This study was approved by the FMT-HVD Research Ethics Commitee (process #492.892/2013). Participants read and signed the informed consent form before enrollment. All patients were treated according to Brazilian Ministry Health protocols (29).

Biological Sample Collection and Clinical Data

The quantification of inflammatory molecules was performed with ∼4 mL of peripheral blood collected at three different times (T0, T1, and T2) by venipuncture in tubes containing EDTA (BD Vacutainer® EDTA K2). In addition, for the measurement of fibrinogen in the first 24 h, peripheral blood samples were collected in tubes with sodium citrate (BD Vacutainer® sodium citrate). After collection, the samples were centrifuged (1,200 g for 5 min.) for the acquisition of a plasma aliquot (500 uL) and storage in a freezer at −80°C, with a view to subsequent measurement. The clinical-epidemiological data of the patients (gender, age, previous history of snakebite, area of occurrence, severity classification of the case, and affected anatomical region) were systematically collected.

Fibrinogen Quantification

Fibrinogen was quantified based on the Clauss method (30), using the Fibrinogen-C Kit (HemosIL®, Instrumental Laboratory Company, USA. Kit code: 0020301100) in an ACL TOP 300CTS coagulation analyzer (Werfen Instrumentation Laboratory, Barcelona, Spain). The test was carried out according to the manufacturer's protocol.

Inflammatory Molecules Level Quantification by CBA

The measurement of chemokines and cytokines in patients' plasma samples was performed using the CBA (Cytometric Bead Array) Flow Cytometry technique with the BD™ CBA Human Chemokine Kit (Code: 552990, BD® Biosciences, San Diego, CA, USA) and BD™ CBA Human Th1/Th2/Th17 Cytokine Kit (Code: 560484, BD® Biosciences, San Diego, CA, USA) following the guidelines described by the manufacturer. The samples were acquired using a FACS Canto II Flow Cytometer (Becton, Dickinson and Company, San Jose, CA, USA) at the HEMOAM. FCAP-Array™ software (v3) was used to calculate the concentrations in pg/mL of each molecule.

Statistical Data Analysis

The clinical data of patients and physical/electronic records, and the results of measurements of fibrinogen, cytokines, chemokines were entered into a database developed using

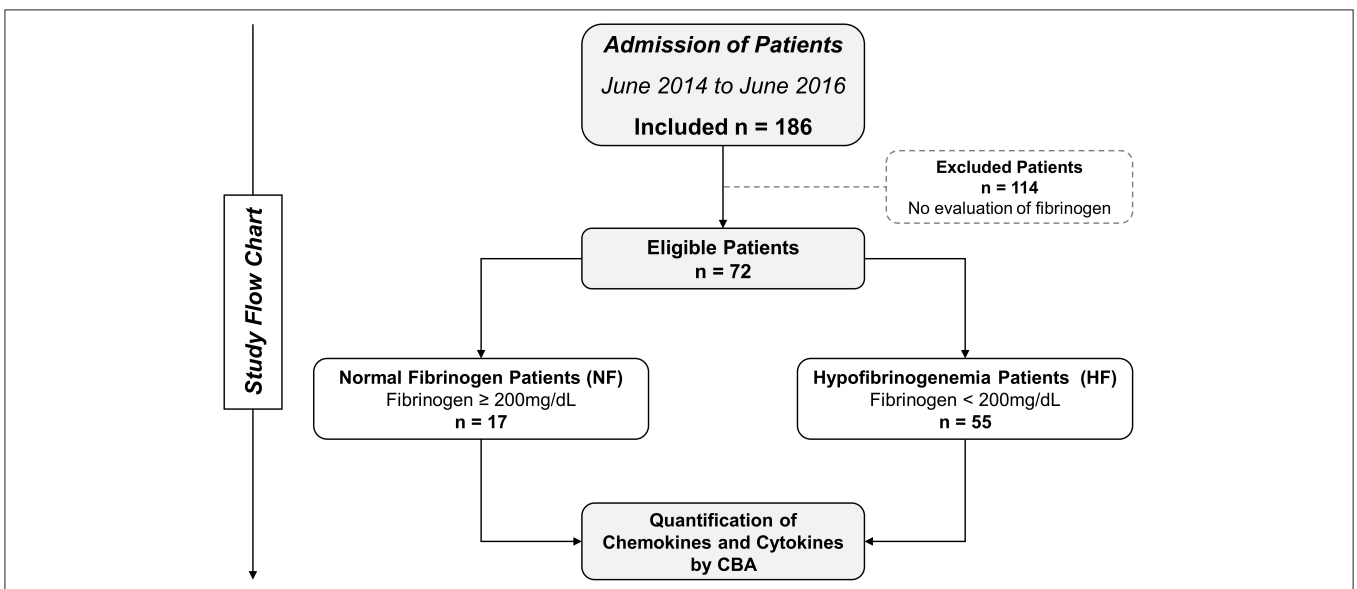

FIGURE 1 | Flowchart of study. Seventy-two patients were eligible and followed up until discharge. These patients were divided into two groups: normal fibrinogen (NF) and hypofibrinogenemia (HF), according to their fibrinogen consumption.

Microsoft Excel. Statistical analyses were performed using the GraphPad Prism (v5.0) and Stata (v13.0) software. Initially, tests were performed to verify normality of data using the Shapiro-Wilk test, which showed results with non-parametric distribution. The comparisons of values between two groups of data were performed using a Mann-Whitney test, while for comparisons of variables with three or more groups, a Kruskal-Wallis test was used, followed by Dunn's *post-test*, for multiple comparisons between groups. The elaboration of networks and a demonstration of complex interactions between the fibrinogen, chemokines, and cytokines evaluated in the study were performed based on the association of these markers in each clinical group (31). Spearman's correlation test was carried out and subsequently the construction of the networks with the Cytoscape 3.7.2 software (Cytoscape Consortium San Diego, CA, USA) were done, following the recommendations and instructions described by the manufacturer. The levels of statistical significance defined in all tests were $p < 0.05$.

RESULTS

Clinical and Epidemiological Baseline of the Patients

The clinical and epidemiological characteristics of the patients showed that the male gender was most common, and median age was statistically higher in *B. atrox* patients, when compared to controls. **Table 1** summarizes the clinical and epidemiological characteristics of the individuals included in the study. Most *B. atrox* patients had no previous history of snakebites and bites had occurred in rural areas. The main anatomical site of the snakebite was the lower limbs and bite classification was mild occurred in *B. atrox* groups. The time between the snakebite and the antivenom therapy administration was no different in patients.

TABLE 1 | Demographic and clinical characteristics of patients and individuals included in the study.

Demographic and clinical characteristics	Control group	*B. atrox* patients		*p-value*
		Normal fibrinogen	Hypo-fibrinogen	
	n = 50	*n* = 17	*n* = 55	
Gender (*n*, Male/Female)	34/16	14/3	47/8	0.248
Age (Years, median and [IQR])	30 [23–42]	45 [27–55]	39 [29–57]	**0.006**
Previous snakebite (*n*, Yes/No)	-	1/16	11/44	0.271
Occurrence zone (*n*, Rural/Urban)	-	15/2	50/5	0.665
Anatomical site of the Snakebite (*n*, Upper/Lower limb)	-	1/16	14/41	0.099
Accident Classification (*n*, Mild/Severe)	-	15/2	12/7	0.127
Time from Snakebite to Antivenom (hours, median [IQR])	-	4 [3–7]	4 [3–8]	0.591

Values in bold show a statistically significant difference.

B. atrox Snakebite Patients With Hypofibrinogenemia (HF) Presented Similar Plasmatic Levels of Inflammatory Molecules When Compared to Those in the Normal Fibrinogen (NF) Group

Figure 2 demonstrates the production dynamics of inflammatory molecules in the control group (CG), *B. atrox* snakebite with normal fibrinogen (NF) and hypofibrinogenemia (HF) before antivenom administration (T0). The inflammatory molecules CXCL-8, CCL-5, CXCL-9, CCL-2 CXCL-10, and IL-6 were higher in patients with *B. atrox* snakebite (NF and HF groups) when compared to the CG group. In addition, TNF, IFN-γ, and IL-4 showed low concentrations in these patients. Furthermore, profile of the circulating inflammatory molecules is similar between the NF and HF snakebite groups, with HF patients presenting higher concentrations of CCL-5 and lower IFN-γ.

Production Dynamics of Inflammatory Molecules in *B. atrox* Snakebite Patients With Normal Fibrinogen (NF) and Hypofibrinogenemia (HF) After Antivenom Therapy

Differences in plasma concentrations of inflammatory molecules were evaluated before antivenom administration and 24 h after antivenom administration (T0 vs. T1), in the NF and HF *B. atrox* snakebite groups. This analysis shows that the elements had different behavior in the first 24 h after treatment. **Figure 3**, it can be noted that there was a significant decrease for CXCL-9 and CXCL-10 in both groups. CXCL-8, CCL-2, and IL-10 showed a significant decrease only in the HF *B. atrox* snakebite patients. In addition, it can be noted that IL-17A showed an upward trend in *B. atrox* groups, and this increase was only statistically significant in the HF group (**Figure 3**).

Administration of Antivenom Therapy Had a Positive Effect in the Profile of Circulating Inflammatory Molecules in Follow-Up of *B. atrox* Snakebite Groups

Figure 4 shows a complementary view of the results of **Figure 3**, where the plasma concentration of the inflammatory molecules and the possible temporal variations (T0, T1, and T2) between groups were compared. The administration of antivenom had a positive effect and presented a similar profile of circulating inflammatory response in follow-ups of *B. atrox* snakebite groups and the levels of molecules analyzed.

Network Correlation of Inflammatory Molecules in HF *B. atrox* Snakebite Patients Are Directly Affected by Fibrinogen Levels

The correlation networks between the inflammatory molecules allow us to understand the interaction between them and the mutual influence, which is exercised, thus allowing a clearer visualization of the polarization of the response profiles at the

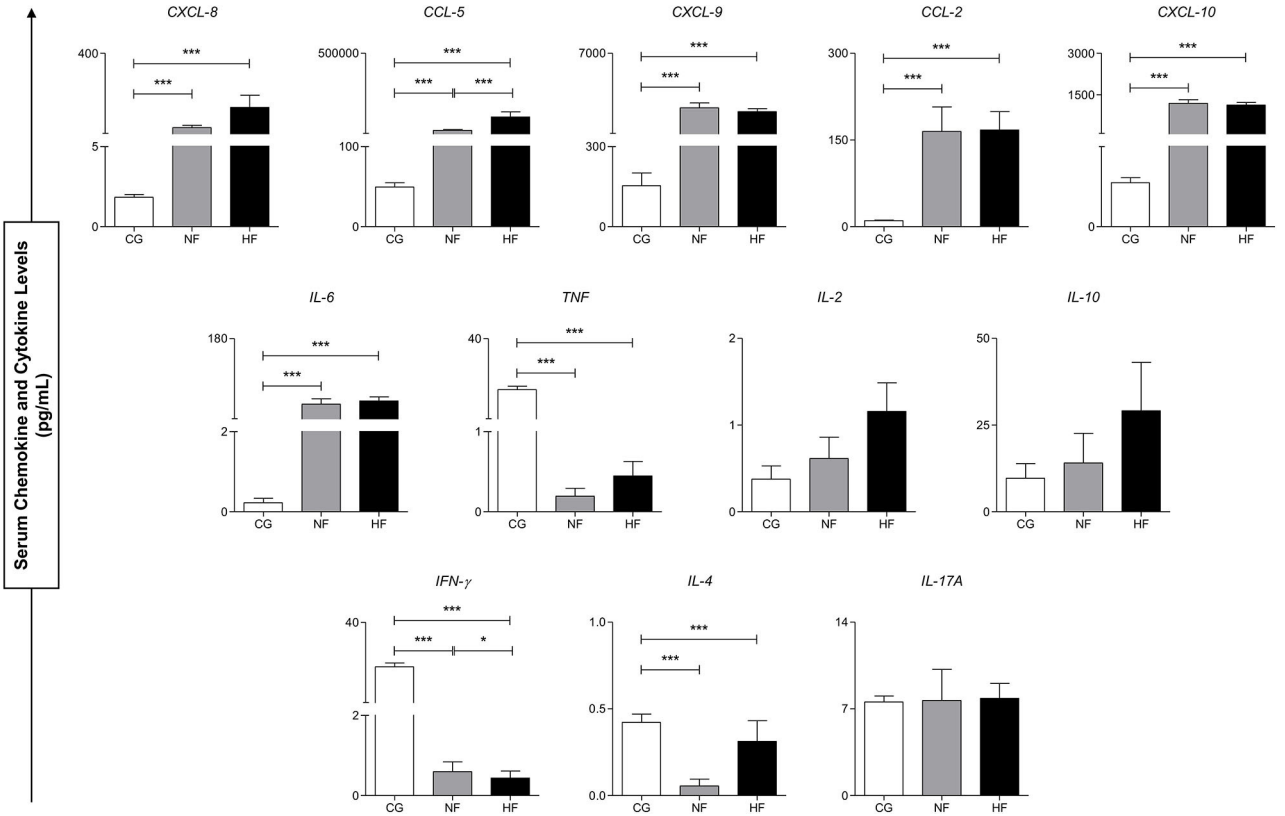

FIGURE 2 | Serum concentrations of circulating molecules of control groups (CG), normal fibrinogen (NF) and hypofibrinogenemia (HF) at T0. *$p < 0.05$; ***$p < 0.0001$.

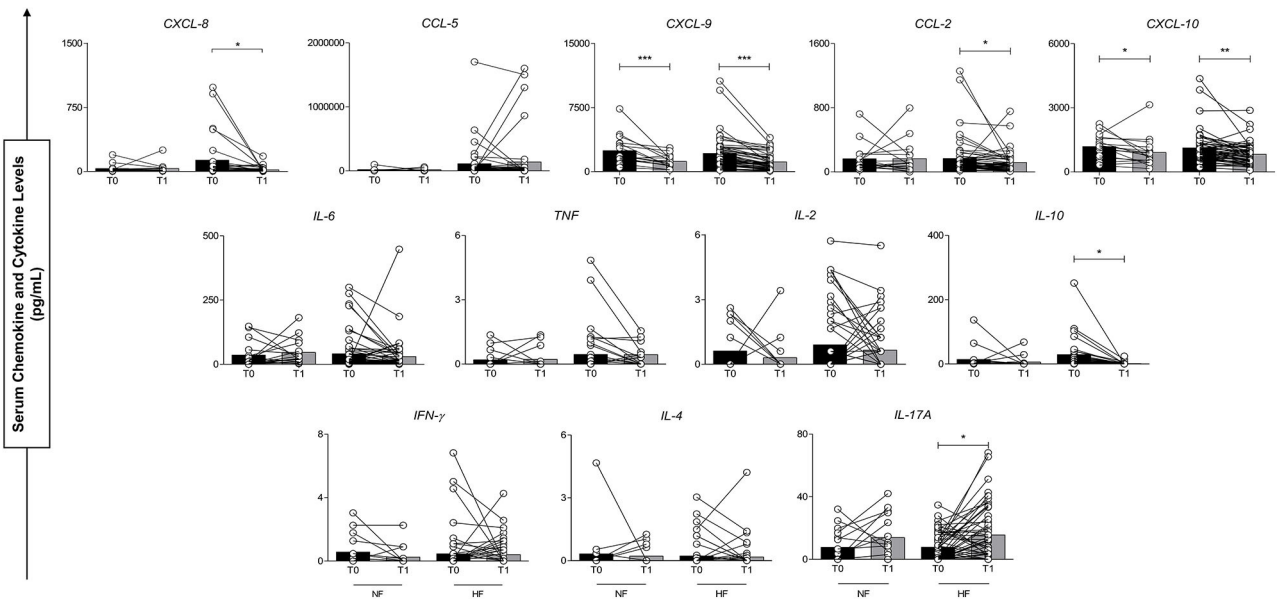

FIGURE 3 | Serum concentrations of circulating molecules of groups with normal fibrinogen (NF) and hypofibrinogenemia (HF) at T0 and T1. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$.

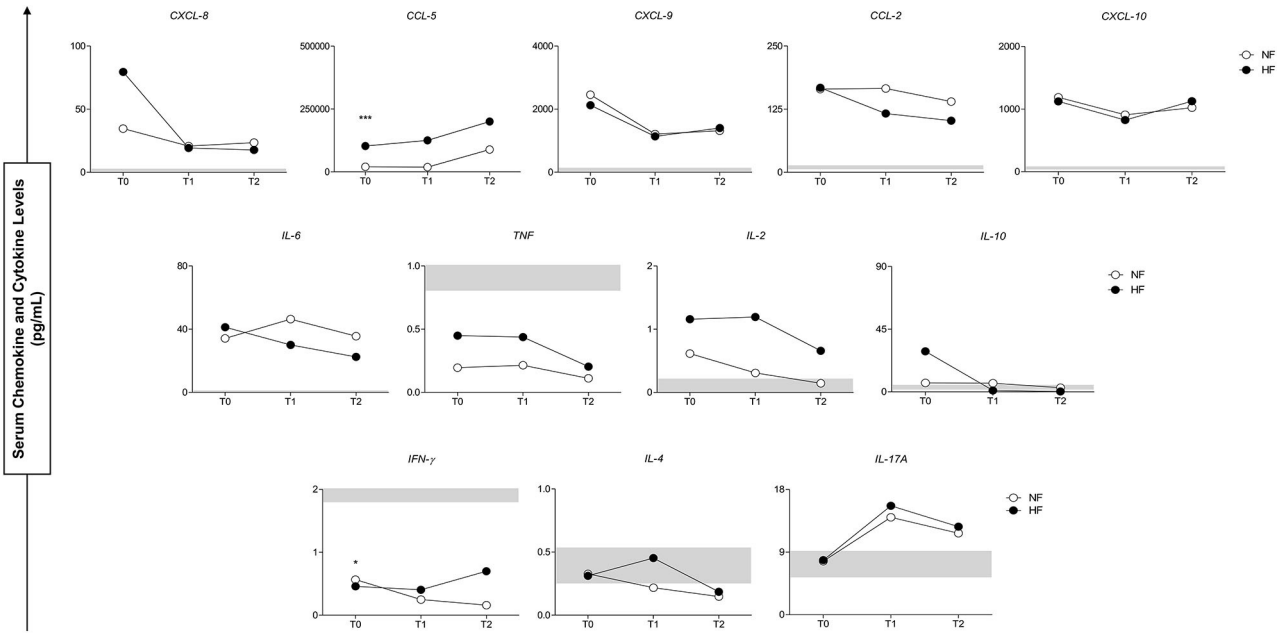

FIGURE 4 | Serum concentrations of circulating molecules of groups with normal fibrinogen (NF) and hypofibrinogenemia (HF) in follow-up of study (T0–T2). At the bottom, the interquartile range (25–75) of the serum concentrations in the CG group is shown as the baseline parameter. *$p < 0.05$; ***$p < 0.0001$.

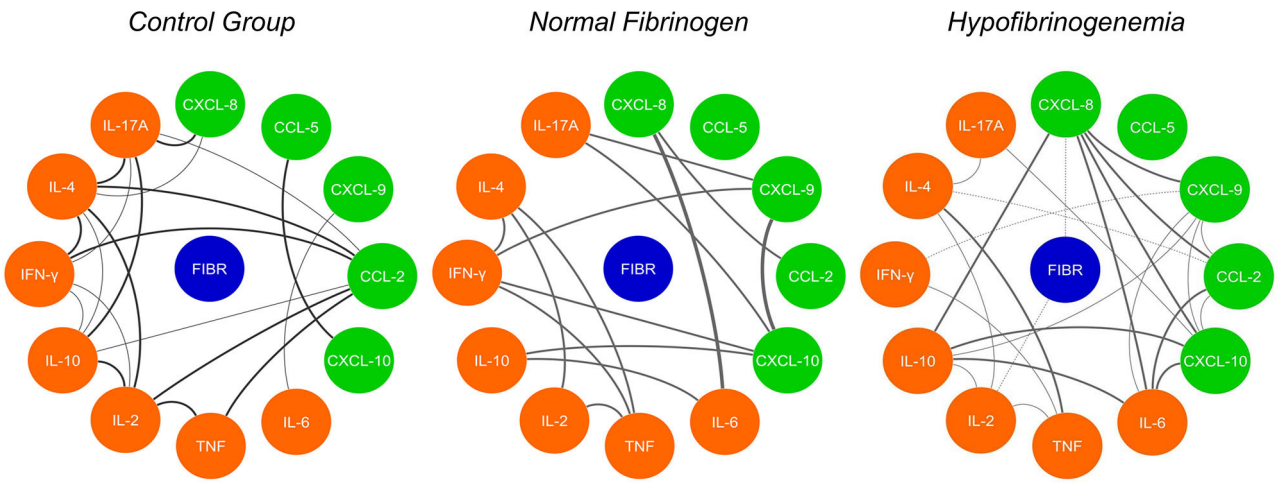

FIGURE 5 | Network correlation of soluble molecules shows interactions occurring throughout the fibrinogen levels and interaction of the molecules in the control group (CG). Each group of colored nodes is used to identify chemokines (green), cytokines (orange), and fibrinogen (blue). Dashed lines indicate negative correlation and continuous lines in black, positive correlation, while thickness shows correlation strength. The correlation index (r) was used to categorize the correlation strength as weak ($r \leq 0.35$), moderate ($r \geq 0.36$–$r \leq 0.67$), or strong ($r \geq 0.68$).

serum level, as shown in **Figure 5**. The CG network shows positive correlations between chemokines and cytokines, which is taken as a normal response in healthy individuals without any inflammatory process. Comparing the existing correlations in the groups of *B. atrox* snakebites patients (NF and HF), we noticed a great difference in the profile of circulating plasmatic molecules and in the number of correlations existing between them at the time of admission. Correlations in the NF *B atrox* snakebite group at T0 demonstrate an acute inflammatory response and strong and moderate correlations occur between chemokines (CXCL-8, CXCL-9, CCL-2, and CXCL-10), inflammatory cytokines (IL-6 and TNF). There is a predominant positive stimulus of the Th1 profile molecules (IFN-γ), but with the participation of the Th2 profile (IL-4) and Th17 profile with the cytokine IL-17A, which plays a stronger inflammatory role. There is also a regulatory process

with the participation of the IL-10 and IL-2 molecules. While in the HF *B. atrox* snakebite group, it is possible to observe a predominance of a chemotactic process with positive and negative, moderate, and strong correlations between chemokines (CXCL-8, CXCL-9, CCL-2, and CXCL-10), both among these molecules as with cytokines (IL-10, IL-6, IFN-γ, IL-4, and IL-17A), with a more polarized profile for Th2 response (IL-4). When analyzing fibrinogen using inflammatory molecules, we observed that CXCL-8 and IL-2 show negative correlations with this hemostatic factor (**Figure 5**).

DISCUSSION

The complex composition of toxins present in the *Bothrops* venom is responsible for a varied pathological response, which is represented in the diverse clinical manifestations observed in patients. Hemostatic and inflammatory disorders can be considered important pathophysiological effects and are associated with the effects of different components of the venom on different cellular and molecular targets. Evidence of crosstalk between coagulation and inflammation can lead to an enhancement of the effects and to greater damage (25). Thus, the present clinical study shows for the first time the association between inflammation and coagulation in patients who were victims of *B. atrox* snakebites.

The chosen hemostatic parameter was the concentration of fibrinogen. Fibrinogen is an acute phase protein, produced by the liver, and its serum concentration may increase in inflammatory and infectious conditions which are associated with vascular damage. In addition, this product of the coagulation cascade was identified as a significant risk factor and as an inflammatory process modulator in various pathological conditions, thus showing their participation in both coagulation and inflammation. Furthermore, the upregulation of the acute phase response proteins is mediated by IL-6 (32–35). *Bothrops* venoms are composed of hemostatically active molecules where, among them, we have pro-coagulant toxins capable of activating coagulation factors and thrombin-like toxins, which are capable of directly cleaving fibrinogen into fibrin. Among the toxins isolated from the venom of *B. atrox*, serine proteinases with thrombin-like activity and metalloproteases able to activate factors II, V, X, XIII, and VIII have already been described (36–40). The intravascular action of these toxins contributes to the formation of fibrin and the consequent consumption of fibrinogen. In addition, *B. atrox* venom toxins present fibrin(ogen)olytic and thrombolytic activity which also contribute to coagulopathy (41). Among the hemostatic changes observed in patients from *B. atrox* snakebites, blood incoagulability is accompanied by low plasma levels of fibrinogen and increased levels of fibrin/fibrinogen degradation products, which characterize an intravascular pro-coagulant effect (10, 42). Our data show that 76.4% of snakebite patients have hypofibrinogenemia, which is close to values found in a previous study (26).

The action of the *B. atrox* venom (BaV) or isolated toxins are responsible for the activation of the inflammatory response by mechanisms that involve direct leukocyte activation or signaling by VAMPs or fragments of cell damage or hydrolysis of extracellular components (43–45). As a consequence of this leukocyte stimulation, migration of neutrophils, monocytes, and macrophages to the lesion site is observed, as well as the synthesis and release of several inflammatory mediators, such as chemokines (CXCL-8, CXCL-1, and CXCL-2), components of the complement system (C1q, C3a, C4a, and C5a), cytokines (IL-12p70, TNF-α, IL-1α, IL-1β, IL-6, IL-10, and INF-γ) and lipid mediators (PGE2, LTB4, and CysLeucotrienes) (6, 43, 44, 46, 47). In the present study, we observed a significant increase in CXCL-8, CCL-5, CXCL-9, CCL-2, CXCL-10, and IL-6 among the groups exposed to the venom (NF and HF), previously to antivenom therapy (T0), when compared to the control group (CG). In addition, decreased concentrations of TNF and IFN-γ were observed. These findings demonstrate a high chemotactic and inflammatory response to exposure to venom in patients and corroborate data previously described in *B. atrox* snakebites, which shows a plasma increase in inflammatory mediators CXCL-8, CCL-5, CXCL-9, CCL-2, CXCL10, IL-6, TNF-α, IL-1β, and IL-10 (27). We observed in our study a low production of INF-γ, which could be associated with the main cell type producing this molecule (NK cells). This would not be activated by BaV during the first 8 h and would end up not constituting and participating in this inflammatory process (6).

The relationship between coagulation and inflammation in the pathogenesis of vascular diseases has become a pathophysiological mechanism of great focus in basic and clinical research. The elucidation of the mechanisms involved in this relationship has helped in the development of new therapeutic strategies, as well as in the diagnosis of pathologies involving the inflammation/coagulation axis through the discovery of biomarkers (48).

In regards to the aspects in which inflammation induces coagulation changes, the inflammatory response is capable of modulating different hemostasis events such as: (i) activation of coagulation due the increased expression of intravascular tissue factor (Factor III), (ii) activation of platelets and thrombus formation through thrombo-inflammation, (iii) decrease in the expression of endogenous anticoagulants such as anti-thrombin, and (iv) reduction of fibrinolytic function. These events induce consumption coagulopathy, which is responsible for the installation of thrombotic and hemorrhagic conditions (24, 49). The present study shows that patients of *B. atrox* snakebites presented increased levels of mediators CXCL-8, CCL-5, CXCL-9, CCL-2, CXCL-10, and IL-6. These mediators have been described to be associated with thrombotic pathologies, and act in the modulation of platelet function, expression of coagulation factors and fibrinolysis (50–53).

Among the mechanisms associated with the consumption of fibrinogen, the tissue factor (TF) plays an important role. TF is a transmembrane glycoprotein, in which the extracellular domain interacts with Factor VIIa, forming the extrinsic tenase complex (Factor III/Factor VIIa), capable of activating factor X and triggering coagulation (54). The intravascular expression of TF by endothelial cells and monocytes is induced by different stimuli, since pro-inflammatory molecules IL-6, CCL-2, and CXCL-8 are

described as inducing increased TF expression (55, 56). *Bothrops* snake venoms and its isolated toxins have already been described as being responsible for inducing TF expression, which is associated with pro-inflammatory events induced by the venom and its components (57–60). Recently, the proinflammatory action of a type-C lectin isolated from *Bothrops jararacussu* venom was demonstrated inducing a monocyte pro-coagulant character by increasing TF expression (61). Interestingly, a recent study by our research group also found that patients who were victims of *B. atrox* envenoming had increased levels of TF in the circulation and was associated with systemic bleeding and blood incoagulability (unpublished data).

When comparing the levels of mediators between the NF and HF groups, the chemokine CCL-5 and cytokine IFN-γ were elevated and decreased, respectively, in the group of patients with fibrinogen consumption. Chemokine CCL-5 is a chemoattractant for monocytes, produced mainly by activated $CD8^+$ T cells. Its role in the coagulation process involves the modulation of different pathways, and studies show that increased levels of the chemokine in patients with ischemic myocardial pathologies are associated with the formation of atherosclerotic plaques by the process of thromboinflammation (53). The expression of TF in this mechanism is associated with the CCL-5 pathway, since studies show that the activation of CCR-5 (CCL-5 receptors) are responsible for the expression of TF (62), as well as activated CD8 + T cells which also promote expression of TF in monocytes (63). Another function is related to the ability of CCL-5 to induce platelet degranulation and aggregation. These activated platelets serve as substrates for the formation of coagulation complexes and clotting activation, culminating in the consumption of fibrinogen and leading to the formation of intravascular thrombus (64).

Previously it has been shown that the levels of circulating soluble inflammatory molecules are higher before the administration of the antivenom, demonstrating an acute response to snakebites of the genus *Bothrops* (27) which agrees with our results which allowed us to observe the drop in levels of CXCL-9 and CXCL-10 in both evaluated groups. IL-10, CCL-2, and CXCL-8 showed a significant decrease only in the HF group. In addition, IL-17A showed an upward trend in both groups, this increase was statistically significant only in the HF group. With regard to this cytokine, we emphasize that IL-17 is a pro-inflammatory molecule that activates macrophages, fibroblasts, and stromal cells, including the expression of ICAM-1 and cytokine secretion (IL-6, CXCL-8, IL-11, factor granulocyte colony stimulator [G-CSF]), prostaglandin E2, and nitric oxide) (65–67). Ding et al. reported that IL-17A promotes the pathogenesis of deep vein thrombosis (DVT), improving platelet activation and aggregation, neutrophil infiltration, and activation of endothelial cells in murine models (68).

The correlation network analyses between the molecules evaluated in the present study show that, in the group of patients with hypofibrinogenemia, it was possible to observe a predominance of a chemoattractive profile, with correlations between chemokines CXCL-8, CXCL-9, CCL-2, and CXCL-10. Leukocyte chemoattraction has an important relevance in the pathogenesis of thrombotic events. Circulating cells are recruited to the site of thrombus formation through interactions with platelets and endothelial cells, and can be induced to express TF and release pro-inflammatory and pro-coagulant molecules, which influence various aspects of thrombus formation, including activation and platelet adhesion and activation of intrinsic and extrinsic coagulation pathways (69). Furthermore, we observed that the mediators CXCL-8 and IL-2 have a correlation with the consumption of fibrinogen, both of which have already been described because they are capable of inducing an intravascular coagulation process (70, 71).

In addition to the ability of inflammation to induce coagulation, the relationship is bidirectional, so that coagulation is also capable of modulating inflammatory activity. In this case, several agents are responsible for this action as coagulation factors and products of the action of these factors (48). Among the mechanisms, coagulation factors with proteolytic function are responsible for interacting with specific cells through their receptors to induce the activation of signaling pathways. Protease-Activated Receptors (PARs), expressed in endothelial cells, mononuclear cells, platelets, and others, have an important function because they are targets of the action of thrombin, TF/VIIa factor complex and Xa factor. The production of cytokines IL-6, IL-2, CXCL-5, CXCL-8, CCL-2, TNF-α, IL-1β, and IFN-γ by these coagulation factors through activation of PARs have already been reported (72–75). Therefore, it is important to consider the participation of factors Xa and thrombin in the inflammatory process observed in our *B. atrox* snakebite patients, since activators of factor X and prothrombin are present in the BaV (38, 39). The increase in CCL-5 levels in patients who presented hypofibrinogenemia may be associated with a possible action of the generated thrombin and FXa, since these factors are responsible for the expression of this chemokine in fibroblasts, platelets, and endothelial cells (76–78). Still, the chemotactic response in HF patients associated with the CXCL-8 and IL-2 correlation to fibrinogen consumption could be associated with thrombin activity, which is capable of inducing leukocyte chemotaxis (79).

Another mechanism which involves the ability of coagulation to modulate inflammation is the action of fibrinogen and its degradation products. The cleavage of fibrinogen by thrombin or by thrombin-like toxins can generate fibrin, which forms the polymer that comprises the clot. This cleavage produces fibrin peptides, such as fibrinopeptide B, which can act as chemoattractions for leukocytes and, thus, independently modulate inflammatory responses (32). It is important to note that these pro-inflammatory functions are a product of fibrin/fibrinogen signaling through binding sites that do not overlap with those involved in the coagulation cascade. The integrin receptor CD11b/CD18 (also called Mac-1, complement receptor 3 or αMβ2) is a representative example. This receptor is expressed by leukocytes of the innate immune system, mainly circulating monocytes, tissue-specific macrophages, and microglia residing in the central nervous system (CNS). Fibrin/fibrinogen signaling through CD11b/CD18 has been shown to activate pro-inflammatory pathways, such as NF-κB, which results in the local production of inflammatory cytokines, such as TNF-α and IL-1β (32). Studies show that fibrinogen and

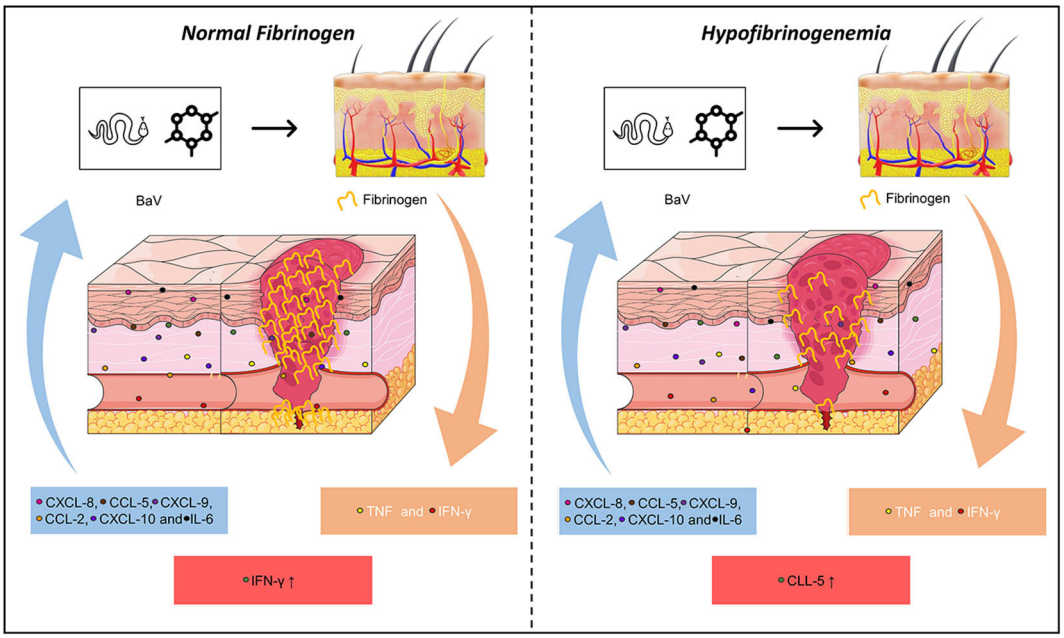

FIGURE 6 | Correlating fibrinogen consumption and profiles of inflammatory molecules in *Bothrops atrox* snakebite patients. Schematic presentation of inflammation-coagulation crosstalk in patients with normal fibrinogen (NF) and hypofibrinogenemia (HF).

fibrin are able to induce the production of IL-6 and TNF-α in blood mononuclear cells (80).

The study had some limitations related to the sample size of patients with normal fibrinogen dosage, quantification of other factors involved in the coagulation cascade (e.g., Tissue Factor) and patients' venom levels were not measured. However, we would like to emphasize that the results presented here help to understand this type of systemic complication; one which is common in the field of snakebites and of great clinical importance.

CONCLUSION

Our results suggest that number of interactions of CXCL-8, CXCL-9, CCL-2, IL-6, and IFN-γ in HF patients are directly affected by fibrinogen levels, which may be related to the inflammatory response and coagulation mutual relationship induced by *B. atrox* venom (**Figure 6**). In addition, CXCL-8 and IL-2 in HF *B. atrox* snakebite patients may be associated with the inflammation-coagulation axis. Furthermore, the hemostatic complication that occurs in patients suffering from *Bothrops* snakebites is multifactorial and the increase in this inflammatory response was reflected in the high concentrations of IL-6, IL-17A, and chemoattractive profile, which is possibly influenced by the coagulation factors activated by the venom. As BaV stimulates inflammation and activation of hemostatic factors, and these in turn contribute to the development of the inflammatory response, we have the result of a cycle in which increased vascular permeability, hypofibrinogenemia, bleeding, and subsequent morbidity occurs in patients. The present study is the first report on inflammation-coagulation crosstalk involving *B. atrox* snakebite patients, supporting the better understanding of envenomation's pathophysiology mechanisms and guides the search for novel biomarkers with therapeutic perspectives.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee at the FMT-HVD (approval number 492.892/2013). Patients were treated according to the recommendations of Brazilian Health Ministry. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IW, AC, and WM designed and performed the experiments, analyzed data, and wrote the manuscript. IW and AC analyzed data. HI and AC performed the experiments. WM, JS, MS, and AMM revised the manuscript. IW, HI, AC, WM, ML, LF, AM, AT, JS, IS, and SO conceived and supervised the project, designed the experiments, interpreted the data, wrote, and revised the manuscript. All authors read and approved the final manuscript.

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Pioneering Study on *Rhopalurus crassicauda* Scorpion Venom: Isolation and Characterization of the Major Toxin and Hyaluronidase

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Scorpionism is responsible for most accidents involving venomous animals in Brazil, which leads to severe symptoms that can evolve to death. Scorpion venoms consist of complexes cocktails, including peptides, proteins, and non-protein compounds, making separation and purification procedures extremely difficult and time-consuming. Scorpion toxins target different biological systems and can be used in basic science, for clinical, and biotechnological applications. This study is the first to explore the venom content of the unexplored scorpion species *Rhopalurus crassicauda*, which inhabits exclusively the northernmost state of Brazil, named Roraima, and southern region of Guyana. Here, we pioneer the fractionation of the *R. crassicauda* venom and isolated and characterized a novel scorpion beta-neurotoxin, designated Rc1, and a monomeric hyaluronidase. *R. crassicauda* venom and Rc1 (6,882 Da) demonstrated pro-inflammatory activities *in vitro* and a nociceptive response *in vivo*. Moreover, Rc1 toxin showed specificity for activating $Na_V1.4$, $Na_V1.6$, and $BgNa_V1$ voltage-gated ion channels. This study also represents a new perspective for the treatment of envenomings in Roraima, since the Brazilian scorpion and arachnid antivenoms were not able to recognize *R. crassicauda* venom and its fractions (with exception of hyaluronidase). Our work provides useful insights for the first understanding of the painful sting and pro-inflammatory effects associated with *R. crassicauda* envenomings.

Keywords: scorpion venom, *Rhopalurus crassicauda*, toxin, electrophysiology, nociception, neurotoxin, pro-inflammatory toxin

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INTRODUCTION

Venomous animals possess the capacity to develop a wide array of compounds with different biological effects inside a specialized apparatus to inject the venom into the preys (1, 2). In Brazil, accidents caused by venomous animals are a frequent neglected disease, with blind spots regarding general education and proper approach in most countries (3, 4). Scorpionism is included in this

scenario, in which *Tityus serrulatus* species is responsible for most of the accidents in the country, reaching numbers of over 100,000 reports in 2017 (5–7). In spite hereof, there are still many neglected accidents caused by other scorpion species in the Brazilian biome (6), such as the scorpion *Rhopalurus crassicauda*, which make the data underestimated.

Described in 1947 (8), *R. crassicauda* species inhabits exclusively the northernmost state of Brazil named Roraima and southern region of Guyana (3), although species of the same genus are found in other regions of the country (9, 10). However, *R. crassicauda* taxonomic is complex and controversial. For instance, the enigmatic species was also referred as *R. pintoi* in spite of the deep differences between both original descriptions. Further, it was treated as a subspecies of *R. laticauda*, which was restored as *R. crassicauda* as the valid species (11). Recently, the species was again considered a *R. laticauda* species (12). In this study we will keep the original taxonomic classification endorsed by Lourenço (2002) – R. *crassicauda* (11), since the researcher performed *R. crassicau*da collections around Boa Vista, Roraima, where actual samplings for the present work were conducted.

Albeit underreported accidents are a problem throughout Brazil, Roraima stands out for its scarce investment in research, the high number of indigenous people (more than 40% of the state is considered indigenous areas), and the vast number of Venezuelan migrants (3). Moreover, Roraima is a very poor region and has been overlooked by the Brazilian government and richest states (i.e., located mainly in south and southeast regions). Although the envenomings caused by scorpions in Roraima have been increasing in wide scale (32, 139, and 288 cases in 2007, 2017, and 2019, respectively) (13), human envenomings caused by *R. crassicauda* and its venom composition remain unexplored (unmatched data in the main academic databases), even though more than 70 years passed since the species description.

Scorpion venoms can trigger several clinical effects and their toxins can target different biological systems (5). Thus, several signs and symptoms can be observed in victims stung by scorpions such as pain, myosis, bradycardia, cardiac arrhythmias, arterial hypotension, increased lachrymal, nasal, salivary, pancreatic, gastric and bronchial secretions, diaphoresis, tremors, piloerection, and muscle spasms, increases blood amylase levels, mydriasis, cardiac arrhythmias, tachycardia, arterial hypertension, acute pulmonary edema, cardiac failure, and even circulatory shock following death (14–16). Concerning *R. crassicauda*, although there is no report about the signs and symptoms, physicians in Roraima have not faced severe intoxication by victims stung by this species, although intense pain and mild paresthesia are always reported by patients.

In Brazil, the use of specific antivenom is indicated to treat all severe scorpion envenomings (5, 17), and the antivenom administration is mandatory in case of envenoming in children under 7 years-old or adults, mainly elderly, with previous health problems such as hypertension and cardiovascular diseases. Nevertheless, besides all the potential adverse effects produced by horse-derived antivenoms (i.e., anaphylactic reaction and serum sickness), and the larger than necessary dose of equine antibodies (about 70% of the antibodies are considered unspecific) (18), the Brazilian scorpion antivenom is produced exclusively against *T. serrulatus* species and could not be used to treat accidents caused by other scorpion genera (e.g., *R. crassicauda*), whilst there are more than 160 different documented scorpion species in the country (7). This study is the first one to explore the venom content of the neglected scorpion species *R. crassicauda* from an overlooked and poor state of Brazil – Roraima. In particular, an effort was made to isolate and characterize a novel scorpion neurotoxin (Rc1), and a spreading factor (hyaluronidase) from its venom.

RESULTS

Scorpions and Venom Milking

A total of 23 specimens of *R. crassicauda* scorpions, collected in the region of Boa Vista (**Figure 1**), were kept in the *vivarium* for venom milking. In order to standardize venom milking, our research group successfully built a restraining electrical device coupled to a dimmer potentiometer. After different electrical stimulation tests in the scorpion telson, the voltage of 18 V proved to be the best and thus suitable for *R. crassicauda* milking. During the initial stages of electrical stimulation, a colorless, watery venom was obtained; subsequently, the milked venom was a viscous fluid. From a total of 115 milky scorpions (one milky droplet extracted), 9.2 mg of proteins was estimated in the soluble crude venom, corresponding to an average of 80 μg of proteins per scorpion.

Venom Fractionation and Enzymatic Activities

To isolate the toxins, the *R. crassicauda* venom was submitted to reversed-phase fast protein liquid chromatography (RP-FPLC) on a C18 column (10 × 250 mm) and the major peak (fraction P8, **Figure 2A**) was re-chromatographed on a different C18 column (2.1 × 250 mm; **Figure 2B**). The resulting pure toxin, named Rc1, represented 24% of the total protein of the soluble crude venom.

A Tricine-SDS-PAGE (16.5%) electrophoresis was used to evaluate the complexity of the components present in *R. crassicauda* venom compared to *T. serrulatus* venom, as well as the purity profile of the eluted fractions (**Figure 3**). Non-reduced venom and peak 9 (P9) showed a single translucent band of ∼45 kDa in the hyaluronan-based gel (**Figure 3, lanes 20** and **21**), indicanting hyaluronidase activity. On the other hand, under reduction conditions, P9 presented a molecular mass of ∼54 kDa (**Figure 3**, lane 14). The major peak P8 revealed a unique protein band of ∼6.5 kDa (**Figure 3**, lane 5).

Non-enzymatic activities of PDE and PLA_2 were detected on *R. crassicauda* venom in the tested concentrations (5 and 65 μg/well, respectively, data not shown).

Molecular Mass and Sequence of Rc1

The mass spectrum of Rc1 showed an average ion *m/z* 6,883.3 Da and the ion *m/z* 3,443.1 Da $[(M + 2H)^{2+}$; **Figure 4A**]. The first 27 N-terminal amino acids from Rc1 were determined by Edman degradation method as KGGYPVDSKGCKISCVINNEYCSRDCT. In addition, Rc1

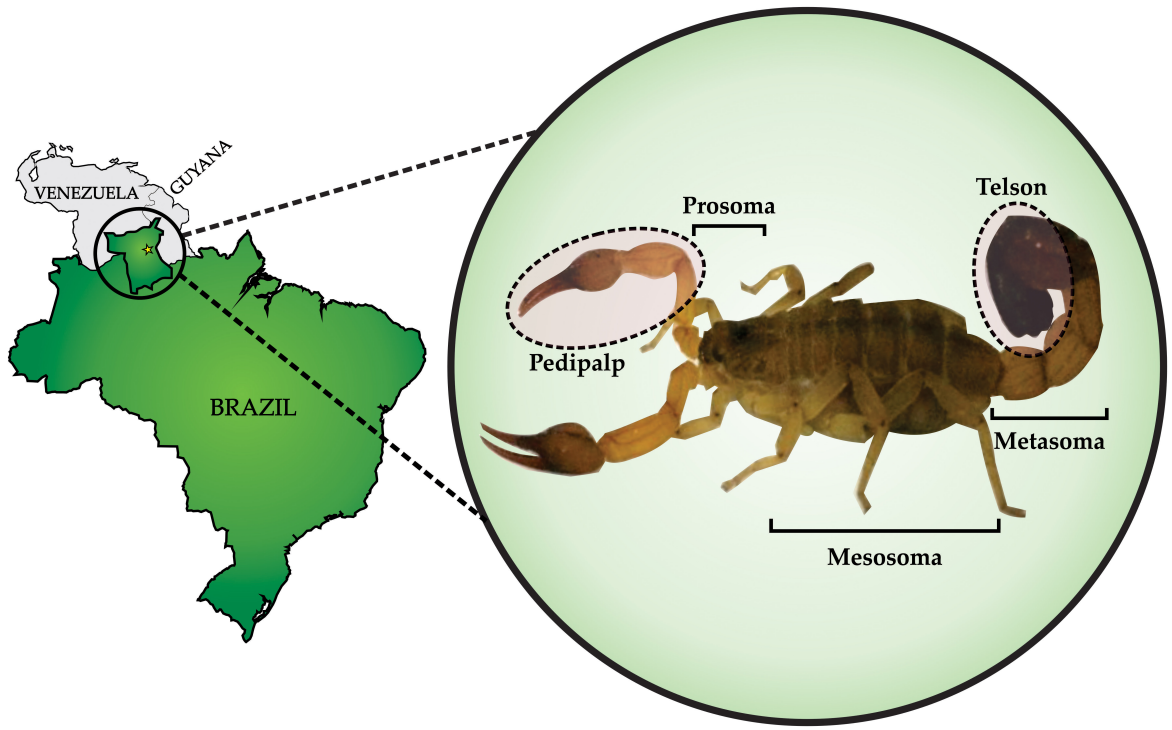

FIGURE 1 | *Rhopalurus crassicauda*: collection and morphology. Left panel shows Roraima, the northernmost state of Brazil. The yellow star indicates the capital city of the state, Boa Vista, where *R. crassicauda* scorpions were collected. Zoom view in the right panel shows the *R. crassicauda* species (scorpion length – 10 cm). Photo taken of a specimen kept in the scorpion *vivarium* of the research group.

internal peptides (**Supplementary Table 1**) were determined by *de novo* sequencing resulting in a sequence coverage of ∼80%, considering the peptides masses in comparison to Rc1 molecular mass determined by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF; **Figure 4A**). Seven Cys conserved residues were recovered from 8 estimated Cys residues. The total sequence obtained for Rc1 (the protein sequence data will appear in UniProt Knowledgebase under the accession number C0HLR6) presented 60% identity with Css9 beta-neurotoxin from *Centruroides suffusus* (**Figure 4B**).

Antivenom Cross-Reactivity

The Brazilian scorpion (ScA) and arachnid (ArA) antivenoms were not able to recognize the soluble crude venom of *R. crassicauda* (**Figures 5A,B**). However, ScA was able to recognize venom fraction P9, which presents hyaluronidase (**Figure 5C**).

Venom and Major Toxin Effects

Both *R. crassicauda* venom and Rc1 toxin (100 and 50 μg/mL, respectively) demonstrated no cytotoxic effects on J774.1 cells (data not shown).

Rhopalurus crassicauda venom (100 μg/mL) increased IL-6 production (**Figure 6B**), whereas Rc1 stimulated TNF-α production. Interestingly, Rc1 was able to increase two-fold the TNF-α levels when compared to Ts1 (**Figure 6A**), being a potent inflammatory toxin. Moreover, NF-kB signaling pathway was activated by *R. crassicauda* venom and Rc1 stimuli, as observed with *T. serrula*tus venom and Ts1, indicating that both scorpion venoms, and major toxins activate intracellular pro-inflammatory pathways (**Figure 7**).

Rc1 activity was also tested on 6 different voltage-gated sodium channels expressed in *Xenopus laevis* oocytes. It was investigated if Rc1, at a concentration of 1 μM, could modulate the voltage dependence of the steady-state activation and inactivation curves. Rc1 altered the activation process of Nav1.4, Nav1.6 channels, and of the insect channels $BgNa_v1$ (**Figure 8**). Application of 1 μM Rc1 shifted the midpoint of activation from −29.7 ± 0.1 mV in control to −48.5 ± 0.5 mV for Nav1.6 channels ($n = 4$). For BgNav1 channels, an alteration of the $V_{1/2}$ from −37.7 ± 0.3 mV to −64.8 ± 0.5 mV after application of Rc1 was observed ($n = 6$). For Nav1.4 channels, a minor but still significant modulation of activation was noted since $V_{1/2}$ values yielded −22.9 ± 0.1 mV and −27.2 ± 0.2 mV in control and in presence of Rc1, respectively. The steady-state inactivation curves were not significantly altered in the presence of Rc1. Rc1 did not show activity on Nav1.1, Nav1.2, and Nav1.5 channels (**Figure 8**).

In vivo, although *R. crassicauda* induced a significant increase in mice paw licking and lifting during the first 15 min (in all tested concentrations), the spontaneous nociception behavior was considerably lower when compared to *T. serrulatus* venom (**Figure 9A**). The same was observed to Rc1 toxin, which needs four-fold more toxin to induce similar nociceptive behavior of Ts1 (**Figure 9B**).

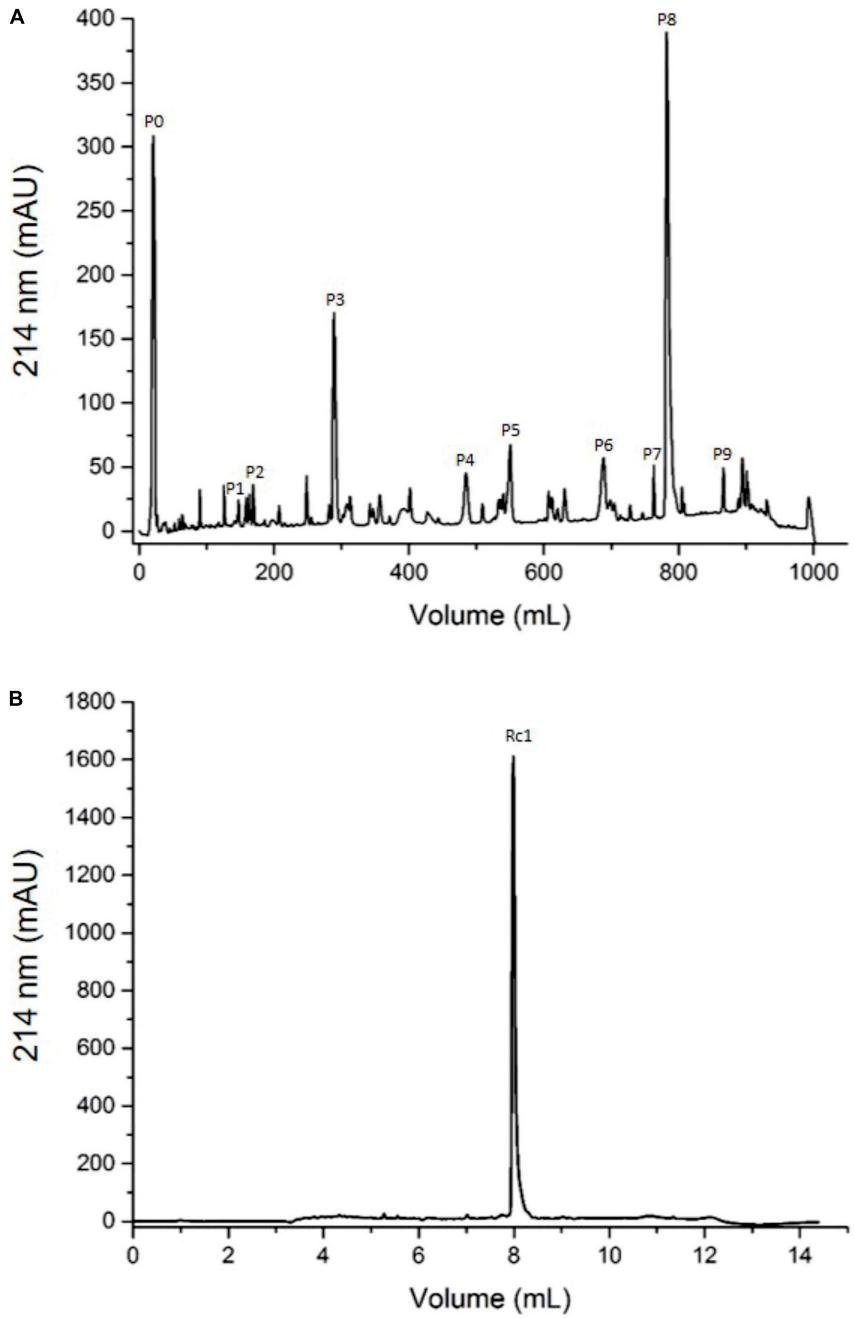

FIGURE 2 | Chromatographic profiles of *R. crassicauda* venom using RP-FPLC system. The protein elution was carried out in a segmented concentration gradient from 0 to 100% of solution B (80% ACN in 0.1% TFA) and absorbance was monitored at 214 nm. **(A)** *R. crassicauda* venom (2 mg) was eluted using 5 concentration gradient steps on a C18 column (250 × 10 mm, 300 Å, and 5 μm particles), at a flow rate of 5 mL/min. **(B)** P8 (40 μg) was re-chromatographed using 4 concentration gradient steps on a C18 column (250 × 2.1 mm, 300 Å, and 5 μm particles), at a flow rate of 0.5 mL/min.

DISCUSSION

A single venom can contain up to several hundred different components producing diverse pathophysiological effects (19). Thus, studies focusing on the development of new drugs based on novel toxins remain valuable today. In addition, the understanding of a venom content can also elucidate local human envenomings and improve the efficacy of antivenom manufacture (20, 21). Although uncountable venomous species and their venoms have been over studied so far, *R. crassicauda* venom remains still unexplored (11).

In this study, the electrical method of venom extraction appears to be safe and successful for specimens of *R. crassicauda*, i.e., permanent injuries were not noticed neither immediately

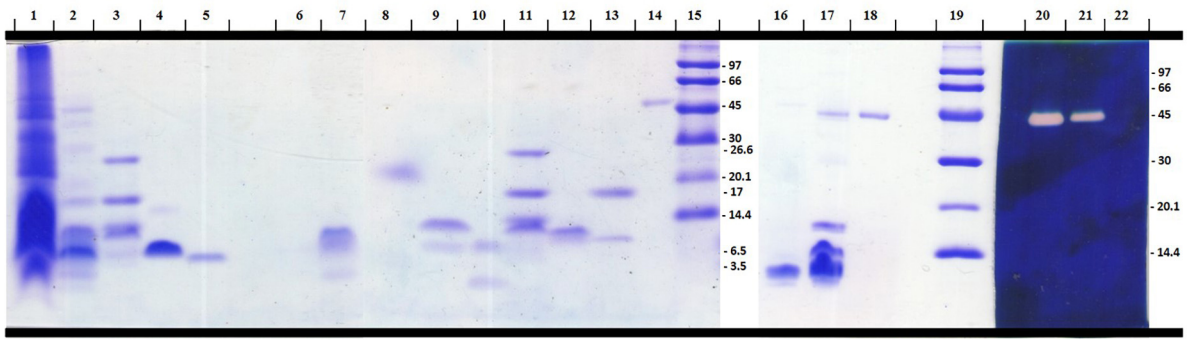

FIGURE 3 | Electrophoretical profile of *R. crassicauda* venom and fractions. Tris-tricine-SDS-PAGE (16.5%) under reducing (Lanes 1–15) and non-reducing (Lanes 16–22) conditions. Lanes 16–22 were incorporated with hyaluronan before gel polymerization. Lanes 1, 16, and 22: *T. serrulatus* venom (18, 16, and 5 μg, respectively). Lanes 2, 17, and 21: *R. crassicauda* venom (18, 5, and 5 μg, respectively). Lanes 3 and 11: molecular mass markers (Sigma M3546). Lanes 15 and 19: molecular mass markers (GE Healthcare 17-0446-01). Lanes 4 and 5: Ts1 and Rc1 (P8), respectively (2 μg). Lanes: 6: P1; 7: P2; 8: P3; 9: P4; 10: P5; 12: P6; 13: P7 (all 2 μg). Lanes 14, 18, and 20: P9 (2 μg). Lanes 1–19 were stained with PlusOne Coomassie Blue PhastGel® R-350. Lanes 20–22 were stained with Stains-all for evaluation of the hyaluronidase activity.

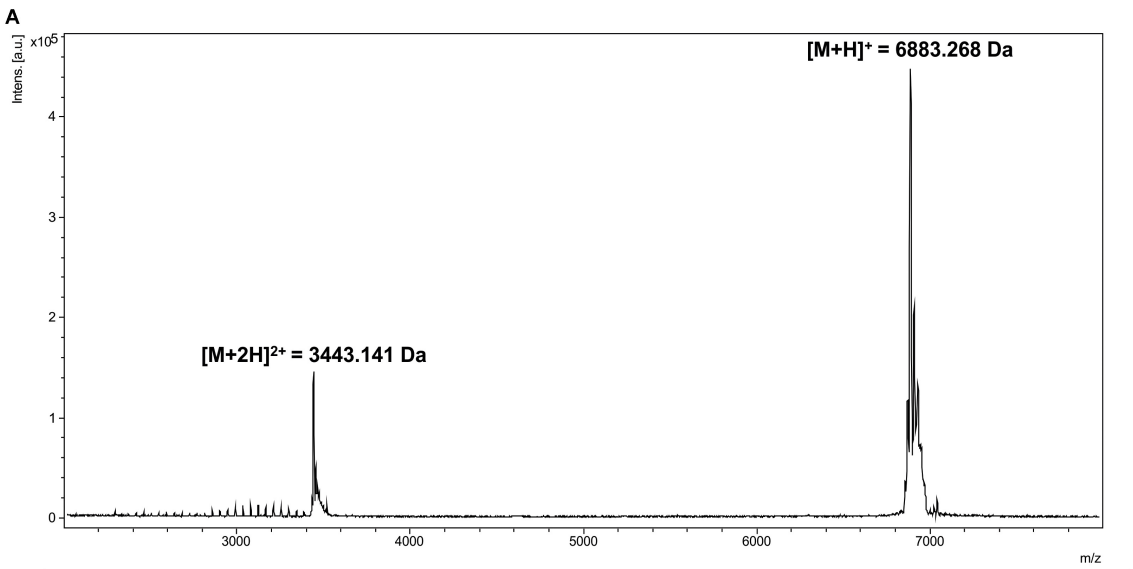

FIGURE 4 | (A) Mass spectrum of Rc1 obtained by MALDI-TOF mass spectrometry in a positive linear ionization mode using DHB matrix. **(B)** Sequence alignments of the Rc1 partial sequence (B3EWP2) with other beta-neurotoxins. Css9 (*Centruroides suffusus*, F1CGT6), CsEv5 (*C. sculpturatus*, P58779), Ts4 (*T. serrulatus*, P45669), Ts1 (*T. serrulatus*, P15226), RjAa2f (*R. junceus*, E7CLP6), and RjAa12 (*R. junceus*, E7CLN6). Conserved residues are in red and Cys residues are shaded in black. Alignment was generated by Clustal Omega server. Identity percentage considered among the aligned residues.

nor within 3 weeks after the milking. The 18 V required for *R. crassicauda* milking demonstrated to be higher than that used in the literature for *T. serrulatus* venom extraction (12 V) (22). The thickening of *R. crassicauda* metasoma probably explains the need for a higher tension (the prefix "crassi" means tick or fat in Latin, hence crassicauda denotes fat-tail).

The purification procedure of *R. crassicauda* venom was able to provide its pure major toxin Rc1 (P8) and hyaluronidase (P9). MS/MS analysis and N-terminal sequencing enabled to cover ∼80% of Rc1 primary sequence. Gaps are due to possible basic regions in Rc1 over digested by trypsin hindering peptide identification. It is interesting to note the presence of small

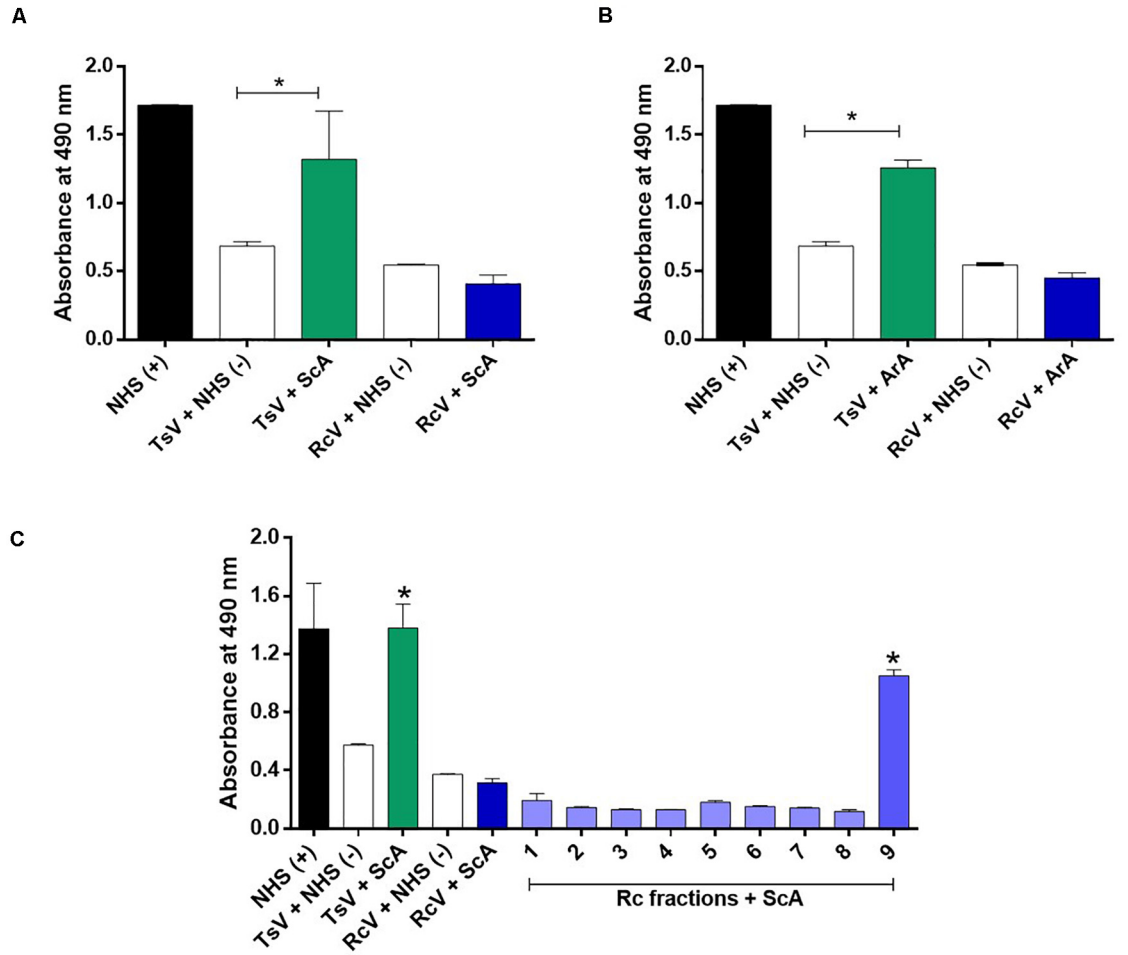

FIGURE 5 | Immunorecognition of *R. crassicauda* venom by Brazilian scorpion and arachnid antivenoms. **(A)** Scorpion antivenom. **(B)** Arachnid antivenom. **(C)** Scorpion antivenom and *R. crassicauda* fractions. The 96-well plates were coated with 2 μg of *R. crassicauda* venom or fractions (1–9) diluted in a solution of 0.05 M carbonate-bicarbonate buffer (pH 9.6). Positive controls (+) were performed with wells coated with non-immune horse serum or TsV, and negative controls (–) were performed by replacing antivenoms with non-immune horse serum. Absorbance was measured at 490 nm. NHS: non-immune horse serum. TsV, *T. serrulatus* venom; ScA, scorpion antivenom; ArA, arachnid antivenom; and RcV, *R. crassicauda* venom. Results are presented as mean ± SD ($n = 3$), which were analyzed by ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$, when compared to the negative respective controls).

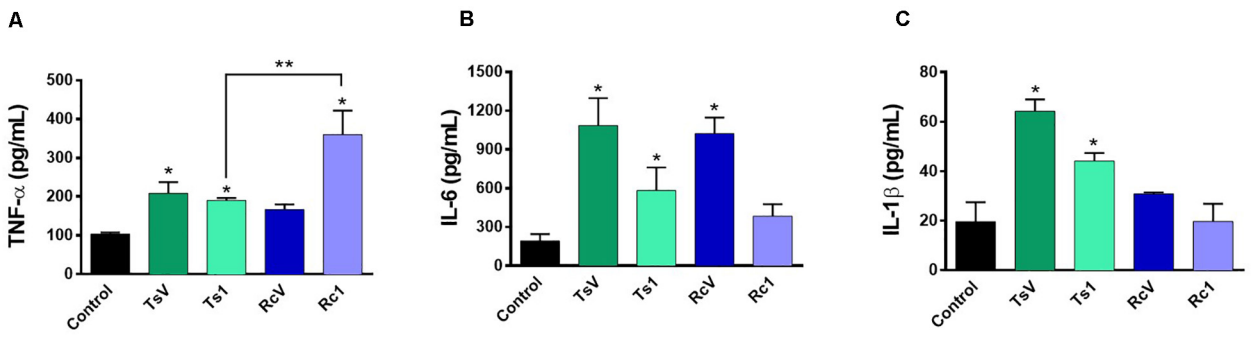

FIGURE 6 | Effect of *R. crassicauda* venom and the major toxin on cytokine levels. J774.1 cells were stimulated with *R. crassicauda* venom (100 μg/mL) or Rc1 toxin (50 μg/mL) for 24 h. As negative control, stimuli with *T. serrulatus* venom (100 μg/mL), Ts1 (50 μg/mL), and unstimulated cells were used. **(A)** TNF-α. **(B)** IL-6. **(C)** IL-1β. TsV: *T. serrulatus* venom. RcV: *R. crassicauda* venom. Results are presented as mean ± SD ($n = 4$), which were analyzed by ANOVA followed by Tukey's *post hoc* test (*$p < 0.05$ when compared to controls; **$p < 0.001$ when compared to Ts1).

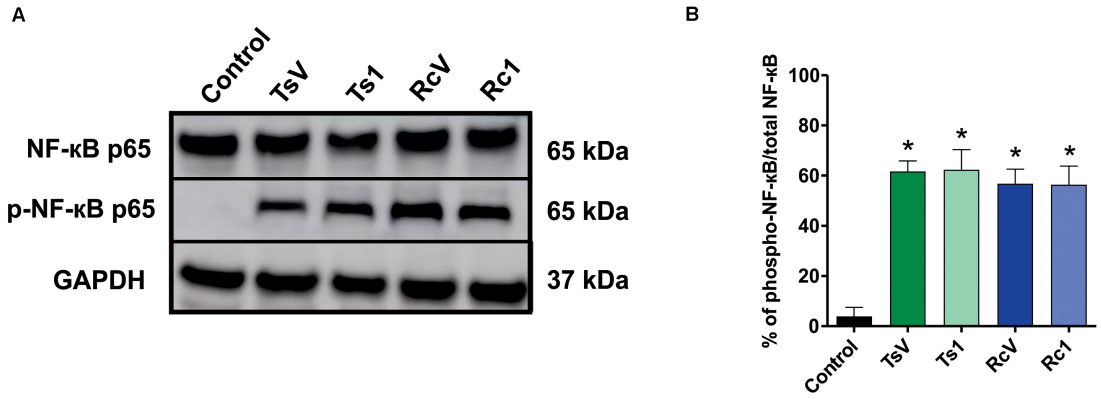

FIGURE 7 | Effect of *R. crassicauda* venom and the major toxin on the NF-kB signaling pathway. **(A)** Western blot of phospho-NF-kB p65 and NF-kB p65 proteins (GAPDH as an internal control). Lane 1 represents control (medium) and lanes 2–5 represent TsV, Ts1, RcV, and Rc1, respectively. **(B)** Percentage of expression of the target protein against the reference protein as quantified by band densitometry. TsV: *T. serrulatus* venom. RcV: *R. crassicauda* venom. Results are presented as mean ± SD ($n = 4$), which were analyzed by ANOVA followed by Tukey's *post hoc* test (*$p < 0.001$ when compared to control).

fragments from the same peptide (**Supplementary Table 1**) which indicates that Rc1 may undergo a proteolytic cleavage by peptidases within the venom gland. In fact, peptidases have been already detected in scorpion venom glands such as those from *Mesobuthus eupeus* (ENA| EF442061.1) and *Hadrurus gertschi* (23). This process might be related to the processing of intracellular proteins as well as to increase venom complexity by producing different proteins/peptides from one single gene (23–26). A mechanism of post-splitting (a post-translational processing) was suggested to Ts19 from *T. serrulatus*, resulting on fragments with split functional activity (26). Moreover, toxin proteolysis is not uncommon to occur in scorpion venoms (24) and a similar fact have also been described in the venom from the ant *Neoponera villosa* (27).

Unfortunately, we could not explore the hyaluronidase sequence due to the low quantity of pure enzyme recovery. Hyaluronidases facilitate the spreading of toxins into the tissues of the prey/victims, since these enzymes hydrolyze hyaluronan of the interstitial matrix (28, 29). P9 is a monomeric hyaluronidase of 54 kDa (reduced) and 45 kDa (non-reduced), as estimated by Tris-Tricine-SDS-PAGE. The observed molecular mass is within the range from 45 to 82 kDa described for scorpion venom hyaluronidases (30, 31). Although there are about 2,200 scorpion species known in the world (32), there are only 12 scorpion hyaluronidases primary sequences deposited in the databanks and 5 enzyme isolation reports (from *Heterometrus fulvipes*, *T. serrulatus*, *Palamneus gravimanus*, *T. stigmurus,* and *Mesobuthus martensii* venoms) (28). Our study is the first one to isolate a hyaluronidase from the scorpion genus *Rhopalurus*, as evidenced as a single band by Tris-Tricine-SDS-PAGE containing hyaluronan.

On the other hand, our study did not detect the phosphodiesterase (PDE) activity in Rc venom. PDEs are exonucleases, possessing the ability to cleave DNA and RNA, as well as other fundamental molecules for physiological processes such as ATP and cAMP (33, 34). These proteins have a molecular mass between 90 and 160 kDa and are widely distributed in snake venoms, although they are commonly found in a few amount on them (35, 36). So far, there are no reports describing PDEs in scorpion venoms. Indeed, the electrophoretic profile of Rc venom did not reveal proteins with high molecular mass over 54 kDa, which corresponds to hyaluronidase.

As such *Rhopalurus junceus* (37), *R. crassicauda* venom showed no PLA_2 activity. Instead Rodríguez-Ravelo et al., using the same species (*R. junceus*) and mass spectrometry analysis, demonstrated that the scorpions from La Poa area, collected in the humid area of Baracoa, Guantanamo Province, showed the presence of phospholipase A_2 (molecular mass within the range from 14 to 19 kDa) (38). The other scorpion species in which this enzyme activity was detected are *Pandinus imperator* (39, 40), *Anuroctonus phaiodactylus* (41), *Scorpio maurus palmatus* (42), *Opisthacanthus cayaporum* (43), *H. fulvipes* (44), and *H. laoticus* (45).

Rc1 exhibited an identity score in the range of 46–60% with scorpion β-neurotoxins from *C. suffusus, Centruroides sculpturatus*, and *T. serrulatus*.β-scorpion toxins bind at a so-called "site-4" of Na_V channels, and shift the voltage dependence of Na_V channel activation toward more negative potentials, promoting spontaneous and repetitive firing (46). Scorpion toxins targeting voltage-gated sodium channels (NaTx) are, in general, composed of 60–76 amino acids cross-linked by four disulfide bounds and are known as long-chain toxins (46). Rc1 presents the positively charged Lys at positions 1 and 12, but, strikingly, it does not show a negatively charged Glu at position 2. All these three residues were shown as determinants of the specificity of β-toxins (47, 48). In any case, the Rc1 electrophysiological findings for the first time reveal the neurotoxic effects of the main Roraima's scorpion venom, which can explain some of the symptoms observed after local envenomings, such as local inflammation and pain.

Indeed, this is the pioneer study showing that *R. crassicauda* venom presents pro-inflammatory activities. The *in vitro* assays demonstrated that this species venom increases levels of IL-6 and that the main toxin Rc1 outstanding increases TNF-α levels. Both

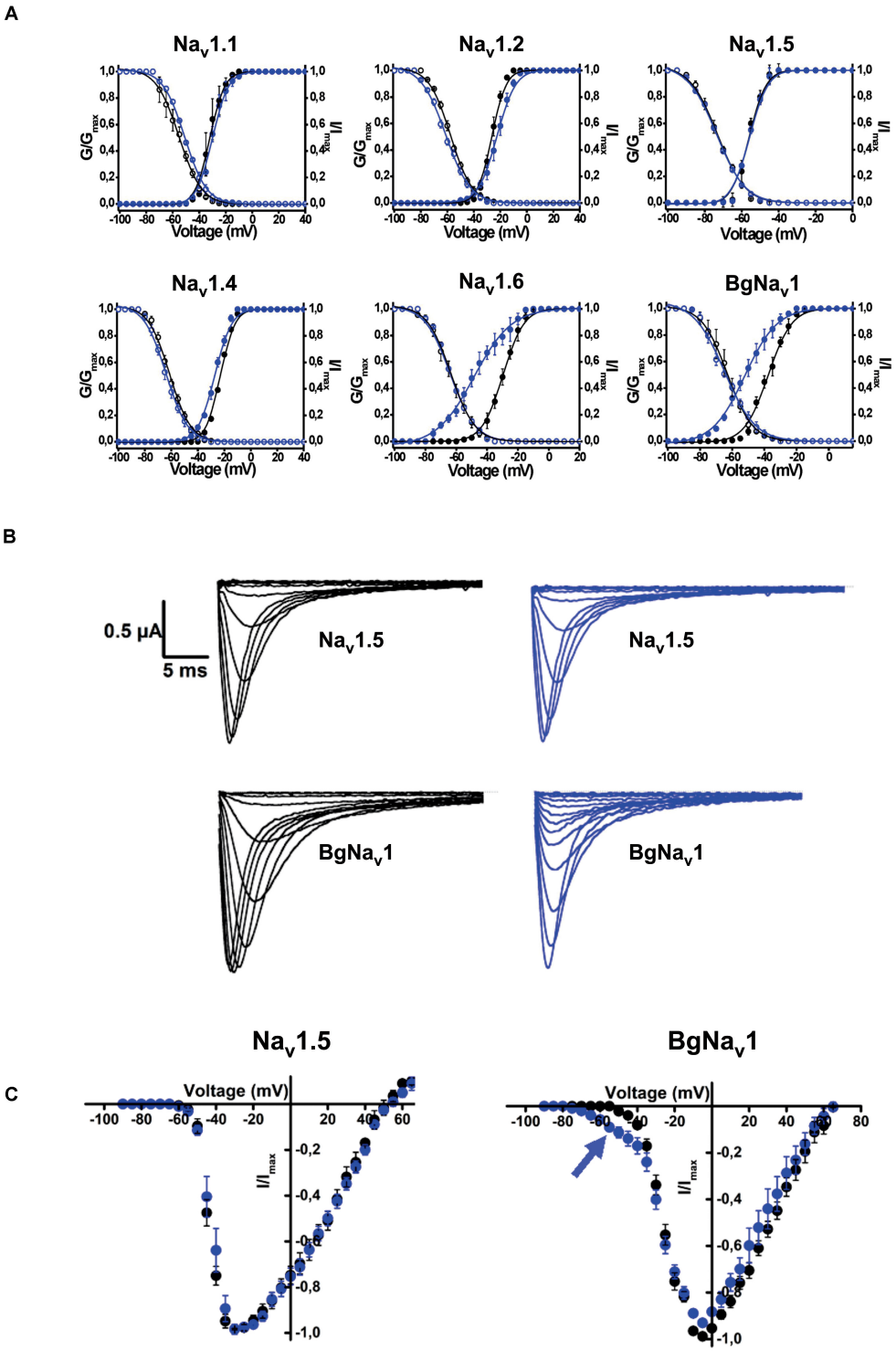

FIGURE 8 | Electrophysiological effects of the major toxin on voltage-gated sodium channels. **(A)** Effects of Rc1 on the voltage dependence of steady-state activation and inactivation curves under control conditions (black symbols) and after the addition of 1 µM of Rc1 (blue symbols), $n = 4$ cells ± SEM. **(B)** Current traces of Nav1.5 (non-effect) and BgNav channels in control (black) and in the presence of Rc1 channels (blue). **(C)** IV curves in control (black) and after application of Rc1 for Nav1.5 (non-effect) and BgNav1 channels.

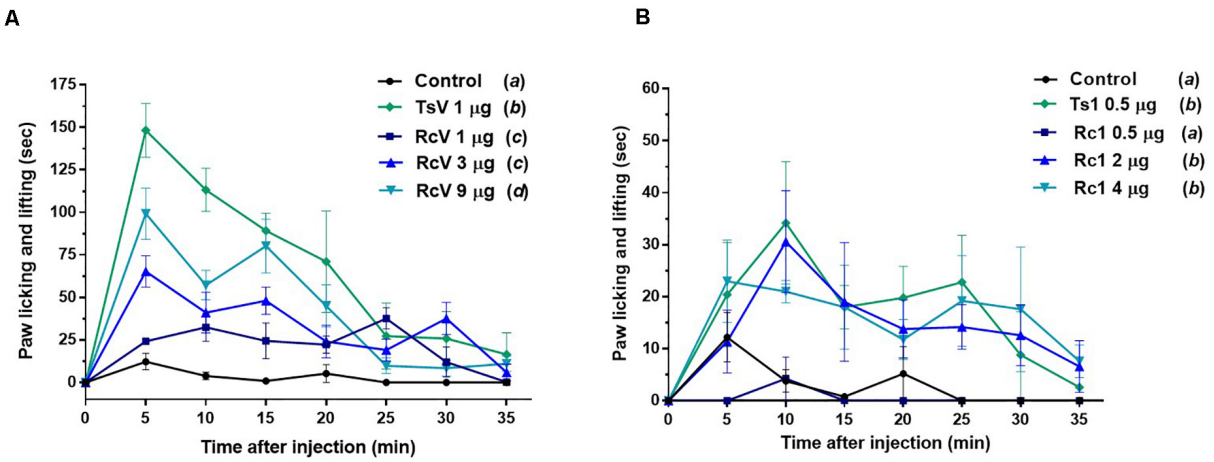

FIGURE 9 | Spontaneous nociception induced by *R. crassicauda* venom and the major toxin. Nociception was assessed by recording the time course of paw licking and lifting behavior after intraplanar (ipl) injections of RcV venom **(A)** or Rc1 **(B)** into C57BL/6 mice right hind paw. *T. serrulatus* venom and its main toxin (Ts1) were used as controls. TsV: *T. serrulatus* venom. RcV: *R. crassicauda* venom. Data are presented as the mean ± SD ($n = 5$), which were analyzed by Two-way ANOVA followed by Tukey's *post hoc* test. Same and different letters represent, respectively, no and statistically significant differences between groups.

IL-6 and TNF-α are cytokines featuring pleiotropic activities. For instance, they can induce synthesis of acute phase proteins, stimulate antibody production and effector T-cell development, as well as elevation of body temperature (49, 50). On the other hand, in the tested concentrations, neither RcV nor Rc1 increased IL-1β, although high levels of this pro-inflammatory cytokine had previous been documented both in patients envenomated by *T. serrulatus* venom (51) and *in vivo* using *T. serrulatus* Ts6 toxin (52). In addition, our study demonstrated that both *R. crassicauda* venom and Rc1 can also activate the NF-kB signaling pathway. This result was consistent with previously reported results for *T. serrulatus* venom and toxins (5, 53–57), thus we supposed that activation of the NF-kB signaling pathway could be one important mechanism of enhancing the immune responses from several scorpion envenomings (53).

The NF-kB signaling pathway that mediates inflammatory responses is the canonical pathway, which is well described elsewhere (58, 59). Scorpion toxins, such as Ts1 (a β-toxin like Rc1), are known to be recognized by *toll like* receptors 2 (TLR2), 4 (TLR4), and CD14, resulting in the activation of NF-kB canonical pathway, which culminates in the production of inflammatory mediators such as cytokines (e.g., IL-6 and TNF-α) and lipid mediators (e.g., PGE_2 and LTB_4) (54).

Moreover, both RcV and Rc1 were not cytotoxic to macrophages in the tested concentration (100 µg/mL). Díaz-García et al. had demonstrated that different fractions of *R. junceus* venom were cytotoxic to A549 and MRC-5 lung cell lines; however, in that study, the authors tested high venom concentrations up to 600 µg/mL (37). In 2019, the same research group demonstrated that *R. junceus* venom inhibited the tumor progression in F3II bearing-mice in a dose-dependent manner (60). As such, *R. princeps* venom has also been explored as an anticancer agent (61).

Based on the greater number of neurotoxins affecting Nav channels and the increase of pro-inflammatory mediators, scorpion venoms can modulate the nociceptive response (5, 62). Factually, scorpions are well-known to cause immediate and localized painful stings, which can be classified as mild, moderate, severe, and very severe, being severe defined as a pain greater than that of a bee sting or equivalent (63, 64). Our studies demonstrated that both *R. crassicauda* venom as well as the toxin Rc1 are able to induce hypernociceptive response in mice, although to a lesser extend when compared to *T. serrulatus* venom and toxin. The peripheral sodium channels $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ are mainly responsible for the pathophysiology of different pain syndromes (65, 66). Indeed, our electrophysiological studies conducted with isolated Rc1 toxin reveals that Rc1 alters the activation process of Nav1.4, Nav1.6, and BgNav1. Thus, the Rc1 action to Nav1.6 channels and the toxin-induced production of inflammatory mediators could explain the painful sting triggered by *R. crassicauda* envenoming. Finally, Rc1 also activated the insect channel BgNav from the cockroach *Blattella germanica*. Effects on insect ion channels are usually observed by scorpion-derived toxins, since insects are the preys of these animals, specially cockroaches, which are known to be their preferred diet (67). In fact, several studies demonstrate the potential of scorpion toxins to be used as insecticides (16, 68). Therefore, Rc1 can be classified as a β-scorpion toxin targeting mammal and insect voltage-gated sodium channels, pro-inflammatory, and painful neurotoxin. There are few studies developed with other *Rhopalurus* species that corroborate with our results. For instance, García-Gómez et al. demonstrated that the venom of *R. junceus* produces a β-effect on sodium channels in F11 cell line (69). Nonetheless, different from our work, many of the literature studies explore scorpion toxins targeting potassium channels (Kvs) (70).

Regarding the cross-reactivity observed on ELISA assays, our results suggest that none of the antivenoms evaluated can recognize *R. crassicauda* venom, indicating selectivity of both antivenoms toward *Tityus* spp. venom components. According

to the Butantan Institute pipeline, ScA is an antivenom specific to *T. serrulatus* scorpion venom, being indicated to the treatment of envenomings caused by scorpion from *Tityus* genus; while ArA is a polyvalent antivenom produced against *T. serrulatus* and two different spider venoms (i.e., *Loxosceles* and *Phoneutria* genera), being indicated for the treatment of envenomings caused by scorpions and spiders. Knowing that *T. serrulatus* scorpions are responsible for most and severe cases of envenomings in Brazil, the ability of the available antivenoms to cross-neutralize venoms from others scorpion species is unknown, especially for accidents caused by another scorpion genus. In this study we did not identify antivenom cross-reactivity with *R. crassicauda* venom, although neurotoxins have been known to present a high degree of similarity (46, 71, 72). However, when we analyzed each venom fraction, the scorpion antivenom was able to recognize fraction 9 (P9), which corresponds to hyaluronidase. Since this enzyme is also found in *T. serrulatus* venom (73) and the ScA is produced against this species venom, it is not surprising that the antivenom can cross-bind to the hyaluronidase from *R. crassicauda* venom. Moreover, most of venom-derived hyaluronidases have demonstrated high sequence identities, specially between the same animal class (28). For instance, *T. serrulatus* hyaluronidase shares a high identity with hyaluronidases from the venoms of *C. sculpturatus* (XP_023226974.1, 76%), *M. martensii* (P86100.2, 72%), and *C. sculpturatus* (XP_023244120.1, 54%). The recognition of just one protein is definitely not enough to inhibit a venom cocktail toxicity and, although additional assays needs to be explored (i.e., *in vivo* lethality inhibition), it is unlikely that the scorpion antivenoms available in Brazil (ScA and ArA) could be used to treat severe cases of *R. crassicauda* envenomings in Roraima.

In conclusion, the present study pioneered the fractionation of *R. crassicauda* venom and successfully isolated and elucidated the major toxin, Rc1, and a hyaluronidase. Furthermore, this work provides useful insights for the first understanding of the painful sting and pro-inflammatory effects associated with *R. crassicauda* envenomings.

MATERIALS AND METHODS

Scorpions and Venom Milking

Rhopalurus crassicauda scorpions were collected in Boa Vista city (latitude 2°49′14.88″ North and longitude 60°40′19.20″ West), Roraima (the northernmost state of Brazil; **Figure 1**). The scorpions were usually caught in the wild, and adults ranging in size from 3 to 5 inches (7–12 cm) were kept in plastic boxes with adequate ventilation. The identification of species were performed through the taxonomic key previously described (11). The animals received water daily, were fed with crickets or cockroaches at least twice a month, and were kept at Medical School of Federal University of Roraima with authorization from the Brazilian Biodiversity Information and Authorization System (SISBIO, http://www.icmbio.gov.br/sisbio/) number 57491.

In total, 23 scorpions were fed 5 days prior to venom milking and each scorpion venom extraction were performed 5 times with intervals of 30–45 days. An extractor with a dimmer potentiometer was developed for this study. The scorpion was placed in the restraining device (an acrylic base with a metallic plate, and a plastic flexible band), the venom gland was firmly held with the pair of forceps, and the platinum electrode was placed against the distal somites. Electrical stimulation was applied for milking, using different electrical pulses (5 to 20 V) during about 10–15 s. Venom was pooled and immediately stored at −20°C.

Reversed-Phase Chromatography of *R. crassicauda* Venom and Tris-Tricine-SDS-PAGE

The pooled desiccated *R. crassicauda* venom was dispersed in 0.5 mL of ultrapure water, centrifuged at 12,000 *g*, 4°C, during 10 min, for removal of insoluble mucus, resulting in the soluble crude venom (supernatant without mucus). The precipitate was resuspended twice under the same conditions and the supernatants were pooled. The protein concentration of the resulting soluble venom without mucus was estimated by NanoDrop^{TM} 2000 (Thermo Scientific, United States) using the extinction coefficient of 1.0. The soluble venom (2 mg of proteins) was applied onto a C18 column (10 mm × 250 mm, 300 Å, 5 μm particles, Jupiter® Phenomenex, United States) equilibrated with 0.1% (V/V) trifluoroacetic acid (TFA). The samples were eluted with a step concentration gradient from 0 to 100% of solution B (80% acetonitrile, ACN, in 0.1% TFA), at a flow rate of 5 mL/min. Absorbance was monitored at 214 nm by the FPLC Äkta Basic UPC-10 Frac-920 system (GE Healthcare, Uppsala, Sweden). The eluted fractions were lyophilized and stored at −20°C until use. The major peak eluted in this chromatographic step was rechromatographed on another C18 column (250 × 2.1 mm, 300 Å, 5 μm particles, Jupiter® Phenomenex, United States), at a flow rate of 0.5 mL/min. The major isolated toxin was designated as Rc1 and submitted to next assays. Protein recovery of Rc1 was calculated by the relative peak area (the fraction peak area divided by total area of all the fractions in the chromatogram), considering both chromatograms. Soluble venoms (*T. serrulatus* venom, TsV, and RcV, 18 μg/well), Ts1 (2 μg/well), and the eluted chromatographic fractions (2 μg/well) were analyzed under reducing conditions by Tris Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Tris-Tricine-SDS-PAGE, 16.5%) (74). The gels were stained with PlusOne Coomassie Blue PhastGel® R-350 (GE Healthcare, Uppsala, Sweden).

Mass Spectrometry Analysis

The molecular mass of Rc1 (0.65 μg) was determined by MALDI with TOF analyzer (RapifleX, Bruker Corporation, Billerica, MA, United States) controlled by flexControl 4.0 software (Bruker Corporation, Billerica, MA, United States). The parameters to obtain data were 10,000 laser shots per spectrum, 500 Hz laser frequency, and the instrument operating in linear positive mode. RapifleX was calibrated with Protein Calibration Standard I (∼4000 and 20000 Da, Bruker Corporation, Billerica, MA, United States). As matrix, 10 mg/mL solution of 2,5-dihydroxybenzoic acid (DHB) was prepared in ACN and 0.1% TFA at 1:1 ratio. Data analysis was performed through

the software flexAnalysis 3.4 (Bruker Corporation, Billerica, MA, United States).

Rc1 (1.5 μg) was also reduced, alkylated, and digested with trypsin (Thermo Fisher Scientific Inc., Waltham, MA, United States) at 1:50 ratio, overnight, under 600 rpm at 37°C. Additionally, a second digestion was performed under similar conditions, but with a different ratio and time (1:100 and 3 h, respectively). The reaction was stopped with 0.5% TFA and the sample desalted. Desalted tryptic peptides were solubilized in 50% ACN and 0.1% TFA solution and analyzed by nLC-MS/MS in a Acquity UPLC® M-Class (Waters, Milford, MA, United States) coupled with a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass spectrometer (Thermo Scientific, Bremen, Germany). They were eluted at a flow rate of 0.6 nl/min using an ACN gradient (3–80%) in 0.1% formic acid for 130 min and immediately submitted to mass spectrometry analysis. MS spectra (400–1750 *m/z*) were acquired with high resolution (70,000 at *m/z* 200) and automatic gain control (AGC) target of 3e6. The twelve most intense ions were subsequently fragmented by HCD in a data-dependent mode. MS/MS spectra (200–2000 *m/z*) were acquired with resolution of 17,500 (at *m/z* 200), normalized collision energy of 25, AGC target of 1e5 and isolation window of ±2 *m/z*. Ions with not assigned or +1 charge were not fragmented. Data were analyzed by PEAKS Studio 7 software (Bioinformatics Solutions Inc., Waterloo, Canada) and peptides sequences were generated by automatic *de novo* sequencing setting the following parameters: parent and fragment mass error tolerance (5.0 ppm and 0.015 Da, respectively) and fixed (cysteine carbamidomethylation) and variable (deamidation of Asn and Gln and oxidation of Met) modifications. All results were manually confirmed to exclude false positives and spectra were also manually investigated.

N-Terminal Sequencing

The N-terminal sequence of Rc1 (33 μg) was determined by Edman degradation (76), using an automated protein sequenator model PPSQ-33A (Shimadzu Co., Kyoto, Japan). The obtained sequence was compared with databases, searching similarities by using Basic Local Alignment Search Tool (BLAST)[1].

Hyaluronidase Activity

The soluble venoms of *T. serrulatus* and *R. crassicauda* (5 μg/well) were analyzed under non-reducing conditions on Tris-Tricine-SDS-PAGE gel (74) and stained with PlusOne Coomassie Blue PhastGel® R-350. To detect the presence of hyaluronidase, a 16.5% separating gel containing 0.4 mg/mL hyaluronan, overlaid by a 5% stacking gel was used (67), which was stained with Stains-all (Sigma Chemical Co., St. Louis, United States) for evaluation of the hyaluronidase activity (76).

Phosphodiesterase Activity

Phosphodiesterase activity in *R. crassicauda* venom (5 μg) was determined in a 96-well plate by using *bis*(p-nitrophenyl) phosphate as substrate, according to the protocol described by Björk (77) and modified by Valério et al. (78), with absorbance reading at 400 nm. As a positive control, snake venom PDE (0.75 μg) was used.

Phospholipase A_2 Activity

The phospholipase A_2 (PLA_2) activity was evaluated according to Habermann and Hardt (79). Briefly, one part of fresh egg yolk was mixed with 3 parts (V/V) of phosphate buffered saline (PBS) and centrifuged at 2,000 *g* for 10 min. Then, 1.25 mL of the supernatant was added to a final 25 mL-suspension containing 1.5% agar and 0.25 mM $CaCl_2$ in PBS and poured into plastic Petri dishes (90 × 15 mm, flat bottom). After layer consolidation, cylindrical holes were performed using a 10 μL-pipet tip. Each well was charged with 50 μL of *R. crassicauda* venom (65 μg) and as controls bovine serum albumin (300 μg) and *Crotalus durissus terrificus* venom (10 μg) were used. The plates were incubated at 37°C for 16 h and the halo diameter corresponding to the phospholipase activity was measured.

Antivenom Cross-Reactivity

ELISA 96-well plate (Costar, Corning, New York, United States) was coated with *T. serrulatus* venom, *R. crassicauda* venom, or *R. crassicauda* chromatographic fractions (2 μg/well) in 0.05 M carbonate/bicarbonate buffer, pH 9.6 (100 μL/well), and incubated overnight at 4°C. Control wells were coated with non-immune horse serum (diluted 1:50 in 0.05 M carbonate/bicarbonate buffer, pH 9.6, 100 μL/well), scorpion antivenom, or arachnid antivenom, which also includes antibodies specific to *T. serrulatus* scorpion venom; Butantan Institute, SP, Brazil). The plates were washed 3 times with PBS pH 7.2, blocked by adding 250 μL of PBS containing 2% (w/V) non-fat dry milk (Molico, Nestlé, Bebey, Switzerland – MPBS), and incubated for 2 h at 37°C. Plates were washed 3 times with PBS-0.05% Tween (PBS-T) and 3 times with PBS. Then, scorpion or arachnid antivenoms (diluted 1:1000 in 1% MPBS) were added, following 1 h incubation at 37°C. The plates were washed as previously and 100 μL of anti-horse polyclonal antibodies conjugated with peroxidase (IgG-HRP, A6917, Sigma-Aldrich, St. Louis, MO, United States, 1:3000 in 1% MPBS) were added to the wells following 1 h incubation at room temperature. The plates were washed again with PBS-T and PBS. In each well, 100 μL of OPD-H_2O_2 (SIGMAFAST™ OPD tablet, Sigma-Aldrich, St. Louis, MO, United States) were added and incubated for 15 min at room temperature for color development. The reaction was stopped with 50 μL of 1 M H_2SO_4 (Merck, Kenilworth, NJ, United States) and absorbance was measured at 490 nm. The assay was carried out in duplicate and the results were analyzed by GraphPad Prism 8.4 software (La Jolla, CA, United States), using one-way ANOVA, followed by Tukey's *post hoc* test.

Cell Line and Culture

Mice macrophages J774.1 cell line (ATCC, Rockville, MD, United States) were cultured in RPMI-1640 medium supplemented with 10% (V/V) fetal bovine serum (FBS), and 1% (w/V) gentamicin, under standard conditions (37°C, 5% CO_2, and 95% humidity). Approximately 2.5 × 10^4 cells diluted in 100 μL of medium were plated per well and incubated

[1]http://blast.ncbi.nlm.nih.gov/Blast.cgi

overnight under the standard conditions. The medium was aspirated and replaced by medium without FBS (100 µl per well) containing *T. serrulatus* venom (TsV), Ts1, *R. crassicauda* venom (RcV), or Rc1 (100 µg/mL), and incubated for 24 h at standard conditions.

Cytotoxicity and Cytokine Levels

The viability of J774.1 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (80). After 24 h of incubation with different stimuli (see cell line culture Section), 5% MTT in RPMI was added to the plated cells. Following 3 h of incubation with MTT, 50 µL of 20% sodium dodecyl sulphate (SDS) in 0.01 M HCl were added and cells were kept at room temperature until complete precipitate solubilization. Absorbance was measured at 570 nm and viability was expressed as the percentage (%) compared to unstimulated cells.

Concentrations of TNF-α, IL-6, and IL-1β were evaluated from the cell supernatants by ELISA using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturers' instructions (R&D Systems, MSP, United States).

Protein Expression by Western Blotting

After removing the supernatants, J774.1 cells were lysed in radioimmunoprecipitation assay buffer RIPA buffer (Merk, Darmstadt, Germany) containing protease and phosphatase inhibitors. Protein quantification was performed using detergent-compatible methodology (DC Protein Assay, Bio-rad, CA, United States). Proteins were separated by polyacrylamide gel electrophoresis (Bolt Bis-Tris 4–12% Plus Gel, Life Technologies, CA, United States) and transferred to 0.22 µm nitrocellulose membrane (GE Healthcare, Madison, WI, United States). The membranes were blocked in Tris buffered saline (TBS) solution containing 0.01% Tween, and 5% non-fat dry milk (Molico, Nestlé, Bebey, Switzerland). Recombinant anti-NF-κB p65 antibody [E379] (Abcam, United States) and Phospho-NF-κB p65 (S536; Abcam, United States) were added at 1:5000 dilution in blocking solution; Anti-GAPDH clone (71.1; Sigma-Aldrich, St. Louis, MO, United States) was added at 1:20,000 dilution. HRP-conjugated antibodies (KPL, Gaithersburg, MD, United States) were used at the dilution of 1:5000. ECL (GE Healthcare, Chicago, IL, United States) was used for band detection. Quantification was performed using Software ImageJ 1.52a (NIH, MD, United States). Data are representative of arbitrary units relative to the control (GAPDH).

Expression of Voltage-Gated Ion Channels in *Xenopus laevis* Oocytes

For the expression of Nav channels (hNav1.1, rNav1.2, rNav1.4, hNav1.5, mNav1.6, the invertebrate channel BgNaV1.1 and the auxiliary subunits rβ1, hβ1, and TipE) in *X. laevis* oocytes, the linearized plasmids were transcribed by using the T7 or SP6 mMessage-mMachine Transcription Kit (Thermo Fisher Scientific, United States). The harvesting of stage V–VI oocytes from anesthetized female *X. laevis* frogs was as previously described (81). Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng/nL by using a microinjector (Drummond Scientific Company, Broomall, PA, United States). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; CaCl2, 1.8; MgCl2, 2; and HEPES, 5 (pH 7.4). This solution was supplemented with 50 mg/L gentamicin sulfate. The use of *X. laevis* was approved by the Ethical Committee for animal experiments of the University of Leuven (P186/2019).

Electrophysiological Assays

Sodium currents were recorded using the two-microelectrode voltage-clamp technique (TEVC) at room temperature (20–25°C). The recordings were processed by a GeneClamp 500 amplifier (Axon Instruments, United States) controlled by a pClamp data acquisition system (Axon Instruments, United States). Whole-cell currents from oocytes were recorded 1–5 days after injection. Currents and voltage electrodes had resistances from 0.8 to 1.4 MΩ and were filled with 3 M KCl. Currents were sampled at 20 kHz and filtered at 2 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a −P/4 protocol. For the assays, Rc1 diluted in ND-96 solution was added directly to the recording chamber to obtain the desired final concentration (1 µM). Experiments were performed at least three times.

For the activation protocols, 100 ms test depolarization, ranging from −90 mV to +70 mV, were applied from a holding potential of −90 mV, in 5 mV increments at 5 s intervals. For the inactivation protocols, double pulses with a conditioning pulse applied from a holding potential of −100 mV to a range of potentials from −90 mV to 0 mV, in 5 mV increments for 50 ms, immediately followed by a test pulse to 0 mV (or −5 mV) were employed. Data were normalized to the maximal Nav current amplitude (Imax), plotted against the pre-pulse potential and fitted using the Boltzmann equation: $I_{Na}/I_{max} = \{(1-C)/(1 + \exp[(V-V_h)/k)]\} + C$, where I_{max} is the maximal I_{Na}, V_h is the voltage corresponding to half-maximal inactivation, V is the test voltage, k is the slope factor, and C is a constant representing a non-inactivating persistent fraction (close to 0 in control).

Nociceptive Assays

Injections of 0.01 mL of *R. crassicauda* venom (1, 3, and 9 µg) and Rc1 (0.5, 2, and 4 µg) into the plantar surface (ipl) of the right hind paw of C57BL/6 mice (male, 18–22g, $n = 5$) were performed. Control groups received ipl injections of TsV (1 µg), Ts1 (0.5 µg), or physiological solution (0.9% NaCl). The time mice spent either licking or lifting/shaking the injected paw was recorded at 5 min intervals for 35 min. All experiments were conducted according to the guidelines of the Ethic Principles in Animal Experimentation of School of Medicine of Ribeirão Preto – University of São Paulo, with the license number 246/2019.

Statistical Analyzes

The experiments were performed at least in triplicate and the results were expressed by standard deviations (SD). The statistical significance of the results was assessed using analysis of variance (one-way ANOVA or two-way ANOVA) followed by Tukey

post hoc test through the GraphPad Prism 8.4.3 software. P values < 0.05 were considered significant.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Comissão de Ética no Uso de Animais (CEUA-FMRP).

AUTHOR CONTRIBUTIONS

MP, KB, JT, TC, and EA designed the research. CA, KB, FC, IO, CB, GA-S, KZ, MR, EP-J, and SP performed the research. MP, KB, IO, GW, SP, LQ, LF, and UZ analyzed the data. All authors contributed to writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02011/full#supplementary-material

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Human Chondrocyte Activation by Toxins From *Premolis semirufa*, an Amazon Rainforest Moth Caterpillar: Identifying an Osteoarthritis Signature

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Pararamosis is a disease that occurs due to contact with the hairs of the larval stage of the Brazilian moth *Premolis semirufa*. Envenomation induces osteoarticular alterations with cartilage impairment that resembles joint synovitis. Thus, the toxic venom present in the caterpillar hairs interferes with the phenotype of the cells present in the joints, resulting in inflammation and promoting tissue injury. Therefore, to address the inflammatory mechanisms triggered by envenomation, we studied the effects of *P. semirufa* hair extract on human chondrocytes. We have selected for the investigation, cytokines, chemokines, matrix metalloproteinases (MMPs), complement components, eicosanoids, and extracellular matrix (ECM) components related to OA and RA. In addition, for measuring protein-coding mRNAs of some molecules associated with osteoarthritis (OA) and rheumatoid arthritis (RA), reverse transcription (RT) was performed followed by quantitative real-time PCR (RT-qPCR) and we performed the RNA-sequencing (RNA-seq) analysis of the chondrocytes transcriptome. In the supernatant of cell cultures treated with the extract, we observed increased IL-6, IL-8, MCP-1, prostaglandin E2, metalloproteinases (MMP-1, MMP-2, MMP-3 and MMP-13), and complement system components (C3, C4, and C5). We noticed a significant decrease in both aggrecan and type II collagen and an increase in HMGB1 protein in chondrocytes after extract treatment. RNA-seq analysis of

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the chondrocyte transcriptome allowed us to identify important pathways related to the inflammatory process of the disease, such as the inflammatory response, chemotaxis of immune cells and extracellular matrix (ECM) remodeling. Thus, these results suggest that components of *Premolis semirufa* hair have strong inflammatory potential and are able to induce cartilage degradation and ECM remodeling, promoting a disease with an osteoarthritis signature. Modulation of the signaling pathways that were identified as being involved in this pathology may be a promising approach to develop new therapeutic strategies for the control of pararamosis and other inflammatory joint diseases.

Keywords: osteoarthritis, toxins, caterpillar, chondrocyte, mediators, cell signaling

INTRODUCTION

There are many venomous moth caterpillars from Order Lepidoptera that can cause severe injuries to humans. The reactions range from urticarial dermatitis, allergic reactions, renal failure, and osteochondritis to intracerebral bleeding (1). Among these venomous caterpillars, the Brazilian moth *Premolis semirufa* (Erebidae family), known as pararama in its larval stage, inhabits rubber plantations found in the Amazon forest and produces a singular clinical manifestation of envenomation (2–4). Pararamosis (pararama-associated phalangeal periarthritis) is a disease caused by contact with *P. semirufa* urticating hairs. This contact causes an intense itching sensation, followed by symptoms of acute inflammation. After repeated contact with this caterpillar, the inflammatory process becomes chronic, leading to joint immobility that is characterized by articular synovial membrane thickening with joint deformities (4–6).

Based on these clinical symptoms of pararamosis, previous studies by our group (7) showed that *P. semirufa* hair extract presents strong proteolytic activity and induces high antibody titer production and an intense inflammatory reaction in the tissues of inoculated mice that is characterized by the presence of macrophages and neutrophils. We also demonstrated using a murine model that the extract promotes activation of T lymphocytes and antigen-presenting cells and increased production of cytokines, such as IL-6, IL-10, IL-12, IL-17, and IL-23 (8). Moreover, the extract activates the alternative and lectin pathways of the complement system, generating biologically active fragments, such as C3a, C4a, and C5a anaphylatoxins in human serum, and direct cleavage of purified complement components such as C3, C4, and C5. These results led us to consider that the complement system plays a role in the inflammatory process seen in humans after envenomation by this caterpillar (9).

Thus, the disease caused in humans by contact with *P. semirufa* caterpillar hairs, in contrast to the manifestations observed due to exposure to other caterpillars, progresses to deformity by osteoarticular changes, with cartilage impairment, as observed in the clinical condition exhibited by joint diseases. The most common and best-investigated joint diseases are osteoarthritis (OA) and rheumatoid arthritis (RA) (10–12). Osteoarthritis is a multifactorial, chronic and degenerative disease of the joints that is characterized by progressive degradation of cartilage and bone damage, and the mechanisms that lead to it have largely been investigated. Chronic and excessive or repetitive mechanical loading of the articular cartilage produces hydrostatic and elastic stress and fluid flow, leading to alterations in chondrocyte morphology. These alterations induce expression of matrix metalloproteinases (MMPs) and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), followed by proinflammatory cytokine production by synovial cells and chondrocytes (13, 14). In addition, endochondral ossification is a characteristic of osteoarthritis. Therefore, the imbalanced expression of MMPs and ADAMTSs play a central role in the first steps of OA, and inflammation is one of the first signals that persists during the whole process, as reviewed by Ripmeester et al. (15). OA progression leads to apoptosis and irreversible calcification of the cartilage matrix. In OA, some cytokines are seemingly produced by cartilage rather than synovial tissue (16, 17). The main pathological feature in the joints is cartilage degradation, accompanied by secondary synovitis (18).

In contrast, RA is a chronic, autoimmune, and systemic disease that affects the joints. This disorder results in synovial inflammation, hyperplasia within the inflammatory pannus, and bone erosion. These processes involve a complex network of interactions between innate and adaptive immunity (19). Additionally, RA is characterized by the production of rheumatoid factors and antibodies that are reactive to citrullinated proteins and by the progressive destruction of synovial and bone articular cartilage. The immune response and cartilage destruction include the production of many cytokines (20) and activation of effector cells and signaling pathways (21).

In view of the clinical similarities between pararamosis and joint diseases, we hypothesized that pararama venom components have the ability to interfere with the phenotype of cells present in the joints, such as chondrocytes, resulting in inflammation and promoting tissue injury. Therefore, to address the inflammatory mechanisms triggered by envenomation, we investigated the effect of *P. semirufa* hair extract on human chondrocytes by evaluating the production of cytokines, chemokines, MMPs, complement molecules, eicosanoids, and extracellular matrix (ECM) components related to OA and RA. In addition, we performed an RNA-sequencing (RNA-seq) analysis of the chondrocyte transcriptome, which allowed us to

identify important pathways related to the inflammatory process of the pararamosis. Collectively, the chondrocyte molecular alterations observed after venom exposure indicate a phenotype signature in pararamosis that is similar to OA and RA. Modulation of the signaling pathways triggered by the caterpillar in human chondrocytes may be a promising approach for the treatment of pararamosis and other inflammatory joint diseases.

MATERIALS AND METHODS

Preparation of *Premolis semirufa* Hair Extract

We collected caterpillars from *Premolis semirufa* Walker, 1856 (22) in areas of a rubber tree plantation of the city of São Francisco do Pará in Pará, Brazil {{Coord|1|10|08.7|S|47|47|26.3|W|}} and maintained them at the Immunochemistry Laboratory of the Butantan Institute in SP, Brazil. The Chico Mendes Institute for Biodiversity Conservation (ICMBIO) of the Brazilian Ministry of the Environment provided the license for capture, transportation, and maintenance of the animals (permission # 45166-4). Access to the venom was granted by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA), an enforcement agency of the Brazilian Ministry of the Environment (010338/2014-4), and by the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) (registration number A05C092). We carried out the extraction procedure of hair proteins and the determination of their enzymatic activity according to Villas-Boas and colleagues (7).

Chondrocyte Culture and Cell Treatment With *P. semirufa* Hair Extract

Normal human articular chondrocytes that were derived from the knee (NHAC-kn) at the second passage were purchased from Lonza (Lonza Walkersville, Inc.) and cultured in chondrocyte growth medium (Lonza, Walkersville, MD, USA) containing 10% fetal bovine serum (FBS), growth factors and supplements [0.2% R3-insulin-like growth factor-1 (R3-IGF-1)], 0.5% human recombinant fibroblast growth factor-beta [hrFGF-β], 0.1% transferrin, 0.2% insulin, and 0.1% gentamicin/amphotericin-B [GA]-1000 at 37°C with 5% CO_2, according to the manufacturer's instructions. Cells were grown in monolayer cultures, and the medium was changed every 2–3 days. For experiments, we used NHAC-kn at the 6th passage.

Cells were seeded into 96-well plates at a concentration of 5×10^4 cells/mL and incubated at 37°C in an incubator with 5% CO_2. After 24 h, we treated the cells with increasing concentrations of the extract (15, 30, and 60 μg/mL), corresponding to 0.3, 0.6, and 1.2 ng of protein/cell, in serum-free medium. We collected supernatants at 24, 48, and 72 h, centrifuged them at 400 × *g* at 4°C for 20 min, and aliquoted and froze the samples at −80°C for further analysis. As negative and positive controls, chondrocytes were cultured in the presence of phosphate buffered saline (PBS) or 10 ng/mL of interleukin-1 beta (IL-1β/IL-1F2, R&D System, code 201-LB-005), respectively.

Cell Viability Analysis by MTT Assay

We assessed the viability of the attached cells by an MTT assay (23) based on the absorption of the MTT salt, 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (Invitrogen, Carlsbad, CA, USA). Viable cells (metabolically active) metabolize the MTT salt, and its reduction leads to insoluble formazan crystals accumulating in the cytoplasm.

Analysis of Cytokines and Chemokines Produced by Chondrocytes

We assessed the concentration of cytokines and chemokines in chondrocyte culture supernatants by flow cytometry using the following BD Biosciences kits: BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines, BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine and BD™ Cytometric Bead Array (CBA) Human Chemokine. The assays were performed according to the manufacturer's recommendations. The samples were evaluated for the presence of the cytokines IL-1β, IL-6, IL-10, TNF, and IL-12p70, as well as for the chemokines IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10, and the concentration of each factor was determined using FCAP Array 3.0 software (BD Biosciences, San Jose, CA, USA).

Detection of Prostaglandins, Leukotrienes, and Thromboxanes Produced by Chondrocytes

We assessed eicosanoid production by chondrocytes using the Prostaglandin E_2 ELISA Kit – Monoclonal, Leukotriene B_4 ELISA Kit and Thromboxane B_2 ELISA Kit, according to the manufacturer's recommendations (Cayman Chemical, Ann Arbor, MI, USA). The concentration of each eicosanoid was determined according to the manufacturer's recommendations.

Production of Components of the Complement System by Chondrocytes

The supernatants of the chondrocyte cultures were also analyzed for the secretion of complement components. We assessed the concentrations of C1q, C3, C4, C5, and C9 using Complement C1, Complement C3, Complement C4, Complement C5, and Complement C9 Human ELISA kits, according to the manufacturer's recommendations (Abcam, Cambridge, UK). In addition, we built a standard curve on the log-log graph to quantify the component concentrations, with the standard concentration listed on the x-axis and the absorbance on the y-axis.

Evaluation of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

We analyzed the presence of MMPs and TIMPs in chondrocyte culture supernatants by using MMP1, MMP2, MMP3, MMP9, and MMP13 Human ELISA Kits and TIMP1 and TIMP2 Human Simple Step ELISA kits according to the manufacturer's recommendations (Abcam, Cambridge, UK). Moreover, we built a standard curve on the log-log graph to dose each MMP

concentration, with the standard concentration shown on the x-axis and the absorbance on the y-axis.

Evaluation of the Presence of Aggrecanase-1 (ADAM-TS4)

We assessed the presence of aggrecanase-1 in the supernatants of chondrocyte cultures by using the Sensitive Aggrecanase Activity Assay kit, following the manufacturer's recommendations (BioTeZ, Berlin, Germany).

Analysis of the Expression of Aggrecan, Type II Collagen and HMGB1 by High-Content Screening (HCS)

Normal human chondrocytes (NHAC-kn) at the 6th passage were cultured in 96-well microplates (Greiner Bio-One, 655986) at a density of 8×10^3 cells/well in chondrogenic growth medium containing supplements and growth factors (Lonza, Walkersville, MD, USA) at 37°C and 5% CO_2. After 24 h, the cells were treated with increasing concentrations of extract at 12 and 49 µg/mL, corresponding to 0.3 and 1.2 ng of protein/cell, in serum-free medium for 24, 48, and 72 h. These concentrations were calculated based on the quantity of extract (µg) *per* cell and were used throughout all 96-well plate experiments. As negative and positive controls, we cultured chondrocytes in the presence of PBS or 8 ng/mL interleukin-1 beta (IL-1β), respectively.

After the treatments, the cultures were washed with PHEM buffer (2 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES pH 6.9) and fixed for 1 h with cold 4% PFA. The cells were permeabilized with 0.1% Triton X 100 for 5 min, blocked with 1% bovine serum albumin (BSA) for 30 min, and then incubated with primary antibody overnight at 4○C. After washing with PHEM glycine (3×), the cells were incubated with the fluorescent dye at room temperature for 1 h, and the plates were subjected to high content imaging analysis by using MetaXpress High Content Image Acquisition & Analysis Software (Molecular Devices). The primary antibodies used were anti-Aggrecan (Abcam plc, Cambridge, UK) diluted 1:100, anti-collagen II (Abcam plc, Cambridge, UK) diluted 1:100, and anti-HMGB1 (Santa Cruz Biotechnology, Inc., CA, USA) diluted 1:500 and were incubated overnight at 4°C. After washing with PHEM (3×), the cells were incubated with Alexa Fluor 647 goat anti-rabbit and Alexa Fluor 488 rabbit anti-mouse secondary antibodies (Life Technologies, Camarillo, CA, USA) at a 1:1,000 dilution for 1 h at room temperature in the dark. The cells were washed with PHEM (3×), nuclei were counted using Hoechst 33342 (5 µM, Life Technologies, Thermo Fisher Scientific) staining for 1 h, and the stained samples were subjected to high content imaging analysis. The image acquisition and fluorescence intensity measurements were conducted by automatic scanning using MetaXpress software with a 10× objective. For each treatment condition and channel, nine images *per* well, in triplicate, were acquired and analyzed. MetaXpress software (Molecular Devices, Sunnyvale, CA, USA) was used to calculate the stained area using the Custom Module and the fluorescence intensity was calculated using the MultiWaveScoring module.

Transcriptomic Analysis

RNA Isolation

Cells were seeded into 24-well plates at a density of 1×10^5 cells/well in chondrocyte growth medium containing supplements and growth factors (Lonza, Walkersville, MD, USA) and incubated at 37°C and 5% CO_2. After 24 h, the chondrocytes were maintained in serum-free medium with the highest concentration of *P. semirufa* hair extract (60 µg/mL *per* well) for 24 h. In parallel, chondrocytes were maintained in the presence of the same volume of PBS or 10 ng/mL of interleukin 1 beta (IL-1β) as negative and positive controls, respectively. At the end of the treatment period, the growth media was removed, and total RNA was isolated from the cell cultures (total of 1×10^6 cells *per* treatment, in triplicate) by using TRIzol (Life Technologies, Inc., Camarillo, CA, USA) according to the manufacturer's protocol. RNA samples were visualized with an agarose gel, and their concentration was assessed on a Nanodrop 2000c spectrophotometer. The Agilent 2100 Bioanalyzer (RNA 6000 Nano LabChip, Agilent Technologies, Santa Clara, CA, USA) was used to determine the RNA integrity number (RIN). All RNA samples had a RIN > 9.10.

Library Preparation and Sequencing

The messenger RNAs (mRNAs) were purified from the total RNA isolated from the human chondrocyte cultures and used to prepare complementary DNA (cDNA) libraries following the protocol of the *TruSeq RNA Sample Prep Kit V2* (Illumina, San Diego, CA, USA). Briefly, mRNAs were isolated with dT-oligos, purified, and fragmented by heating at 94°C (4 min) in the kit fragmentation buffer. Double-stranded cDNAs were synthesized, end-repaired and A-tailed. Sequencing adapters were then ligated to the cDNA fragments according to the manufacturer's protocol. The cDNA fragments were enriched by 15 cycles of PCR amplification. The quality of the libraries was evaluated by cDNA size distribution, as measured by a 2100 Bioanalyzer with DNA1000 assay (Agilent Technologies, Santa Clara, CA, USA). An ABI StepOnePlus Real-Time PCR System was used to estimate the size of the libraries before sequencing. The cDNA libraries were sequenced on an Illumina HiSeq 1500 System in Rapid Run mode using a paired-end flow cell with a 2*101 bp paired-end configuration.

Quality and Filtering FASTQ Reads

The raw sequencing read contaminants were removed with Bowtie version 2 2.2.5 (24), and Trimmomatic version 0.36 was used to trim and remove reads with low-complexity and homopolymer enriched regions, poly-A/T/N tails, adapter sequences and low-quality bases. Reads were filtered out if more than 90% of them corresponded to a homopolymer or low-complexity regions and if the mean quality score was lower than 25 in a window size equal to 15. After trimming, all reads smaller than 40 bp were discarded. A quality check was performed using FastQC. Next, Hisat2 (25) was used to align reads from each sample against the human reference genome (annotation version 92), generating the count values for the genes that were used in the differential expression analysis, which were further described.

The read quality results using FastQC and map quality plots indicated that the lowest covered library had more than 20 million reads and that the percentages of mapped reads were higher than 90% for all libraries. We also compared the LFC (log2-fold change) of RNA-seq and RT-qPCR for Ext x Ctrl and IL1B x Ctrl (further described). For Ext x Ctrl, the correlation was ∼84%, and for IL1B x Ctrl, the correlation was ∼89%. This accuracy shows the high quality of the RNA extraction, the Illumina Rapid Run sequencing and the mathematical model.

cDNA Synthesis and RT-qPCR

For measuring protein-coding mRNAs, reverse transcription (RT) was performed using SuperScript III according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) followed by quantitative real-time PCR (RT-qPCR). For all genes, oligo-dT primer reverse transcription was performed using 350 ng of total RNA isolated from the human chondrocytes in a 20 μL RT reaction with SuperScript III, followed by qRT-PCR using 5 μL of 8-fold diluted RT reaction in 20 μL of qRT-PCR (ViiA 7 Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA). Transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the results are presented as the relative abundance using the 2−ΔΔCT method (26). The primer sequences are listed in **Supplementary Table 1**.

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Expression Analysis

RNA-seq *in silico* analysis included diverse quality and quantity steps to assess transcriptome expression (**Supplementary Figure 1**). We assessed read quality using FastQC version 0.11.5. After the quality procedures, we used Ensembl Gene ID transcripts to map the reference genome (GRCh38) (annotation version 92). To quantify transcripts, we used featureCounts (27) from subread version 1.6.2, resulting in a table of 58,233 gene IDs as rows, with samples in columns and cells as the raw read counts. Then, we removed genes with low expression, i.e., row sum of expressions < 1, resulting in 18,671 valid transcripts. The data generated for this study were deposited at the Sequence Read Archive (SRA) under SRA accession number PRJNA592966.

By using edgeR version 3.26.8, we calculated the normalized expression table in "counts *per* million" (CPM), which was input to infer the differentially expressed genes (DEGs), which were defined as an absolute value of log2-fold change between two groups greater than one and a false discovery rate (FDR) <0.05. For that, we only compared (a) Ext × Ctrl and (b) IL1B × Ctrl.

One approach that defined the experiment's success was evaluating data clustering using the multidimensional scaling plot (MDS). The main idea was to verify whether the samples clustered well as experimental groups. We observed that the MDS plot showed accurate clustering among the control, extract and IL-1β treatments (data not shown).

Enrichment Analysis

We performed an enrichment analysis with all recognized DEGs from the extract treatment vs. the control comparison. For that, we used two different techniques: (1) Gene Set Enrichment Analysis (GSEA), based on Kolmogorov-Smirnov statistics, and used fast-GSEA (fGSEA version 1.10.1) to calculate Pathway Enrichment Analysis (PEA) and (2) Over-Representation Analysis (ORA) using String-db (version 11.0)/KEGG and MetaCore™ (version 6.36 build 69400/2018). We also used MetaCore to calculate Maps (pathways) and Network Statistics, in addition to other methods. The FDR cutoff was set to 0.05 for each technique.

In the present work, we focused our analysis on genes that are associated with OA and evaluated expression of these factors in the supernatant or in the extracellular matrix of chondrocyte cultures. The preselected genes were *ACAN*, *ADAMTS4*, *BGN*, *C1QA*, *C1QB*, *C1R*, *C1S*, *C2*, *C3*, *C4A*, *C4B*, *C5*, *C7*, *C8A*, *C8B*, *C9*, *CCL2*, *COL1A1*, *COL2A1*, *CXCL8*, *GJA1*, *GJC1*, *HAS2*, *HAS3*, *HMGB1*, *HYAL1*, *HYAL2*, *HYAL3*, *IL18*, *IL1A*, *IL1B*, *IL6*, *KRT19*, *MMP1*, *MMP2*, *MMP3*, *MMP9*, *MMP13*, *PTGES*, *PTGES2*, *SOX9*, *TGFB1*, *TIMP1*, *TIMP2*, *TNF*, and *TP53*.

Statistical Analysis

All reported experiments were performed independently at least twice, and the data are expressed as the mean ± SEM. Statistical comparisons for wet-laboratory experiments were calculated using Student's *t*-test or two-way ANOVA followed by Dunnett *post hoc* tests. For these statistical calculations, we used GraphPad Prism-7 (San Diego, CA, USA) and considered a p-value < 0.05 to be significant.

RESULTS

Human Chondrocyte Activation by *P. semirufa* Hair Extract: Production of Cytokines, Chemokines and Prostaglandin E_2

To assess the effect of *P. semirufa* hair extract on human chondrocytes, we analyzed the viability of these cells after treatment with three extract concentrations (15, 30, and 60 μg/mL) for 24, 48, and 72 h. **Figure 1** shows that the lowest extract concentrations (15 and 30 μg/mL) induced a small reduction in cell viability (9 and 11%, respectively) after 48 h of incubation. After 24 h, the highest concentration (60 μg/mL) induced a reduction of ∼25% in cell viability. Based on these results, we conducted the subsequent experiments using the extract at 15 and 60 μg/mL. The positive control, IL-1β, induced a reduction in cell viability of ∼20% or less over time.

Inflammatory mediator production is an essential event in the progression of joint diseases and possibly in pararamosis. Thus, we evaluated cytokines, chemokines, and eicosanoids in the supernatants of chondrocytes treated with pararama hair extract. **Figure 1** shows that only IL-6, IL-8, and MCP-1 were significantly induced in a dose- and time-dependent manner in cell cultures treated with the extract compared to those of the negative control (buffer). The positive control IL-1β was used to mimic the pathophysiology of joint inflammation and induced the production of the same cytokines and chemokines but at increased concentrations.

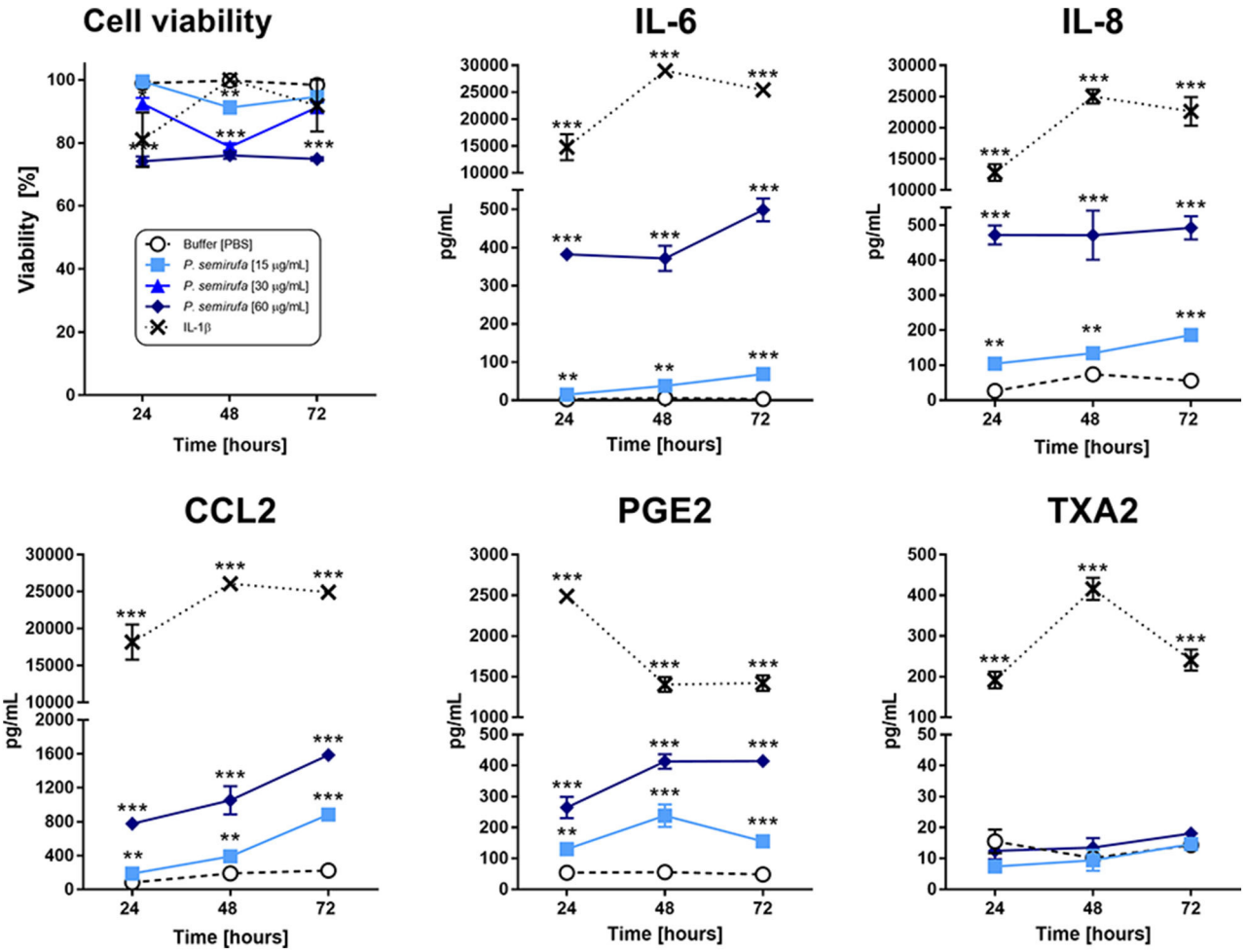

FIGURE 1 | Assessment of cytokines, chemokines, and eicosanoids in chondrocytes treated with the *P. semirufa* hair extract. Chondrocytes were cultured in 96-well plates at a density of 5×10^4 cells/mL and treated with buffer, IL-1β (10 ng/mL) or pararama hair extract (15, 30, or 60 µg/mL) for 24, 48, and 72 h. After each treatment period, we measured the cell viability by an MTT assay. The supernatants were collected from cells treated with buffer ⊖, IL-1β [-x-], 15 µg/mL ■ or 60 µg/mL ◆ pararama hair extract for 24, 48, and 72 h by centrifugation at 400 ×g at 4°C for 20 min to assess the concentration of cytokines, chemokines, and eicosanoids. The results represent two separate experiments performed in duplicate and are expressed as the mean of the concentrations of the molecules ± SEM. The data were analyzed using two-way ANOVA and Dunnett's *post hoc* test. $^{**}p < 0.01$; $^{***}p < 0.001$ vs. the control (buffer treatment).

Figure 1 also shows that only the extract induced the production of prostaglandin E_2 (PGE2). In the positive control (IL-1β), thromboxane A_2 (TXA2) and prostaglandin were detected in the supernatants, and PGE_2 was produced at higher levels than that of the extract. LTB4 was not detected in these treatment conditions (data not shown).

Pararama Hair Extract Induces Chondrocytes to Produce Complement System Components

Considering the importance of the complement system in the inflammatory process, we evaluated the levels of C1q, C3, C4, C5, and C9 in the supernatants of chondrocyte cultures treated with the extract. **Figure 2** shows that the production of C3, C4, and C5 components was significantly higher in cells treated with the extract than in the positive and negative controls (IL-1β and buffer, respectively). Interestingly, the extract treatment reduced the C5 component concentration over time. There was no increase in the production of C1q or C9 by the treated cells (data not shown).

Pararama Hair Extract Induces Chondrocytes to Produce Molecules That Act on the Extracellular Matrix

As mentioned before, MMPs and ADAMTSs are capable of degrading several matrix components, as well as type II collagen. Therefore, we assessed MMP production in the supernatants of the cultures. **Figure 3** shows a significant increase in MMP-1, MMP-2, MMP-3, and MMP-13 in the supernatants of chondrocytes treated with the extract compared to those of the buffer treatment. The positive control (IL-1β)

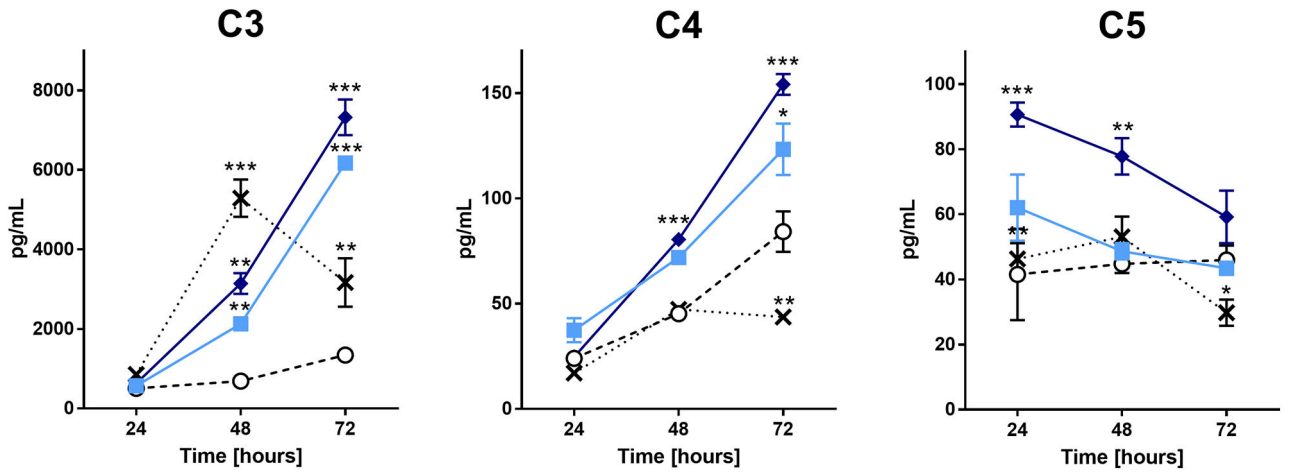

FIGURE 2 | Complement components analysis in the supernatant of chondrocytes treated with the *P. semirufa* hair extract. Chondrocytes were cultured in 96-well plates at a density of 5 × 10^4 cells/mL and treated with buffer -⊖-, IL-1β [-x-], 15 μg/mL -■- or 60 μg/mL -◆- pararama hair extract for 24, 48, and 72 h. After each treatment period, we removed the supernatants, centrifuged them at 400 ×g at 4°C for 20 min, and assessed the concentrations of complement components by ELISA. The results represent two separate experiments performed in duplicate and are expressed as the mean of the concentrations of the complement components ± SEM. The data were analyzed using two-way ANOVA and Dunnett's *post hoc* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the control (buffer treatment).

induced an increase in the tested MMPs. On the other hand, we did not detect any increase in aggrecanase (ADAMTS4) activity or tissue inhibitor of metalloproteinases (TIMPs) in the supernatants of the pararama hair extract- and IL-1β-treated cells compared with those of the buffer-treated cells (data not shown).

Pararama Hair Extract Reduces the Expression of Aggrecan and Type II Collagen and Increases HMGB1 in Chondrocytes

The integrity of both Aggrecan and type II Collagen is important in the structure of healthy cartilage. Therefore, we assessed their presence by a high-content screening (HCS) to evaluate the effects of the extract on these molecules in human chondrocytes. In parallel, we also investigated the presence of high mobility group box 1 (HMGB1), a protein that is associated with inflammatory diseases, such as RA and OA. **Figure 4** shows a reduction in the stained area for both Aggrecan and type II Collagen in cells treated with the extract compared to that of buffer treatment. The Aggrecan reduction was more pronounced after 24 h of treatment, whereas we observed the reduction in type II Collagen at each time point. For HMGB1 protein, there was an increase in the fluorescence intensity within the nucleus after 24 and 72 h of extract treatment.

RT-qPCR Analysis of Chondrocytes Treated With Pararama Hair Extract

In this study, we performed quantitative real-time PCR (RT-qPCR) to investigate the gene expression profile of some molecules associated with OA and RA (**Supplementary Table 1**). The gene expression results, shown in **Figure 5**, are consistent with the results from other experiments, in which we observed an increase in IL-6 and IL-8 in supernatants and a lack of IL-1β following the extract treatment. *IL-1α* and *IL-1β* genes were downregulated following extract treatment and were upregulated by IL-1β treatment; however, *IL-6* and *IL-8* were both upregulated following extract and IL-1β treatments. In addition, *IL-18*, *TNF*, and *TGF-β1* gene expression was downregulated in chondrocytes after extract treatment (**Figure 5**).

MMP-1 and *MMP-3* were upregulated and highly expressed following both extract and IL-1β treatment, with *MMP-13* only upregulated in cells that were treated with IL-1β (**Figure 5**). These results showed a positive correlation with the protein data obtained in IL-1β- and pararama-treated cell supernatants.

The hyaluronan synthase 2 *(HAS2)* gene was upregulated following both extract and IL-1β treatments; however, *HAS3* was not changed by treatment with the extract but was upregulated by IL-1β treatment. In addition, hyaluronidase (HYAL), *HYAL-1*, *HYAL-2* and *HYAL-3* genes were downregulated following the extract treatment but were not modulated in the positive control group (**Figure 5**).

Gene expression analysis showed a reduction in the expression of some inflammatory joint disease markers, such as Aggrecan and types I and II Collagen, after extract and IL-1β treatment. Genes related to intracellular proteins, such as *HMGB1* and *SOX9*, were downregulated following extract treatment but were upregulated in the positive control. However, *P53* showed a distinct behavior; it was downregulated by the extract but was not altered by IL-1β treatment (**Figure 5**).

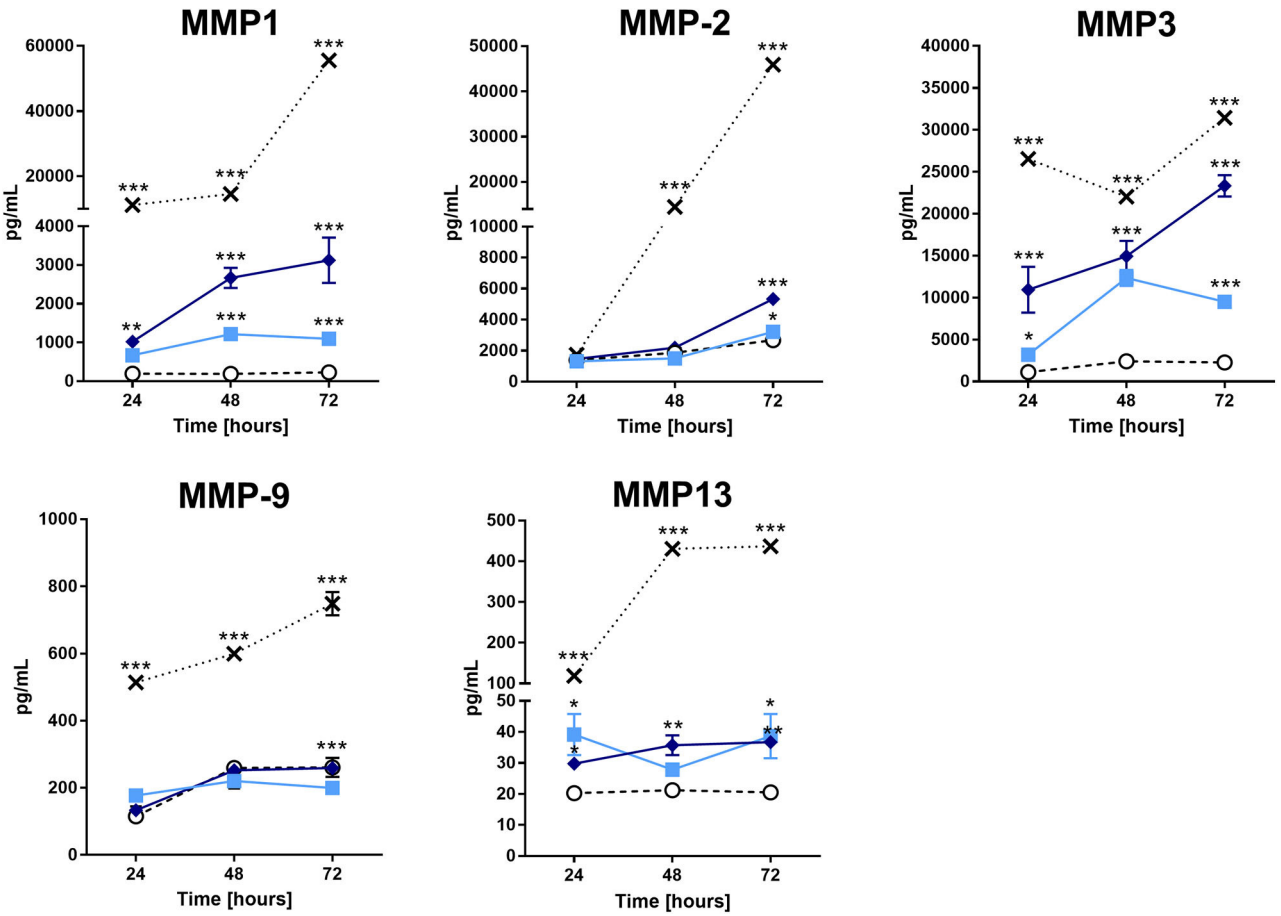

FIGURE 3 | Matrix metalloproteinases analysis in the supernatant of chondrocytes treated with the *P. semirufa* hair extract. Chondrocytes were cultured in 96-well plates at a density of 5 × 10^4 cells/mL and treated with buffer ⊖, IL-1β [-x-], 15 μg/mL ■ or 60 μg/mL ◆ pararama hair extract for 24, 48, and 72 h. After each treatment period, we removed the supernatants, centrifuged them at 400 ×g at 4°C for 20 min, and assessed the concentration of matrix metalloproteinases by ELISA. The results represent two separate experiments performed in duplicate and are expressed as the mean of the concentrations of the metalloproteinases ± SEM. The data were analyzed using two-way ANOVA and Dunnett's *post hoc* test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. the control (buffer treatment).

Transcriptome Analysis of Human Chondrocytes Treated With Pararama Hair Extract

In this study, we performed transcriptomic analysis to elucidate the response of human chondrocytes to *P. semirufa* hair extract treatment after 24 h of *in vitro* stimulation. We used edgeR to normalize the gene expression in "counts per million" (CPM) over the 18,671 valid transcripts and to calculate the differentially expressed genes (DEGs), and here we show only two comparisons: "extract treatment x buffer treatment" (Ext × Ctrl) and "IL-1β treatment × buffer treatment" (IL-1β × Ctrl). There were 3,553 DEGs in the Ext × Ctrl group, of which 1,583 were upregulated and 1,970 were downregulated, and 5,506 DEGs in the IL-1β × Ctrl group, of which 2,601 were upregulated and 2,905 were downregulated.

The gene expression analysis focused on chondrocyte molecules that were predicted to be found in the culture supernatants, on the extracellular matrix or on those genes evaluated by RT-qPCR analyses, totaling 46 selected genes. A broader whole transcriptome data analysis remains to be explored and will be the scope of a future study.

Thus, 13 out of the 46 selected genes were DEGs: *ACAN*, *C3*, *CCL2*, *CXCL8*, *GJA1*, *HAS2*, *IL6*, *MMP1*, *MMP2*, *MMP3*, *MMP13*, *PTGES*, and *SOX9*. We did not detect the transcription of genes coding for *C1QA*, *C1QB*, *C2*, *C7*, *C8A*, *C8B*, *C9*, or *IL18* in the control, extract, or IL-1β treatment groups. We observed the transcription of some other genes, such as *ADAMTS4*, *BGN*, *C1R*, *C1S*, *C4A*, *C4B*, *C5*, *COL1A1*, *COL2A1*, *GJC1*, *HAS3*, *HMGB1*, *HYAL1*, *HYAL2*, *HYAL3*, *IL1A*, *IL1B*, *KRT19*, *MMP9*, *PTGES2*, *TGFB1*, *TIMP1*, *TIMP2*, *TNF*, and *TP53*, but they were not DEGs (**Supplementary Table 2**).

Supplementary Figure 2 shows a heatmap of the selected transcribed genes, in which the hierarchical clustering analysis shows consistent grouping among the treatments. In addition,

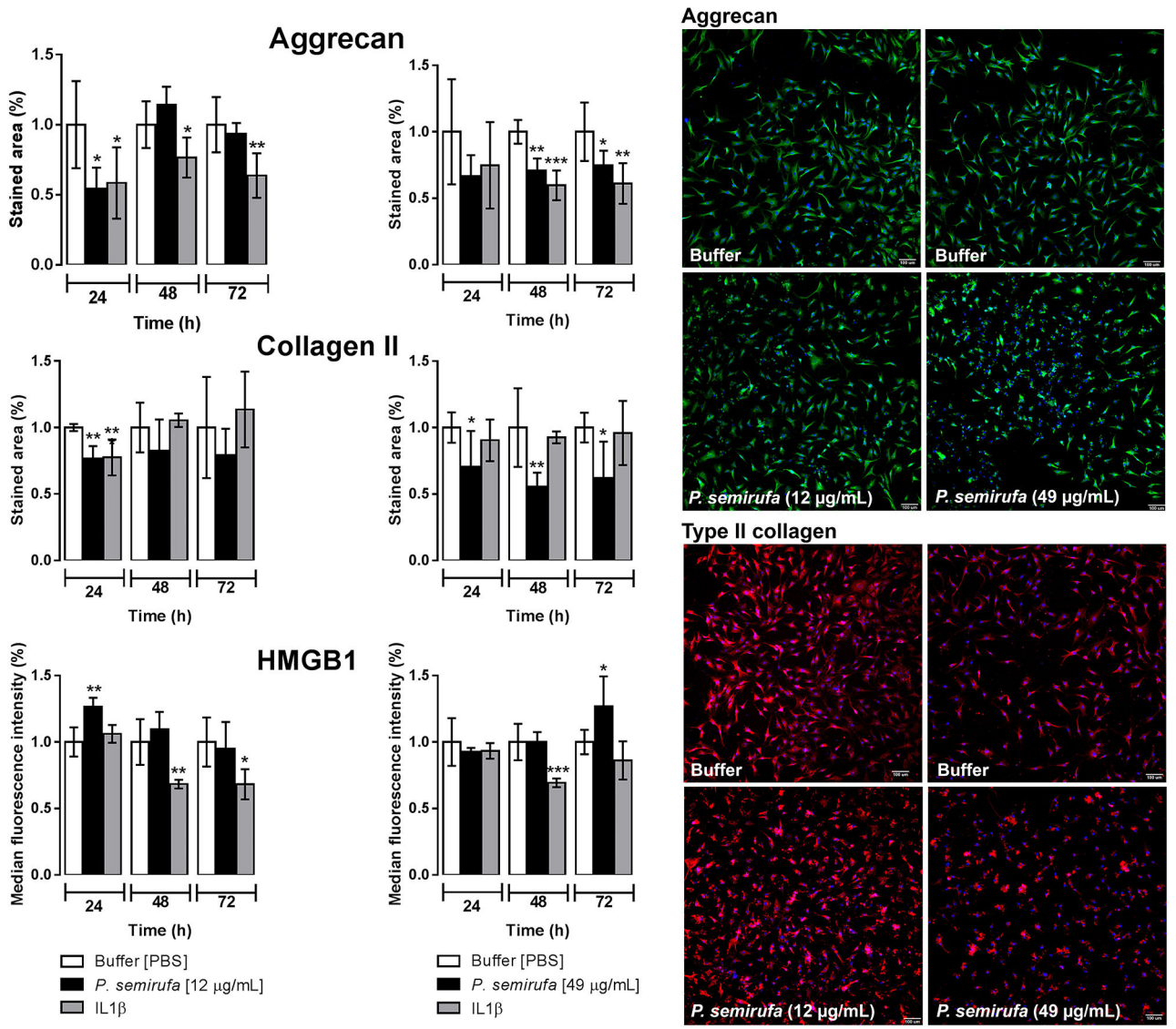

FIGURE 4 | Evaluation of aggrecan and type II collagen production and HMGB1 expression by high-content screening (HCS). Chondrocytes were cultured in 96-well plates at a density of 4×10^4 cells/mL and treated with buffer, IL-1β or pararama hair extract (12 and 49 µg/mL) for 24, 48, and 72 h. After each treatment period, the cells were fixed and blocked. Then, the cells were incubated with anti-Aggrecan, anti-collagen type II or anti-HMGB1 antibodies. In parallel, the cell counts were assessed using Hoechst 33342 staining. The image acquisition and fluorescence intensity measurements were conducted by automatic scanning by using MetaXpress software and a 10× objective, with the Custom Module to calculate the stained area and Multi Wave Scoring Module. For each condition and channel, nine images per well in triplicate were acquired and analyzed. Representative fluorescence microscopy images correspond to the cells obtained after 24 h of treatment. The results were normalized and represent two independent experiments performed in triplicate and are expressed as the mean of the stained area ± SEM or the mean of the median fluorescence intensity ± SEM. The data were analyzed using Student's *t*-test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. the control (buffer treatment).

although IL-1β treatment showed more upregulated genes compared to those of extract treatment, we can see that the response to these treatments was very similar.

We also performed a MetaCore enriched pathway (map) analysis to investigate the relationships between the DEGs and to find the most important signaling pathways in *P. semirufa* hair extract treatment (Extr × Ctrl). MetaCore identified 452 enriched pathways for Ext × Ctrl, of which 260 contained at least one of those selected genes. We selected 19 out of the 260 pathways associated with inflammation and OA (**Supplementary Table 3**), some of which will be further discussed here. This rationale led us to identify pathways associated with the immune response, angiogenesis, ECM remodeling, and release of proinflammatory mediators as the most significant signaling pathways associated with pararamosis pathogenesis (**Figure 6**).

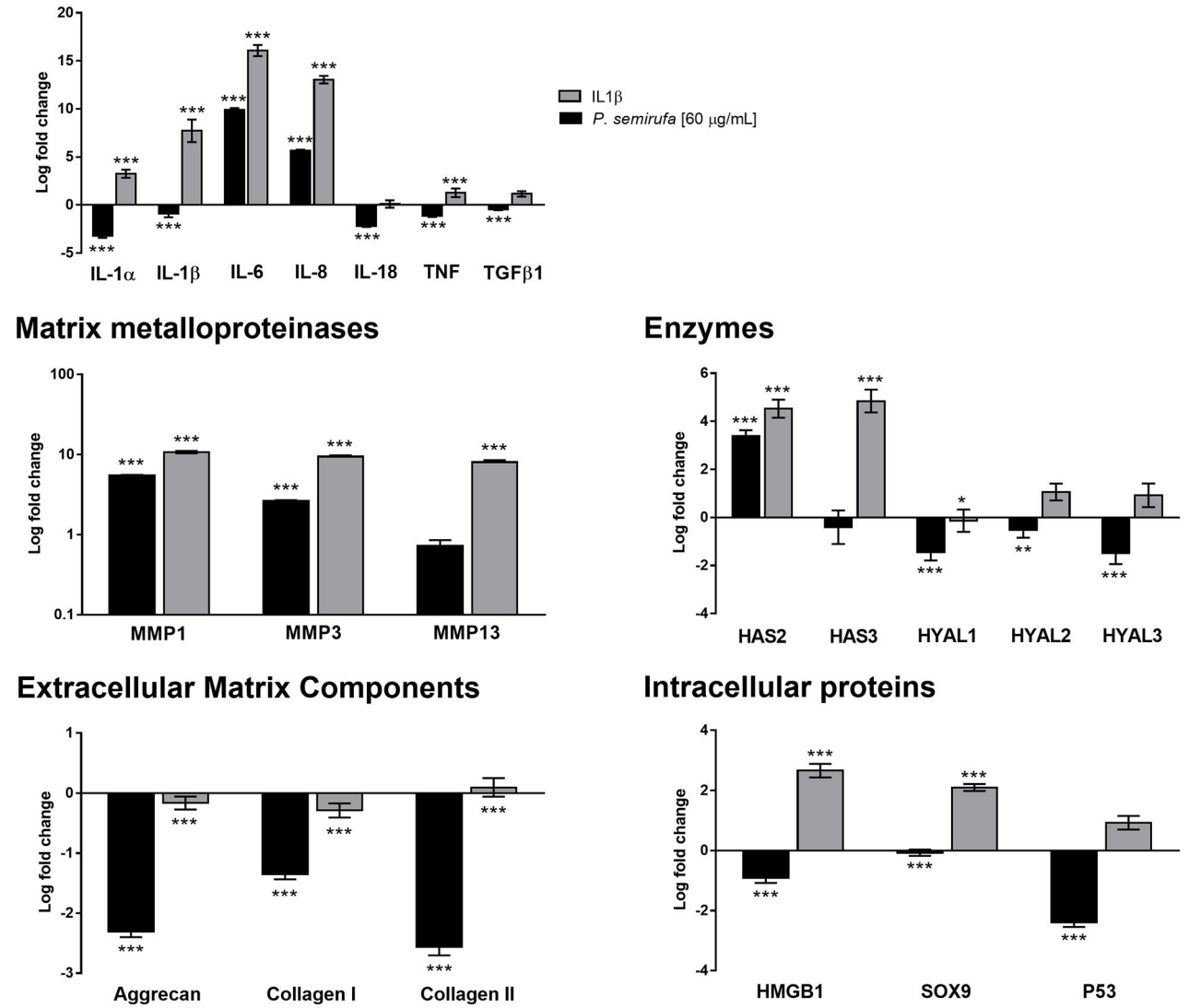

FIGURE 5 | Gene expression profile in chondrocytes treated with the *P. semirufa* hair extract. Chondrocytes (1×10^5 cells/well) treated with buffer, IL-1β or extract (60 μg/mL) were collected, and total RNA was extracted using TRIzol. Relative mRNA quantification was carried out by RT-qPCR. All experiments were performed in triplicate, and the values are presented as the mean ± SEM normalized to *GAPDH* as an endogenous control. The data were analyzed using Student's *t*-test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. the control, which was arbitrarily set to 1 (buffer treatment).

DISCUSSION

Pararamosis is caused by accidental penetration of human subcutaneous tissue by *P. semirufa* caterpillar hairs. Over time, this condition can evolve into osteoarticular deformities due to impaired cartilage resulting from an inflammation of the joints, similar to other joint diseases. Understanding of the molecular mechanisms involved in pararamosis may contribute to establishing more effective therapeutic approaches for this occupational neglected disease, which affects communities such as the rubber tappers of the Amazon rainforest.

This study aimed to assess the effects of the *P. semirufa* hair extract on chondrocytes, an important cell type that is present in the joint and is involved in the onset of joint diseases. We treated these cells with IL-1β, as a positive control, since this is a well-known mediator that is involved in the pathophysiology of joint inflammation (28). We investigated a panel of cytokines, chemokines, MMPs, complement components, eicosanoids, and ECM components related to OA and RA that are potentially produced by chondrocytes in response to the pararama hair extract or IL-1β treatment. Another approach was the transcriptomic analysis of treated cells and the selection and

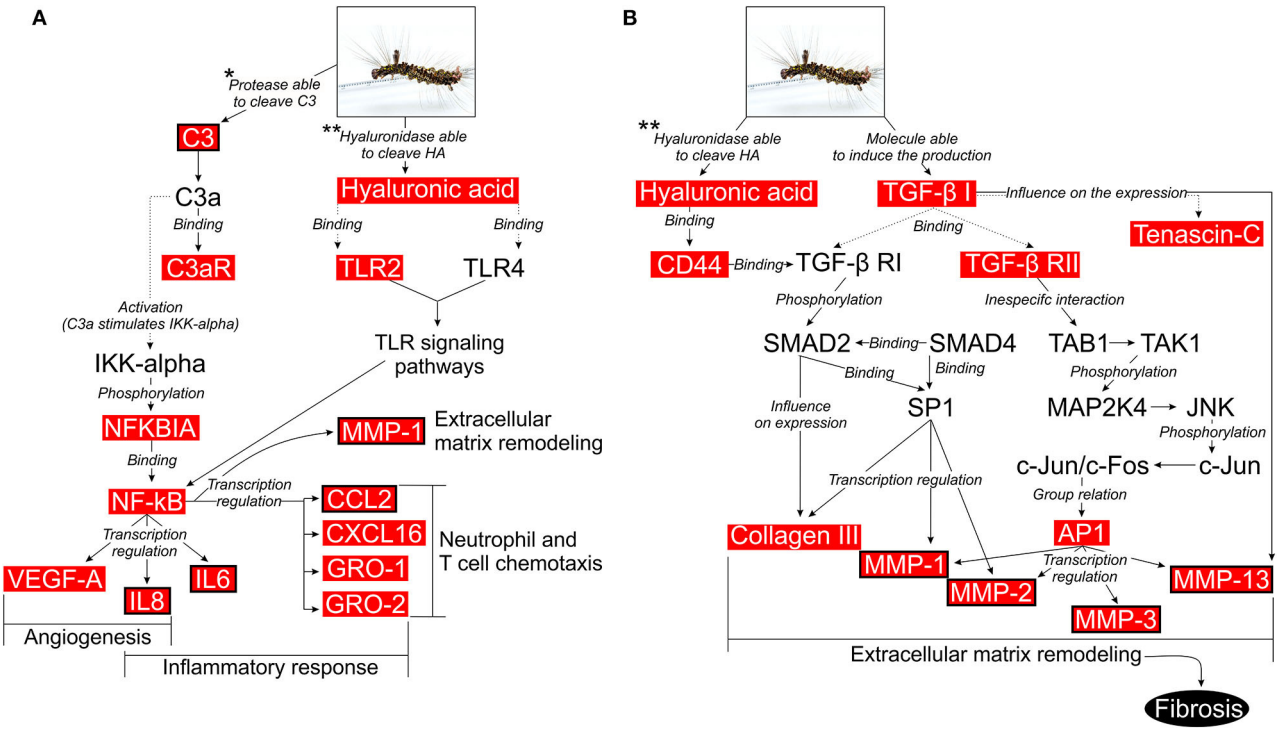

FIGURE 6 | Possible intracellular signaling pathways activated in chondrocytes upon treatment with pararama hair extract. **(A)** Activation of cells by the complement system or by the interaction between low molecular weight hyaluronan and TLR2/TLR4. **(B)** Activation of cells by the interaction between low molecular weight hyaluronan and CD44 or by interaction between TGF-β receptors and TGF-β1. Illustration based on the data obtained with the use of the MetaCore pathway analysis tool (GeneGO/Thomson Reuters) and enriched with DEGs, which are highlighted in red boxes. Red boxes with black borders are the molecules that were validated in this study. Studies that assessed proteases that are able to cleave the C3 component and hyaluronidase that is able to cleave HA in the extract: (**7, *9).

testing of 46 genes involved in OA to verify whether the extract induced a disease with an OA signature.

The analyses of cytokines and chemokines in the supernatant of chondrocytes treated with the *P. semirufa* hair extract showed a time-dependent increase in the levels of IL-6, IL-8, and MCP-1 (**Figure 1**), which was confirmed by RT-qPCR experiments (**Figure 5**). IL-6 and CXCL8 (IL-8) are proinflammatory and angiogenic cytokines that are potent chemoattractants for neutrophils. Several studies have shown increased levels of IL-6 and IL-8 in peripheral blood mononuclear cells or the bone marrow of patients with rheumatoid arthritis (29). Moreover, IL-8 expression is associated with chondrocyte hypertrophy and cartilage destruction in osteoarthritis (30, 31). CCL2 (MCP-1) is a member of the β-chemokine family and, when produced at high levels, it regulates the immune process, triggers chemotaxis, activates macrophages and takes part in the activation of mast cells and the production of leukotrienes (32). Some authors have reported increased MCP-1 levels in the inflammatory process, both in RA and in OA (32, 33).

The extract also induced chondrocytes to produce PGE_2 (**Figure 1**). In patients with OA, PGE_2 expression is elevated, and it is associated with bone degeneration, cartilage metabolism, inhibition of proteoglycan biosynthesis, and joint pain (34–36). PGE_2 is spontaneously released, and its production is induced by cyclooxygenase-2 (COX-2) expression (37). Thus, the extract may modulate chondrocytes to a proinflammatory profile similar to that found in patients with joint diseases, such as OA. In addition, the transcriptome analysis showed that the genes *PTGS2* (prostaglandin-endoperoxide synthase 2 or *COX-2*) and *PTGES* (prostaglandin E synthase) were highly upregulated in chondrocytes treated with the extract.

Additionally, we verified a time-dependent increase in MMP-1, MMP-2, MMP-3, and MMP-13 in the pararama hair extract-treated cultures (**Figure 3**), which was confirmed by RT-qPCR and transcriptome analyses, except for MMP-13 (**Figure 5**, **Supplementary Table 2**). MMPs play an important role in ECM turnover during embryogenesis, morphogenesis, normal tissue remodeling and repair, but in uncontrolled conditions, MMPs contribute to the pathogenesis of several diseases associated with tissue destruction, such as arthritis (38–42).

During OA progression, cytokines and chemokines such as IL-6, IL-8, MCP-1, and CCL5 (RANTES) actively participate in catabolic activities and are involved in cartilage destruction, such as through the production of MMP-1,−3 and−13 (28, 43–48). In addition, other proteinases produced by chondrocytes, such as MMP-2 and MMP-9, may also play a role in the degradation of several matrix components (49). Furthermore, IL-6 is an essential cytokine that triggers osteoclast differentiation and bone resorption (50, 51). Thus, increased release of cytokines and chemokines by chondrocytes treated with the pararama

extract indicates a direct effect of extract component(s) on these cells, activating chondrocytes to produce cytokines and chemokines that may induce the production of MMPs by these same cells, which degrade matrix components. In addition, the downregulation observed in the *SOX9* gene following extract treatment (**Figure 5**) positively correlated with the downregulation of ECM transcription, since *SOX9* is responsible for the transcription of some ECM molecules, such as aggrecan and type II collagen (52).

Complement factors present in the synovial fluid originate from synovial cells, chondrocytes, infiltrating leukocyte or traumatic hemarthrosis (53–57). Analysis of complement components in the supernatants of chondrocyte cultures showed that C3, C4, and C5 were significantly higher in cells treated with the extract than in cells treated with buffer or IL-1β (**Figure 2**). This result suggests that the extract induces complement component production by direct action on chondrocytes or by indirect induction through cytokine production. Despite the increased production of C5, the concentration of this component in the supernatant decreased over time. As the extract contains serine proteases that are capable of cleaving complement components, including C5 (9), a reduction in this component may result from cleavage by the hair extract proteases.

During activation of the complement system, anaphylatoxins C3a and C5a are typical cleavage products that bind to their respective receptors C3aR and C5aR, expressed on a wide variety of cell types and induce inflammatory responses (58, 59). Nozaki et al. (60) identified these anaphylatoxins as proangiogenic factors that induce vascular endothelial growth factor (VEGF) expression in chorion tissue. Notably, VEGF expression has been observed during OA (61). Transcriptome analyses of chondrocytes treated with the extract and MetaCore analyses highlighted a complement activation pathway. Considering that C3 is a DEG and is present in the supernatants of chondrocyte cultures treated with the extract and that the extract proteases are able to directly cleave C3 and generate C3a (9), C3a fragments might bind to C3aR (also a DEG in our transcriptome analysis), activating nuclear factor kappa B (NF-κB) and the production of inflammatory cytokines, such as IL-6, and factors involved in angiogenesis, such as IL-8 and VEGF, thus resembling the events observed in joint diseases (**Figure 6A**).

Sequential events affect the homeostatic integrity of the extracellular matrix during OA progression, including a decrease in the amount of aggrecan and an increase in collagen (62–64). These changes also modify the collagen type composition from type II to type I, thereby affecting the mechanical stability of the extracellular matrix (65, 66). Results from the HCS experiments of chondrocyte cultures treated with the extract, confirmed by RT-qPCR (**Figures 4**, **5**), suggest that the extract induces a reduction in aggrecan and type II collagen (I) by direct cleavage by extract proteases, (II) by inducing proteases expression, such as MMPs, by these cells, or (III) by inhibiting their gene expression. Transcriptome analysis showed that *ACAN (*aggrecan) was a highly downregulated DEG, while *COL2A1* was not a DEG but was a downregulated gene with low expression (**Supplementary Table 2**).

The protein high-mobility group box 1 (HMGB1) induces cytokine production and blood vessel formation and plays an important role in cell proliferation, differentiation, and migration (67). High levels of HMGB1 are observed in inflamed joints and serum of people with RA (68). HMGB1 was detected in chondrocyte nuclei, after 72 h of treatment with the extract (**Figure 4**), though both RT-qPCR, and the transcriptome analyses did not show any increase in *HMGB1* expression, perhaps due to differences in the treatment times used in the experiments.

Hyaluronic acid (HA), a polymer composed of glucuronic acid and N-acetyl glucosamine, is produced by hyaluronic acid synthases (HAS), expressed in fibroblast-like cells in the synovial lining and cartilage chondrocytes. HAS2 is the major isoform responsible for HA production in cartilage (69, 70). In chondrocytes, HA retains proteoglycans, such as aggrecan, and interweaves with collagen, providing a protective load-bearing surface (71, 72). Binding of HA to its primary receptor CD44 induces TGF-β receptor (TGFBR) activation, disturbances in cell adhesion to extracellular matrix components, inflammation, development, tumor growth, and metastasis (73, 74). RT-qPCR revealed an increase in *HAS2* expression and a reduction in *HYAL1*, *HYAL2,* and *HYAL3* expression after extract treatment (**Figure 5**). Transcriptome analysis showed an increase in the expression of *HAS2*, *HAS2-AS1* (anti-sense), *CD44*, and *TGFBR2*. We found a significant hyaluronidase activity in the hair extract (7), which may act on hyaluronic acid present in the chondrocyte ECM and activates the TGF-β pathway by increasing the HA-CD44-TGFBR interaction. TGF-β receptors interact with their ligand, TGF-β1, which is highly expressed in chondrocytes (75–77). We detected a slight increase in *TGF-β1* expression in treated cells (**Supplementary Table 2**). This interaction leads to the phosphorylation of SMAD2, which interacts with SMAD4. The SMAD2-SMAD4 complex translocates to the nucleus, where it modulates the transcription of TGF-β regulated genes, such as COL3A1 (Type III collagen) (**Figure 6B**). COL3A1 is a DEG that is highly expressed in chondrocytes treated with the extract, is related to the fibrosis process, and its expression is more pronounced in OA cartilage (78).

TGF-β receptors TGF-β1 interactions also activate MAP kinase pathways, such as the extracellular signal-regulated kinase (ERK) 1/2 pathway and the c-Jun N-terminal kinase (JNK) pathway. In the latter pathway, the transcription factor activator protein 1 (AP-1) is considered a key factor for MMP expression (79). MAP kinases are involved in MMP gene transcription (80), and in our study, they may be involved in the transcription of genes for matrix metalloproteinases such as MMP-1, MMP-2, MMP-3, and MMP-13 (**Figure 6B**). Tenascin-C (TNC), a hexameric glycoprotein component of the ECM, is a highly expressed molecule that participates in this pathway and was upregulated in extract treated chondrocytes. TNC interacts with over 25 different molecules, such as pathogenic components, matrix constituents, soluble factors, and cell surface proteins (81). TNC is a key molecule in tissue remodeling, and its deregulated increased expression is linked to joint diseases, including OA and RA. Thus, we again identified a proinflammatory profile induced by the extract in chondrocytes.

Low molecular weight hyaluronan (LMW-HA) is increased in joints in OA and has been shown to interact with TLR2/TLR4 in chondrocytes (82). In our model, this association also

occurred in pararama hair extract treated chondrocytes. In fact, our transcriptomic analysis showed upregulation of the TLR2 in treated chondrocytes. This ligand and receptor interaction activates NF-κB, which is responsible for the transcription of chemokines *(IL-8, MCP-1, CXCL16, GRO1, and GRO2*), cytokines *(IL-6)*, and *MMP-1* by chondrocytes. These molecules contribute to chemotaxis, activation of inflammatory cells and ECM remodeling (**Figure 6A**). These factors were all highly expressed DEGs in chondrocytes that were treated with the extract.

In conclusion, our data shows that pararama hair extract induces chondrocyte inflammation, with the production of il-6, il-8, mcp-1, pge2, and complement components such as c3, c4, and c5. In addition, cartilage degradation and extracellular matrix remodeling features, such as increased expression of mmp1, mmp2, mmp3, and mmp13, and reduced type ii collagen and agrecan, were also observed. Transcriptomic and bioinformatics analyses of these cells indicated that the extract can activate important pathways related to the inflammatory process of joint diseases, such as the inflammatory response, chemotaxis of immune cells and extracellular matrix remodeling. Since the phenotype found in the human chondrocytes, treated with the extract, resembles those seen in joint diseases, such as oa, these data highlight the oa signature in pararamosis that should be further investigated in order to determine strategies to treat this and other joint diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov, PRJNA592966.

AUTHOR CONTRIBUTIONS

IV-B and DT designed research. IV-B, AC, CD-P, and KM performed the experiments. GP, IJ, AC-T, and DT contributed with biological material, reagents, and analytic tools. IV-B, FL, CD-P, CM, KM, and DT analyzed data. IV-B, FL, CM, and DT wrote the paper. All authors contributed to the article and approved the submitted version.

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The Crotoxin:SBA-15 Complex Down-Regulates the Incidence and Intensity of Experimental Autoimmune Encephalomyelitis Through Peripheral and Central Actions

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Crotoxin (CTX), the main neurotoxin from *Crotalus durissus terrificus* snake venom, has anti-inflammatory, immunomodulatory and antinociceptive activities. However, the CTX-induced toxicity may compromise its use. Under this scenario, the use of nanoparticle such as nanostructured mesoporous silica (SBA-15) as a carrier might become a feasible approach to improve CTX safety. Here, we determined the benefits of SBA-15 on CTX-related neuroinflammatory and immunomodulatory properties during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis that replicates several histopathological and immunological features observed in humans. We showed that a single administration of CTX:SBA-15 (54 μg/kg) was more effective in reducing pain and ameliorated the clinical score (motor impairment) in EAE animals compared to the CTX-treated EAE group; therefore, improving the disease outcome. Of interest, CTX:SBA-15, but not unconjugated CTX, prevented EAE-induced atrophy and loss of muscle function. Further supporting an immune mechanism, CTX:SBA-15 treatment reduced both recruitment and proliferation of peripheral Th17 cells as well as diminished IL-17 expression and glial cells activation in the spinal cord in EAE animals when compared with CTX-treated EAE group. Finally, CTX:SBA-15, but not unconjugated CTX, prevented the EAE-induced cell infiltration in the CNS. These results provide evidence that SBA-15 maximizes the immunomodulatory and anti-inflammatory effects of CTX in an EAE model; therefore, suggesting that SBA-15 has the potential to improve CTX effectiveness in the treatment of MS.

Keywords: crotoxin, SBA-15, mesoporous silica, experimental autoimmune encephalomyelitis (EAE), IL-17, neuroinflammation, motor impairment

INTRODUCTION

Multiple sclerosis (MS) is a quite frequent neurological disorder diagnosed in young adults. The common symptoms include, among others, fatigue, visual impairment, movement and coordination problems, cognitive dysfunction and pain (1). This chronic inflammatory disease is mediated mainly by auto-reactive T-lymphocytes, being classified as an autoimmune condition. The hallmarks of MS are myelin and neuroaxonal lesions, resulting from the increase in the blood-brain barrier (BBB) permeability by pro-inflammatory cytokines and chemokines action, which allows the influx of immune cells into the central nervous system (CNS) (2, 3). In addition to the increased infiltrating cells, activation and proliferation of resident glial cells are also observed (4–6). It is well known that T helper (Th) 17 plays a key role in the MS development, being involved in the BBB breach, glial cell activation and pro-inflammatory cytokine release, such as interleukin (IL) 17. The importance of Th17 cells in the development of the disease was also demonstrated in the experimental autoimmune encephalomyelitis (EAE) model, an animal model of MS (7–9).

Studies have shown that the EAE model reproduces neuroinflammation, axonal demyelination and motor impairment observed in MS, encouraging its use in studies aiming to understand the mechanisms involved in the course of the disease as well as to evaluate possible therapies (10, 11). Additionally, within this model, the first symptom to emerge is pain (hypernociception) followed by motor impairment (clinical signs) (12–14), which allows for the independent evaluation of both symptoms (15, 16). Treating these symptoms remains a challenge; current therapies are focused on modulating disease progression and evidence of a definitive cure is lacking (17, 18).

Natural products have been considered a rich source of compounds for the development of potential drugs for the treatment of pain and other disorders, such as neurodegenerative diseases (19, 20). Crotoxin (CTX), a neurotoxin from the *Crotalus durissus terrificus* rattlesnake, is the main compound responsible for the venom high toxicity. Despite the toxic effect, in low doses, it has prolonged anti-inflammatory, immunomodulatory, antitumoral and antinociceptive activities, making it a potential drug for chronic pain and inflammatory diseases (21–25). However, its toxic effect may comprise its usefulness in treating debilitating diseases or its ability to be administered in high doses.

Reports have demonstrated the great potential of mesoporous materials in the field of nanoscience originating from their particular characteristics such as unique porous structures and adsorption properties (26–30). Among these materials, mesoporous silica SBA-15 has been studied as a carrier agent, due to its physicochemical and structural properties, allowing it to act as a delivery system, aiding the release profile, and improving the immune response (31–34). Additionally, studies show that the SBA-15 surface allows for high drug-loading, which may reduce the toxicity of compounds (35, 36). Although the mechanisms by which SBA-15 decreases the toxicity of compounds is not clear, the controlled release of them may contribute to that. In accordance, previous studies from our group have demonstrated that SBA-15 allows an increase of CTX dose with non-toxic effects (37).

Considering that, in the present study, we proposed to improve the effect of CTX on the EAE model through its conjugation with silica SBA-15 and to investigate its effect on central and peripheral important sites for disease development and maintenance. Of interest, skeletal muscle contractility properties were also investigated, since peripheral alterations induced by EAE are poorly reported. Hence, the effect of the treatment with CTX conjugated to SBA-15 in the EAE model was evaluated and compared to unconjugated CTX using different schedules of treatment [single or 5 administrations, being 1 dose *per* day for 5 consecutive days] through behavioral and functional assays and molecular biology tools.

MATERIALS AND METHODS

Animals

Experiments were performed on female C57BL/6 mice (38) (wild type, 18-22g), aged 8-12 weeks, from the animal facility of Butantan Institute (Sao Paulo, Brazil). Mice were group-housed in 5 to 6 animals per cage and kept under a 12-h light/dark cycle (lights on at 6 am) with food and water *ad libitum*. The animal room was kept under 21 ± 2.0°C and humidity (50% ± 10% RH). All behavioral tests were performed between 7:00 am and 5:00 pm. The experimental procedures performed on animals were approved by the Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 1320/14 and 7986010819), and performed in accordance with the National Council of Animal Experimentation Control (CONCEA) regulation and with the guidelines for the ethical use of conscious animals in pain research published by the International Association for the Study of Pain (39). Behavioral tests were performed with 8 animals per group; histological and functional skeletal muscle assays included 4-5 animals per group; interleukins expression or release and $TCD4^+$ cell frequency were analyzed using 5-6 animals per group.

Induction of EAE and Clinical Signal Scale

Mice were randomly immunized subcutaneously (s.c.) with incomplete Freund's adjuvant (*#F5506, IFA; SIGMA, St Louis, USA*) containing 4 mg/ml of *Mycobacterium tuberculosis* (#BD-231141, *H37Ra; Difco*™) and 200 µg of MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK peptide, Proteimax, Brazil) for the induction of EAE. Immediately after immunization and 48 h later, the animals received intraperitoneal (i.p.) injection of 300 ng of pertussis toxin (#P7208, *Sigma-Aldrich*). Control groups consisted of animals injected with CFA (incomplete Freund's adjuvant containing 4 mg/ml of *Mycobacterium tuberculosis*) and pertussis toxin or naïve. Clinical scores were recorded daily by a blind experimenter and when a score of 3 or higher was observed, water and food were made available to the base of the cage to be easily accessed. Clinical scores were

TABLE 1 | Clinical EAE scores.

Score	Observation
0	No symptom
0.5	Partial loss of the tail tone
1.0	Complete loss of tail tone, with no evidence of hind limb weakness
1.5	Evidence of hip weakness upon ambulation (slightly wobbly)
2.0	Hip weakness and hind limb paresis
2.5	Partial hind limb paralysis (from one of the limbs)
3.0	Complete hind limbs paralysis, but capable of moving around the cage
3.5	Complete hind limbs paralysis and difficult to moving around the cage (forelimbs paresis)
4.0	Complete hind limbs paralysis and partial paralysis of the forelimbs, but still responsive (consider euthanasia)
4.5	Complete hind limbs paralysis and paralysis of the forelimbs, decreased responsiveness (consider euthanasia)
5.0	Immobile and unresponsive, moribund or death (immediate euthanasia)

classified as follows in **Table 1** and reached the peak between the 14^{th} and 18^{th} day after immunization.

Crotoxin (CTX)

Crotoxin was purified from *Crotalus durissus terrificus* venom obtained from Laboratory of Herpetology, Butantan Institute, by anion-exchange chromatography as previously described (40, 41). Dr. Sandra Coccuzzo Sampaio from the Laboratory of Pathophysiology, Butantan Institute, kindly supplied CTX.

SBA-15 Synthesis

The synthesis recipe and adsorption characterization of the SBA-15 were analogous to those reported elsewhere (42). Small-angle X-ray scattering [SAXS] characterization was performed as previously described (43).

Preparation of the Complex (CTX:SBA-15) and Treatment

The CTX was diluted in phosphate-buffered saline (PBS) pH 7.4, slowly added to the silica SBA-15 (1:10) and held for 24 h at 2°C to 8°C, with occasional stirring.

In the *in vivo* assays, the following compounds were used: 40 μg/kg of CTX (22, 44) or 54 μg/kg of CTX:SBA-15 administered by subcutaneous (s.c., 200 μl) route, since SBA-15 conjugation enabled an increase of 35% of CTX non-toxic dosage, as previously demonstrated by our group (37). The animals were treated with 5 doses (1 administration/day for 5 consecutive days) or with a single administration, starting from the 5^{th} day post-immunization, based on previous results from our group which considered the onset of the first symptom of the disease to start the treatment (pain threshold alteration that occurred in the 4^{th} day after immunization) (45). PBS (s.c., 200 μl) was used as vehicle control.

Behavioral Test for Mechanical Hypernociception Determination—Electronic von Frey

In a quiet room, animals were placed individually inside acrylic cages on an elevated wire grid and the plantar surface of the paw was stimulated with a pressure-meter which consisted of a hand-held force transducer fitted with a 0.5 mm^2 polypropylene tip (*electronic von Frey anesthesiometer, Insight Equipment's Ltda., Ribeirão Preto, SP, BRA*). Mice were first habituated to the experimental environment (room and apparatus) for at least 20 min. The mechanical nociception test consisted of applying a rising perpendicular force to the hind paw followed by a clear flinch response after a paw withdrawal. Paw stimulation is repeated until the animal shows three similar measures (46). The animals were submitted to pain sensitivity analysis before (baseline measurement) and every day after immunization with MOG_{35-55} by a blind experimenter until the first clinical signs of motor alterations appeared.

Ex Vivo Skeletal Muscle Contractility Properties

The contractile properties (tetanic force) of the extensor digitorum longus muscle (EDL) were evaluated as previously described (47). Briefly, animals were euthanatized at the peak of EAE, the entire hindlimb was collected and the EDL muscle was isolated and placed in an organ bath containing 20 ml of Tyrode solution (2 mM $CaCl_2$, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 137 mM NaC_l, 24 mM $NaHCO_3$, 11 mM glucose, pH 7.4) at 25 °C perfused with carbogen (95% O_2 + 5% CO_2). The muscle was tied by the tendons to an apparatus attached to a force transducer (Grass Instruments model FT03, USA). The muscle optimal length was determined and the EDL was stimulated to contract isometrically through electrical field stimulation (Grass Instruments S-88 Grass-stimulator). The force transducer was recorded and analyzed using a PowerLab system (AD Instruments, USA). Forces are expressed in grams and normalized by the EDL weight.

Skeletal Muscle Cross-Sectional Area (CSA)

The skeletal muscle cross-sectional area was assessed as previously described (48). Briefly, after euthanasia at the peak of EAE, the tibialis anterior muscle was collected, frozen, and stored in liquid nitrogen. Following that the muscle was fixed and cross-sectioned (10 μm sections) using a cryostat. Sections were submitted to HE-staining according to the procedure previously described (49). The myofiber cross-sectional area (μm2) was evaluated at 200 magnification and analyzed (Image Pro-Plus, NHI, USA). An investigator who was blind to the animal identity performed all of the analyses.

Interleukins mRNA Analysis by Real-Time PCR

After euthanasia, the lymph nodes (pool of inguinal and mesenteric) and tissue from the region that included the lumbar segments of the spinal cord were collected. A time course of IL-17 mRNA expression induced by EAE was evaluated in both lymph nodes and spinal cord (3^{rd}, 7^{th}, 10^{th}, and 14^{th} day). The timepoint of mRNA expression increasing was determined in each region, and it was selected for cytokine release evaluation and $TCD4^+$ cell frequency analysis. The total RNA was isolated using the Illustra RNAspin Mini Kit (*GE Healthcare*), and the preparation was suspended in 20 μL of H_2O

(RNA free). RNA concentrations were determined using the NanoVue® apparatus (GE Healthcare Life Science) and 500 ng was transcribed into cDNA by SuperScript™ III reverse transcriptase *(#18080044, Thermo Fisher Scientific)*, according to the manufacturer's protocols. Quantitative real-time PCR was performed using gene-specific primers. Real-time PCR amplification mixtures contained 2 µl cDNA, 5 µl Fest SYBR™ Green Master Mix *(#4385612, Thermo Fisher Scientific)*, 2.2 µl H_2O (RNA free), and 0.8 µM specific PCR primers. Reactions were carried out in a StepOnePlus™ Real-Time PCR system (*Thermo Fisher*) thermocycler. The results were analyzed using the method of quantitative relative expression $2^{-\Delta\Delta Ct}$ (50). The primer sequences were as follows in **Table 2**.

Cytokine Levels Evaluation by MULTIPLEX

The spinal cord from the region comprised between lumbar segments was collected at the peak of the disease. Spinal cords were individually homogenized in buffer containing PBS (0.01 M, pH 7.4), Tween 100 (0.1%), cocktail protease inhibitor, and phosphatase (*1:300, Sigma-Aldrich, EUA*). The homogenate was centrifuged at 10,000*g* for 5 min at 4°C. An aliquot of the supernatant was separated for the determination of proteins quantification by the Coomassie colorimetric method (*Bradford Reagent – Thermo Scientific, Rockford, IL, USA*) (51). The samples were normalized (1.2 µg/µL) and the concentration of the cytokines was determined by MULTIPLEX, xMap method, using the commercial kit (*#MCYTOMAG, Millipore, USA*). Reading was performed by the equipment Luminex 200 - Software xPonent/Analyst version 4.2.

Cell Analyses by Flow Cytometry

The lymph nodes (pool of inguinal and mesenteric) were collected on the 7th day after immunization. The cells were isolated by flushing the tissue through a cell strainer (70 µm) with RPMI medium, followed by centrifugation at 450 g for 5 min at 4 °C and the pellet was resuspended in 3 ml of RPMI medium. In a 24-well plate, the cells were cultured at a concentration of 1×10^6 cells in 500 µl in RPMI medium with or without MOG_{35-55} (1 µg/ml) and BD GolgiStop™ Protein Transport Inhibitor (provided in the kit or Cat #554724) for 12 h. After 8 h, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for 4 h. The cells were stained for surface markers and intracellular cytokine with Mouse Th17/Treg Phenotyping kit *(#560767, BD Biosciences)* according to the manufacturer's directions. Samples were analyzed using a FACS Canto II Flow Cytometer (*BD Biosciences, San Diego, USA*) after the acquisition. Data analysis was performed using FlowJo VX.0.7r2 (*Tree Star, Ashland, OR*). The strategy for analysis and representative dot plots of the experiments are shown in the **Supplementary Material**.

TABLE 2 | Cytokine levels evaluation by MULTIPLEX.

Gene	Forward sequence	Reverse sequence
Ppia	AGCGTTTTGGGTCCAGGAAT	AAATGCCCGCAAGTCAAAAG
HPRT	CGTCGTGATTAGCGATGATGA	CCAAATCCTCGGCATAATGATT
IL-17	GCGTGTCCAAACACTGAGGCCA	ATTGCGGTGGAGAGTCCAGGGT
IL-10	CCCAAGTAACCCTTAAAGTCCTG	GCTGGACAACATACTGCTAACC

Protein Analysis by Western Blotting

At the peak and the 26th day after EAE, the spinal cord was dissected, the tissue from a region that included lumbar segments was collected and individually homogenized in buffer containing Hepes-NaOH (1 M, pH 7.9), NaCI (1,54 M), EGTA (200 mM), Triton-X 100 (1 %), cocktail protease inhibitor and phosphatase (1:300, Sigma-Aldrich, EUA). The homogenate was centrifuged at 15,000*g* for 10 min at 4°C. An aliquot of the supernatant was separated for proteins quantification by Coomassie colorimetric method (Bradford-Thermo Scientific) (51).

Protein samples were separated (30 µg) by electrophoresis on polyacrylamide gel SDS-PAGE (10% or 12%) and transferred to nitrocellulose membranes. Incubation consisted of 1 h at room temperature with blocking solution containing 5% blocking agent (GE Healthcare) dissolved in Tris-Buffered Saline - 10% Tween 20 (TBS-T) followed by incubation with primary antibodies Anti-GFAP (1:5,000, #Ab53554, *Abcam*), anti-CD11b (1:1,000, #Ab9485, *Abcam*), anti-MBP (1:500, #ab40390) or anti-GAPDH (1:5,000, #Ab75476, *Abcam*) overnight at 4°C in TBS-T/5% bovine serum albumin (BSA). Then, membranes were washed 3 times for 5 min in TBS-T and incubated for 1 h with secondary antibodies conjugated with peroxidase (GE Healthcare Life Science) at 1:5,000 in 5% blocking agent (GE Healthcare Life Science) dissolved in TBS-T. Bands were visualized using ECL (Pierce) solution with a digital image capture system and an optical density was determined by image lab software (UVITEC Cambridge).

Histological Evaluation

At the peak of EAE clinical signs, the spinal cord was collected and fixed in 10% (w/v) paraformaldehyde (PFA) for at least 24 h, at room temperature. Following fixation, the tissues were transferred to 70% ethyl alcohol, dehydrated, embedded in paraffin, and sectioned (5 µm) using a microtome. The sections were stained with hematoxylin and eosin (HE) according to the standard protocol. The microscopic images were obtained using a DMLB microscope (Leica Microsystems) coupled to a camera (DFC420; Leica Microsystems, Switzerland) with software LAS version 4.5 (Leica Microsystems). The histological images from the spinal cord of each animal were blindly scored from 0 to 4 (inflammatory score), as follows: 0, no infiltration of inflammatory cells; 1, mild cellular infiltration; 2, moderate infiltration; 3, severe infiltration; and 4, massive infiltration (52, 53).

Data Analysis

Statistical analysis of behavior test for mechanical hypernociception was carried out using a two-way analysis of variance (ANOVA) followed by Tukey's post-test, whereas one-way ANOVA followed by Tukey's t-test applied for AUC and biomolecular assays. All data are expressed as mean ± SEM. Statistical significance was set at $p < 0.05$. These analyzes were performed at Prism software, version 6.0 (GraphPad Software, La Jolla, CA).

RESULTS

A Single Administration of CTX:SBA-15 Improves the Clinical Condition Induced by the EAE Model

We previously described that the repetitive administration of CTX (40 μg/kg), starting from the 5th day post-immunization (5 consecutive doses 40 μg/kg) was effective in reducing EAE-induced clinical signs (45). Since SBA-15 conjugation enables an increase of 35% in CTX non-toxic dosage, a dose of 54 μg/kg was used in the present study (37). CTX:SBA-15 induced analgesic effect during the observed period when compared to the EAE group (**Figure 1A**). No differences were detected between unconjugated CTX and CTX:SBA-15 groups. Regarding the clinical scores, each animal was evaluated from the 9th to 26th day after immunization, (**Figures 1B–E**). The area under the curve (AUC) of clinical signs (individually calculated but represented as the mean of the group) (**Figure 1C**), the incidence of clinical signs (**Figure 1D**), and the maximum score reached by each animal (**Figure 1E**) were analyzed. The animals with EAE had the onset of clinical signs on the 9th day after immunization (**Figure 1B**) and reached the peak between the 14th to 18th day after immunization. Unconjugated CTX and CTX:SBA-15 delayed the appearance of clinical signs (12th to 14th day after immunization), which had a milder clinical picture during the whole period when compared to the EAE + PBS group (**Figures 1B, C**). In addition, the disease incidence, as well as the intensity of the clinical signs were 25% lower in both treated groups (**Figures 1D, E**).

Since silica SBA-15 potentiates the immune response and prolongs the effect of various compounds, mice were treated with a single administration of unconjugated CTX or CTX:SBA-15 on the 5th day after immunization. Interestingly, CTX in both conditions induced a long-lasting analgesic effect (**Figure 1F**), however, only CTX:SBA-15 delayed the manifestation of clinical signs from the 10th to the 14th day and improved the clinical scores (**Figures 1G, H**), also decreasing the intensity of motor impairments (**Figure 1J**). Among the animals treated with the complex CTX:SBA-15, only 13% of the animals at the outset and 60% at the peak of the disease had manifested the disease, showing around 30% less incidence of EAE (**Figure 1I**). Given these results, only a single administration of unconjugated CTX or CTX:SBA-15 was used for the next experiments.

CTX:SBA-15 in a Single Administration Prevents the Muscle Impairment Induced by the EAE Model

Since the EAE group treated with CTX:SBA-15 showed a decrease in the intensity of motor impairments, functional and morphological properties of skeletal muscle were also investigated at the peak of the disease (**Figure 2**). EAE animals displayed reduced body mass when compared to control groups (Naive and CFA). These changes were accompanied by decreases in the Soleus, Plantaris, Gastrocnemius and tibialis anterior muscle mass in animals (**Supplementary Figure 1**). Of interest, a single administration of CTX:SBA-15, but not unconjugated CTX, was sufficient to prevent the weight and skeletal muscle loss seen in EAE animals (**Supplementary Figure 1**). In addition, the tibialis anterior muscle atrophy was apparent at the single fiber level, measured by myofiber cross-sectional area. The treatment with CTX:SBA-15, but not unconjugated CTX, showed a protective effect against EAE-induced skeletal muscle atrophy, preserving myofibers cross-sectional area of tibialis anterior muscle as compared to EAE group (**Figures 2A, B**).

To evaluate the skeletal muscle contractile property, we assessed the maximal tetanic absolute force of the extensor digitorum longus muscle (EDL). As expected, the EAE showed a reduction of this property, when compared to control groups. The treatment with CTX:SBA-15, but not unconjugated CTX, showed a protective effect against EAE-induced skeletal muscle functional loss (**Figure 2C**). Thus, these findings reinforce that the treatment with a single administration of CTX:SBA-15 prevents EAE-induced motor impairment.

CTX:SBA-15 Decreases Peripheral and Central IL-17 Expression

Considering that a single administration of the CTX:SBA-15 complex improves the clinical signs and muscle functions, we investigated its effect in peripheral lymphoid organs (lymph node) and at the CNS (spinal cord), regions that are deeply involved in the disease development. Firstly, the time course of IL-10 (**Supplementary Figure 2A**) and IL-17 mRNA expression in lymph nodes (**Figure 3A**) were evaluated in EAE animals. The results showed that EAE induced a significant increase in pro-inflammatory cytokine IL-17 mRNA expression in the lymph node, on the 7th day after immunization (**Figure 3A**); no differences were detected in other time points or IL-10 mRNA expression (**Supplementary Figure 2A**). Considering these results, the effect of the treatments on the expression of Th17 cells at the same time point was evaluated, through the $CD4^+$/$Foxp3^+$(**Supplementary Figure 2B**) or $CD4^+$/$IL\text{-}17^+$ (**Figure 3B**) expression by flow cytometry (gating strategy analysis and representative Dot Plots, **Supplementary Figure 3**). The results showed an increase of Th17 cells in the EAE and EAE + CTX groups, while the EAE animals treated with CTX:SBA-15 showed a significant reduction of Th17 cells (**Figure 3B**). No difference was observed in Treg cell analysis (**Supplementary Figure 2B**).

Taking into account the effect of CTX:SBA-15 on the lymph node, and the relevance of interleukin IL-17 as a key player in the development of the disease, we investigated if this interference would also occur at the CNS (spinal cord). Our data shows that EAE induces an increase in the IL-17 mRNA expression in the spinal cord, at the peak of the disease (**Figure 3C**). In addition, it was observed that EAE mice showed an increase in the IL-17 interleukin release at this same period by multiplex, while a single administration of CTX or CTX:SBA-15 significantly reduced this interleukin to normal levels (**Figure 3D**). Both mRNA expression and multiplex analyses were also performed for interleukin IL-10 (mRNA and cytokine level). An increase of IL-10 mRNA expression in the spinal cord was only observed at the peak of the disease in EAE animals, but there was no

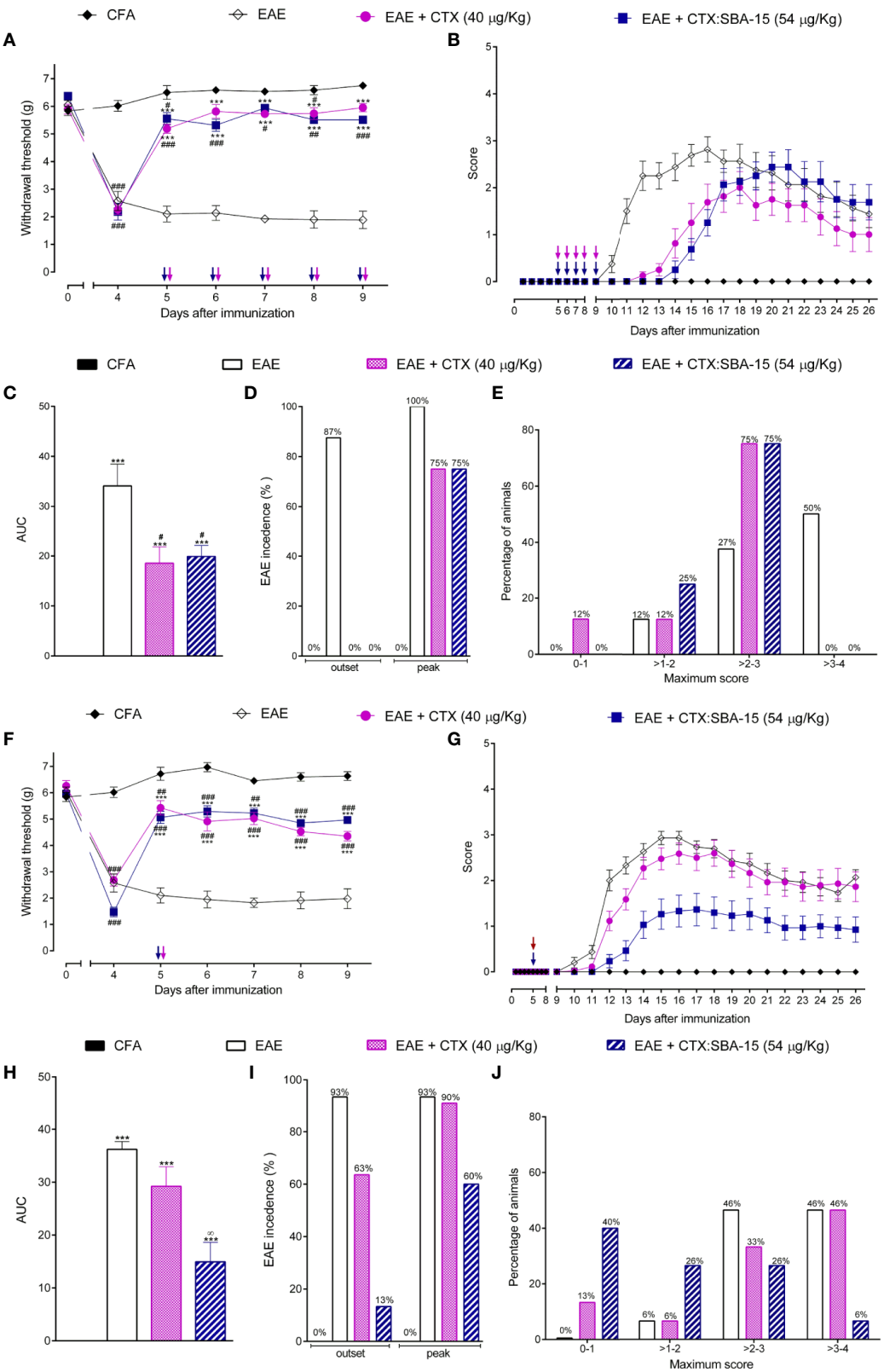

FIGURE 1 | Continued

FIGURE 1 | Effect of CTX:SBA-15 treatment on EAE in rodents. Nociceptive threshold was daily assessed throughout the experimental protocol (day 0) **(A, F)**. Animals were daily evaluated for the onset of clinical signs according to the scale parameters graded from 0 to 5 **(B, G)**. Statistical analysis of clinical signs was made by area under the curve analysis **(C, H)**, the incidence of the disease was shown in percentage (%) of animals showing clinical score equal to or greater than 1 in the group **(D, I)** and the maximum score reached for each animal on the evaluated period was shown in percentage (%) **(E, J)**. Results are expressed as mean (± SEM) n = 8 animal per group. Data are representative of two independent experiments. ***p < 0.001 indicates significant difference when compared to the EAE group. $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 and $^{\#\#\#}$p < 0.01 indicates a statistically significant difference when compared to CFA group. ∞p < 0.01 indicates a statistically significant difference when compared with EAE + CTX group. Two-way ANOVA test was used for behavior analysis, and a One-way ANOVA test was used for AUC, both followed by Tukey's test.

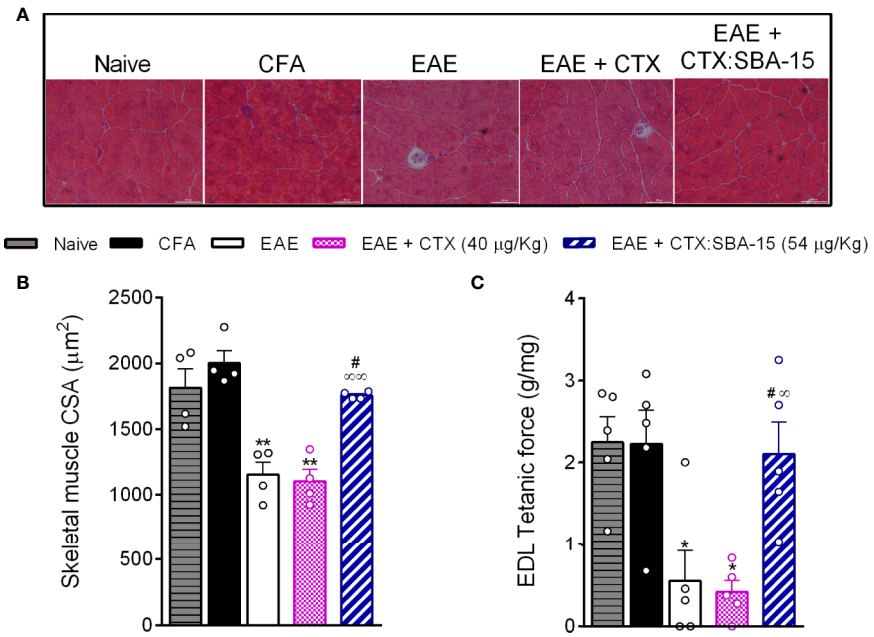

FIGURE 2 | Effect of CTX: SBA-15 treatment on EAE-induced changes in muscle morphology and function. On the peak of the disease, the tibialis anterior muscle was collected for morphological analysis of the cross-sectional area **(A, B)** and the long digital extensor muscle (LDE) for muscle function **(C)**. Results are expressed as mean (± SEM) n = 4–5 animal per group. *p < 0.05 and **p < 0.01 indicates a statistically significant difference when compared to the naive and CFA groups. $^{\#}p$ < 0.05 indicates a statistically significant difference when compared to EAE group. ∞p < 0.05 and ∞∞p < 0.01 indicate statistically significant differences when compared to EAE + CTX group. One-way ANOVA test was used, followed by Tukey's test.

difference in the interleukin levels (**Supplementary Figures 2C, D**).

CTX:SBA-15 Single Administration Reduces the Neuroinflammation in the Spinal Cord

Considering that the EAE model induces a massive immune cell infiltration to the CNS, together with glial cells activation/proliferation, it was next evaluated whether CTX:SBA-15 treatment could also attenuate those activities in the spinal cord. Cell infiltration was evaluated using histological scores in spinal cord sections stained with HE. The results indicate that the number of infiltrating cells was increased in spinal cord sections of the EAE group, mainly observed in the dorso-lateral and ventral areas of spinal cord, when compared to control groups (CFA and naïve), while unconjugated CTX and CTX:SBA-15 decreased EAE-induced cellular infiltration (**Figures 4A, B**). Of interest, CTX:SBA-15 treated mice displayed a more pronounced drop in cell numbers when compared to unconjugated CTX (**Figure 4B**).

Through western blotting assays, at the peak of the disease, a significant increase of microglial/macrophage marker (CD11b) was observed in the EAE animals when compared to controls groups (CFA and naïve) (**Figure 4C**), while no differences were observed for the analysis of GFAP expression at this period (data not shown). Although there is no difference in the relative expression of CD11b marker when comparing unconjugated CTX and CTX:SBA-15 groups, CTX:SBA-15 in a single administration, but not unconjugated CTX, partially reduces the EAE-induced CD11b expression (**Figure 4C**). At the 26th day after immunization a significant increase of astrocyte marker (GFAP) was observed in EAE-PBS-treated animals (**Figure 4D**); in contrast, no difference was observed in CD11b expression (data not shown). Again, unconjugated CTX did not interfere with this expression, while the animals treated with CTX:SBA-15 show reduced GFAP expression (**Figure 4D**) compared to the

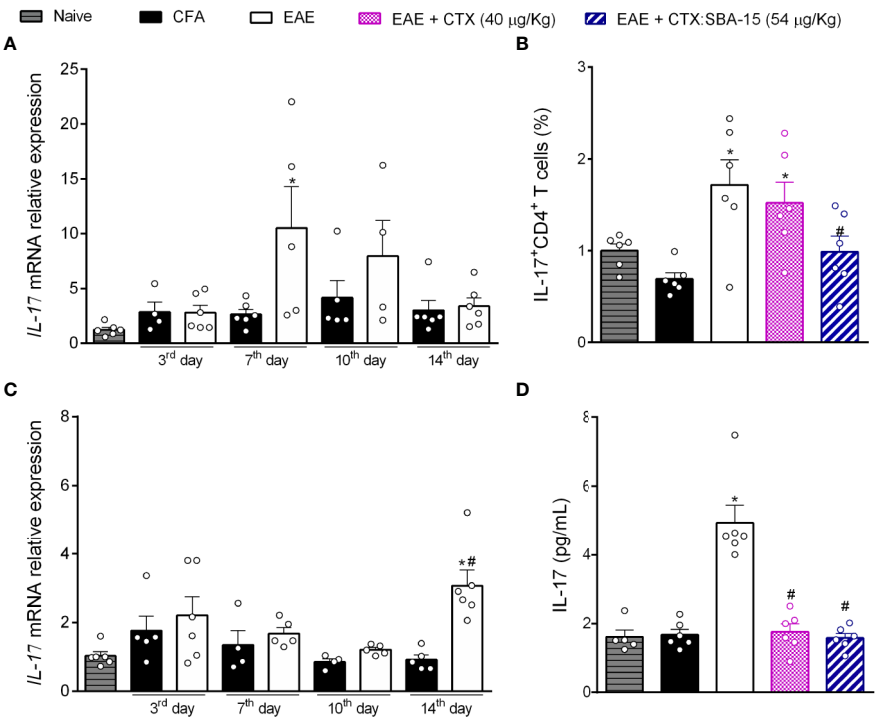

FIGURE 3 | The treatment with CTX:SBA-15 decreases the expression of Th17 cells and IL-17 cytokine induced by EAE. The lymph node **(A)** and spinal cord **(C)** samples were collected at indicated times for mRNA for IL-17. At 7th day after immunization, the cells from lymph nodes were extracted and stained for extracellular marker, for $CD4^+$, and intracellular cytokine IL-17 **(B)**. The results were evaluated by flow cytometry and analyzed in FlowJo software. At the peak of disease the samples from the spinal cord were processed to the protein assay for IL-17 **(D)** assessed by MULTIPLEX. All data are expressed as mean (± SEM) n = 5–6 animal per group. *$p < 0.05$ indicates a statistically significant difference when compared to the Naive and CFA group. #$p < 0.05$ indicates a statistically significant difference when compared to the EAE. One-way ANOVA test was used, followed by Tukey's test.

EAE group, despite of the fact that there is no difference in this expression between unconjugated CTX and CTX:SBA-15 groups.

DISCUSSION

MS is a chronic inflammatory autoimmune disease that especially affects the CNS. Demyelination lesions and neuroinflammation mostly on the brain and spinal cord are the pathological hallmarks of this disease (2, 54, 55). Considering the large variability of clinical manifestation and disease progression, the treatment is complex, based on drugs that slow the progression and manage the symptoms. Unfortunately, there is no cure for this condition yet (17, 18, 56).

To better understand the pathology and the sensorial and motor alterations, as well as to find new therapeutic compounds, previous data from our group confirmed the antinociceptive and immunomodulatory effects of CTX in the MS model (EAE) induced in mice. These results showed that CTX, when administered in 5 consecutive doses, controlled both pain sensitivity and clinical signs development, while when administered in a single administration, it interfered with the pain sensitivity without any effect on clinical signs (45). However, CTX is a neurotoxin, and its use, especially in high doses or in chronic schedules, is limited by its toxic effect, mainly for debilitating conditions. Hence, taking advantage of the silica SBA-15 beneficial effects as a carrier agent (37, 57), we previously demonstrated that SBA-15 attenuates CTX-induced toxic effects by increasing in 35% the CTX lethal dose 50% (LD_{50}) (37). Thus, this work aimed to improve the immunomodulatory effect of CTX through its adsorbing to SBA-15 in the EAE model. Both treatments with unconjugated CTX or CTX:SBA-15, administered in 1 or 5 consecutive doses, induced analgesic effect in the EAE model during the evaluated period. Corroborating previous results about CTX, regarding the motor alterations (clinical sings), 5 consecutive administrations of unconjugated CTX delayed the manifestation of clinical signs and lowered the intensity of motor impairment (45) which was also observed with CTX:SBA-15 treatment. In contrast, when administered in a single administration, while unconjugated CTX does not affect the motor symptoms, the CTX:SBA-15 complex delayed the onset of symptoms and attenuated the intensity of clinical signs. A recent clinical report suggests that starting the treatment during the early symptoms with a highly efficient drug is the best option to prevent the progression of the irreversible lesions and change the disease course (58). However,

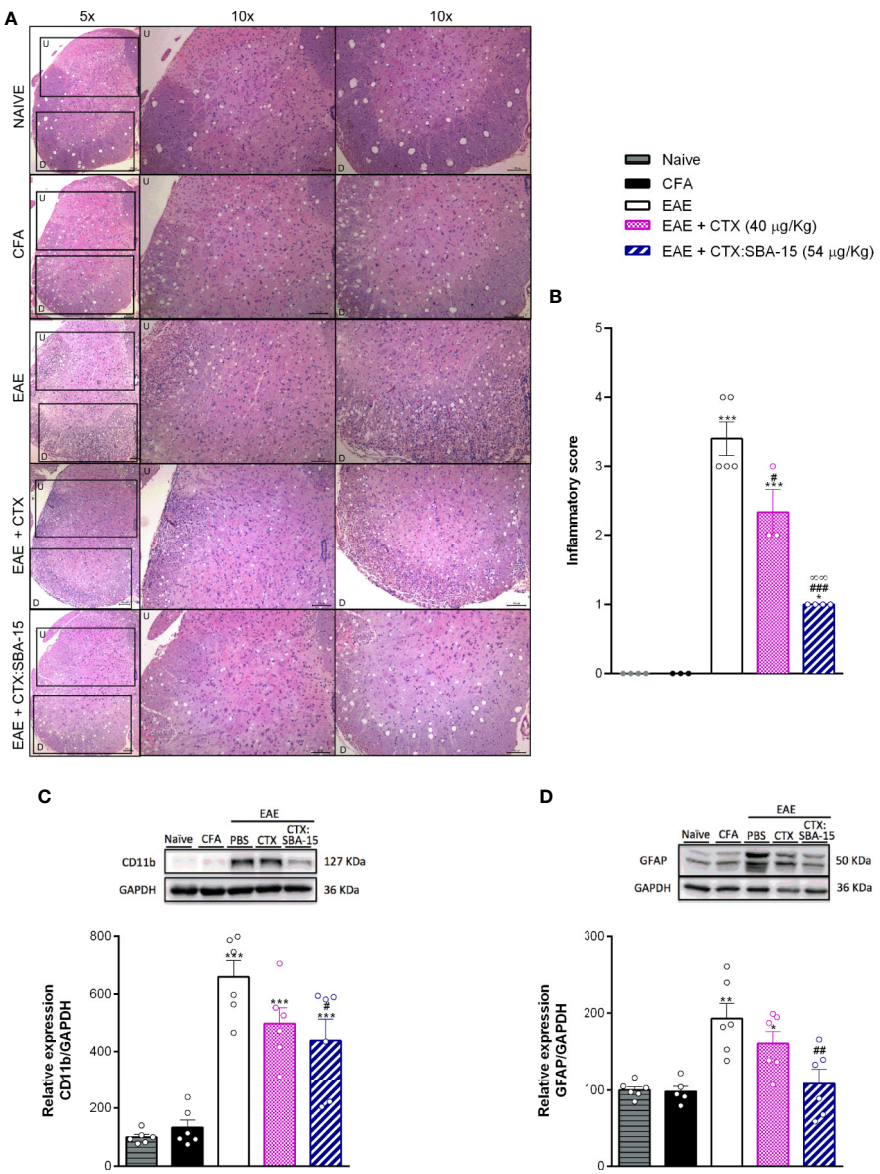

FIGURE 4 | Effect of the treatment with CTX:SBA-15 on neuroinflammation. Animals were treated with CTX (40 μg/kg, s.c.) or CTX:SBA-15 (54 μg/kg, s.c.) in a single administration on the 5th day after immunization with MOG_{35-55} in CFA. The spinal cord of the animals was collected at the peak **(A–C)** or at 26° after immunization **(D)**. The cell infiltration analysis was performed by hematoxylin and eosin (HE) staining. Representative sections of cell infiltration are shown **(A)** as well histological scores **(B)** of whole spinal cord. Upper (U) and down (D) indicate the magnified corresponding figures. Scale bar 100 μm (n = 4-5) animals per group). The expression of microglia, CD11b **(C)**, or astrocytes, GFAP (D), markers, were analyzed by Western Blotting assay (n = 6 animals per group). All data are expressed as the mean (± SEM). ***p<0.001, **p<0.01 and *p<0.05 indicates a statistically significant difference when compared to the Naive and CFA group. #p<0.05, ##p<0.01 and ###p<0.001 indicates a statistically significant difference when compared to the EAE. ∞∞∞p<0.01 indicates a statistically significant difference when compared to EAE + CTX group. One-way ANOVA test was used, followed by Tukey's test.

this has never been seen with a single administration treatment for MS patients or even in animal models of MS.

Although MS has been considered as mainly a neuroinflammatory disease with demyelination and additional axonal damage in the CNS, lower motor activity and slow conduction in peripheral sensory and motor nerve fibers have also been observed (59–61). Reports have shown that EAE induces atrophy in addition to disruption in morphology (14, 62) and disfunction in skeletal muscles (63). Our results demonstrate that mice with EAE showed a reduction in body weight, in part as a consequence of the muscular mass loss and atrophy. Surprisingly, the treatment with CTX:SBA-15, but not with unconjugated CTX alone, prevents EAE-induced muscle atrophy and functional loss, maintaining the health contractile

properties of the EDL muscle. It was described that the atrophy observed in the EAE model is related to sudden muscle inactivity due to neurological injuries, which may be linked to T cell infiltration and microglial activation (14, 60, 63). The present results confirm the effectiveness of a single administration of CTX:SBA-15 in the prevention of EAE-induced motor impairment, probably as a result of the decrease in the immune response and neuroinflammation.

Classical MS immune responses are initiated with antigen-bearing dendritic cells, which migrate to lymphoid organs, such as the lymph nodes, leading to activation and polarization of naive $CD4^+$ T cells (64, 65). Increasingly clinical and pre-clinical studies demonstrate the importance of Th17 cells in the MS pathogenesis. High levels of IL17 in the serum and cerebrospinal fluids were found in patients with MS, which may be related to the disease progression and the extension of the lesions measured by magnetic resonance imaging. Th17 contributes to the neuroinflammation by recruiting immune cells and increasing the release of the pro-inflammatory cytokines, in addition to inducting BBB breach (66–69). Our results demonstrating that single administration of CTX:SBA-15 reduces IL-17 levels and inhibits the Th17 cell recruitment point out that CTX:SBA-15 complex may exert its long-lasting effects through IL-17 inhibition. It was previously demonstrated that the inhibition of Th17 cells in lymph organs can reduce the severity of EAE, in addition to the fact that knockout mice ($Il17a^{-/-}$ mice) are partially resistant to EAE induction (70).

The neuroinflammation induced by MS, in particular by Th17 cells, leads to an increase in BBB permeability, which allows the influx of inflammatory cells into the CNS, activating resident glial cells, such as microglia and astrocytes. Also, astrocytes may contribute to immunomodulation and protection of neurons against cytotoxins and oxidants, and microglia/macrophages activation plays a fundamental role in neuroinflammation. These events mediate the recurrent episodes of demyelination and axonal lesion (4, 67, 71). Some evidence suggests that there is integration between T cells and microglia, as well as astrocytes, which have receptors for IL-17, and the inhibition of those cells can reduce the intensity of EAE (72–75). Our results, showing that CTX:SBA-15 decreased the infiltrating cell in the CNS and the central expression of glial cells induced by EAE, reinforce the ability of SBA-15 in enhancing the immunomodulatory effects of CTX. Besides that, CTX:SBA-15 prevents the EAE-induced decrease in the myelin basic protein (– MPB) expression, observed in a later period in the EAE groups treated with PBS or even with unconjugated CTX (**Supplementary Figure 4**), suggesting that CTX:SBA-15-induced downregulation in neuroinflammation and immune response would interfere in the demyelination process.

The presented data corroborate the mechanism of unconjugated CTX, as previously described by our group (45). On the other hand, the increase in CTX dose together with silica properties promoted even higher benefits in the EAE model with a single administration in the early phase of development. In addition, this work evidenced the CTX:SBA-15 control over peripheral important functional and morphological parameters of skeletal muscle altered by the disease, as well as its effect on central inflammation.

The properties of nanostructured silica in to carry, to protect, and to deliver the entrapped antigens or compounds are attributed to its hexagonal nanostructured pores, which protect compounds against proteolysis, organic solvents, high shear stresses, and low pH (57, 76, 77). Previous data from our group demonstrated that silica does not alter some of the mediators known to be responsible for the antinociceptive effect of CTX (37). Considering that, the fact that once encapsulated and kept entrapped in the silica, the compound may have its release controlled (34, 78) could be a possible explanation for the SBA-induced CTX effect potentiation in EAE animals. However, the lack of pharmacokinetic studies is a limitation of the present study, and future assays should be performed to address this question. Importantly, silica is an inert particle and, based on the present results, would have its use possibly expanded to other therapeutic compounds in order to decrease the required treatment schedule, primarily for medicines with undesirable effects.

In summary, one single administration of the complex CTX:SBA-15 in the early phase of development can ameliorate the EAE-induced neuroinflammation, through the prevention of peripheral Th17 cells recruitment, reduction of cells infiltrate to the CNS, decrease in spinal cytokine IL-17 expression and glial cells activation, hence reducing the intensity and incidence of clinical signs, and preserving muscle mass and function. Thus, on the whole, our results showed that SBA-15 when used as a carrier, besides enabling the increase in CTX dose, can improve the immunomodulatory and anti-inflammatory effects of this toxin, suggesting that the complex CTX:SBA-15 could be a potential formulation for the control of sensitivity, such as pain, motor alterations, atrophy and loss of motor function. Moreover, these findings suggest that SBA-15 can be a useful tool for new therapeutic formulation for several biological compounds.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care Committee of the Butantan Institute (CEUAIB, protocol number 1320/14 and 7986010819).

AUTHOR CONTRIBUTIONS

MS'A, GP, and OS'A designed the study. MS'A, AG, and FL performed the experiments and analyzed the data for study

design. NT helped with EAE induction. MS'A and MR performed and analyzed the skeletal muscle experiments. MS'A and AB performed and analyzed the PCR assays. MB, LK and WC helped with the collection, preparation, and strain of cells for flow cytometry assay. OR helped with analysis and interpretation of Flow Cytometry data. JF and VZ helped with the design study and results interpretation. MS'A and GP wrote the original draft with input from OS'A, LK, JF, and VZ. GP and OS'A obtained the funding for the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.591563/full#supplementary-material

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frontiers in Immunology

ORIGINAL RESEARCH
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Discovery of a Recombinant Human Monoclonal Immunoglobulin G Antibody Against α-Latrotoxin From the Mediterranean Black Widow Spider (*Latrodectus tredecimguttatus*)

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Widow spiders are among the few spider species worldwide that can cause serious envenoming in humans. The clinical syndrome resulting from *Latrodectus* spp. envenoming is called latrodectism and characterized by pain (local or regional) associated with diaphoresis and nonspecific systemic effects. The syndrome is caused by α-latrotoxin, a ~130 kDa neurotoxin that induces massive neurotransmitter release. Due to this function, α-latrotoxin has played a fundamental role as a tool in the study of neuroexocytosis. Nevertheless, some questions concerning its mode of action remain unresolved today. The diagnosis of latrodectism is purely clinical, combined with the patient's history of spider bite, as no analytical assays exist to detect widow spider venom. By utilizing antibody phage display technology, we here report the discovery of the first recombinant human monoclonal immunoglobulin G antibody (TPL0020_02_G9) that binds α-latrotoxin from the Mediterranean black widow spider (*Latrodectus tredecimguttatus*) and show neutralization efficacy *ex vivo*. Such antibody can be used as an affinity reagent for research and diagnostic purposes, providing researchers with a novel tool for more sophisticated experimentation and analysis. Moreover, it may also find therapeutic application in future.

Keywords: envenoming, spider toxins, latrotoxin, monoclonal antibodies, phage display, widow spiders, *Latrodectus tredecimguttatus*, toxin neutralization

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INTRODUCTION

The sight of a spider causes fear and anxiety in a large number of people (1, 2). Despite their fearsome disposition, most spiders are not a threat to human health, and their bites only induce minor toxic effects (3). A handful of spider species do, however, possess a venomous bite that can cause severe damage to humans (3, 4). The unfortunate victim suffering from envenoming from these species may experience significant pain, systemic illness, or dermonecrosis (4). One such group of spiders are the widow spiders (*Latrodectus* spp.) that have a worldwide distribution with 32 recognized species (5, 6). The clinical syndrome resulting from envenoming from *Latrodectus* spp. is called latrodectism and is characterized by diaphoresis and gradually developing local and regional pain that last for hours to days (5). In case of systemic envenoming, which occurs in about one-third of cases, nonspecific symptoms, such as nausea, vomiting, headache, and fatigue, are common, but latrodectism is fortunately rarely life-threatening (4).

These symptoms are caused by the action of toxic proteins present in the venom of *Latrodectus* spp. Their venoms contain a high number of different toxins, among which α-latrotoxin (α-LTX) is the key toxin that specifically affects vertebrates (7, 8). The gene for α-LTX from the Mediterranean black widow spider (*L. tredecimguttatus*) encodes a precursor protein that undergoes proteolytic cleavage during venom maturation, resulting in the active α-LTX of 130 kDa that appears to be responsible for the clinical manifestations in human victims (9, 10). All *Latrodectus* species likely express a form of α-LTX with high functional similarity to the ortholog from *L. tredecimguttatus*, which is reflected by the limited variability (>90% nucleotide identity), conserved cysteine residues, similar lengths, and posttranslational processing signals of α-LTX sequences across species (11), which may explain the similarity of clinical manifestations observed from bites by various widow spiders (12).

Pharmacologically, α-LTX binds to multiple membrane proteins on presynaptic neuronal and neuroendocrine nerve terminals and induce spontaneous massive neurotransmitter release through vesicle exocytosis via both Ca^{2+}-dependent and independent mechanisms (13, 14). Due to this function, α-LTX has played a fundamental role as a tool in the study of neurotransmitter release in vertebrates (15), although some questions about its mechanism of action remain unanswered (13). Currently, neurotransmitter exocytosis induced by α-LTX is believed to involve an initial step of α-LTX binding to extracellular cell surface membrane proteins, which has been studied *in vitro* using α-LTX. Three structurally distinct receptors have been identified to this date: (i) neurexin 1α, a neuronal protein with a single transmembrane domain (16, 17), (ii) latrophilin 1, also called CIRL (calcium-independent receptor of α-latrotoxin), a G protein-coupled receptor (18, 19), and (iii) protein tyrosine phosphatase σ (20). Once bound to a receptor, α-LTX oligomerization ensues, followed by membrane insertion and formation of a non-selective cation channel (21, 22), which leads to Ca^{2+} entry and vesicle exocytosis. How this release of neurotransmitters causes the clinical manifestations previously described is yet to be elucidated (4). The diagnosis of latrodectism is purely clinical, combined with the patient's history of spider bite. No analytical assays exist to detect widow spider venom in blood, urine, or at the bite site (4, 23).

Antibodies are widely used in many diagnostic and therapeutic applications, and having a monoclonal antibody targeting α-LTX could be of utility in both the research setting as an affinity agent, when latrotoxins are employed for experimentation, and in the clinical setting when widow spider bites are suspected. Murine monoclonal antibodies against α-LTX have been produced and successfully applied in studies of the mode of action of the toxin (24–27); they are nowadays used for its affinity purification. Phage display technology has previously been used to discover recombinant human monoclonal antibodies against animal toxins. Still, to the best of our knowledge, no recombinant human monoclonal antibody has ever been reported against a spider toxin. Here, we report the discovery of the first recombinant human monoclonal immunoglobulin G (IgG) antibody (TPL0020_02_G9) that binds α-LTX and show *ex vivo* neutralization efficacy. This affinity agent may find applications in research, as well as it may be of utility in future therapeutic purposes due to its fully human, recombinant, and monoclonal nature.

MATERIALS AND METHODS

Reagents

Lyophilized native α-LTX (>98% purity) was obtained from Alomone Labs (Jerusalem, Israel, LSP-130). 6,7-Dinitroquinoxaline-2,3-dione (DNQX, an AMPA receptor antagonist) and D(−)-2-amino-5-phosphonopentanoic acid (D-APV, an NMDA receptor antagonist) were obtained from Tocris Bioscience. All other chemicals used for preparation of the artificial cerebrospinal fluid (ACSF) and pipette solutions were obtained from Sigma-Aldrich.

Biotinylation of Toxin

α-LTX was reconstituted in phosphate buffered saline (PBS). Biotin linked to *N*-hydroxysuccinimide (NHS) *via* a PEG_4-linker (EZ-Link™ NHS-PEG_4-Biotin, No-Weigh™ Format, Thermo Scientific, #21329) was added to the toxin solution at a molar ratio of 1:3 (toxin:biotin) and left to react at room temperature for 45 min. Buffer exchange columns (Vivacon 500, Sartorius, 5,000 Da Molecular Weight Cut-Off) were employed for purification of the biotinylated toxins. Protein concentration was assessed based on absorbances measured on a NanodropLite Spectrophotometer.

Phage Display Selection and Assessment of Polyclonal Output

For phage display selection, the IONTAS phage display library λ was employed. This library is a human antibody phage display library with a clonal diversity of 1.6×10^{10}, with antibodies in the form of single-chain variable fragments (scFvs), which was constructed from B lymphocytes collected from 43 non-immunized human donors (28).

Selections and primary screenings were performed as described previously (28, 29) with the following modifications: In short, for the selections, biotinylated α-LTX (5 μg/ml) was indirectly immobilized on streptavidin-coated (10 μg/ml) MaxiSorp vials. A deselection process utilizing streptavidin-coated Dynabeads was performed on the library before commencing the first selection round to deselect phages displaying streptavidin-recognizing scFvs. In the second and third selection round, neutravidin was used instead of streptavidin to limit further accumulation of potential streptavidin binders. Three rounds of selections were carried out, and the selected phage outputs were then evaluated for antigen binding. The evaluation was carried out similarly to the selections by testing whether the output phages, purified by sequential polyethylene glycol precipitation (28), from the second and third selection round displayed increased binding to either biotinylated α-LTX (5 μg/ml) indirectly immobilized on streptavidin-coated (10 μg/ml) MaxiSorp vials, streptavidin coated vials, or vials blocked in 3% (w/v) skimmed milk in PBS. Following binding of the phages to the immobilized antigens (α-LTX, streptavidin, or milk proteins), phages were eluted using 100 μg/ml trypsin and added to cultures of *E. coli* TG1 cells with a measured optical density of 0.5 at 600 nm and shaken at 150 rpm at 37 °C for 1 h. Then, the cultures were plated and incubated overnight at 30 °C on 2xTY medium plates supplemented with 2% glucose and 100 μg/ml ampicillin. The following day, the number of colony-forming units was determined.

Subcloning and Primary Screening of scFvs

The scFv genes from selection round two and three were sub-cloned from the phage display vector using *NcoI* and *NotI* restriction endonuclease sites into the pSANG10-3F vector for expression of soluble scFvs (30) and transformed into *E. coli* strain BL21(DE3) (New England Biolabs). For each selection round, 267 individual scFv clones were picked, expressed in 96-well format, and scFv-containing supernatants were tested for binding to MaxiSorp plates coated with 5 μg/ml α-LTX, as previously described (29). For binding detection, a monoclonal scFv ELISA was performed, using a 1:20,000 dilution of ANTI-FLAG M2-Peroxidase (HRP) antibody (Sigma-Aldrich, #A8592) in 3% (w/v) skimmed milk in PBS and o-phenylenediamine dihydrochloride (OPD) solution (Sigma-Aldrich, #SLBP6518V) according to the manufacturer's protocol. Upon initial screening, 39 scFvs were selected and sequenced (Eurofins Genomics sequencing service) using the S10b primer (GGCTTTGTTAGCAGCCGGATCTCA). The antibody framework and CDR regions were annotated and analyzed to identify unique clones.

Conversion of scFv to IgG1 Format

Antibody variable domains were codon-optimized for expression in human cells and designed with *NheI* and *AvaI* restriction sites at the 5' and 3' ends. Variable domains were synthesized and cloned into expression vectors containing the constant domain sequences of the respective human IgG1 heavy chain or human λ light chain. Following sequence verification, plasmids were prepared in sufficient quantity for transfection using Plasmid Plus purification kits (Qiagen). HEK 293 (human embryonic kidney 293) mammalian cells were passaged to the optimum stage for transient transfection. Cells were transiently transfected with heavy and light chain expression vectors and cultured for a further 6 days at 37 °C with shaking at 140 rpm. Cultures were harvested by centrifugation at 4,000 rpm and filtered through a 0.22-μm filter. The first step of purification was performed by Protein A affinity chromatography with elution using citrate at pH 3.0, followed by neutralization with 10% (v/v) 1 M Tris at pH 9.0. The antibody was exchanged into PBS at pH 7.2 using a PD10 desalting column (GE Healthcare). Antibody concentration was determined by UV spectroscopy, and the antibodies were concentrated as necessary. Antibody purity was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC). SEC was performed on a Superdex® 200 5/150 GL column (Cytiva, #28-9909-45) connected to an Agilent 1100 system. The mobile phase was PBS, pH 7.2 (Gibco, #20012-019). Analysis was conducted at room temperature using a flow rate of 0.2 ml/min. 5 μl sample at 1 mg/ml was injected and peaks were detected using a UV absorption of 280 nm. Endotoxin testing was performed on a *Charles River* Endosafe® nexgen-MCS™ platform according to manufacturer's instructions using matching cartridges with sensitivity to 0.05 EU/ml.

Monoclonal IgG ELISA

To test if the previously determined binding of the scFv was retained through reformatting to the IgG format, a monoclonal IgG ELISA was set up. A MaxiSorp plate was coated either with α-LTX (5 μg/ml) or *L. tredecimguttatus* whole venom (10 μg/ml) overnight, and the following day it was blocked with 3% (w/v) skimmed milk in PBS. Subsequently, the antibody was diluted in 3% (w/v) skimmed milk in PBS in a dilution series and added in triplicates to the plate. The dilution series was constructed from a monoclonal IgG stock of TPL0020_02_G9 with a concentration of 1 mg/ml and included the following concentrations: 2,000 ng/ml, 1,000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, and 31.25 ng/ml. To test specificity of TPL0020_02_G9, the binding to wells coated with streptavidin, neutravidin, and wells blocked with 3% (w/v) skimmed milk in PBS was tested. In the controls, the highest IgG concentration (2,000 ng/ml) was used. For detection of binding, a 1:10,000 dilution of Anti-Human IgG (Fc-specific)-Peroxidase antibody (Sigma-Aldrich, #A0170) in 3% (w/v) skimmed milk in PBS was utilized along with a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate kit (Thermo Scientific, #34021). Absorbance was measured at 450 nm in a Multiskan FC Microplate Photometer (Thermo Scientific).

Rat Brain Slice Preparation

Wistar rats (16–18 days old, males and females) were used in this study. All experiments were performed in accordance with the European Directive 2010/63/EU and were approved by the Local Bioethics Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences.

Rat brain was removed and chilled to 2–4 °C in a solution containing: 125 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 0.1 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 3 mM $MgCl_2$, and 10 mM D-glucose (pH 7.4). Coronal slices (250 μm thick), comprising the medial prefrontal cortex (mPFC), were made using a vibrotome

(7000smz-2, Campden Instruments) and incubated in ACSF containing: 125 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 1 mM $MgCl_2$, and 10 mM D-glucose (pH 7.4; T = 22–24 °C, 305–308 mOsm/L). All solutions were aerated with carbogen (95% O_2 + 5% CO_2).

Electrophysiological Recordings

Whole-cell patch clamp recordings were performed using an EPC-10 patch clamp amplifier (HEKA Elektronik GmbH, Germany). For recordings, slices were perfused with ACSF solution (T = 22–24 °C) at a rate of 2 ml/min. Pyramidal cells in the mPFC (L2/3) were visualized with an upright microscope (BX51WI, Olympus, Japan) equipped with differential interference contrast optics. The pyramidal cells had triangular bodies and apical dendrites and were recognized according to previously described electrophysiological criteria (31). The patch pipettes (2.5–3.5 MΩ) were made from borosilicate glass (WPI) using a p-97 puller (Sutter Instruments). The pipette solution contained: 135 mM KSO_3CH_3, 5 mM NaCl, 0.2 mM EGTA, 10 mM Hepes, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM phosphocreatine (pH was adjusted to 7.3 with KOH, 295 mOsm/L). Access resistance was typically 15–20 MΩ and remained stable during the experiments (≤ 20% increase) for the cells included in the analysis. The signals were filtered at 10 kHz and sampled at 20 kHz. Reagents were applied to the perfusion ACSF.

α-LTX was dissolved to 0.5 mg/ml in 50% glycerol and aliquots were kept at −20 °C. 15 µl of α-LTX solution were then diluted with 275 µl of ACSF, incubated for 2 h at room temperature, diluted by ACSF to 1 nM, and applied to the slices. In preincubation experiments with IgG TPL0020_02_G9, α-LTX was first diluted with 75 µl of IgG solution (1 mg/ml) in PBS, and then with 200 µl of ACSF. The mixture was likewise incubated for 2 h at room temperature, diluted by ACSF to 1 nM α-LTX concentration, and applied to the slices.

Data Analysis and Statistics

The offline data analyses of recorded spontaneous excitatory post-synaptic currents (spontaneous EPSCs) were performed using Clampfit 10.2 software (Molecular Devices) and Origin 9.1 (OriginLab Corp.). The experimental data are presented as means ± SD. The data were normally distributed (Shapiro-Wilk test). The statistical significance of the differences in effects of α-LTX and α-LTX in the presence of IgG was evaluated by Student's two-tailed unpaired t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Phage Display Selection and Screening of scFvs

scFv-displaying phages from the IONTAS phage library λ were used to select antibody fragments against biotinylated α-LTX from *L. tredecimguttatus*. Three rounds of selections were performed to enrich the pool of phages binding the toxin (**Figure 1**). The scFv-encoding genes in the accumulated phages were isolated from both the second and third selection round, sub-cloned into the pSANG10-3F expression vector (30), 267 clones were picked from each selection round, and the scFvs were expressed in solution. Recombinant monoclonal scFvs were tested for their binding ability to directly coated α-LTX (**Figure 2**). Using a cut-off value of 0.5 in absorbance, 39 binders were selected for further characterization by DNA sequencing, yielding six unique scFvs with a distribution of 31 binders comprising one scFv (named TPL0020_02_G9), four binders comprising another scFv, and four unique binders.

Conversion From scFv to IgG1 Format and Characterization by Monoclonal ELISA

The scFv that yielded the highest binding signal in the monoclonal scFv ELISA, TPL0020_02_G9, was selected for conversion to IgG1 format. Following expression by transient transfection in HEK 293 cells, the antibodies were purified by affinity protein A chromatography. The purity of the IgG was determined by SDS-PAGE and SEC, which showed >97% purity (**Figure 3**). The endotoxin level was determined as < 0.05 EU/mg by LAL chromogenic endotoxin assay. An ELISA was performed to confirm the retention of binding of the IgG, named TPL0020_02_G9, to α-LTX (**Figure 4A**). The ELISA confirmed that the IgG was able to bind α-LTX, even at the lowest dilution of 1:32,000 (31.25 ng/ml), indicating further titration might be possible. The specificity of TPL0020_02_G9 was confirmed, as control experiments showed no binding to neutravidin, streptavidin, or milk proteins at the highest IgG concentration of 1:500 (2,000 ng/ml). TPL0020_02_G9 was furthermore shown to retain binding signals when the purified α-LTX coating was exchanged for *L. tredecimguttatus* whole venom (**Figure 4B**), although, unsurprisingly given the low percentage of α-LTX in whole venom (32), the signal decreased.

Functional *Ex Vivo* Analysis of α-LTX and its Activity Neutralized by the Discovered IgG

We studied the effects of IgG TPL0020_02_G9 on the capability of α-LTX to enhance the frequency of spontaneous EPSCs recorded from brain neurons. We performed whole-cell voltage clamp recordings from pyramidal cells in the mPFC of rat brain slices. The neurons were voltage clamped at −80 mV. At this voltage spontaneous inward synaptic currents of 12.1 ± 1.6 pA amplitude were recorded, and the average frequency was 3.3 ± 1.2 Hz (n = 17) in the control (**Figure 5A**). In the presence of 1 nM α-LTX, an increase of the frequency of spontaneous EPSCs was observed (**Figure 5B**). The threshold for α-LTX effect was set as three times the frequency SD value of the control. On average, 5.8 ± 0.8 min (n = 9) were required to reach this threshold level (**Figure 5D**). The effect of α-LTX developed sharply and after 12.2 ± 3.9 min (n = 9) the spontaneous EPSC frequency exceeded its control value by more than 4 times (**Figures 5A, C, D**). In most cases, further increase in the EPSC frequency prevented its correct calculation due to a strong overlap between individual spontaneous EPSCs (**Figure–5A**). The spontaneous EPSCs were completely abolished by the application of 10 µM DNQX and 100 µM D-APV

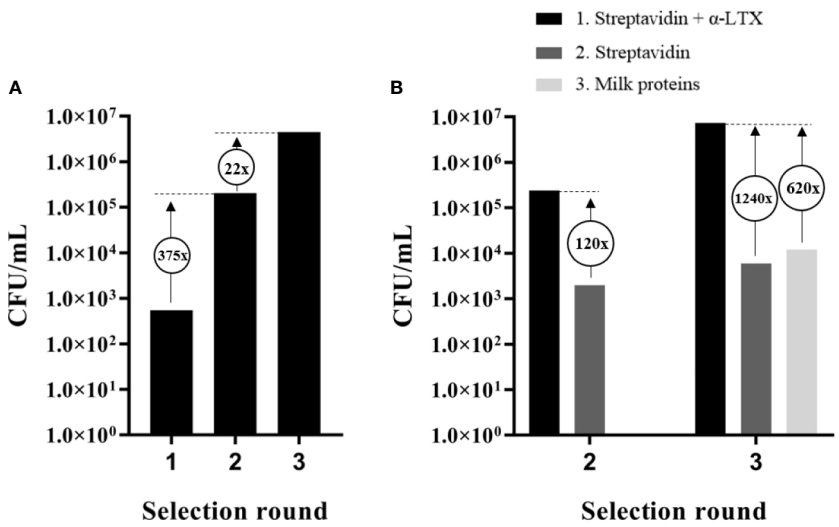


FIGURE 1 | Output and assessment of phage display selections against α-LTX. **(A)** Accumulation of scFv-displaying phages from the IONTAS phage display library λ with affinity to α-LTX over three rounds of selection. An increase in CFU/ml of 375-fold and 22-fold respectively were observed between the selection rounds. **(B)** CFU/ml was determined for output phages from selection round two and three for binding to either α-LTX, streptavidin, or milk proteins. An increase of 30-fold between the second and third selection round was observed in CFU/ml for the phages that bound streptavidin-captured α-LTX. Only few phages with affinity to streptavidin or milk proteins were accumulated compared to phages with affinity to α-LTX.

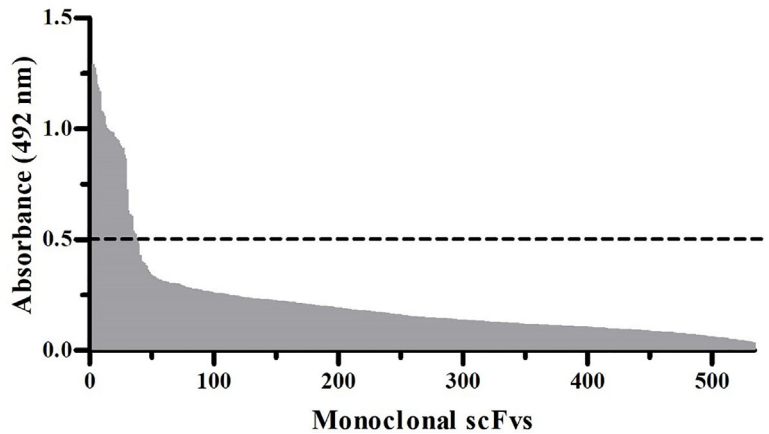


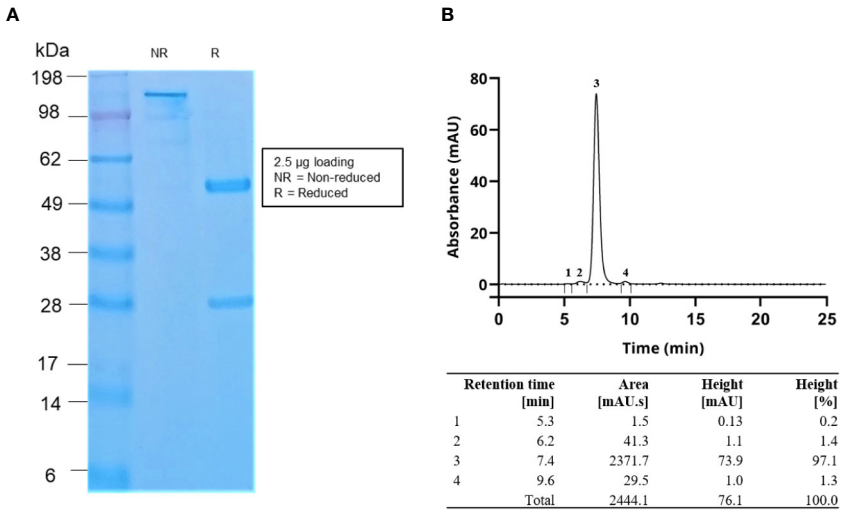
FIGURE 2 | Monoclonal scFv ELISA signals against α-LTX. In total, 534 scFv clones were expressed in solution and screened for their ability to bind directly coated α-LTX. scFvs displaying a binding signal above the cut-off absorbance value of 0.5 (dotted line) at 492 nm were considered hits.

(**Figure 5A**), indicating that those EPSCs were mediated by ionotropic glutamate receptors. Our data on α-LTX action are in good agreement with previous results on α-LTX action [e.g., (33)].

Preincubation of α-LTX with IgG TPL0020_02_G9 at 1:10 ratio (w/w) did not completely inhibit the action of the toxin but markedly slowed down the development of its effect (**Figure 5**). The onset time of the effect (exceeding the 3 × SD threshold) increased to 19.1 ± 4.8 min (n = 8). Further frequency increase was almost as sharp as in control experiments without the antibody. A four-fold spontaneous EPSC frequency increase in the control took place after 12.2 ± 3.9 min (n = 9); in the presence of the IgG, this time increased to 24.6 ± 3.1 min (n = 8; **Figures 5B–D**). Based on these observations, we conclude that the IgG significantly delays the toxic effects of α-LTX.

DISCUSSION

Here, we report the discovery of the first recombinant human monoclonal IgG antibody against a spider toxin, which showed neutralization efficacy *ex vivo*. Due to its human, recombinant, and monoclonal nature, this antibody could potentially find utility as a



	Retention time [min]	Area [mAU.s]	Height [mAU]	Height [%]
1	5.3	1.5	0.13	0.2
2	6.2	41.3	1.1	1.4
3	7.4	2371.7	73.9	97.1
4	9.6	29.5	1.0	1.3
	Total	2444.1	76.1	100.0

FIGURE 3 | **(A)** SDS-PAGE gel image and **(B)** SEC data of the purified IgG antibodies. The SEC data demonstrated that the antibody purity was >97%. Flow rate in SEC was 0.2 ml/min.

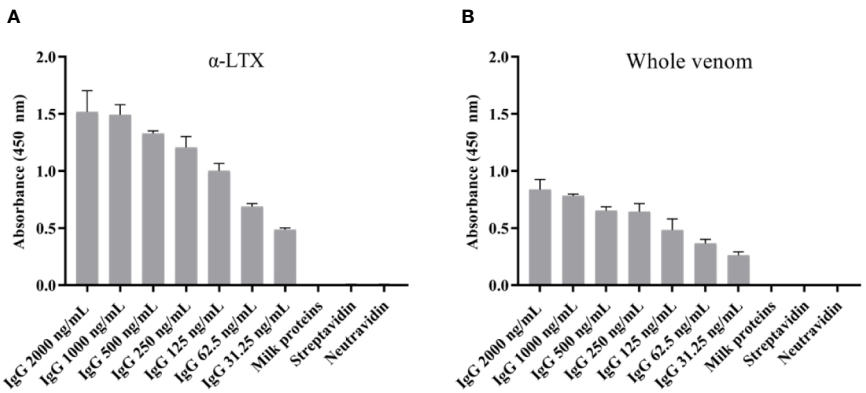


FIGURE 4 | Monoclonal IgG ELISA signals against **(A)** α-LTX and **(B)** *L. tredecimguttatus* whole venom. The binding capability of TPL0020_02_G9 to α-LTX was assessed using different concentrations of IgG. The binding specificity was evaluated by testing the binding of the IgG to three controls (binding to milk proteins, streptavidin, and neutravidin) using the highest IgG concentration (2,000 ng/ml). Each column represents the average of triplicate measurements with error bars indicating the standard deviation.

therapeutic, but might also find application as an affinity reagent for research or diagnostic purposes (23). By utilizing antibody phage display, we were able to discover six unique human scFvs recognizing α-LTX from *L. tredecimguttatus*. The most promising of these six scFvs was converted to a fully human IgG1 with the purpose of increasing stability and to have an Fc fragment to increase its (therapeutic) utility. The IgG TPL0020_02_G9 was confirmed in ELISA to have retained the specificity and strong binding for α-LTX of the scFv and was also shown to retain binding when tested against *L. tredecimguttatus* whole venom. Importantly, we have found that the IgG is capable of decreasing α-LTX activity as tested in rat brain neurons, thereby demonstrating its ability to neutralize α-LTX *ex vivo*.

TPL0020_02_G9 is a recombinant human monoclonal IgG1 antibody, which possesses several advantages compared to existing animal-derived polyclonal or monoclonal antibodies. In regards to monoclonal antibodies, only antibodies of murine origin derived using hybridoma technology or phage display technology have been reported for α-LTX (24–27, 34, 35). However, due to their heterologous nature, such antibodies are not optimally suited to be developed into therapeutics for human recipients. Moreover, as a result of the recombinant origin of

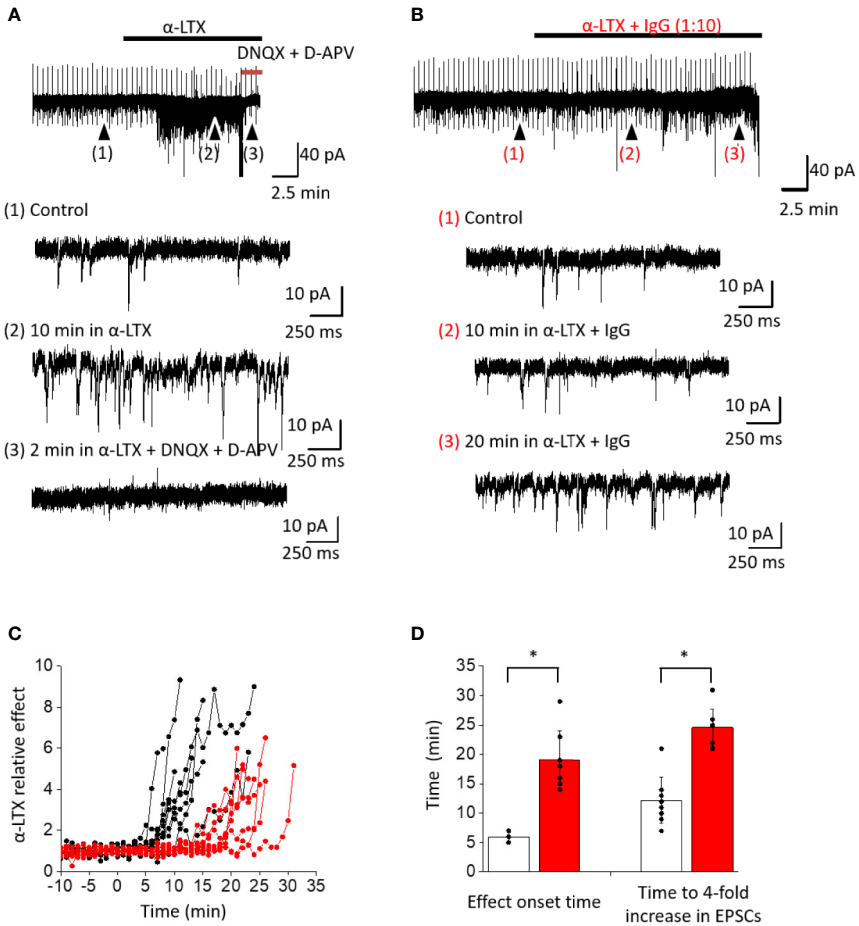


FIGURE 5 | Effect of α-LTX and its mixture with IgG TPL0020_02_G9 at 1:10 ratio (w/w) on spontaneous EPSC frequency in pyramidal neurons from mPFC. **(A)** Representative recording of α-LTX action. Time course of the experiment and expanded parts in control (1), after 10 min of α-LTX application (2), and after 10 μM DNQX and 100 μM D-APV application (3) are shown. α-LTX causes a strong increase of spontaneous EPSC frequency, and selective antagonists of ionotropic glutamate receptors abolish this effect. **(B)** Representative recording of α-LTX action in the presence of IgG TPL0020_02_G9. Strong frequency increase is seen only after 20 min of application. **(C)** Time development of α-LTX effect and its modulation by IgG. Spontaneous EPSC frequencies are normalized to the average control values. The development of effect is strongly delayed in the presence of IgG (red traces) compared to control with α-LTX alone (black traces). **(D)** Characteristic times when the 3 × SD threshold is reached or spontaneous EPSC frequency is increased four-fold for α-LTX alone (white bars) and in the presence of the IgG (red bars). The differences are significant ($p < 0.05$, unpaired t-test).

TPL0020_02_G9, continuous production of this antibody is independent of laboratory animals as well as spider venom. This is particularly beneficial, as venom collection is a very labor-intensive process, which requires "milking" of a large number of spiders as each spider only produces small amounts of venom (36). Most importantly, the recombinant origin of TPL0020_02_G9 ensures reproducibility, which is often a concern with animal-derived antibodies (37). This is in line with the recent recommendation in May 2020 from the European Commission's Joint Research Centre on non-animal-derived antibodies. This recommendation urges to stop using animals for the development and production of antibodies for research, regulatory, diagnostic, and therapeutic applications by recognizing the scientific validity of non-animal-derived antibodies (38). Non-animal-derived antibodies derived from naïve display libraries, like the one used in this study for phage display selection, facilitates the selection of antibodies for specificity and affinity (39). In addition, they can be used repeatedly for the discovery of antibodies against different antigens in contrast to the necessary new immunization scheme for animal-derived polyclonal and monoclonal antibodies. The defined sequence of TPL0020_02_G9 makes it possible to engineer the antibody into various immunoglobulin isotypes, species, and to introduce specific modifications in its sequence. Furthermore, the human IgG1 format is strategically chosen, as it is compatible with therapeutic use in humans.

The antibodies discovered in this study were selected for their ability to bind to α-LTX from *L. tredecimguttatus*. However, given the high degree of homology of α-LTX isoforms across spider species (11), with α-LTX from the Australian redback spider *Latrodectus hasselti* sharing >90% amino acid identity to

α-LTX from *L. tredecimguttatus* (40), it is likely that the discovered IgG may be cross-reactive towards other α-latrotoxins, although this hypothesis has yet to be tested. If cross-reactive, the IgG may find broader application as an affinity reagent in research, as well as a potential biotherapeutic against widow spider bite envenoming in future.

AUTHOR CONTRIBUTIONS

Conceptualization, AL. Methodology, SF, LL, MN, AV, CS, MC, MF, and AL. Validation, SF, AV, CS, and AL. Formal analysis, SF, LL, AV, CS, and AL. Investigation, SF, LL, MN, CS, MC, MF, and AL. Resources, MN, MF, and AL. Data curation, SF, LL, MN, and CS. Writing, original draft preparation, SF and AL. Writing—review and editing, SF, LL, MN, AV, CS, MC, MF, and AL. Visualization, SF, LL, MN, CS, and AL. Supervision, AL.

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In Vitro Tests for Assessing the Neutralizing Ability of Snake Antivenoms: Toward the 3Rs Principles

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There is an urgent need to strengthen the implementation of the 3Rs principle (Replacement, Reduction and Refinement) in the use of experimental animals in toxinological research and in the assessment of the neutralizing efficacy of snake antivenoms. This is a challenging task owing to the inherent complexity of snake venoms. The state of the art on this topic is hereby reviewed, with emphasis on the studies in which a correlation has been observed between *in vivo* toxicity tests and *in vitro* surrogate assays, particularly in the study of lethal activity of venoms and its neutralization. Correlations have been described with some venoms-antivenoms when using: (a) enzyme immunoassays, (b) hemagglutination, (c) enzyme assays (proteinase, phospholipase A_2), (d) *in vitro* coagulant effect on plasma, (e) cell culture assays for cytotoxicity, (f) functional assays for assessing neurotoxicity *in vitro*, (g) use of hens' eggs, and (h) antivenomics. Additionally, the routine introduction of analgesia in these assays and the design of more 'humane' protocols for the lethality test are being pursued. It is expected that the next years will witness a growing awareness of the relevance of the 3Rs principles in antivenom testing, and that new *in vitro* alternatives and more 'humane' experimental designs will emerge in this field.

Keywords: neutralization, lethality assays, *in vitro* assays, analgesia, 3Rs, antivenoms, snake venoms

INTRODUCTION

Snakebite envenoming exerts a heavy toll in terms of mortality and disabilities on a global basis (1). Owing to their public health relevance, the World Health Organization (WHO) included these envenomings as a category A disease in its list of Neglected Tropical Diseases in 2017 (2), and a resolution on the subject was adopted at the World Health Assembly in 2018 (3). More recently, the WHO launched a global strategy to prevent and control these envenomings, aimed at reducing by 50% the number of deaths and amputations due to this disease by the year 2030 (4). This strategy is based on four pillars, one of which is to 'ensure safe, effective treatment'.

The centerpiece in the therapy of snakebite envenomings is the timely administration of safe and effective antivenoms, which are preparations of IgGs or IgG fragments prepared from the plasma of horses or other animals immunized with venoms of one snake species (monospecific antivenoms) or several species (polyspecific antivenoms) (5). Upon parenteral administration in envenomed patients, antivenom antibodies bind to venom components in the circulation or in tissue compartments and contribute to their elimination. Generally, antivenom therapy is complemented by ancillary treatments which vary depending on the pathophysiology of envenomings (1). Antivenom efficacy is evaluated at the preclinical level by assessing its capacity to neutralize the lethal action of venoms in animal models, usually mice (5, 6). This is the gold standard of antivenom efficacy which is required before antivenoms are introduced into clinical use and as part of the routine quality control of antivenoms by manufacturers and regulatory agencies. The basic protocol for these neutralization assays involves the incubation of venom and antivenom prior to administration in animals. Another experimental option, which is not routinely used in quality control laboratories but which better mimics the actual circumstances of a snake bite, is the rescue-type assay, in which venom is injected first and antivenom is administered afterwards. In addition to lethality, depending on the toxicity profile of venoms, the assessment of neutralization of other toxic activities is also recommended, such as hemorrhagic, myotoxic, dermonecrotic, defibrinogenating, and *in vitro* coagulant activities, depending on the venom (5, 6). Except for the *in vitro* coagulant activity, the rest of these assays involve the use of high numbers of mice, with the consequent suffering and distress inflicted in these animals because of the toxic action of venoms.

There is a growing awareness on the need to significantly reduce the number of mice used in antivenom assessment, as well as the pain and distress involved in these tests, along the philosophy of the 3Rs (Replacement, Reduction and Refinement) proposed by Russell and Burch (7). A significant amount of work has been devoted by many groups to the search of *in vitro* alternatives to these animal tests, and to the refinement of these assays. Owing to the high variability of snake venom composition and mechanisms of action, no simple generalizations can be made regarding the implementation of these alternative tests. However, there are examples of *in vitro* assays which show a good correlation with the *in vivo* tests, and further work is urgently needed in this field. The present review presents the state of the art in the development of *in vitro* tests for antivenom preclinical efficacy assessment. The review focuses mostly on studies in which the correlation between *in vitro* and *in vivo* tests was evaluated.

THE CHALLENGE OF FINDING SUITABLE *IN VITRO* TESTS FOR ASSESSING ANTIVENOM EFFICACY

One of the main challenges for finding suitable *in vitro* tests that would substitute *in vivo* experiments in the evaluation of antivenoms has to do with the complexity of snake venoms and snakebite envenomings. In some cases, the toxic profile of venoms depends on the action of one or few toxins which induce a single toxicological effect, e.g., the action of some neurotoxic elapid venoms which act by blocking the neuromuscular junctions. Thus, once these components are identified, it is feasible to develop immunochemical or functional *in vitro* tests to study the ability of antivenoms to react and neutralize these venoms. However, for many snake venoms this is not the case, as the overall pathophysiology of envenoming is the result of the combined action of several toxins acting on different tissues or physiological systems (1), a fact that complicates the development of *in vitro* surrogate tests. Toxins may act synergistically or additively (8) and have complex toxicokinetic and toxicodynamic profiles which play a role in the *in vivo* assays. Moreover, effects such as cardiovascular or renal alterations, as well as local tissue damage, involve multifactorial processes difficult to reproduce *in vitro*.

In addition, there is a growing body of evidence indicating that the pathophysiology of many envenomings derives not only from the direct action of toxins on tissues, but also from endogenous processes in the organism, such as inflammatory cascades resultant of the action of toxins or the generation of damage-associated molecular patterns (DAMPs) from affected tissues, which contribute to the pathophysiological alterations (9, 10). Thus, the study of snake venom composition and mechanisms of action, and the identification of the main toxins responsible for the predominant toxicological effects provide relevant information for the knowledge-based design of alternative *in vitro* assays that correlate with *in vivo* toxicity tests.

ASSESSMENT OF ANTIVENOM NEUTRALIZING EFFICACY AT DIFFERENT STAGES DURING THE MANUFACTURING PROCESS

The quality control of antivenoms, in terms of assessing their neutralizing efficacy against medically-relevant snake venoms, is generally carried out at two stages: (a) in-process, i.e. along the plasma fractionation procedures for generating purified IgG or F $(ab')_2$ preparations, and (b) in the final product, before the antivenom is released for medical use in the health systems. The in-process quality control is carried out by the manufacturer, whereas the quality control of the final product is done by the manufacturer and, in some countries, by the national regulatory agencies as well.

Generally, the final quality control of antivenoms necessarily involves the test for the neutralization of lethal activity of venoms in mice, which is the gold standard for antivenom efficacy assessment (5). On the other hand, the in-process quality control of antivenom efficacy offers opportunities for the implementation of *in vitro* tests aimed at detecting whether there is a loss of neutralizing antibodies during plasma fractionation. However, many manufacturing laboratories

routinely use the mouse lethality assay for these in-process quality control analyses. It is necessary to develop *in vitro* assays which correlate with the *in vivo* tests for the in-process quality control of antivenoms. This will greatly reduce the number of mice utilized during the manufacturing process. Likewise, the follow up of the development of neutralizing antibody titers in the plasma of horses along the immunization scheme, in order to establish the best time for starting the bleeding protocols, could be done by using *in vitro* tests that offer a good correlation with the *in vivo* potency assays, hence reducing the need for the latter.

THE ORIGINS OF *IN VITRO* TESTING OF ANTIVENOMS

Since the dawn of antiserum therapy for snakebite envenomings, the assessment of the neutralizing potency of antivenom was based on the ability to abrogate the lethal action of venoms in various animal models (11, 12). In addition, even at those early times of antivenom development, scientists were searching for *in vitro* tests for assessing antivenom efficacy. Albert Calmette, one of the founders of snake antivenom therapy, described the parallelism between neurotoxicity and indirect hemolysis in neurotoxic (elapid) venoms, and between hemorrhagic activity and proteolysis in viperid venoms (11). Based on such parallelism, he developed laboratory assays to assess the neutralization of hemolytic and proteolytic activities of venoms by antivenoms and described the relationship with the neutralization of *in vivo* toxicity (11). In his book of 1907 *Les Venins, les Animaux Venimeux et la Sérotérapie Antivenimeuse*, referring to these *in vitro* methods, he states (page 269) 'These various control methods make it possible to verify exactly the activity of *sérums antivenimeux* without it being necessary to use animal testing' (11). Likewise, Vital Brazil, working in São Paulo, Brazil, described experiments on the neutralization of *in vitro* coagulant and proteolytic activities of venoms, and on the formation of precipitates when venoms and antivenoms were allowed to react in a test tube (12).

Ahuja and Brooks (13) described an *in vitro* hemolysis test for assessing the neutralizing potency of cobra antivenom in India, which correlated with the neutralization of lethality. In South Africa, Paul A. Christensen studied several *in vitro* activities of venoms (hemolysis, rennin-like effect, gelatinase and anticoagulant activities) and their neutralization by antivenoms. He found no correlation between the neutralization of lethality and *in vitro* hemolysis in the case of *Naja flava* (now *Naja nivea*) venom (14). As will be described later, no generalizations can be made regarding the possible substitution of *in vivo* toxicity tests by *in vitro* assays, owing to the great variability in the composition and action of snake venoms.

ENZYME IMMUNOASSAYS

Theakston et al. (15) introduced the enzyme-linked immunosorbent assays (ELISAs) for the quantification of venom and antivenom. An ELISA was then used to quantify antivenom antibodies in several commercial antivenoms used in Africa and some rabbit experimental antivenoms and this was correlated with the neutralization of lethality in mice (16). A good correlation was described when using the venoms of the African species *Bitis arietans*, *Echis carinatus* (now *E. ocellatus*), *Naja haje* and *N. nigricollis*. Similar descriptions of significant correlation between ELISAs and *in vivo* neutralization of lethality have been described for a monospecific *Naja naja kaouthia* antivenom (17), monospecific *Crotalus durisus terrificus* antivenom (18), bispecific *Bothrops alternatus* and *B. pubescens* antivenom (19), and monospecific *Daboia siamensis* antivenom (20).

In contrast, poor correlation between ELISA and neutralization of lethality was described for the bothropic antivenom manufactured in Brazil when tested against the venom of *Bothrops jararaca* (18, 21) and a monospecific *Micrurus nigrocinctus* antivenom toward its homologous venom (22). Hence, the feasibility of using ELISAs for assessing antivenom potency must be made on a case by case basis. An explanation for the lack of correlation in the case of some venoms and antivenoms is that proteins that do not play a role in toxicity may be highly immunogenic and, therefore, the immune response detected by ELISA may reflect antibody titers against toxicologically irrelevant components. This is illustrated in the case of the venom of the black mamba *Dendroaspis polylepis*, whereby antivenoms show highest antibody titers against high molecular mass non-toxic metalloproteinases, whereas titers against neurotoxins are lower (23).

A solution to this situation is the identification and isolation of venom components having the highest toxicity in a venom, by assessing the 'toxicity score' of venom fractions (24). Once these toxins are identified, ELISAs can be developed for the quantification of antibodies against them. This increases the likelihood of correlation between immunoassays and the *in vivo* neutralization of lethality. This concept has been proven in the case of antivenom against *Naja naja siamensis*, since a higher correlation was observed when immunoassays were carried out using a purified α-neurotoxin, as compared to crude venom (17). Similarly, a higher correlation was described for the Brazilian bothropic antivenom when using a hemorrhagic fraction of the venom of *B. jararaca* as compared to crude venom, but not when using a phospholipase A_2 (PLA_2)-rich fraction (21, 25). The growing body of information of snake venom proteomes, together with the identification of key toxins, provides valuable evidence for the setting of these more directed ELISAs.

In the cases of venoms whose predominant toxins represent a high percentage of venom composition, ELISAs using crude venoms are likely to give a good correlation with *in vivo* toxicity tests. This is the case of the venom of the South American rattlesnake *C. d. terrificus*, in which the potent neurotoxin crotoxin comprises 60% of the venom (26). Similarly, the venom of the cobra *Naja kaouthia* has a high concentration of α-neurotoxins which display the highest toxicity score (27). It is necessary to explore medically relevant venoms and their corresponding antivenoms to establish in which cases good

correlation between ELISA and neutralization of lethality can be achieved by using crude venoms or when it is recommended to use purified toxins.

PASSIVE HEMAGGLUTINATION AND HEMAGGLUTINATION INHIBITION

A method based on passive hemagglutination and its inhibition was developed for testing a monospecific *Naja naja siamensis* antivenom using glutaraldehyde treated sheep erythrocytes coupled with toxin 3, a neurotoxin from this venom (28). A similar method was used by Pradhan et al. (29) to assess whether it correlates with the *in vivo* neutralization of lethality. Erythrocytes treated with glutaraldehyde and then with tannic acid were coupled with *Naja naja* venom and then incubated with varying dilutions of the antivenom. Also, inhibition of hemagglutination was carried out by incubating antivenom with venom, followed by addition to venom-coated erythrocytes. A good correlation between these tests and the *in vivo* neutralization of lethality was observed. It remains to be seen whether this method works only for these α-neurotoxin-rich venoms or also for other venoms having a different toxin composition.

NEUTRALIZATION OF *IN VITRO* ENZYMATIC ACTIVITIES

Snake venoms are rich in hydrolytic enzymes. The proteomic analyses of viperid venoms have revealed a predominance of snake venom metalloproteinases (SVMPs), phospholipases A_2 (PLA_2s) and serine proteinases (SPs), with variations between and within species (1). In turn, elapid venoms are generally rich in PLA_2s (1). These enzymes are responsible for some of the main pathophysiological effects in envenomings. SVMPs induce hemorrhage and coagulopathies (30, 31), PLA_2s are responsible for muscle necrosis and neurotoxicity, depending on the enzyme (32, 33), and serine proteinases induce defibrinogenation and hypotension (31, 34). Therefore, the study of *in vitro* activities associated with these enzymes has been pursued and correlated with *in vivo* toxicity.

Several studies have demonstrated a correlation between the neutralization of *in vitro* coagulant activity of venoms, associated with the action of procoagulant SVMPs and serine proteinases, and neutralization of lethality. This was described for *Calloselasma rhodostoma* venom and a monospecific antivenom (35) by using sheep plasma (for assessing *in vitro* coagulation) and intraperitoneal injection in mice (for assessing lethality). Similar findings were reported in the case of *Bothrops jararaca* venom and the Brazilian bothropic antivenom, whereby *in vitro* coagulant activity was assessed by rotational thromboelastometry (ROTEM) on chicken plasma, and lethality was studied in mice by the i.p. route (36). A correlation was also described between neutralization of *in vitro* coagulant activity and neutralization of lethality (i.p. route) in the case of *Bothrops asper* venom and a polyspecific Costa Rican antivenom (37).

To further expand these observations, we have assessed the correlation of these activities based on data included in two publications which evaluated many antivenoms. In a study carried out in Latin America in which seven polyspecific viperid antivenoms were assessed against venoms of six species of *Bothrops* sp. (38), a significant correlation was found ($R = 0.492$, $p = 0.0011$, $n = 41$). Two publications evaluated the neutralization of these effects by seven antivenoms against venoms of *Echis ocellatus* from various locations in sub-Saharan Africa (39, 40). A significant correlation between neutralization of lethality and *in vitro* coagulant activity was observed ($R = 0.7643$, $p = 0.0009$, $n = 15$). These findings support the view that the study of neutralization of *in vitro* coagulant activity could be a surrogate test for estimating the neutralizing ability of viperid antivenoms. Additional studies with other venoms and antivenoms are required to further substantiate this correlation. It is necessary to standardize the conditions of the *in vitro* coagulant assay, including the type of plasma used and the assessment of clot formation. In order to standardize the performance of this *in vitro* test in quality control laboratories, it is recommended that reference antivenoms be prepared and run in parallel every time an antivenom is being evaluated for its efficacy.

Other studies have shown correlation between neutralization by antivenoms of PLA_2 activity *in vitro* and neutralization of lethality in mice in the cases of venoms of *Bothrops asper* (41), *Crotalus durissus terrificus* (42), and *Micrurus nigrocinctus* (22), using simple indirect hemolytic assays for the determination of PLA_2 activity. Further studies are necessary to assess whether these *in vitro* enzymatic assays correlate with lethality in a larger number of venoms and antivenoms. There are venoms in which the main toxicity is due to presynaptically-acting neurotoxic PLA_2s (43). Such are the cases of *Oxyuranus scutellatus*, *Crotalus durissus*, and *Bungarus* sp venoms, characterized by the presence of the potent PLA_2 neurotoxins taipoxin, crotoxin and bungarotoxin, respectively (44). It is likely that the neutralization by antivenoms of PLA_2 activity *in vitro* of these venoms or purified β-neurotoxins correlates with the neutralization of lethality. Owing to the simplicity and low cost of these *in vitro* assays, they could be highly convenient for introduction in antivenom manufacturing laboratories to assess the development of immune response in horses and for in-process analysis of the neutralizing potency of antivenoms, with the consequent reduction in the number of mice.

SURROGATE TESTS FOR THE STUDY OF NEUTRALIZATION OF OTHER TOXIC ACTIVITIES

The complexity of the pathophysiology of snakebite envenomings calls for a more comprehensive evaluation of the neutralizing ability of antivenoms, involving not only the neutralization of lethality, the gold standard of preclinical

antivenom efficacy, but also of hemorrhagic, defibrinogenating, myotoxic and dermonecrotic activities, which play key roles in envenomings by diverse snake species (6). The WHO has established the neutralization of lethality as the 'essential' test for the preclinical evaluation of antivenoms and, depending on the venom, additional 'supplementary' tests are recommended when new antivenoms are developed or when an existing antivenom is being distributed to a new geographic region (5) (**Figure 1**). For example, in the case of most viperid venoms, neutralizations of hemorrhagic, myotoxic, and defibrinogenating activities are recommended. Likewise, assessment of antivenoms against venoms of necrotizing spitting cobras (*Naja* sp.) should include the neutralization of dermonecrotic activity (6). Since these supplementary tests involve the use of mice, the search for alternative *in vitro* assays is necessary.

Hemorrhagic activity by viperid venoms is predominantly due to the action of SVMPs on the basement membrane that surrounds and provides support to endothelial cells in capillary blood vessels. In particular, the hydrolysis of type IV collagen is critical for microvessel disruption (30, 45, 46). Inhibition of venom metalloproteinase activity by chelating agents or peptidomimetic inhibitors results in the abrogation of hemorrhage (47–49). Hence, *in vitro* inhibition of proteinase activity of venoms may constitute a surrogate alternative for assessing the neutralization of hemorrhagic effect. A significant correlation between neutralization of hemorrhage and of hydrolysis of casein *in vitro* was shown for the polyspecific viperid antivenom manufactured in Costa Rica when tested against ten venoms (50). A higher correlation is expected if physiologically relevant substrates, such as basement membrane components, are used as substrates, a hypothesis to be tested. An ELISA-based assay was developed for the quantification of gelatinase activity of viperid venoms. It is based on the addition of venoms to gelatin-coated wells in plates, followed by incubation. Then, anti-gelatin antibodies are added followed by a conjugate and color development (51). Activity was higher in viperid venoms, as compared to elapid and 'colubrid' ones. Activity was abolished by EDTA, indicating that it is due to SVMPs. Whether this assay offers a good correlation with hemorrhagic activity of venoms and its neutralization by antivenoms remains to be determined. Likewise, a high correlation was described between an ELISA using a monoclonal antibody raised against the PIII hemorrhagic SVMP jararhagin and the hemorrhagic activity of individual venoms of *Bothrops jararacussu* (52). This could be the basis of an ELISA aimed at assessing the neutralizing ability of antivenoms against hemorrhagic venoms.

Coagulopathy, i.e. defibrinogenation, is a common consequence of envenomings by viperids and some elapids and 'colubrids' and contributes to the systemic hemorrhage characteristic of these envenomings (1, 31, 53). Defibrinogenating effect is tested *in vivo* by determining the minimum dose of venom that renders blood unclottable in experimental animals (54, 55). Defibrinogenation is the consequence of the consumption of clotting factors owing to the action of procoagulant enzymes in venoms, i.e., factor X activators, prothrombin activators and thrombin-like enzymes (31, 56). Therefore, the *in vitro* coagulant activity of venoms is likely to be a surrogate test for *in vivo* defibrinogenating effect. Indeed, a relationship was shown between the ability of a polyspecific antivenom to neutralize *in vitro* coagulant and *in vivo* defibrinogenating activities of five viperid venoms (55).

Myotoxic activity of snake venoms is predominantly due to the direct action of PLA_2s, and PLA_2 homologs, on the plasma membrane of muscle fibers (43, 57). However, no correlation between inhibition of PLA_2 activity and of myotoxicity is expected because in many venoms enzymatic phospholipid degradation is mostly due to non-toxic enzymes, as in the case

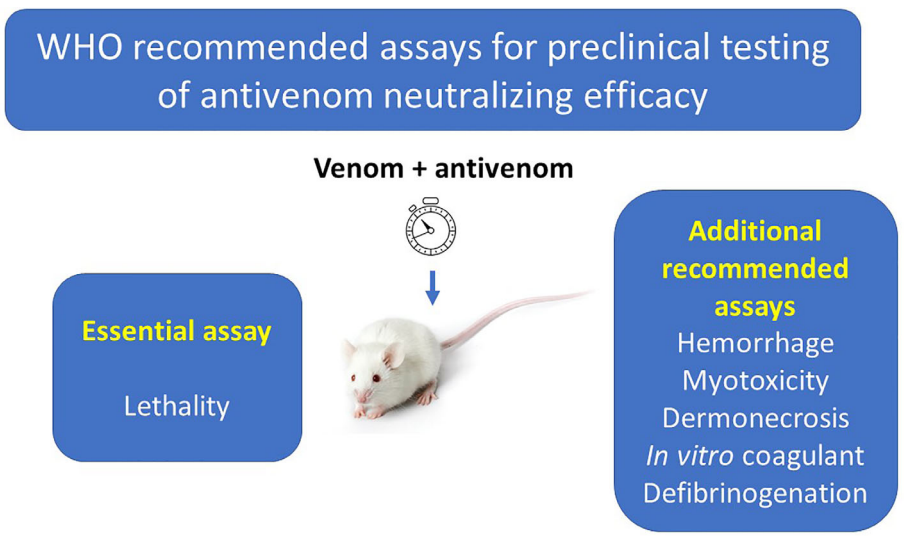

FIGURE 1 | Assays included in the WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins for the assessment of antivenom preclinical efficacy. The WHO divides these assays into the 'essential assay', which is the analysis of the neutralization of lethal activity of venoms, and 'additional recommended assays', which assess the neutralization of other toxic activities, depending on the toxicological profile of the venom under study.

of *Bothrops asper* which has an acidic PLA_2 with high enzymatic activity but being devoid of myotoxicity (58). An alternative is the assessment of cytotoxicity on muscle cell lines, i.e., myoblasts and myotubes of the C2C12 line. Myotubes are good models of mature muscle fibers and are highly susceptible to myotoxic PLA_2s (59). The correlation between neutralization by antivenoms of *in vivo* myotoxicity and *in vitro* cytotoxicity on myotubes must be studied. Likewise, the assessment of cytotoxicity in cell culture systems could become a surrogate assay for the analysis of dermonecrosis, a clinically significant effect of envenomings by spitting cobras in Africa and Asia (1, 53). The myogenic cell line C2C12 was used to assess cytotoxicity by venoms of five species of *Naja* sp. from Africa and its neutralization by a polyspecific antivenom (60), but whether this assay correlates with *in vivo* dermonecrosis remains to be investigated. A cell culture test using human keratinocytes was developed to study the cytotoxic action of *Naja* sp. venoms and its neutralization by recombinant antibodies (61). Since these venoms induce demonecrosis, this *in vitro* test could be of value to assess the neutralizing efficacy of antivenoms. Cytotoxicity on kidney cell lines has been used in the analysis of nephrotoxic effects of venoms and toxins (62) and must be explored as a surrogate test for assessing antivenom efficacy, although venom-induced nephrotoxicity is of a multifactorial pathogenesis which also involves the effects of hemodynamic alterations (63).

EX VIVO AND *IN VITRO* ASSESSMENT OF NEUROTOXICITY

Neuromuscular paralysis leading to respiratory arrest is one of the predominant effects of snakebite envenomings, particularly those caused by species of the family Elapidae, but also by some species of the family Viperidae (1, 53). It results from the action of a variety of neurotoxins at the neuromuscular junctions. Post-synaptically acting polypeptides of the three finger toxins (3FTx) family (α-neurotoxins) act by binding with high affinity to the cholinergic nicotinic receptor (AChR) at the motor end-plate of muscle fibers (64). Neurotoxicity is also due to the action of PLA_2s at the nerve terminal (β-neurotoxins), by hydrolyzing phospholipids of the plasma membrane, inducing a calcium influx and the consequent alteration of the neurotransmitter exocytotic machinery (65). Other types of neurotoxins include the dendrotoxins, present in mamba (*Dendroaspis* sp) venom, which are inhibitors of the voltage-dependent potassium channels (66). Neurotoxins play a key role in the lethality of snake venoms.

Ex vivo neuromuscular preparations have been used by several groups to study the neurotoxic effect of venoms and isolated toxins. The most often used preparations are the chick biventer-cervicis and the mouse phrenic-diaphragm. Once dissected out, these are placed in a bath containing a physiological solution, and muscle twitches are evoked by electrically stimulating the nerve (67). Neurotoxicity is evidenced by the blockade of evoked muscle contractions. This system has been used to assess the ability of antivenoms to neutralize the neuromuscular blocking effect [see, for example, Barfaraz and Harvey (68); Camargo et al. (69), Silva et al. (70)]. In the majority of these studies, the correlation with neutralization of lethality *in vivo* was not investigated, although it is likely that, owing to the relevance of neuromuscular paralysis in the overall toxicity of these venoms, such correlation is likely to occur. Herrera et al. (71) described a relationship between the neutralization of lethality and *ex vivo* neuromuscular blocking activity of the venom of taipan (*Oxyuranus scutellatus*) by two antivenoms. This system is also useful to assess the myotoxic effect of venoms (67). These tests, however, require a specialized laboratory, and are therefore difficult to adapt to the routine quality control analysis of antivenoms. In addition, being *ex vivo* tests, they involve the use of animals.

An alternative to assess the inhibition of post-synaptically acting α-neurotoxins is an assay that quantifies the binding of these neurotoxins to purified AChR, such as those from the electric organ of fish, such as *Torpedo californica* (72). Non-radioactive variations of this assay have been described, which have great potential for antivenom evaluation *in vitro*. The basic set up of these procedures is based on the binding of purified AChR to α-neurotoxin bound to wells in microplates. After a washing step, antibodies against AChR are added, followed by conjugated secondary antibodies (73, 74). This procedure allows the detection of α-neurotoxins in venoms by a competition step whereby the venom is incubated with AChR before the addition to the α-neurotoxin coated plate (74).

These procedures have been adapted for the study of the ability of antivenoms to bind α-neurotoxins and thus to inhibit their binding to AChR (22). An adaptation of this assay was used to assess its correlation with venom LD_{50} of 20 elapid snake venoms, as well as the correlation of the neutralizing efficacy of an antivenom with the inhibition of AChR binding. In both cases a significant correlation was found, especially in venoms containing a predominance of α-neurotoxins (75). Owing to its simplicity and high-throughput nature, this assay could be adapted to antivenom development and quality control laboratories in the case of elapid neurotoxic venoms rich in α-neurotoxins (**Figure 2**). A potential limitation to the widespread implementation of these assays is the availability of purified AChR. This could be circumvented by the use of mimotopes and peptides derived from AChR which bind to α-neurotoxins (76, 77), as this will avoid the need to obtain the receptor from rays or eels.

For venoms in which β-neurotoxins predominate, a possible *in vitro* alternative would be the neutralization of PLA_2 enzymatic activity of the purified predominant neurotoxins. Examples are taipoxin in *Oxyuranus scutellatus* (78), β-bungarotoxins in *Bungarus* sp. (79), and crotoxin in *Crotalus durissus* (26) venoms. In the case of venoms such as those of *Bungarus* sp. and *Micrurus* sp., which present both α- and β-neurotoxins, the two assays (AChR binding and PLA_2 activity) can be used. In the cases of venoms, such as those of *Dendroaspis* sp, rich in other types of neurotoxins, i.e. dendrotoxins (80), an as yet unexplored possibility would be the use of patch-clamp methods using oocytes expressing relevant receptors, such as voltage-dependent potassium channels (81) in the case of dendrotoxins. These, however, require electrophysiology facilities which are not readily available in antivenom quality control laboratories.

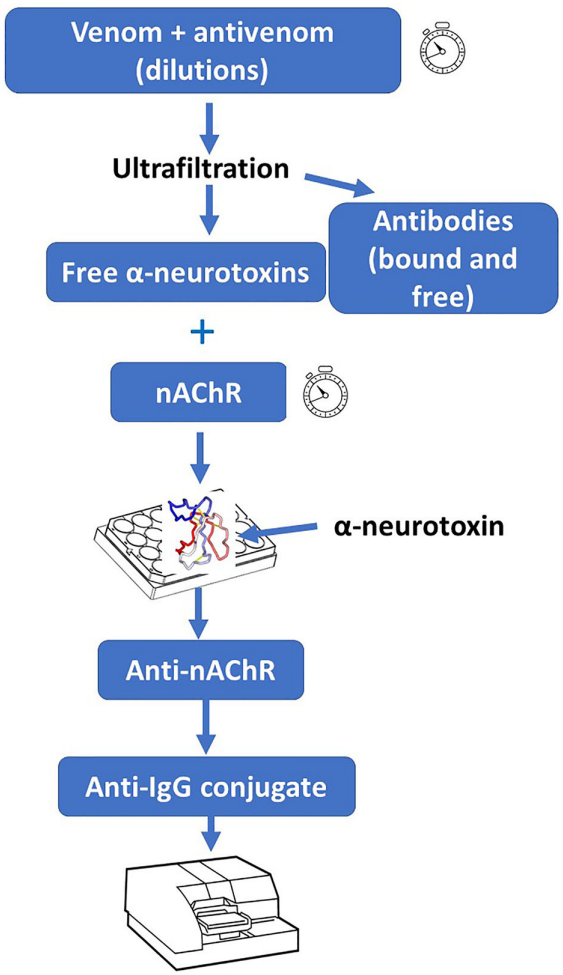

FIGURE 2 | *In vitro* assay for the assessment of the ability of antivenoms to bind to post-synaptically acting α-neurotoxins from snake venoms. A solution containing a fixed concentration of venom is incubated with various dilutions of antivenom. Then, antibodies (both free and venom-bound) are removed from free low molecular mass toxins (including neurotoxins) by ultrafiltration. The filtrate (containing these toxins) is incubated with purified nicotinic acetylcholine receptor (nAChR). Afterwards, the preparation is added to plate wells that had been coated with a purified α-neurotoxin. Upon incubation and washing, anti-nAChR antibodies are added, followed by washing and addition of conjugated anti-IgG antibodies. After adding the corresponding substrate, the absorbance is recorded. The nAChR preparation, which is obtained from the electric organ of fish, could be substituted by synthetic peptides containing the binding site for α-neurotoxins. For details of this procedure, see Ratanabanangkoon et al. (74).

HEN'S EGGS AS A MODEL FOR TESTING VENOM TOXICITY AND NEUTRALIZATION BY ANTIVENOMS

The use of hen's eggs at a developmental stage when no reflex pain arcs have yet developed was proposed as a model to assess venom toxicity and neutralization by antivenoms (82, 83). Venom solutions are applied to filter paper discs and then placed over the yolk sac membrane of shell-less eggs, followed by incubation at 37°C. This model was initially proposed for the study of the hemorrhagic activity of viperid venoms and showed a good correlation with the *in vivo* intradermal rodent assay (82). The model was then applied to the study of venom-induced lethality (83). The death of the embryo was assessed by observing the cessation of heart beats, followed by the submergence of the yolk sac membrane into the yolk (83). This model, however, cannot be applied for the study of neurotoxic venoms owing to the incipient development of neuromuscular junctions at this developmental stage in the chick embryo. The model was also used for assessing its correlation with *in vivo* toxicity, i.e. lethality, in the analysis of neutralization of nine venoms by antivenoms (84). A high correlation was found, suggesting the feasibility of using this system for evaluating antivenoms preclinical efficacy, except for neurotoxic venoms, for the reason indicated above. The model is more economic than those performed in mice and is also more convenient from the 3Rs perspective.

ANTIVENOMICS

The application of -Omics technologies has had a high impact in the study of snake venoms, providing novel and relevant clues for understanding their evolution and composition in their ecological and medical contexts (85). In particular, the field of proteomics as applied to venoms, i.e. 'venomics' (86), has shed light on the complexity of these toxic secretions (87, 88). An application of the study of venom proteomes to the field of antivenoms is 'antivenomics', a translational venomics applied to the fine characterization of the ability of antivenoms to recognize different components in venoms.

Antivenomics methodologies have evolved through three 'generations'. The baseline for antivenomic analysis is the proteomic characterization of venoms, with identification of the proteins and peptides after separation by reverse phase HPLC and one-dimension SDS-PAGE, and their quantification and classification in different protein families (86, 89). 'First generation' antivenomics was based on the incubation of venom and antivenom, followed by precipitation of immunocomplexes, and analysis of the supernatants containing venom proteins not recognized by antivenom antibodies (90). In 'second generation' antivenomics, the ability of antivenom antibodies to recognize venom components is assessed by affinity chromatography, whereby antibodies are bound to the chromatographic matrix and venom is passed through the columns. Hence, bound (reactive) and unbound (non-reactive) venom components are identified (91). The percentage of non-reactive venom component is then estimated based on the comparison between the areas under the peak of bound and unbound fractions, allowing a quantitative assessment of immune reactivity. In turn, 'third generation' antivenomics, which also uses affinity chromatography, enables the determination of the maximal binding capacity of antivenom antibodies for a particular toxin and also allows the quantification of the

percentage of venom-specific antibodies in the whole antivenom (92).

Even though antivenomics is not a functional test in terms of neutralization of venom activities, it can shed valuable information for understanding the preclinical efficacy of antivenoms. The relative weight of venom components in the overall toxicity of a venom can be studied by determining the 'toxicity score' for each component, which takes into consideration the toxicity of each toxin and its relative abundance in the venom (24). Once the most relevant toxins in a venom are identified, the ability of antivenoms to recognize these components can be quantified through antivenomics, hence providing indirect evidence of efficacy of the antivenom.

It has been suggested that an antivenom is effective when it is able to immunocapture 20–25% of venom components (93), and the WHO guidelines for production, control and regulation of antivenoms indicate that an immunocapture capability of ≥25% of venom proteins generally correlates with a good outcome in the *in vivo* neutralization tests (5). Therefore, these guidelines recommend the use of antivenomics as a first screening test for the neutralizing ability of antivenoms, before moving to the *in vivo* tests (5). As indicated above, the application of antivenomics to the analysis of the ability of antivenoms to recognize the most toxic components in a venom, as identified by the toxicity score, further potentiates the analytical power of this *in vitro* method. This underscores the relevance of studying snake venoms from a functional 'toxicovenomics' approach, i.e., by combining venomics with characterization of toxicity profiles of individual venom fractions (88). **Table 1** summarizes the information available on *in vitro* assays that have shown correlation with *in vivo* tests in the assessment of antivenom neutralizing ability.

TOWARD REFINING THE MOUSE LETHALITY TEST

The Introduction of Prophylactic Analgesia

Animal tests to assess venom toxicity and neutralization by antivenoms, particularly the mouse lethality assay, are associated with pain and distress, which may last for prolonged time intervals, as has been shown for crude venoms (94), and purified myotoxic PLA_2s (95) and hemorrhagic SVMPs (96). The algogenic effect of venoms is due to the action of venom peptides and proteins that directly activate nociceptive (pain sensing) neural pathways, as well as by the action of endogenous inflammatory mediators released in tissues as a consequence of venom actions, which stimulate nociceptive receptors in neurons (94, 97). Despite the evident suffering induced in laboratory animals when assessing venom toxicity and neutralization by antivenoms, the scientific community in Toxinology, as well as antivenom manufacturers, have been slow at introducing interventions aimed at refining these tests with the use of analgesia. One reason might be the possibility that analgesia affects the results of the tests, although this assumption has not received experimental support. Hence, it is time to consider the routine use of precautionary analgesia in these tests, along the lines indicated by the WHO (5).

The analgesics such as buprenorphine (98), morphine and tramadol (99, 100) have been shown to be effective analgesics when used in experiments involving venoms that cause local tissue damage and death. No differences in the extent of local hemorrhage, edema and myonecrosis induced by venom of *Bothrops asper* in mice were observed in mice pre-treated with morphine and tramadol, as compared to controls not receiving analgesia (99). The analgesic effect of these drugs can be readily evaluated by using the Mouse Grimace Scale (MGS) (101) and the mouse exploratory activity (102), which enable the quantification of pain. It was shown that morphine and tramadol are effective in reducing pain in several models of envenoming by the venom of *B. asper* (100). Likewise, the use of tramadol did not alter the results of the estimation of antivenom potency in the case of *B. asper* venom and a polyspecific antivenom (37). It is necessary to expand these observations to other venoms to assess whether similar results are obtained. In that case, the routine use of analgesia should be promoted in research and quality control laboratories.

The duration of the action of these analgesics in mice must be considered. It has been estimated that it is between 2 and 3 h for morphine (103, 104) and up to 6 h for tramadol (105), whereas the action of buprenorphine in the rat lasts for 6–12 h (106). Hence, in experiments to assess lethality and its neutralization, which usually last for 24 h, there is a need of subsequent administrations of the analgesic. In the case of neurotoxic venoms, it is likely that opioid analgesics, such as the ones described, affect the outcome of the test. In these cases, the use of milder analgesics, such as paracetamol, could be considered.

The Modification of the Protocol for the Lethality Test

The routine methods to estimate the LD_{50} of venoms and the ED_{50} of antivenoms usually last 24 or 48 h, depending on the route of injection (5, 6). Such prolonged time intervals involve much pain and distress in mice. Consequently, efforts are being carried out to make these tests less distressful. It is recommended that, before the assessment of venom LD_{50} or antivenom ED_{50}, a range-finding test is done, in which only one mouse per venom or venom/antivenom level is used. In this way, the range of doses to be used in a complete experiment, which usually works with five to seix mice per group, can be selected without having to sacrifice too many mice (5). When the i.p. route is used in these tests, a 48 h observation period is established (5, 6). However, our unpublished observations at Instituto Clodomiro Picado reveal that the number of mice dead at 24 h is the same as at 48 h, hence not justifying observations beyond 24 h.

A more drastic shift in the protocol to assess venom LD_{50} and antivenom ED_{50} uses a maximum observation period of 8 h [see, for example, Barber et al. (107)]. In this methodology, envenomed animals are observed at regular time intervals, e.g., every hour, and the severity of envenoming is graded according to a pre-established set of parameters. Animals that are severely affected at any time interval, i.e., are moribund, are euthanized, and all animals surviving at the end of the 8-h observation period are also euthanized. This modification of the classical methodology reduces the extent of animal suffering, although it may affect the precision of the results, as it has been observed that mice that appear

TABLE 1 | Summary of the *in vitro* and *ex vivo* assays that have shown correlation with *in vivo* toxic activities of snake venoms in the assessment of the neutralizing ability of antivenoms.

Type of assay	Applications
Enzyme immunoassays (EIA)	Correlation with neutralization of lethality in some venoms and purified toxins
Passive hemagglutination	Correlation with neutralization of lethality in some venoms
Phospholipase A_2 activity	Correlation with neutralization of lethality in some venoms
In vitro coagulant activity on plasma	Correlation with neutralization of lethality in some venoms. Correlation with defibrinogenating activity
Proteinase activity	Correlation with neutralization of hemorrhagic activity in some venoms
Cytotoxic activity on cells in culture	Possible correlation with neutralization of myotoxic and dermonecrotic activities of venoms*
Nerve-muscle preparations for assessing neuromuscular blockade	Possible correlation with neutralization of lethal and neurotoxic activities of venoms and isolated neurotoxins*
Binding to nicotinic acetyl choline receptors	Correlation with the neutralization of the lethal activity in venoms rich in post-synaptically acting α-neurotoxins
Antivenomics	Correlation with the neutralization of toxic components identified in venoms through proteomics and the toxicity score (toxicovenomics)

**In these cases, there have not been studies correlating observations in vitro and in vivo on the neutralization of these toxic activities; however, based on the mechanism of action of myotoxins, cytotoxins and neurotoxins, such correlation is highly likely.*

moribund may then recover. A balance needs to be made between the need to refine the lethality test and the need to ensure the robustness of the test for assessing antivenom efficacy. This urges the development of studies to assess the correlation between the results of these improved protocols and those of classical protocols.

CONCLUDING REMARKS

There is an urgent need to develop *in vitro* assays that correlate with *in vivo* toxicity tests in the study of venoms and in the assessment of the neutralizing ability of antivenoms, along with the 3Rs paradigm (**Figure 3**). This goal must be strengthened by research funding agencies and agendas, regulatory agencies and diverse stakeholders related to antivenom development, manufacture and quality control. This is a challenging task owing to the great complexity of the composition and mechanisms of action of venoms. A research-based, case by case analysis is needed in order to determine which is the most appropriate *in vitro* assay for each venom-antivenom system, providing the highest correlation with *in vivo* toxic activities, particularly lethality.

The best way to proceed along this line is to harness the growing body of information emerging from the study of venom toxicology and composition, which allows the identification of the most relevant toxic activities and toxins in each venom. This will facilitate the development of immunochemical or *in vitro* functional tests, enzymatic or otherwise, in substitution of animal-based assays. In turn, this calls for a closer collaboration between researchers in the biochemistry and pharmacology of venoms and toxins with professionals and technicians in antivenom production and quality control laboratories. Likewise, the regular use of analgesia in toxicity tests should be actively promoted in toxinological research and antivenom manufacture. It is expected that such initiatives will lead, in the short term, to a significant reduction in the number of animals used in research and antivenom development and potency evaluation, as well as in the suffering inflicted to those animals in the *in vivo* assays.

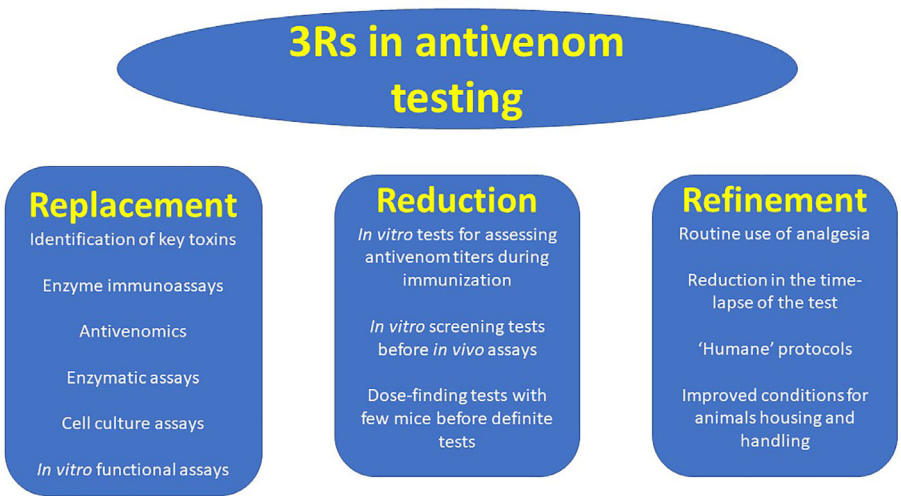

FIGURE 3 | The 3Rs principles, as applied to the evaluation of the neutralizing ability of antivenoms. The search for Replacement, Reduction and Refinement (3Rs) should be actively pursued in the field of antivenom potency testing. Some examples of the implementation of these principles in antivenom testing are shown.

DATA AVAILABILITY STATEMENT

Inquiries on the sources of information used in this review can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JG prepared the first version of this manuscript. MVa, AS, MH, MVi, GS, AS, CH, and GL revised and contributed to the content of the manuscript. All authors revised the final version of the work and agreed with its content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mesenchymal Stromal Cell-Based Therapies as Promising Treatments for Muscle Regeneration After Snakebite Envenoming

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Snakebite envenoming is a global neglected disease with an incidence of up to 2.7 million new cases every year. Although antivenoms are so-far the most effective treatment to reverse the acute systemic effects induced by snakebite envenoming, they have a limited therapeutic potential, being unable to completely neutralize the local venom effects. Local damage, such as dermonecrosis and myonecrosis, can lead to permanent sequelae with physical, social, and psychological implications. The strong inflammatory process induced by snake venoms is associated with poor tissue regeneration, in particular the lack of or reduced skeletal muscle regeneration. Mesenchymal stromal cells (MSCs)-based therapies have shown both anti-inflammatory and pro-regenerative properties. We postulate that using allogeneic MSCs or their cell-free products can induce skeletal muscle regeneration in snakebite victims, improving all the three steps of the skeletal muscle regeneration process, mainly by anti-inflammatory activity, paracrine effects, neovascularization induction, and inhibition of tissue damage, instrumental for microenvironment remodeling and regeneration. Since snakebite envenoming occurs mainly in areas with poor healthcare, we enlist the principles and potential of MSCs-based

therapies and discuss regulatory issues, good manufacturing practices, transportation, storage, and related-procedures that could allow the administration of these therapies, looking forward to a safe and cost-effective treatment for a so far unsolved and neglected health problem.

Keywords: advanced therapy medicinal products, mesenchymal stromal cells, snakebite, envenoming, muscle regeneration

INTRODUCTION

Snakebite envenoming (SBE) is a public health problem affecting as many as 2.7 million people every year all around the world, most of whom live in poorly developed tropical and subtropical countries (1). Since 2017, SBE is considered a highest priority neglected tropical disease and the lack of enough antivenom supply in affected countries lead to thousands of deaths per year (2). Local damage, such as myonecrosis, dermonecrosis, hemorrhage, and edema, is only partially neutralized by antivenoms, at most. This is a noteworthy situation, considering that local damage can lead to permanent disability (3). Therefore, there is a current and urgent need of: i) providing adequate antivenom supply and ii) developing new comprehensive or complementary treatments that help to neutralize both systemic and local damage (and then permanent sequelae) (4).

Among the various tissue damage effects induced by SBE, myotoxicity-induced local myonecrosis (5) and hemorrhage (6) could be the most severe ones. Impaired skeletal muscle regeneration (SMR) (7, 8) may be associated or not to low response to antivenom (9).

SMR is a complex process with three overlapping steps (10), all of them affected by SBE. Mesenchymal stromal cells (MSCs) based therapies have shown to induce muscle regeneration in a variety of models in preclinical and clinical studies, mainly by anti-inflammatory activity, paracrine effects, revascularization induction, and microenvironment remodeling (11–14). In a recent publication, our group could demonstrate that adipose derived allogenic MSCs revert macrophage activation, cytokine storm and hyperinflammatory state associated with COVID-19 showing that MSCs stops tissue damage and promotes recovery (15). This promising landscape, considering the nature of the local and systemic damage induced by SBE, and the matching benefits of MSCs based therapies drive us to propose that this kind of treatment could be effective in reverting the induced tissue damage caused by SBE that do not respond to antivenom treatment, in particular by improving SMR.

Here, we will discuss the biological basis of local and systemic effects induced by SBE and the well-described benefits of MSCs based therapies in similar processes. Furthermore, preliminary results of MSCs' secretome treatment upon muscle damage caused by *Bothrops atrox* venom are presented, supporting our approach. Also, we will discuss about regulatory issues, good manufacturing practices, transportation, storage, and related procedures that will be necessary to effectively translate this proposal into treatments for this neglected medical problem.

SNAKEBITE ENVENOMING (SBE) RELEVANCE

SBE is a public health problem with at least 1.8–2.7 million of cases worldwide per year with mortality estimations ranging from 81,410 to 137,880 deaths (1). In the developing world, tropical and subtropical Africa, Asia, Oceania, and Latin America, SBE is considered a major public health problem with higher human morbidity and mortality ratios due to factors such as the scarcity of antivenom, poor health services, and problems in the transportation of patients (16). Furthermore, different types of sequelae are commonly presented after SBE, from psychological trauma to physical disabilities, with 400,000 of the surviving victims suffering from permanent sequelae (17–20). For instance, the annual number of amputations due to SBE in sub-Saharan Africa alone was estimated from 5,908 to 14,514 (21).

Currently, SBE is a Category A neglected tropical disease, according to the World Health Organization (WHO), which remarks the importance of SBE in terms of incidence and severity, especially for developing countries (2). Moreover, a Road Map for a globally coordinated response to SBE was recently published by WHO, with the ambitious goal of reducing snakebite deaths and disability by 50% before 2030 (4).

One of the main obstacles in providing adequate treatment for SBE is that venom composition is heterogeneous. As an example, in the two main venomous families (Viperidae and Elapidae), we may find a great diversity of toxin families, such as snake venom metalloproteinase (SVMP), phospholipase A2 (PLA2), snake venom serine proteinase (SVSP), three-finger toxin (3FTx), C-type lectin-like protein (CTL), L-amino acid oxidase (LAO), low molecular mass myotoxins (Myo), and others. This wide range of components contained in snake venoms; depending on the snake species, lead to complex local and systemic symptoms (22).

While the gold standard for treating SBE is based on specific antivenom, which have proved to be effective against systemic symptoms and can save the lives of affected people, SBE also leads to long-term sequelae and disabilities, many of them caused by local damage which is mainly neither prevented nor reversed by antivenoms (9). Despite the variety of toxins, their resulting local tissue damage usually includes myonecrosis, dermonecrosis, hemorrhage, and edema; and also can be accompanied by concomitant infection, compartment syndrome, and bite's site complications (23). Moreover, local damage caused by SBE is frequently followed by poor tissue regeneration associated with tissue loss and dysfunction, leading to permanent sequelae as well as consequent social and psychological implication (8, 17, 20). It is

noteworthy that SBE affects predominantly people at the economically productive age, increasing the economic impact of permanent disability (24). Even more, local damage could also lead to death when necrotizing fasciitis, a soft-tissue infection characterized by rapidly progressing inflammation and necrosis of subcutaneous fascial tissues, is developed (25), which makes the local damage and necrosis per se a life-threatening situation.

Local damage induced by SBE, particularly myonecrosis, is currently a worldwide unsolved and neglected medical problem. Recently proposed therapies, such as anti-inflammatory treatment for general local effects (26) or photobiomodulation (PBM) by low-level laser therapy for the sensory effects (27), are still insufficient, showing no significant advantage on SMR. However, PBM with light emitting diode (LED) in red and infrared wavelengths showed to be a more promising therapy, since it could reduce the extent of myotoxicity, edema, inflammatory infiltrate, and hyperalgesia in mice after SBE (28). Moreover, the accelerated tissue regeneration induced by PBM therapies has already been stated, and further studies are encouraged (29).

Another promising therapy regarding local damage is based on nanobodies (recombinant single-domain antigen-binding fragments from camelid heavy chain-only antibodies). Nanobodies are proposed to be used as antivenoms, showing notable advantages such as rapid diffusion, high-biodistribution due to their small size and no Fc region (responsible for adverse reactions in antivenom therapy) in their structure (30). A recent study demonstrated their efficacy in neutralizing both local tissue hemorrhage and myonecrosis in mice after SBE, when applied a mixture of selected specific nanobodies (31), showing the potential of this therapy over Fab or IgG- based antivenoms in resolving local damage.

In addition to antivenom, new therapies focused on local damage caused by SBE are required to prevent the consequent sequelae and disabilities. Since the effects caused by SBE are so heterogeneous, it is difficult to design a specific therapy for each particular toxin action. This is the reason why we propose that Advanced Therapy Medicinal Products (ATMPs) (comprising somatic cell therapies, gene therapies, and tissue-engineered products according to the EU Directive No 1394/2007) may be an alternative treatment for SBE, due to their properties. Specifically, we hypothesize that mesenchymal stromal cells (MSCs), activated by local signals from the venom-injured area, will display a broad range of therapeutic effects including anti-inflammatory, regenerative and disruptive of the complement-inflammation-coagulation crossroad, promoting not only tissue regeneration but also re-vascularization of the affected area. We also presume that cell-free MSCs-based therapies could also present these listed benefits, with the additional advantage of its production logistics being more adequate for use in low-income countries, which are the most affected by SBE.

LOCAL MYONECROSIS INDUCED BY SNAKEBITE ENVENOMING

Local damage caused by SBE results in myonecrosis as one of their main complications. Although not yet completely understood, the current knowledge on the pathogenesis of snakebite-induced myonecrosis and its unsuccessful healing have been well described in a recent review (7).

Several snake venom components from the most medically relevant venomous snake families (Viperidae and Elapidae) contribute to myonecrosis, inducing both myotoxic and hemorrhagic damage. Muscle damage induced by SBE is a consequence of a direct action of myotoxins (such as PLA2, 3FTx, LAO, and Myo) upon the plasma membranes of muscle cells and an indirect effect in vascular degeneration and ischemia, in essence due to local hemorrhages caused by hemorrhagic toxins (such as SVMP, SVSP, and CTL) (32). Additionally, the unbalanced inflammatory response provoked by these toxins contributes to further tissue damage and impaired regeneration processes (33–35). All of these described deleterious effects are usually presented together in SBE victims, where myotoxins PLA2s and hemorrhagic toxins SVMPs are the primarily acting venom components (36).

PLA2s and PLA2 homologs, well-established mediators of myonecrosis (37, 38), interact with yet unidentified "acceptors" in skeletal muscle cell plasma membrane, causing rapid membrane disruption by both catalytically-dependent and independent mechanisms. For instance, a catalytically-active myotoxic PLA2 predominantly induces phosphatidylcholine hydrolysis, acting only on the external monolayer of the sarcolemma; whereas a catalytically-inactive PLA2 homolog does not depend on phospholipid hydrolysis to disrupt plasma membrane (39). Interestingly, cell membrane cholesterol content (inversely related to membrane fluidity) is a relevant proposed parameter for myotoxic effects due to PLA2s, considering that cell membrane cholesterol depletion (and increased membrane fluidity) promotes membrane damaging of myoblasts by this type of myotoxin (40). Finally, membrane perturbation caused by PLA2s induces a rapid influx of extracellular calcium which provokes hyper-contraction of myofilaments, mitochondrial calcium uptake (with mitochondrial damage due to calcium overload), and the activation of calcium-dependent intracellular proteinases (calpains) and even endogenous PLA2s themselves; defects that lead to myonecrosis (41).

SVMPs, zinc-dependent toxins can act directly at the microvascular level, causing hemorrhage (6, 42, 43). A unifying model explains their action mechanism (41). The hydrolyzation of basement membrane components of capillary vessels, especially type IV collagen, induces mechanical instability of the capillary (44, 45). Then, hemodynamic forces, such as wall hydrostatic pressure and shear stress, provoke the distention and disruption of the capillary wall, leading to extravasation (46). This damage indirectly contributes to myonecrosis by the restriction of oxygen and nutrients to muscle tissue (ischemia) (47). However, its most deleterious effect could come after myonecrosis, when hemorrhage hampers the regeneration process.

Although myotoxins such PLA2s are directly responsible for myonecrosis while hemorrhagic toxins such SVMPs indirectly contribute to this damage, it is remarkable how muscle regeneration in the presence of both types of toxins is significantly compromised, while in the presence of only PLA2,

muscle recovers from myonecrosis without important abnormalities (8, 48). Considering that microvascularization is one of the determinant factors leading SMR (10), damaged microvasculature (specifically induced by hemorrhagic toxins) could be the main (but not the only) reason for poor muscle regeneration after SBE, in particular after Bothrops sp. (Viperidae snake) envenoming (38, 49, 50).

However, other considerations beside myotoxic effects and microvascular damage must be taken into account when analyzing myonecrosis caused by SBE and its following regeneration. Damage of intramuscular nerves, degradation of muscle cell basement membrane, degradation of the extracellular matrix, and deleterious effects on myogenic cells are also involved (7). The **Figure 1**, summarizes the main hypothetical factors that determine the poor outcome in skeletal muscle regeneration after myonecrosis induced by viperid venoms, associated to viperid toxins and the steps of the SMR which they affect.

SKELETAL MUSCLE REGENERATION (SMR)

SMR is defined as the multi-step process required for the formation of new myofibers or myofiber segments after necrosis. Three consecutives but overlapping stages are described (**Figure 2**) (10):

1. The inflammatory reaction, characterized by the infiltration of specialized cells (macrophages, neutrophils, etc.) which act as scavengers of necrotic debris and activate regulatory cells.
2. The regenerative phase, consisting in the activation, proliferation, differentiation, and fusion of satellite cells.
3. The remodeling-repair phase, including the maturation of newly formed myofibers and remodeling of regenerated muscle.

Inflammatory Reaction

The inflammatory reaction is initiated by neutrophils in their early recruitment after myonecrosis and its induction is followed by macrophage activation (51). Even though phagocytes classical role was meant to be scavengers of necrotic debris, now it is known that they perform a more active role, orchestrating the inflammatory reaction and also promoting muscle regeneration (52, 53). The discovery of direct and constant heterocellular surface apposition over large areas and long linear distances between macrophages and myogenic cells throughout all stages of myogenesis reinforced their proposed importance not only at the inflammatory reaction but also at the regenerative phase (54).

A proper SMR depends on classical phagocytosis, which could allow the following stages of SMR after the inflammatory reaction; however, a precise balance between induced pro- and anti-inflammatory factors is also required (55). Here, macrophage subpopulations, M1 pro-inflammatory macrophages and M2 anti-inflammatory macrophages, play a pivotal role. While M1 macrophages release inflammatory mediators, including interleukin IL-1b, IL-12, and nitric oxide (NO) (56); M2 macrophages release anti-inflammatory mediators, including IL-4, IL-10, and transforming growth factor-beta (TGF-β) and promote remodeling of the extracellular matrix (ECM) and stimulate angiogenesis (57, 58). M2 macrophages also secrete insulin-like growth factor-1 (IGF-1) that activate muscle precursor cells (satellite cells) supporting their growth and fusion to form new muscle fibers (59). The benefit of using allogeneic MSC comes from the MSC-induced transdifferentiating of M1 (pro-inflammatory) into M2 (anti-inflammatory) macrophages.

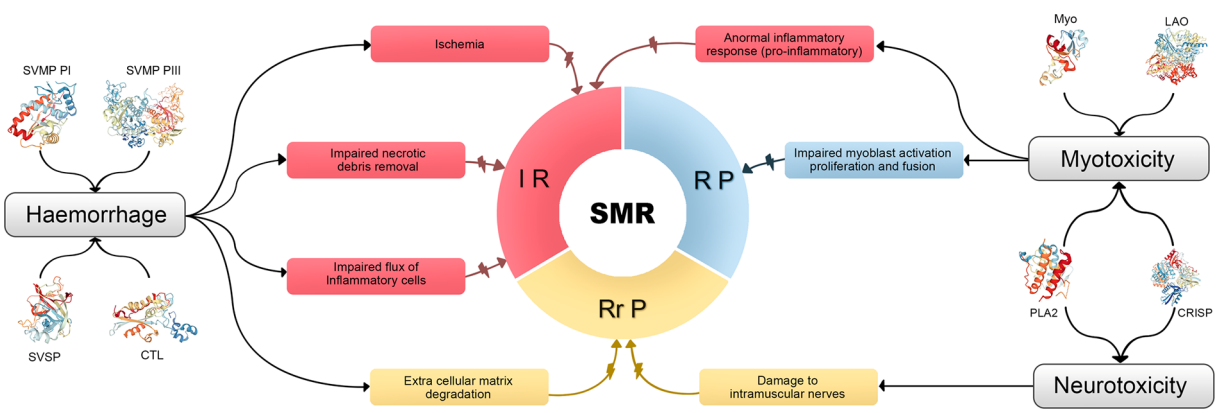


FIGURE 1 | Summary of the main hypothetical factors that determine the poor outcome in skeletal muscle regeneration after myonecrosis induced by viperid venoms, associated to viperid toxins and the steps of the SMR which they affect. Different venom components induce hemorrhage, myotoxicity, or/and neurotoxicity. These deleterious effects impair the normal SMR acting on all their three steps. IR, inflammatory reaction; RP, regenerative/phase; RrP, remodeling-repair phase; CRISP, cysteine-rich secretory protein [Protein Data Bank accession ID (PDB ID): 3MZ8]; CTL, C-type lectin-like protein (PDB ID: 1IXX); LAO, l-amino acid oxidase (PDB ID: 2IID); Myo, low molecular mass myotoxin (PDB ID: 4GV5); PLA2, phospholipase A2 (PDB ID:1TGM for the monomer and PDB ID:3R0L for the dimer); SVMP, snake venom metalloproteinase (PDB ID:3DSL for class PIII and PDB ID: 1ND1 fo class PI); SVSP, snake venom serine proteinase (PDB 1D:1OP0).

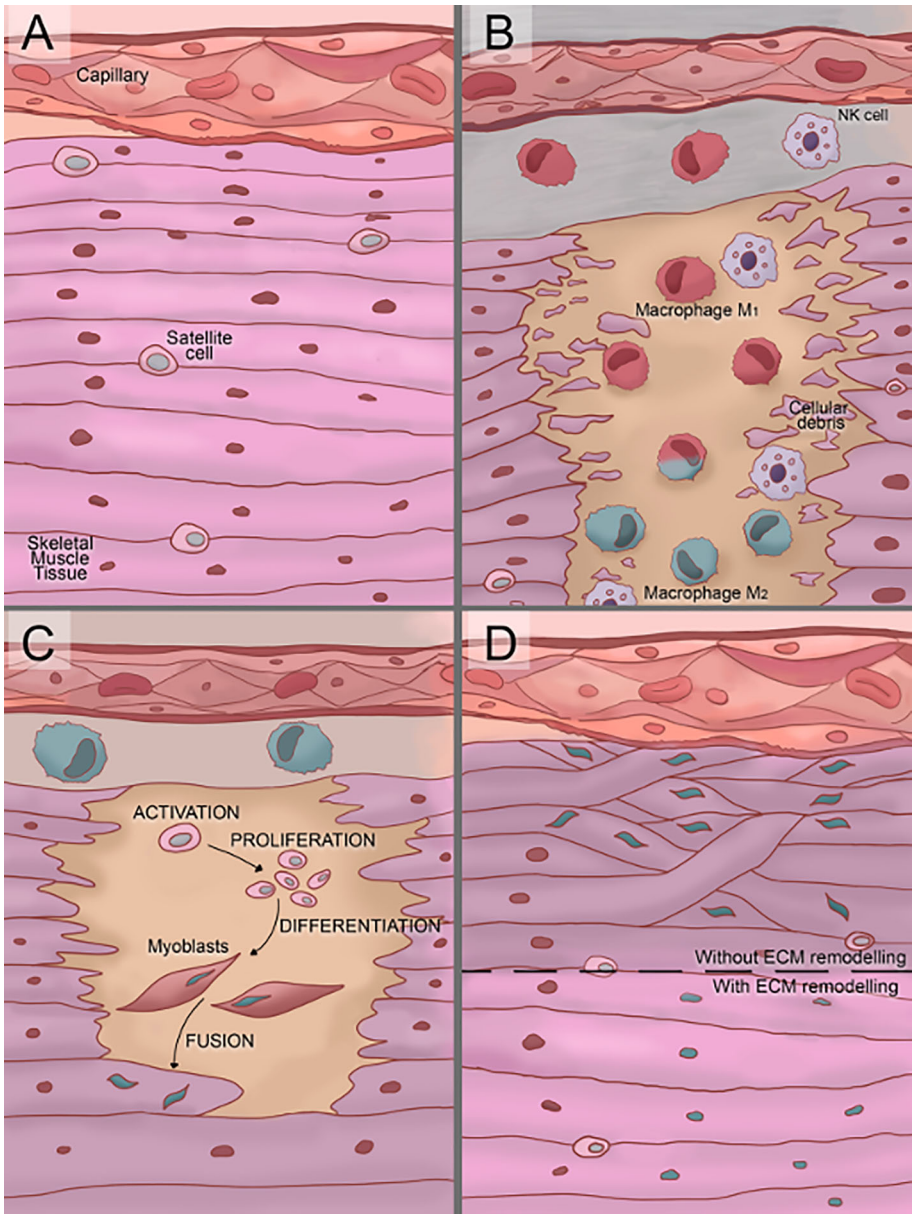


FIGURE 2 | Illustration of the three phases of skeletal muscle tissue regeneration. **(A)** In normal conditions, satellite cells are located between the sarcolemma and basement membrane of terminally-differentiated muscle fibers. **(B)** Soon after damage, the inflammatory reaction starts. First, the cells of the immune system (neutrophils and macrophages) infiltrate the damaged tissue produce a proinflammatory stage. Over time, an anti-inflammatory stage starts to replace the proinflammatory one, in this transition, proinflammatory M1 macrophages switch to anti-inflammatory M2 macrophages. **(C)** Once the proinflammatory stage starts to decay, the regenerative phase starts with the satellite cells activation. Then, activated satellite cells proliferate and differentiate into myoblast and finally myoblasts fuse into myotubes. **(D)** The remodeling/repair phase consists in the extra-cellular microenvironment (ECM) remodeling and maturation of the new myofibers. If an inadequate remodeling/repair phase occurs, the myotubes grow unorganized generating dysfunctional muscle tissue.

Neutrophils are the first to reach the site of snake venom-induced tissue damage. They stimulate an inflammatory environment and after stopping myonecrosis, they participate in the regeneration of damaged tissue. The resolution of inflammation and tissue regeneration are mediated by the remotion of necrotic material and the release of chemokines, cytokines, and growth factors (60). In cancer, two different subtypes of neutrophil populations have been described (61). The N1 subtype is pro-inflammatory, mainly with phagocytic and cytotoxic activity. While, the N2 subtype is induced by TGF-β and a low level of IFN-β, reducing inflammation and releasing growth factors such as VEGF, promoting angiogenesis (61, 62). Is widely known that MSC produce TGF-β (63) and, recently, it was reported that MSCs stimulate the polarization to N2 subtype

(64). Finally, MSC based therapies would favor the anti-inflammatory neutrophils subtype and tissue repair, in synergy with other regulatory cell populations also stimulated.

The Regenerative Phase

The regenerative phase of SMR includes the activation, proliferation, differentiation, and fusion of satellite cells and ends with new functional myofibers. Satellite cells are the muscle precursor cells and they are required for a successful skeletal muscle regeneration (65, 66).

After muscle injury, satellite cells need to be activated and undergo a rapid proliferation for muscle regeneration. This activation is induced by different signals such as the generation of sphingosine-1-phosphate in the inner side of the plasma membrane of the satellite cell (67) or the increased NO synthase activity which generates more NO and probably induces the indirect release of hepatocyte growth factor (HGF) from the ECM (68, 69). In addition, several pro-myogenic stimuli that activate intrinsic pathways that stimulate proliferation are required, including IGF1, HGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), tumor necrosis factor-α (TNFα) and β (TNFβ), platelet-derived growth factor-AA (PDGF-AA) and BB (PDGF-BB), vascular endothelial growth factor (VEGF) and also the implementation of a highly specialized, epigenetic and regulatory gene expression program (70–72). For instance, specific patterns of miRNAs (gene expression regulatory molecules) for regeneration and differentiation suggested they are likely involved in the process of satellite cell proliferation, differentiation, and skeletal muscle regeneration in general (73).

Activated satellite cells need to be differentiated into myoblast and consecutively form myotubes by fusion. For example, a required satellite cells activation factor such as HGF inhibits muscle cell differentiation (74). It was described that as well as the rapid proliferation stage is controlled by Notch signaling (75), Wnt signaling controls the differentiation phase (76). Different proteins such dysferlin, myomarker, Eps15 homology domain-containing proteins (EHD), and annexins have been associated with membrane myoblast fusion and myotube formation, undoubtedly the muscle cell communication through paracrine signaling, especially by exosomes (77), plays an important role for the correct satellite cell activation, differentiation, and maturation (78). Moreover, not only the protein cargo (such as VEGF or IGF1) is involved, but also miRNA cargo. More than 170 different miRNAs were found within muscle exosomes including miR-1, miR-133a, miR-133b, miR-206, which regulate myogenic differentiation/myoblast proliferation (79–82).

The Remodeling-Repair Phase

Growth and maturation of newly formed myofibers may vary according to various factors from the type of damage to the involvement of blood vessels. However, it is remarkable that, after myogenesis, presence of the nerve is required. When neuromuscular connections are not reestablished, regenerating myofibers remain atrophic (83). Besides, another crucial factor for successful muscle regeneration is the maintenance of the basal lamina of muscle fibers, because remnants act as scaffolds to guide satellite cell divisions after injury (84). Also, the mechanical loading is essential for the subsequent maturation of myotendinous junctions and muscle remodeling; if immobilization is too prolonged, regenerated myofibers remain atrophic and their orientation is more disordered (10). Furthermore, mechanical loading enhances the ability of myoblasts to promote an M2-like macrophage phenotype following exposure to ECM scaffolds and M2-like macrophages promote myoblast chemotaxis and differentiation while lacking weight-bearing impairs muscle remodeling (85). This link between the inflammatory reaction and the remodeling-repair phase reaffirms muscle regeneration steps are overlapping and each one affects over the others.

In addition, a remarkable consideration for a proper SMR is the capillarization damage. Microvasculature would be a critical factor that transcends only one step of the SMR due to capillaries, satellite cells, and muscle remodeling appear to be intimately linked and capillarization would be necessary for appropriate necrotic debris scavenge, satellite cells function, systemic cytokines delivery, the transportation of muscle-derived cytokines, or for cell-cell interactions between satellite cells and endothelial cells (86).

However, even with all the current knowledge about the SMR, a well-described process, there is not much information about its occurrence after SBE. A successful SMR with proper inflammatory reaction, regenerative phase, and remodeling-repair phase require many factors such as the removal of necrotic material, the presence of intact blood supply and innervation, or the permanence of the basement membrane surrounding necrotic fibers (10). Unfortunately, after SBE, injured tissues do not provide the ideal conditions for SMR. Envenomed muscle tissue shows inhibited myoblast cell proliferation and fusion into myotubes (87). Moreover, the reported microvascularization and innervation damage after SBE may hamper a proper SMR (8). The implementation of a comprehensive treatment that could counteract the negative effects of SBE would be necessary to allow the normal SMR process and MSC-based therapies could accomplish these characteristics.

MESENCHYMAL STROMAL CELLS-BASED THERAPIES

MSC are multipotent stromal cells from different tissue sources that can be differentiated into a variety of cell types. The International Society for Cellular Therapy has proposed three minimum criteria to define MSCs (88): (i) adherence to plastic, (ii) specific surface antigen presence/absence, and (iii) expression of multipotent differentiation potential. Therefore, MSC-based therapies imply the direct or indirect use of MSC for therapeutic purposes. While cell-based therapies directly apply MSC to patients, cell-free therapies may use MSC conditioned medium containing the secretome (as "all the factors secreted by a cell") or MSC derived exosomes (89), a part of this secretome that can be further isolated (90).

MSCs can be obtained from several sources (91). While source and purification protocol can modify MSC properties (92), in general, MSCs share several characteristics such as the fact that hypoxic culture enhances their proliferation (93) and that they have shown a well-studied therapeutic potential both in animals and humans (94–96).

Four main properties have been proposed to explain MSCs therapeutic potential (**Figure 3**) (14): (i) the ability to secrete multiple bioactive molecules (proteins, mRNAs and miRNAs) capable of stimulating regeneration and inhibiting inflammation, (ii) the lack of immunogenicity and the ability to perform immunomodulatory functions, (iii) the ability to home to sites of inflammation following tissue injury when injected intravenously, and (iv) the ability to differentiate into various cell types.

A relevant consideration when considering MSC-based therapies is their well-reported safety (97). Even though, some clinical trials have often shown only moderate success, which is usually attributed to low engraftment or low retention rates of cells as it is the case of most of MSC-based cardiovascular therapies, MSC-based therapies have been extensively reported to improve angiogenesis in both preclinical and clinical trials (13, 98–101). Moreover, reports of MSCs ability to induce tissue microenvironment remodeling directly over ECM components (12, 102) and MSCs immunomodulatory potential (103) reinforce the proposition that paracrine effects govern the therapeutic potential of MSC but also prompt the theory that MSC-based therapies could enhance SMR after SBE (**Figure 4**).

The therapeutically effect of MSC administrated by intrarterial or intravenous infusion or local injection has been

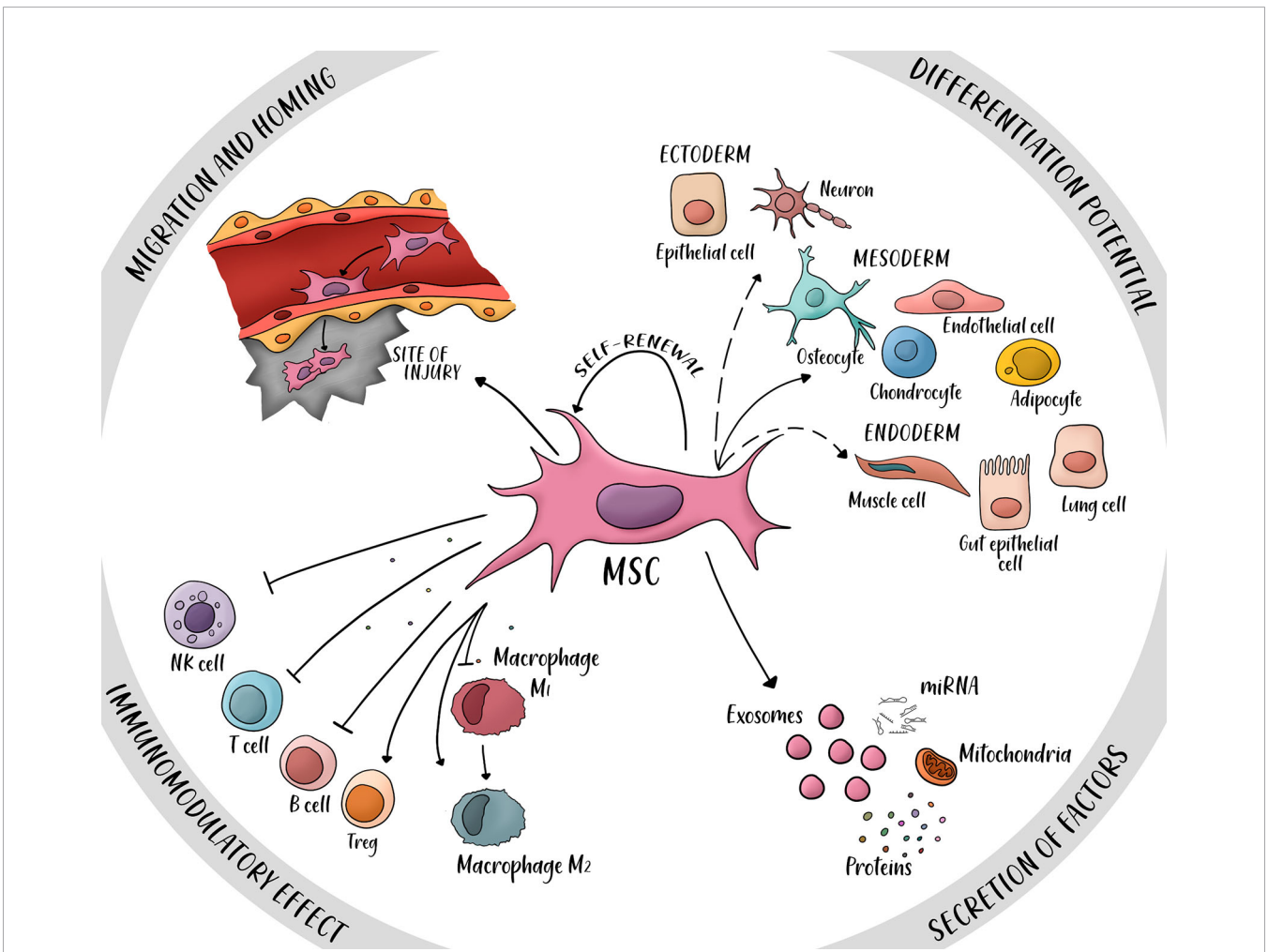


FIGURE 3 | Four main properties of mesenchymal stem cells (MSC). MSCs have a potential for differentiation into ectoderm cells (neurons, epithelial cells), mesoderm (osteocyte, endothelial cell, chondrocyte, and adipocyte), and endoderm (muscle cells, gut epithelial cells and lung cells). Secretion of factors such as proteins, miRNAs, mitochondria, and exosomes can promote repair of damaged tissue and immunomodulatory potential. The immunomodulatory effect is mainly immunosuppressive, several secreted cytokines inhibit the activity of natural killer cells (NK cells), T cells and B cells; other cytokines activate the proliferation of regulatory T cells (T reg) and the switch from macrophage M1 (pro-inflammatory) to macrophage M2 (anti-inflammatory). The property of migration and homing is possible by the expression of specific ligands and receptors in the site of injury. Dashed arrows: Controversial transdifferentiation *in vivo*.

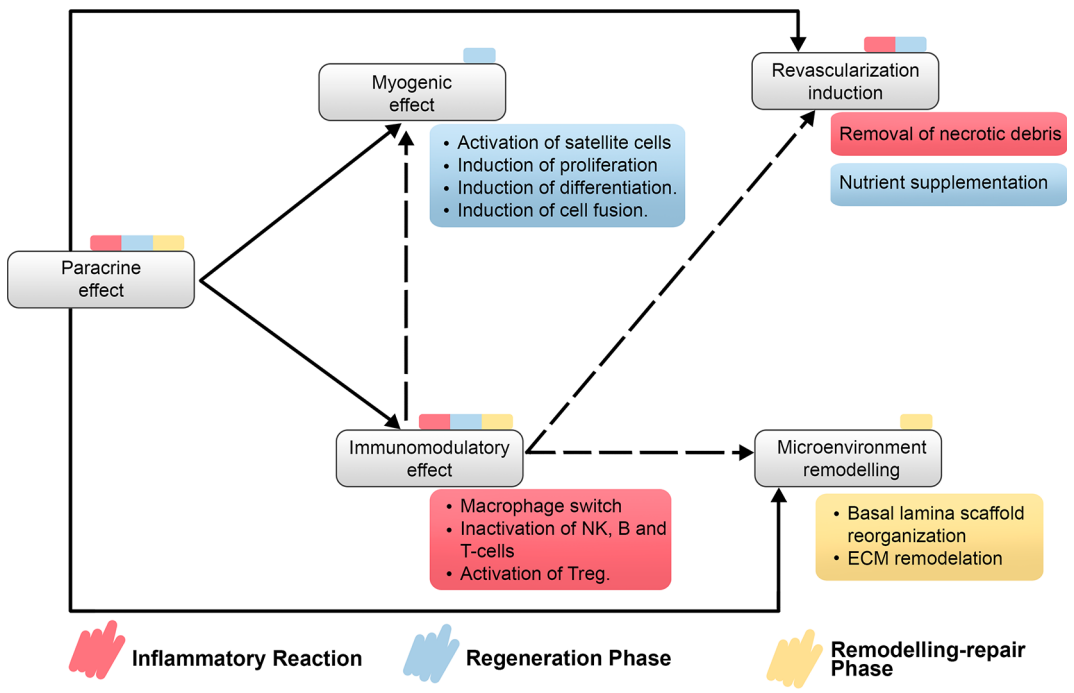


FIGURE 4 | Relationship on main properties of MSC-based therapies regarding the recovery of the impaired SMR after myonecrosis induced by snakebite envenomation. It is detailed the direct and indirect relationship between properties of MSC-based therapies and which step or steps of SMR would be benefited by each property. Treg, regulatory T cells; NK cells, natural killer cells.

demonstrated in humans for more than 400 reported Clinical Trials (ClinicalTrials.gov) including our group's experience with more than 200 patients suffering multiple sclerosis (104), Chronically Limb Ischemia (99, 105–107), and COVID-19 (15). Moreover, a meta-analysis of randomized controlled trials, showed that MSC-cell based therapy increase ulcer healing, angiogenesis, and reduce amputation rate (100), although the mechanisms involved in tissue regeneration in the ulcers healing is an aspect that should be further studied.

Our group has designed a protocol for the isolation, characterization, and longtime culture of MSC derived from visceral fat using a xeno-free and human component free culture media (XANADU media, Patent pending). This protocol rejuvenates mitochondria in MSC with the advantages of i) not incorporating Fetal Bovine Serum (FBS) and avoiding the possibility of animal-to-human contamination and free of Human Platelet Lysate, which increases fibrinogen and prothrombosis. MSC obtained and expanded with this media express low Tissue factor and high tPA/PAI-1 ratio. Using XANADU media, adipose-derived human MSC expanded during eight consecutive passages (56 days, every 7 days), showing MSC characteristic morphology, without signs of replicative senescence (**Figure 5** panel A). The culture maintained a low doubling time (ranging from 45 to 70 h) similar to that obtained using a chemically defined commercial medium (MQD-commercial medium) and significantly less than those obtained with FBS-supplemented medium (FBS control medium) (**Figure 5**). The expansion of the adipose-derived human MSC in XANADU media shows a rate of proliferation suitable for its large-scale production ("biobanking"), due to the fact that the cumulative doubling of its population was significantly higher (25.6 h) than that obtained with control medium (14.21 h), (**Figure 5**) and similar to that obtained in commercial xeno-free media (**Figure 5**).

A preliminary study using a human cytokine antibody array shows that cells grown in XANADU media secrete paracrine factors with capacity for regulation of tissue regeneration and modulation of immune response, that may be relevant in the context of SBE (**Figure 6**). In particular, the enrichment of TIMP-1 and TIMP-2, natural inhibitors of matrix metalloproteinases (MMP) with an important role in setting the right balance for promoting tissue remodeling (7); Angiogenin, a powerful stimulator of angiogenesis; and RANTES, MCP1, ENA78, Gro-a, IL6, IL8, Eotaxin, GCP2 which are regulatory chemokine's of the immune response.

Considering that SBE occurs primarily in developing countries, the benefits of treating the local damage with a cell-free MSC-based therapy are valuable. Therapeutic effects of MSCs are mostly mediated by their ability to produce bioactive molecules such as cytokines, growth factors, and extracellular matrix proteins, extracellular vesicles, and miRNAs. Since MSC-CM and MSC-exo carry the wide spectrum of secreted bioactive molecules, they are rational alternatives with several advantages compared to the direct use of MSC (108). MSC-EV has shown to

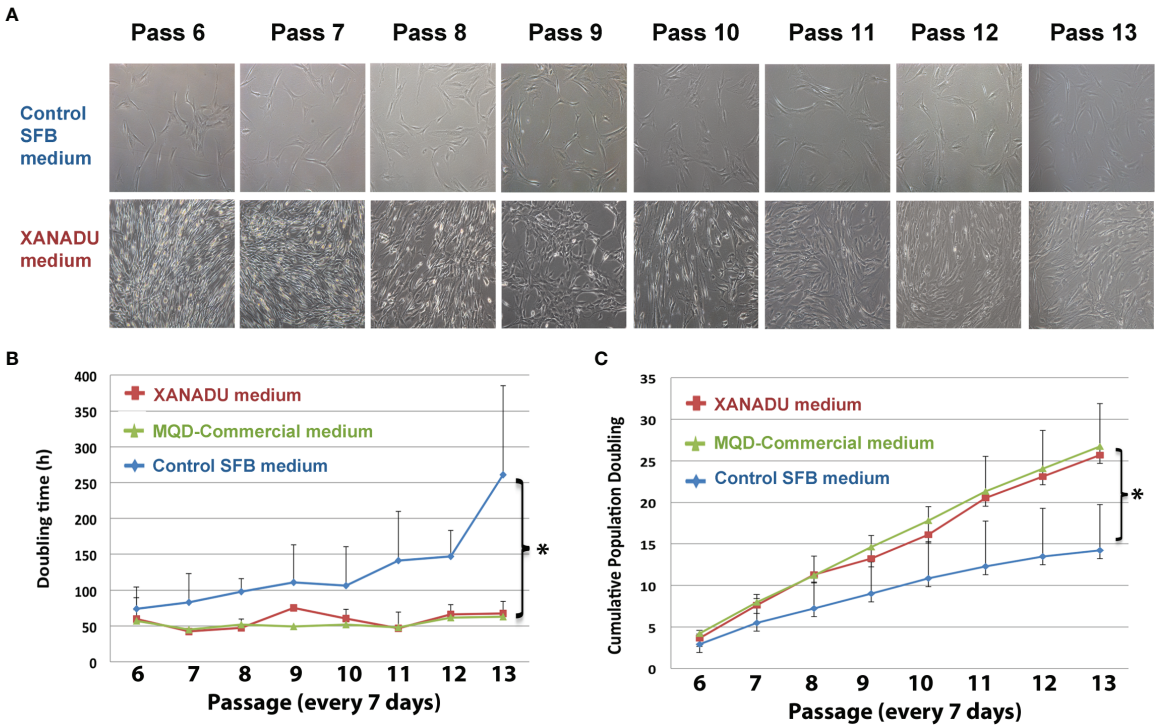


FIGURE 5 | hMSCs derived from omentum adipose tissue, grown for long periods in chemically defined media have optimal morphological and proliferative properties. The hMSC were cultivated in control medium supplemented with fetal bovine serum (Control SFB medium) on adherent plates and XANADU chemically defined medium and commercial MQD on plates functionalized with vitronectin for eight consecutive passes. **(A)** Morphology and culture density of hMSC. **(B)** Duplication time. **(C)** Cumulative doubling of the population. The figures in **(A)** are representative of three independent experiments. The data of **(A, B)** are the mean plus the deviation of at least three independent experiments. (*) $p \leq 0.05$ when compared to the control SFB medium.

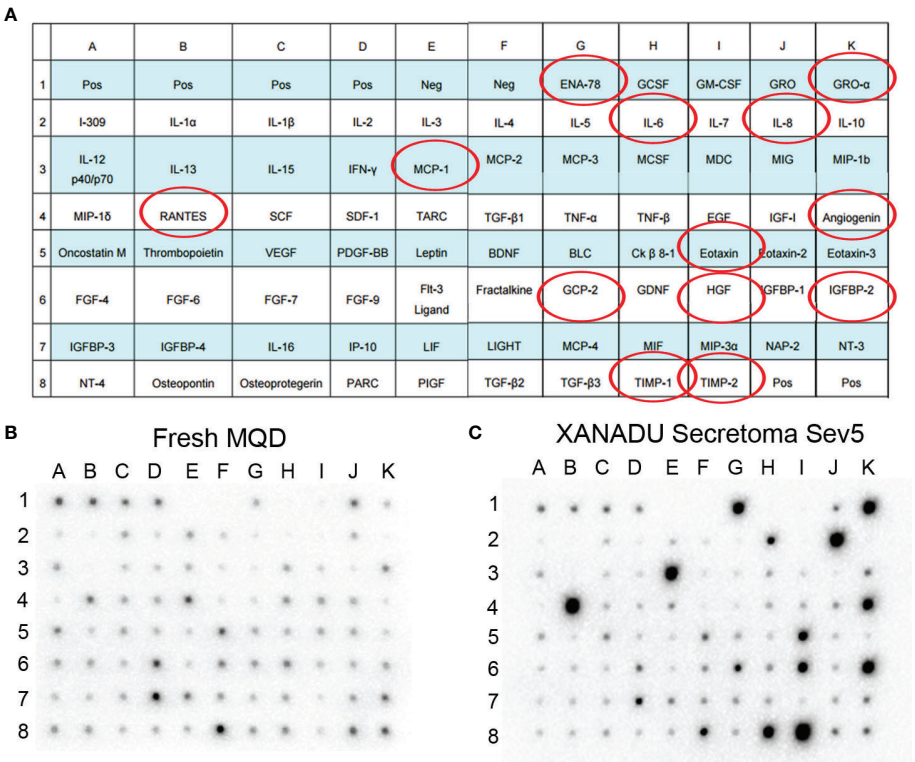
induce accelerated SMR *in vitro* and *in vivo* by enhancing not only angiogenesis but also myogenesis (109). Therefore, cell-free MSC-based therapies appear to share the main properties of MSCs plus several advantages (110, 111). Some of them are (89, 112):

1. Reduced risks associated with engraftment.
2. Their lower immunogenicity compared with living cells.
3. Reduced possibilities of ectopic tissue development.
4. Significant lower cell number required for the treatments.
5. Their more cost-effective use of controlled laboratory condition (e.g., bioreactors) with easier and more productive procedures).
6. The possibility of being modified to desired cell-specific effects.
7. Their easier evaluation for safety, dosage, and potency.
8. Their convenient storage and transportation without altering their properties and without further precautions such as cryoprotectors.

Moreover, in the future, molecular engineering could modify the cargoes of MSC-S and MSC-EV to contain specific miRNAs, proteins, or surface markers to facilitate myogenesis and regeneration (78).

As stated above, bioactive molecules such proteins and miRNA are the effectors of the paracrine action produced by MSC (as well as MSC-S and MSC-EV). Some of these biomolecules can induce myogenesis by direct regulation of myogenic differentiation/myoblast proliferation, property that would enhance SMR acting at the regenerative phase (113). More important, antimicrobial peptides such as LL-37, hepcidin and β defensins inhibit microbial contamination in the injured tissue (therefore the life-threatening fasciitis) (114, 115). Some other molecules induce immunomodulation, revascularization, and microenvironment remodeling (14, 116–118), properties that would enhance SMR acting at all three steps of the process. These bioactive molecules appear to be signals with general effects on muscle cells, endothelial cells, and immune cells as we concern. Their effects improve the endogenous SMR in a comprehensive way which is ideal for treating local damage induced by SBE.

Immunomodulation is a property of MSC-based therapies. MSC express highly immunomodulatory markers such as CD73 (ecto-5- nucleosidase), IL-10, IDO, and other cytokines and interleukins, which inhibits the proliferation of T helper 2 and CTL lymphocytes, the effector function of inflammatory cells such as neutrophils and induce the proliferation of regulatory T cells (119–122). Also, this property induces the switch of



	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1b
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF-α	TNF-β	EGF	IGF-I	Angiogenin
5	Oncostatin M	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck β 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Pos	Pos

FIGURE 6 | Secretoma of hMSC cultured in chemically defined medium. The hMSCs derived from omentum adipose tissue were isolated and cultured in XANADU chemically defined medium (hMSC- line Sev5). The obtained secretome was used for the assay of cytokine array. Both the fresh non-secretoma medium and the secretion-enriched medium of hMSC-line Sev5 were incubated on membranes of the Human Cytokine Antibody Array (Abcam) kit, following the manufacturers' recommended protocol. The red circles indicate the cytokines that are significantly increased in the secretion-enriched XANADU after culture of the MSCs for 5 days compared to the fresh medium. The figure is representative of three independent experiments.

macrophages from pro-inflammatory phenotype M1 to anti-inflammatory phenotype M2 (123), a key event during the inflammatory reaction of SMR. It is clear that during inflammation every type of regeneration is severely hampered, if it is even possible, so the macrophage switch is required for an efficient SMR; however, the role of M2 and M2-like macrophages transcend from the inflammatory reaction to the regenerative phase. They become activators of satellite cells by secreting bioactive molecules, which would mean that MSC-based therapies improve the regeneration by direct paracrine signaling but also by indirect paracrine signaling through macrophage secretions. This fact could imply a longer-lasting effect generated by MSC-S or MSC-EV even when these therapies do not maintain any living cellular medicaments in the damaged place, and then bypassing macrophage effectors. Moreover, immunomodulation is linked with the remodeling phase too by promoting remodeling of the ECM that returns to the regenerative phase of SMR because M2 macrophages need to interact with ECM to promote myoblast chemotaxis and differentiation. Also, angiogenesis is improved by M2 macrophages which made immunomodulation a property involved in all the three overlapping steps of SMR and also a particularly effective property against SBE. Immunomodulation helps to counteract SBE damage by preparing the damaged tissue with an appropriate anti-inflammatory microenvironment, activating precursor cells, recovering the ECM and inducing angiogenesis after microvasculature damage.

Revascularization induction is another key property of MSC-based therapies that could help to improve SMR after SBE. Immunomodulation can help to induce angiogenesis and, it has been well-described as an effect of MSC-based therapies by our group (**Figure 7**). The presence of microvasculature in the place of local damage is necessary primarily due to its transportation implications. The inflammatory reaction implies the removal of necrotic debris and the regeneration phase needs a supply of nutrients during the activation, differentiation, and fusion of satellite cells. Also, the delivery of cytokines needs the presence of blood vessels as "highways". Additionally, endothelial cells induce the regenerative phase by interacting with satellite cells. It is remarkable how the loss of microvasculature is a determinant reason why the SMR is impaired after SBE. Therefore, the positive effect of MSC-based therapies by enhancing angiogenesis and promoting neoangionesis could be one of the most important properties of these kinds of therapies.

Microenvironment remodeling induced by MSC-based therapies could be a decisive property for SMR after SBE. Considering that venom components completely disrupt the

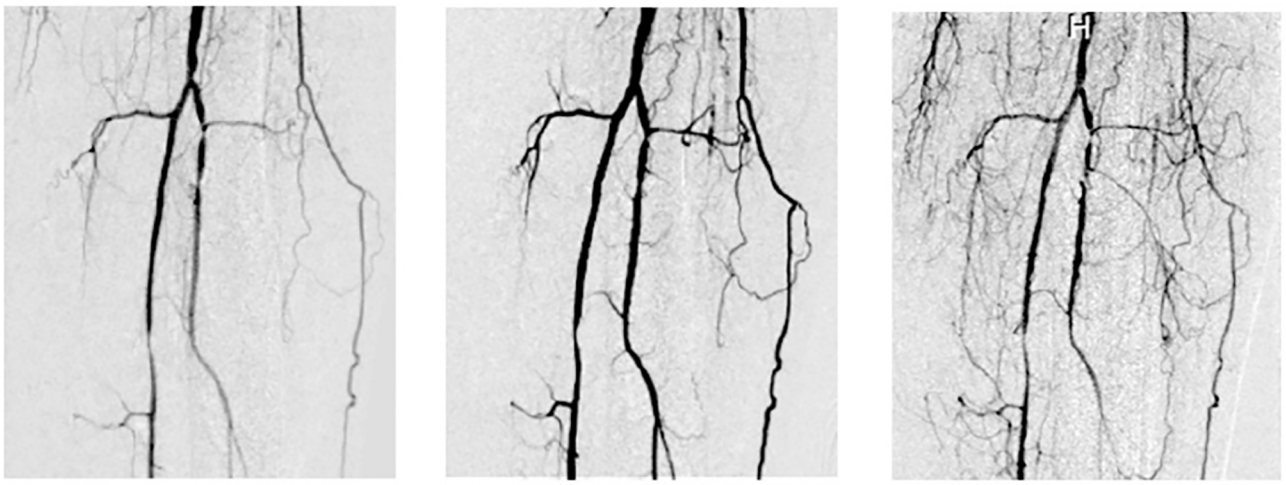


FIGURE 7 | Vasculogenesis after Intrarterial adipose-derived MSC administration (intrarterial 1 x 106 cell/kg). Left: basal, middle: 6 months after and right: 12 months after intrarterial administration in a type 2 diabetic patient with a Grade 6 Rutherford (from Soria B. 2016. La Nueva Biología y sus Aplicaciones Médicas, with permission) (124).

microenvironment of the damaged tissue, the remodeling phase may be impossible to perform without further stimulus. Although this step was enumerated as the last one of the three, the fact that SMR is an overlapping process means that it is necessary not only for a final result but also during the regenerative phase. Satellite cells need scaffolds to guide their divisions after injury and, even when newly myofibers are already differentiated and fused, their growth and maturation depends on ECM. By inducing microenvironment remodeling, MSC-based therapies cover a specially affected consideration after SBE and would improve the SMR till functional myotubes.

EXPERIMENTAL SECTION: ASSESSING THE POTENTIAL OF MSC-BASED THERAPY IN ENVENOMING TREATMENT

Aiming to further demonstrate that MSC's derived products can have a therapeutical effect upon snake envenomings, we performed a pilot study using the secretome of adipose-derived human MSC cultured in XANADU culture media after intramuscular injection of *Bothrops atrox* venom in mice. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Brazilian Council for the Control of Animal Experimentation (CONCEA). The protocol was approved by the Ethics Committee in Animal Use from the Federal University of Minas Gerais (protocol 321/2018-CEUA/UFMG).

As stated above tissue damage, coagulopathies and hyperinflammation restricts the initiation of the regeneration process. **Figure 8** shows preliminary data on the acute effect of secretome administration on the muscle damage. Twenty 18–20 g female Swiss mice were divided in four groups of five animals each, according to the table below (**Figure 8**). As a negative control, a group of animals was injected with 50 µl of PBS by both intramuscular (i.m) and intravenous (i.v) routes (G1). To confirm the muscle damage caused by *Bothrops atrox* venom, a group received 50 µg of it diluted in 50 µl of PBS inoculated intramuscularly in the gastrocnemius muscle and, after 15 minutes, PBS by i.v route (G2). To simulate the treatment with MSC-based therapies we are suggesting in this review, a group of mice was inoculated with 50 µg of *B. atrox* venom diluted in 50 µl of PBS i.m. and, 15 minutes after the venom injection, 50 µl of the secretome was injected in the animal's tail vein (G3). Finally, to assess whether the secretome alone had any effect by its own, another group received PBS i.m and secretome i.v (G4). Blood was collected from all animals by tail vein puncture 3 h, 72 h and one week after the injections. The sampled blood was tested for its creatine kinase (CK) activity, using CK-NAC kit, Labtest, Belo Horizonte. Brazil, as a biomarker of muscle injury. The test was made in duplicates and statistical analysis was performed using one-way ANOVA and Bonferroni post-test in GraphPad Prism software.

The results of this preliminary study (**Figure 8**) showed that animals from the groups inoculated with *B. atrox* venom had elevated CK levels three hours after venom inoculation, indicating muscle damage, as expected. This elevation was statistically significant ($p \leq 0.05$) when compared to the control PBS-PBS. However, the group that received intravenous injection of secretome, 15 minutes after the venom injection, returned to CK levels comparable to controls 72 h after venom inoculation, whereas the group that received venom and intravenous PBS still presented higher levels at this time point. These results indicate a potential beneficial effect of secretome from MSC upon muscle damage caused by snake venom. They can be interpreted as an indication that the MSC secretome seems to reduce the extent of acute myonecrosis and that this

GROUPS	Intramuscular injection (T0)	Intravenous injection (T15)
G1 (PBS+PBS)	PBS	PBS
G2 (BaV+PBS)	*B. atrox* venom (50 µg)	PBS
G3 (BaV+SEC)	*B. atrox* venom (50 µg)	Secretome (50 µl)
G4 (PBS+SEC)	PBS	Secretome (50 µl)

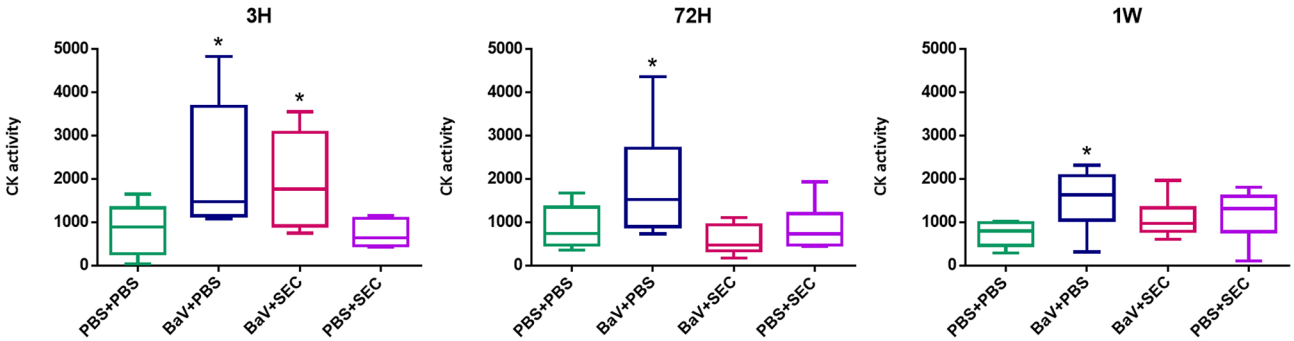


FIGURE 8 | Preliminary assay. Creatine kinase levels of mice sera 3 h, 72 h, and 1 week after inoculation. Group PBS+PBS was injected with 50 µl of PBS by both intramuscular (i.m) and intravenous (i.v) routes. Group BaV+PBS received 50 µg of *Bothrops atrox* venom diluted in 50 µl of PBS and only PBS by i.v route. Group BaV+SEC was inoculated with 50 µg of *B. atrox* venom diluted in 50 µl of PBS i.m. and 50 µl of MSC secretome injected in the animal's tail vein. Group PBS+SEC received PBS i.m and secretome i.v. * = $p \leq 0.05$ when compared to the control PBS-PBS.

may have an impact on reducing the extent of acute muscle damage and, probably, would favor a more successful regenerative response. The intravenous injection of secretome alone appears to have no effect on CK levels. These are still preliminary data that will be better and more deeply explored in the future but are an indication that the use of cell-free MSCs-based therapies have a promising potential as an alternative treatment for local damage caused by SBE. Pre-clinical and clinical trials of these therapies against snake venom are also mandatory before starting the translation process in a pilot study.

In contrast, in certain processes such as Crohn's complex fistulae, diabetic ulcers, and COVID-19, coagulation is impaired, inflammation exacerbated, M1 macrophages activated and releasing IL-1b, IL-12, and NO. The role of MSC (and here of the secretome in SBE) is to transform pathological inflammation into a physiological response (**Figure 9**). We anticipate that this approach will open a new avenue on tissue regeneration.

THE TIME TO ACT IS NOW: MSC-BASED THERAPIES FOR LOCAL DAMAGE INDUCED BY SBE

Advanced Therapy Medicinal Products (ATMP), including cell therapy, needs to meet some requirements to assure safety, efficacy, and quality in their use. While regulations specifics may vary among different locations, Good Manufacture Practices (GMP) are virtually a universal requirement for the production and application of ATMP such as MSCs based therapies (125–127). GMP manufactured cellular medicaments implies the definition of several variables such as cell dose and frequency, donor, cell source, culture process, isolation, and expansion. Related logistic such as storage (cryopreservation), transportation (cold chain), and quality tests are also necessary (96). For this specific case, considering that most SBE affected people reside in poor areas of developing countries, it is relevant to understand the viability of these alternatives.

As stated above, MSC-based therapies appear to be safe (97). Moreover, even when only moderate success or even failures could discourage their application, recent success and deeper comprehension of stem cell biology have provided a rationale pathway to MSC regulatory approval (128). Even for countries without any legislation about ATMP, as most developing countries, robust results based on pre-clinical and clinical trials following GMP would guarantee the application of these therapies. Therefore, the performance of pre-clinical and clinical trials is the key step for the future implementation of these alternatives.

It is a relevant question whether the application of these therapies is actually feasible. As GMP is not an extended practice in developing countries and the requirement of these ATMPs for the treatment of local damage induced by SBE implies the presence of the medication at remote places, it seems nearly impossible to perform them without an exorbitant local economic inversion. Luckily, the existence of cell-free therapies (MSC-S and MSC-EV) may improve this situation critically, by allowing the importation of ATMPs from foreign GMP compliant laboratories with relatively easy and cheap logistics. The absence of rejection problems allowing a status of universal compatibility of these therapies, together with their advantages at freeze-drying, packaging, and transportation (108) made these

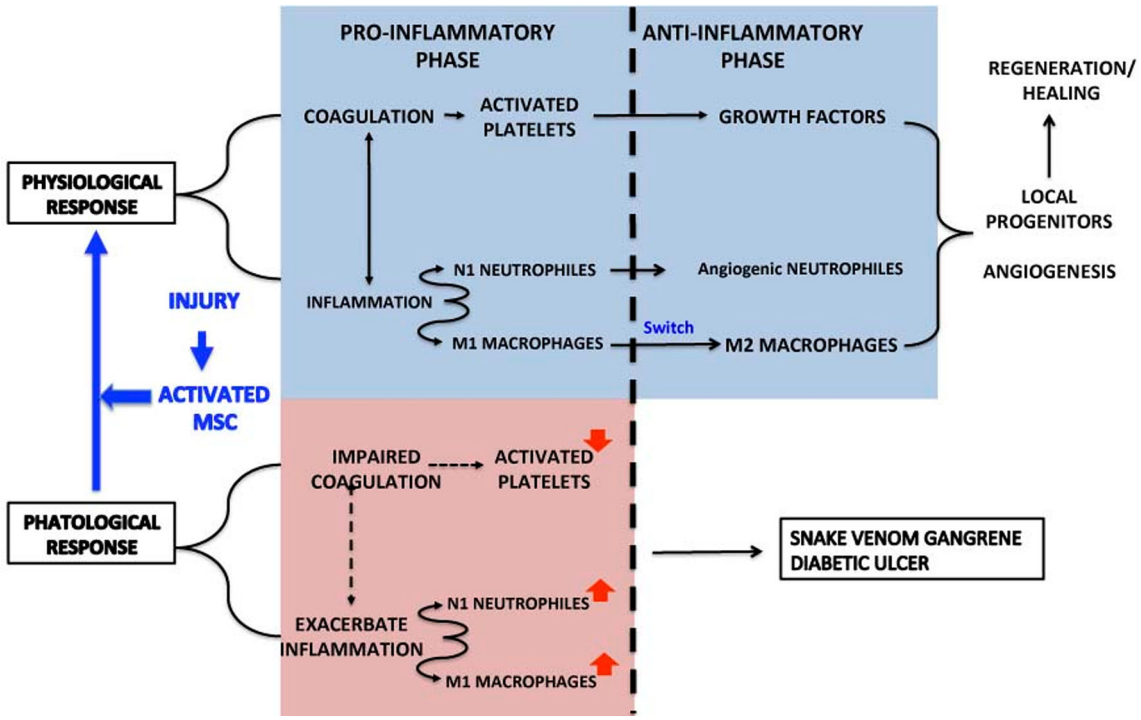


FIGURE 9 | Summarizes the physiological and pathological response in the inflammation-regeneration crossroad. Under physiological circumstances the response to an injury activate platelets, N1 neutrophils and M1 macrophages. Platelets not only promote coagulation to stop hemorrhage but release growth factors that, in cooperation with Angiogenic neutrophils and M2 macrophages, contribute t tissue regeneration through the mobilization of local progenitors, angiogenesis and ECM remodeling.

types of MSC-based therapies in promising prospects, even at precarious situations, because reduces costs and increase the applicability of these ATMPs not only to capital cities but to any level 2 hospital or superior.

Even when legal approval and extended application of MSC-based therapies for local damage caused by SBE is certainly years away from now, the characteristics and properties of MSC-based therapies, especially of cell-free therapies, for enhancing SMR while counteracting SBE effects would help millions of people who may avoid disabilities through the implementation of these treatments.

CONCLUDING REMARKS

In this article, we have proposed an alternative treatment for SBE, a highest priority neglected tropical disease affecting millions of people every year worldwide. Since the most affected people by SBE is part of the economically productive population suffering from bites during, ironically, their working time, giving them a therapy against a considerable risk of disability would impact both at public health and economic levels. Moreover, the WHO has reaffirmed the importance of this kind of proposals by announcing their goal of reducing snakebite disability by 50% before 2030.

While gold-standard treatments against SBE are antivenoms, which are effective against systemic symptoms, they can only neutralize partially local damage. MSC-based therapies could cover the lacking aspects related to SMR after SBE by counteracting the microvasculature and microenvironment damage as well as enhancing SMR after myonecrosis at every step of the regeneration process. Furthermore, MSC-S and MSC-EV could be excellent alternatives of cell-free therapies (with clear logistic advantages for the current case and the support of its preliminary successful results) since the positive effect of MSC-based therapies seems to depend on the secretion of bioactive molecules, property that both therapies share.

In summary, MSC-based therapies appear to be ideal, comprehensive alternatives for enhancing SMR after SBE due to their ability to act at all the three overlapping steps of SMR by paracrine effects, myogenic induction, immunomodulation, revascularization, and microenvironment remodeling, as well as they are likely to counteract the specific damages provoked by SBE. The development and optimization of MSC-based therapies for local damage induced by SBE could improve the quality of life of millions of people, especially people in developing countries, contributing to reduce public health burden and economic impact of this neglected tropical disease.

AUTHOR CONTRIBUTIONS

JT, CC-O, and BS conceived the concept of the paper. ES-C, CP-R, JT, CG-D, and BS wrote the first draft that was circulated, and all

the authors contributed with different sections. CC-O, CG-D, and TCSA designed and executed the preliminary *in vivo* assay. AH prepared secretome samples and answered the second round of revisions. All the authors contributed to the acquisition, analysis, and interpretation of data for the work, revising it critically for important intellectual content, final approval of the version to be published, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CC-O, CG-D, BS, and JT edited and submitted the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Treatment of Chronic Venous Ulcers With Heterologous Fibrin Sealant: A Phase I/II Clinical Trial

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Background: Heterologous fibrin sealant (HFS) consists of a fibrinogen-rich cryoprecipitate extracted from *Bubalus bubalis* buffalo blood and a thrombin-like enzyme purified from *Crotalus durissus terrificus* snake venom. This study evaluated the safety and immunogenicity of HFS, estimated the best dose, and assessed its preliminary efficacy in the treatment of chronic venous ulcers (CVU).

Methods: A phase I/II non-randomized, single-arm clinical trial was performed on 31 participants, accounting for a total of 69 active CVUs. All ulcers were treated with HFS, essential fatty acid, and Unna boot for 12 weeks. The outcomes assessed were: (1) primary safety, immunogenicity analyses, and confirmation of the lowest safe dose; (2) secondary promising efficacy by analyzing the healing process. Immunogenicity was evaluated using the serum-neutralizing (IgM and IgG) and non-neutralizing (IgA and IgE) antibody techniques against the product. The immuno-detection of IgE class antibodies was assessed using dot-blot assay before and at the end of treatment. Positive samples on dot-blot assays were subsequently analyzed by western blotting to verify the results.

Results: No severe systemic adverse events related to the use of HFS were observed. Local adverse events potentially related to treatment include ulcer pain (52%), peri-ulcer maceration (16%), peri-ulcer pruritus (12%), critical colonization (8%), peri-ulcer eczema (4%), the opening of new ulcers (4%), and increased ulcerated area 4%). Neutralizing and non-neutralizing antibodies did not show significant deviations at any of the evaluated time points. Blot assays showed that all patients presented negative immunological reactions, either before or after treatment, with the thrombin-like enzyme component. In addition, two participants showed a positive immunological reaction to the cryoprecipitate

component, while another two were positive before and during treatment. Regarding the secondary outcomes of preliminary efficacy, a total healing and significant reduction of the area was observed in 47.5 and 22%, respectively. A qualitative improvement was observed in the wound beds of unhealed ulcers.

Conclusions: The investigational HFS bioproduct proved to be safe and non-immunogenic with a good preliminary efficacy for the treatment of CVU, according to the protocol and doses proposed. A multicentric phase III clinical trial will be necessary to verify these findings.

Keywords: varicose ulcer, biological dressings, fibrin tissue adhesive, fibrin glue, fibrin sealant

INTRODUCTION

Chronic venous ulcers (CVUs) are responsible for approximately 70% of chronic ulcers of the lower limbs. They affect a large portion of the adult population, causing significant economic and social impacts and reducing the quality of life (1). The main clinical treatment used for the healing of CVUs is compression therapy, which diminishes the effects of chronic venous hypertension on the macro- and microcirculation (2). Furthermore, local treatment with compressive therapy is also essential, since it promotes the preparation of the ulcer bed, contributing favorably to the evolution of the healing process (3).

The pharmaceutical industry is continuously researching and developing new products for the treatment of CVUs. Thus, research studies range from the simplest coverage, namely solutions for hygiene and antisepsis, to the most complex types of dressings, which actively interfere in the various stages of healing (4). Among several substances used, fibrin sealant acts as a reference for topical use since it is a compatible biological and biodegradable material whose activities reproduce the final cascade blood coagulation stages, which are composed of fibrinogen and thrombin (5). In the presence of small amounts of calcium and factor XIII, thrombin converts fibrinogen into insoluble fibrin, forming a stable fibrin network. Commercial fibrin sealants are also supplemented with aprotinin, an antifibrinolytic agent, to slow down clot fibrinolysis (6, 7).

Fibrin sealant plays an important role in hemostasis and the healing process, given that the network of fibrin and the matrix of proteins associated with it not only promotes angiogenesis, collagen synthesis, and wound contraction, but also contributes to accelerated re-epithelization (8–10). By remaining in the ulcer bed for at least four days and totally degrading within 10 days, fibrin sealant is an excellent scaffold for incorporating and facilitating cell growth and the release of growth factors and antibiotics (11, 12).

Currently, there are three basic types of fibrin sealants: (1) autologous (obtained from the individual's own thrombin and cryoprecipitate), (2) homologous (thrombin and cryoprecipitate obtained from "pool" of human plasma donors), and (3) heterologous (obtained from animal compounds). The amount of autologous fibrin sealant obtained from the individual's own thrombin and cryoprecipitate is low, making it unfeasible for its use. Similarly, homologous fibrin sealants can promote the transmission of infectious or parasitic diseases, despite the precautions taken by manufacturers to reduce the risk of transmitting viruses (13, 14). The use of commercial sealants in the treatment of UVs, although very useful in healing, has the inconvenience of having a high cost, due to the prolonged time of use and the large amount that must be applied to cover each wound.

Since the 1990s, the Center for the Study of Venoms and Venomous Animals (CEVAP) in São Paulo State University (UNESP) (São Paulo, Brazil) has been researching and developing a new fibrin sealant. Through *in vitro* and *in vivo* studies using different applications and species, the heterologous fibrin sealant (HFS), which comprised of a fibrinogen-rich cryoprecipitate extracted from buffaloes blood and a thrombin-like enzyme (a serine protease named gyroxin) purified from the venom of South American rattlesnakes, has been standardized (15, 16). The lack of human products in the composition of this fibrin sealant eliminates the risk of transmission of infectious and parasitic diseases carried by human blood (15, 17). In addition to its sealant, hemostatic, and adhesive functions, this new bioproduct also provides an excellent scaffold for stem cells (18, 19) and can be used as a drug delivery system (20, 21).

Previous HFS studies have demonstrated its low toxicity and non-mutagenicity (19), combined with preclinical studies in animals (15, 17) and preliminary studies in humans (22, 23), encouraging the use of this bioproduct in phase I/II clinical trials. In light of these favorable results, the primary objective of the present study was to evaluate the safety of the bioproduct: (i) by clinical evolution, laboratory exam results, and the existence of local and systemic adverse events; (ii) by the analysis of its immunogenicity; (iii) to estimate the lowest safe dose. As secondary objectives, this study evaluated the preliminary efficacy of the bioproduct by analyzing the evolution of the healing process and wound epithelization, and evaluating its capacity to diminish the ulcerated area and prepare the wound bed.

PATIENTS AND METHODS

Study Design and Regulatory Agencies

A phase I/II, non-randomized, single-arm clinical trial on participants with active CVUs was carried out between September 2015 and March 2017.

The clinical protocol was approved in advance by the National Commission on Ethics in Research (CONEP) (Certificate of Presentation of Ethical Appreciation no. 37410814.4.0000.5411) (v8, approved on 12/16/2014) and the National Health Surveillance Agency (ANVISA), whose Consent Record of Sealant Study II was approved on 06/05/2015 (no. 0498468154) (proc. no. 25351010938201571). This trial was registered on 11/11/2014 in the Brazilian Clinical Trials Registry (ReBEC) at http://www.ensaiosclinicos.gov.br/. The first participant was enrolled on 09/09/2015 (Universal Trial Number (UTN) U1111-1163-9824 and register number RBR-9mbdj3). The public access URL is available at http://www.ensaiosclinicos.gov.br/rg/RBR-9j7qqr/. The participants originated from the Chronic Ulcers Ambulatory Unit at the Dermatology Service of the Hospital of Clinics at the Botucatu Medical School, São Paulo State University (UNESP) (São Paulo, Brazil) and were treated at the Clinical Research Unit (UPECLIN) of the same university. All participants consented to participate by signing the terms of free and informed consent (TFIC).

The data from all the participants were collected by five physician researchers (dermatologists) (LPFA, SRCS, MRCS, ABCOL, and GRH), and the data were registered and stored in an electronic formulary (Eletronic Case Report Form (eCRF)) developed specifically for this study (http://www.crf.fmb.unesp.br/selante/login.php?accesscheck=%2Fselante%2Findex.php) by the support team at UPECLIN. eCRF generated spreadsheets in Excel format, which were used for statistical analysis.

Eligibility Criteria

The participants who met the eligibility criteria for the study were treated with HFS, essential fatty acid, and Unna boot for 84 days. Thirty-one participants, of both sexes, with chronic ulcers of venous etiology in the lower limbs were selected for the study, provided they met the inclusion and exclusion criteria described below.

Inclusion Criteria

a. Sign the terms of free and informed consent (TFIC);
b. Be at least 18 years of age, for both sexes;
c. Have chronic venous disease with CVU evidenced by one or more of the following signs:
    i. Hyperpigmentation of the distal third of a lower limb;
    ii. Stasis eczema;
    iii. Lipodermatosclerosis;
    iv. Varicose veins.
d. Have at least one ulcer, whose evolution time is between a minimum of 6 weeks and a maximum of 5 years;
e. Present a sum of ulcer areas of both limbs between 2 and 60 cm^2;
f. Have at least one ulcer with an area greater than 2 cm^2;
g. The following medications were not taken within the two weeks prior to the initiation of treatment:
    i. Venotonics;
    ii. Pentoxifylline;
    iii. Fibrinolytic drugs.
h. Be available to attend the UPECLIN once per week to complete the treatment.

Exclusion Criteria

a. Have ulcers in the lower limbs from other etiologies (hematological, neoplastic, infectious, or other causes);
b. Take anticoagulants;
c. Have an infected ulcer, that is, associated with erysipelas, cellulitis, or lymphangitis
d. Have an ulcer with critical colonization, that is, a large quantity of exudation and/or a fetid odor and/or bed staining/wound coloration yellowish and/or greenish and/or opaque red and/or tissue of friable granulation.
e. Present necrosis in the ulcer bed;
f. Have an ulcer with a devitalized tissue covering the entire bed;
g. Have venous ulcer associated with peripheral arterial disease characterized as an ankle brachial index (ABI) < 0.9;
h. Being unable or unwilling to adhere to compressive treatment of the lower limb for seven days;
i. Have a prior history of allergy to Unna boot treatment;
j. Have a prior history of allergy to treatment with essential fatty acid;
k. Have suspicion or confirmation of pregnancy;
l. Have coagulogram values outside the limits of normality (TTPA >1.25 and prothrombin activity time <70% or >100%)
m. Be a woman of fertile age not utilizing secure contraceptive methods.

Observations:

1. Participants with an infected ulcer, with critical colonization, with the presence of necrosis, and with devitalized tissue covering the entire bed could be included after the adequate treatment of these conditions.
2. Women of fertile age who do not use secure contraceptive methods could be included if they agreed to the use of at least one reliable contraceptive method.
3. Participants could only be included once. Those who participated in the study and had their ulcers healed were not eligible for this study again if there were recurrences.

Discontinuation Criteria

After inclusion in the study, participation was discontinued if the individual:

a. Removed or withdrew from the terms of consent (TFIC);
b. Initiated the use of an anticoagulant during the study;
c. Presented coagulogram values outside the limits of normality (TTPA >1.25 and prothrombin activation time <70 or >100%) with clinical significance.
d. Presented infection associated with ulcer(s) (erysipelas, cellulitis, or lymphangitis) during follow-up. In these cases,

the participant was included in the routine treatment of the dermatology service.

e. Had critical colonization during the follow-up, according to the researcher's discretion. The only procedure allowed for the treatment of colonization was superficial surgical debridement, without local anesthetic, for a maximum of three times during the study and before the dressing was applied. If there was a need for topical and/or systemic antibiotics, participation was terminated;
f. Utilized other treatments not recommended in the protocol;
g. Removed the Unna boot within less than five days after its application;
h. Became pregnant;
i. Had distal pulses not palpable and ABI < 0.9;
j. Presented a severe adverse event, according to the researcher's discretion;
k. Presented clinically significant local adverse events, such as severe pain, eczema, enlarged area, or the opening of a new ulcer, at the researcher's discretion.

Production of Investigational Product (HFS)

Purification of Thrombin Like-Enzyme

CEVAP maintains a serpentarium for the breeding and milking of specimens with authorization and registration as a scientific breeder for research purposes at the Brazilian Institute of the Environment and Natural Resources (IBAMA) (protocol number 02001.005670/90–77), in addition to having authorization for the management of wild fauna (no. 3507.7263/2012-SP). Standard operational protocols (SOPs) are followed rigidly in environments where *Crotalus durissus terrificus* snakes are housed for venom production, according to the international good management practices to ensure the quality and purity required for the production of biopharmaceuticals (17, 24). Further information on CEVAP is provided at https://youtu.be/CPcs4ity-Uw.

First, the venom was extracted and filtered. Then, the protein dosage was evaluated, followed by lyophilization and storage in a refrigerator between +4 and +8°C. Subsequently, the venom was subjected to fractionation *via* high-performance liquid chromatography (HPLC). Lastly, the purity of the thrombin-like enzyme (serine protease, gyroxin) component was evaluated using sequencing and mass spectrometry as described by Barros et al. (25). Further information on HFS is provided at https://youtu.be/y6ho6M0amA8.

Extraction and Processing of Cryoprecipitate

Bubalus bubalis buffaloes are ideal for the large-scale production of cryoprecipitate (11, 15). Specimens housed at Céu Azul Farm, located in the municipality of Pereiras (São Paulo, Brazil) were kindly provided by Mr. Aristides Pavan. CEVAP researchers carried out sanitary management practices on a monthly basis, including vaccinations, deworming, isolation and quarantine when necessary, the protection of animals against vectors of infectious diseases, diagnostic serological tests against zoonoses, and an annual evaluation of the hypersensitivity test against tuberculosis (Mantoux or PPD), in addition to clinical examinations performed by a veterinarian. These actions are recommended by the Secretary of Agriculture and Supply for the State of São Paulo by the Department of Animal Health of the Secretary of Agricultural Defense of the Ministry of Agriculture, Livestock, and Supply (MAPA), Brazil, and by the World Health Organization (WHO), and were conducted under the coordination of the Guilherme Shin Iwamoto Haga (CRMV/SP 19621) veterinarian. All animals were microchipped in order to maintain the biosafety and traceability of the extracted cryoprecipitate. The academic bases for processing and traceability were published by Pontes et al. (26) and Ferreira Jr. et al. (27). Details of the formulation are protected by patents no. BR 10 2014 011432 7 and BR 10 2014 011436-0 (28, 29).

Formulation of the Product

Each dose of the medication for topical use was packaged and distributed in three bottles (**Figure 1A**): (1) diluent vial containing 0.6 mL of calcium chloride (white stripe); (2) component 1 vial containing 0.4 mL of the thrombin-like enzyme (red stripe); (3) component vial 2 containing 1 mL of cryoprecipitate (black stripe). All vials were stored in a freezer at −20°C until use. The composition of the product is described in detail in patents no. BR 10 2014 011432 7 and BR 10 2014 011436-0 (28, 29).

Application of Product in Ulcers

The product was applied to each participant once a week for 12 weeks. Prior to application, packages containing the three vials were thawed by removing from the freezer 15 min before application. After cleaning the wounds with 0.9% physiological saline, the product was applied to the ulcerated areas.

The diluent vial component (white stripe, 0.6 mL) was mixed into a fraction 1 vial (red stripe, 0.4 mL) and taken up in a 1-mL syringe. The fraction 2 vial (black stripe: 1 mL) was used to fill a second 1-mL syringe. Both syringes were connected to a duo needle-free valve extension line (Y-connector) (**Figure 1B**). Then, the contents of the two syringes were applied concurrently over the entire bed of the ulcer, mixing the two HFS components. Total polymerization was expected to occur for approximately 3–5 min, ultimately appearing as a colorless gel (**Figure 1C**). Afterwards, gauzes containing essential fatty acids were placed in a sufficient quantity to cover the ulcerated surface, and the dressing was finished with an Unna boot bandaging half and half, from the foot to immediately below the knee (**Figure 1D**). Participant were instructed to not remove the dressing and to wear it for seven days until their return visit to the clinic.

Outcomes

The outcomes were classified into primary and secondary outcomes.

Primary Outcomes

These outcomes were related to the safety and confirmation of the proposed dose.

- Evaluation of local adverse events (AEs): pain, itching, maceration of the ulcer edge, eczema, local infection, critical

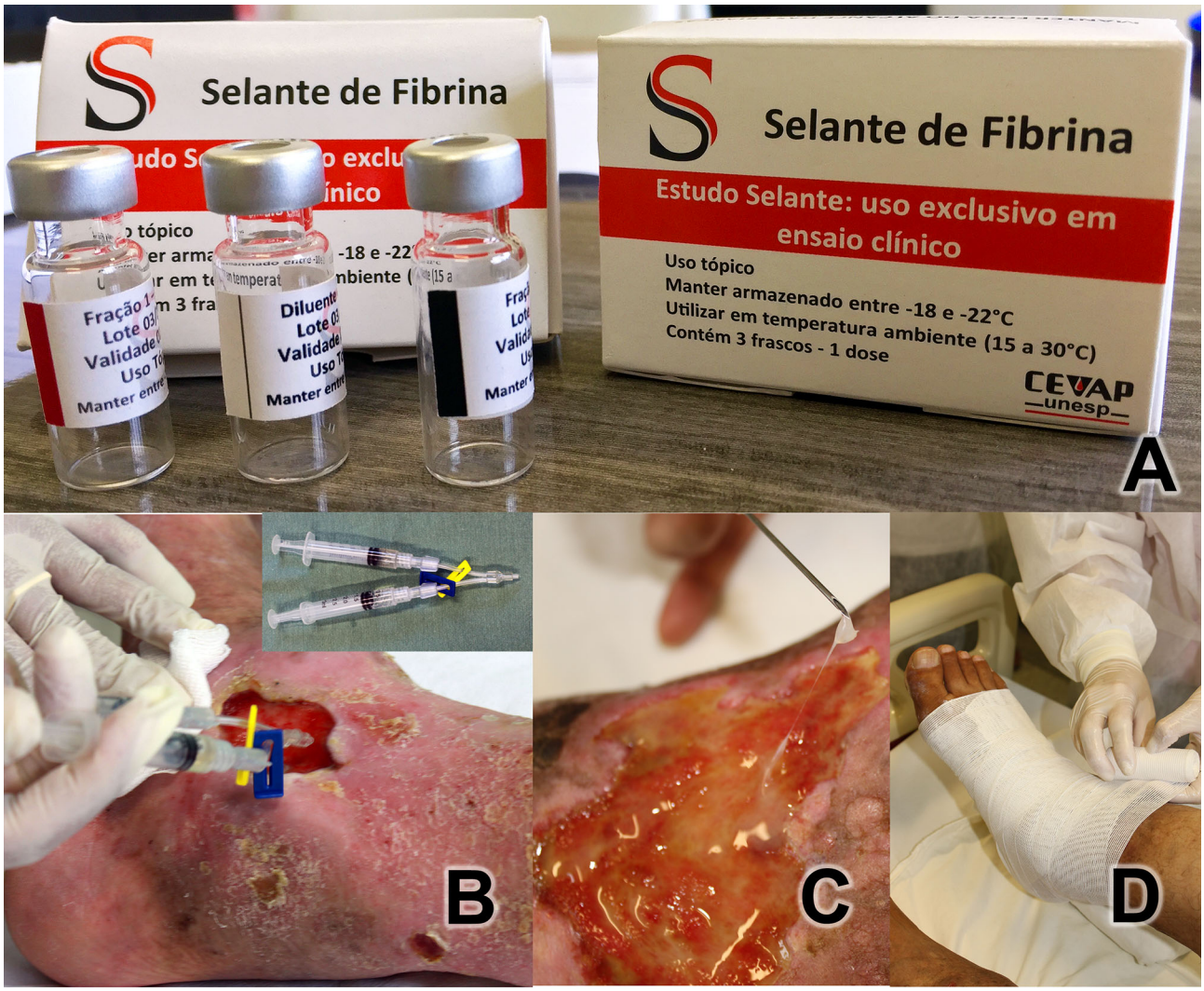

FIGURE 1 | **(A)** Heterologous Fibrin Sealant Package with the three bottles. Diluent vial (white stripe) containing 0.6 mL of calcium chloride; Fraction 1 vial (red stripe) containing 0.4 mL of serine protease extracted from the venom of *Crotalus durissus terrificus*; Fraction 2 vial (black stripe) containing 1 mL of cryoprecipitate rich in fibrinogen and coagulation factors extracted from buffaloes. **(B)** Application investigational product on the ulcerated bed. Y-connector using two syringes (in detail) with 1 mL of components each one. **(C)** Detail of fibrin adhered to the ulcer. **(D)** After covering the ulcer bed with gauze soaked in essential fatty acids, the Unna boot was applied.

colonization, increased ulcer extension, and opening of a new ulcer.

- Evaluation of systemic AEs: potential blood clotting disorders in addition to clinical systemic alterations.
- Evaluation of laboratory alterations: Complete blood count, erythrocyte sedimentation rate (ESR), prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen and C-reactive protein (CRP) were determined according to the protocol proposed and approved by the Ethics Commission.
- The minimum necessary dose of the investigational product to cover a maximum ulcer surface area of 60 cm^2 per participant was evaluated and confirmed, as proposed in the clinical protocol.
- Immunogenicity: performed using nephelometry, dot blot, and western blotting. For this purpose, blood was collected from all participants; the serum was aliquoted and stored at −80°C until the exams were performed.

The AEs were classified according to the guidelines of the International Conference for Harmonization (ICH) (30), which considers AE to be any undesired clinical occurrence in a clinical research participant, who receives a pharmaceutical product that does not necessarily have a causal relationship with this treatment. It is worth mentioning that AE registration was performed over 12 weeks of follow-up. Thus, an AE can be any unfavorable and unintended sign (including an abnormal laboratory finding, symptoms, or disease temporally associated

with the use of a product under investigation, considered to be related or not to it). Pre-existing conditions that worsened during the study were also reported as AEs. Any changes in laboratory tests were considered systemic AE as well as systemic changes detected by clinical history (primary outcomes).

The causal relationship between the investigational product and AE was determined by the researchers' judgment at the time of clinical care and classified as:

- Probable: The temporal relationship is well defined and there is no other possible causal factor. In this case, there is an almost certain relationship between the reaction and the medication.
- Possible: The temporal relationship between the event and medication administration is well defined, but there is another possible causal factor.
- Remote: The relationship with the drug is unlikely, but it cannot be definitively ruled out.
- Not related: The temporal relationship between the event and the ingestion or administration of the medication is non-existent or doubtful or, yet, there is another factor that can respond as a causal factor of the reaction.

Nephelometry, Dot Blot, and Western Blotting

Using nephelometry, the total IgA, IgE, IgG, and IgM types were dosed at zero time (T0 – screening), that is, before the initiation of treatment, and again at T1 (30 days), T2 (60 days) after the start of treatment, and T3 (90 days) at the end of treatment (31). Furthermore, the antibodies of the specific IgE class, which would have been produced by the patients against the cryoprecipitate and/or against the thrombin-like enzyme (serine protease), the main sealant components, were investigated in the serum before the beginning and at the end of the treatment. Thus, a potential turning point and sensitization of participants to the investigational product was sought. To this end, the dot blot assay was performed according to the immunodetection procedures proposed by Santos et al. (32). Samples that were positive in dot blot tests were subsequently subjected to western blotting tests for structural identification of the allergen responsible for sensitization (32). In the dot blot assay, 4 μL of each component (cryoprecipitate or thrombin-like enzyme – serine protease) was immobilized on a 2 × 2 cm nitrocellulose membrane. Once immobilized, they were incubated with the participants' serum. In this case, those who eventually developed antibodies and were therefore sensitized to the evaluated components will contain specific antibodies in their blood that will appear on the mentioned nitrocellulose membrane. From this stage, the use of secondary anti-IgE antibody (Abcam) followed by the streptavidin conjugate (HRP) (Abcam) was able to signal which patients were sensitized and developed immunogenicity against the components of the sealant.

Secondary Outcomes

These were evaluated according to the preliminary effectiveness of the product by monitoring the evolution of the ulcerated area (diminished area and complete healing or reepithelization) of the evolutionary ulcer-bed characteristics (decrease or absence of devitalized tissues in the ulcer bed during treatment) and of diminution in the exudation quantity.

Statistical Considerations

The sample size was estimated considering examples of phase I/II clinical studies, which aim to evaluate safety and confirm the minimum application dose required. The failure to carry out this study on 10 participants, as recommended in the phase I trials, is justified by the fact that the efficacy outcome is one of the targets, although there are, to date, no data on safety, nor confirmation of an adequate dose for the product under investigation.

For statistical analysis, descriptive summaries and confidence intervals were calculated, and McNemar's test was applied to analyze the evolution of ulcers before and at the end of treatment. Categorical variables are presented as percentages. Continuous variables were assessed for normality using the Shapiro–Wilk test and presented as the means and standard deviation (SD) or the median and quartiles (p25–p75), if indicated.

A significance level of 5% was considered for the analysis, performed using the software SPSS v21.0 (33). Product safety was assessed by observing local and systemic adverse events, in addition to clinical and laboratory parameters.

RESULTS

General Aspects

From September 2015 to March 2017, 31 of the 40 participants evaluated met the eligibility criteria and were included, accounting for a total of 69 active CVUs. Sixteen were male (51.6%); the mean age was 65.9 (± 14.0) years; and the main antecedent was systemic arterial hypertension (61.3%), as shown in **Tables 1** and **2**.

Figure 2 shows the study flowchart. Six participants were discontinued from the study (19.3%) for the following reasons: critical colonization of the ulcer in two participants (6.45%), opening of new ulcers in two participants (6.45%), an increase in the ulcerated area in one participant (3.22%), and loss of follow-up in one participant (3.22%), although his ulcer was practically healed. Therefore, 25 participants completed 12 weeks of follow-up.

The clinical characteristics of all evaluated ulcers are described in **Table 3**. A similar distribution was observed between the limbs and preferential location in the medial region of the leg, with a mean active ulcer time of 1.8 years (± 2.0).

Primary Outcomes

Safety—Adverse Events

Regarding the AEs of the 31 participants included in the study, 85 were observed, of which 61 were considered local and 24 were systemic. Nineteen participants (61.3%) had more than one EA, and nine participants (29%) also had local and systemic EAs. Most AEs were mild or moderate, with only one having pain classified as severe AE. Of the total local AEs, 25 [25/61 (40.1%)]

TABLE 1 | General characteristics of the 31 participants included in the study at baseline.

Identification (initials and N° in the study)	Gender	age	BMI	SAH	DM	DVT	Total number of ulcers	Initial area (cm^2)
ALC16	M	37	34.6	No	No	Yes	3	44.37
AMB03	M	53	26.7	No	No	Yes	2	14.05
BP26	M	52	29.1	No	No	Yes	5	21.99
C-C36	F	67	32.0	Yes	Yes	No	1	6.36
CA23	M	44	27.3	No	No	No	1	17.39
CL12	F	64	32.8	Yes	No	No	2	11.04
CRS33	M	59	36.8	No	No	Yes	5	19.77
E-S01	F	78	24.7	Yes	Yes	No	2	17.27
EEV13	F	83	16.9	No	No	No	2	3.99
FAS05	M	63	26.4	Yes	Yes	Yes	3	9.54
HFS28	F	74	30.3	Yes	No	No	2	47.42
IMS35	F	89	23.9	Yes	No	No	4	28.77
J-M39	M	66	28.5	No	No	No	1	2.01
J-S42	M	86	33.5	Yes	No	Yes	1	51.83
JPF18	M	55	21.8	No	No	Yes	1	11.19
JSF29	M	84	27.8	Yes	No	No	1	15.12
LOF22	M	56	32.4	No	Yes	No	1	14.87
LS15	M	78	29.8	Yes	Yes	No	2	15.54
MIV14	F	70	38.3	Yes	Yes	No	6	38.50
MPG17	F	78	29.7	Yes	Yes	No	3	58.45
NMA30	F	78	30.9	Yes	No	No	2	4.03
NS07	F	73	41.2	Yes	Yes	No	2	52.90
PLB27	F	42	52.2	No	No	No	2	11.37
SVR04	F	65	31.4	Yes	No	No	2	20.66
TAA10	F	87	21.2	Yes	No	No	4	9.61
VAR21	M	63	31.5	Yes	No	No	1	60.00
VAT34	F	51	40.4	No	No	Yes	1	2.93
VD19	M	49	25.1	No	No	No	1	36.43
VDR32	M	68	25.4	Yes	No	No	2	41.15
VLA11	F	75	48.2	Yes	Yes	No	2	7.60
WN37	M	58	26.1	Yes	No	No	2	4.40

M, male; F, female; BMI, body mass index; DM, diabetes mellitus; SAH, systemic arterial hypertension; DVT, deep vein thrombosis. Identification (initials and No. in the study).

TABLE 2 | Baseline clinical characteristics of the 31 participants included in the study and the 25 participants who were followed for 12 weeks.

Variables	All participants included (n=31)	Participants who completed the study (n=25)
Mean age (± SD) in years	65.9 (14.0)	66.0 (14.8)
Mean BMI (± SD)	30.9 (7.5)	30.9 (6.0)
Female n (%)	15 (48.4)	11 (44.0)
Male n (%)	16 (51.6)	14 (56.0)
SAH n (%)	19 (61.3)	15 (60.0)
DM n (%)	09 (29.0)	09 (36.0)
Smoking history n (%)	03 (9.7)	2.0 (8.0)
History of lower limb DVT n (%)	08 (25.8)	08 (32.0)

BMI, body mass index; SAH, systemic arterial hypertension; DM, diabetes mellitus; DVT, deep vein thrombosis.

were classified as possibly related to the investigational product (**Table 4**).

There were 24 systemic AEs detected by laboratory changes, including eosinophilia (one participant), or by medical history, such as arterial hypertension (two participants), sinusitis (two participants), lower back pain (two participants), epistaxis (four events in the same participant), cough (two events in the same participant), epidermoid cyst infection (one participant), acute kidney injury (one participant), headache (one participant), facial paralysis (one participant), diffuse eczema (one participant and related to concomitant moisturizing lotion), cold (one participant), nausea and vomiting (one participant), hypoglycemia (one participant and related to concomitant hypoglycemic medication), chest pain (one participant), and dry mouth (one participant). None were attributable to the product.

There was no statistically significant difference between the averages before and throughout the treatment of subsidiary laboratory tests, such as complete blood count, ESR, VT, TPT, fibrinogen, and CRP (**Supplementary Material 1**).

Safety—Immunogenicity

The serum levels of IgM, IgG, IgA, and total IgE in 30 participants (the material collected from one participant was mislaid) were determined before, during, and at the end of treatment, the results of which are described in **Tables 5**–**8**.

The serum levels of IgM and total IgA were unchanged throughout the treatment with the product. As to the IgG levels, only four participants [EEV13, FAS05, HFS28, and WN37 (**Table 6**)] presented normal levels before treatment.

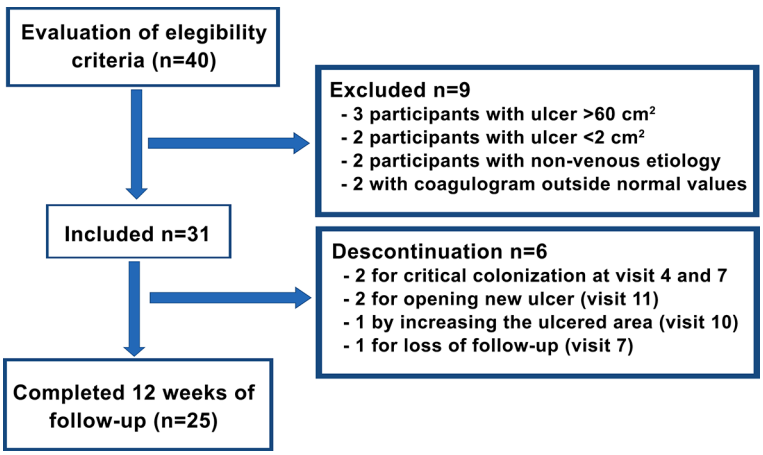

FIGURE 2 | Study flowchart.

TABLE 3 | Distribution of ulcers from all participants included and from those who completed the study according to their clinical characteristics at baseline.

Variables	All participants included (n=31 participants with 69 ulcers)	Participants who completed the study (n=25 participants with 59 ulcers)
Affected lower limb		
-Left	35 (50.7%)	30 (50.8)
-Right	34 (49.3%)	29 (49.2)
Localization		
- Medial region of the leg	38 (55.1%)	35 (59.3)
- Lateral region of the leg	12 (17.4%)	12 (20.3)
- Anterior region of the leg	4 (5.8%)	4 (6.8)
- Posterior region of the leg	1 (1.4%)	0 (0.0)
- Dorsal aspect of the foot	4 (5.8%)	2 (3.4)
- Medial aspect of the foot	6 (8.7%)	4 (6.8)
- Lateral aspect of the foot	5 (7.2%)	2 (3.4)
Active ulcer (years) median (p25-p75)	0.7 (0.3 – 3)	0.7 (0.3 – 3)
Pain (VAS) median (p25-p75)	3.0 (0.0 – 7.0)	3.0 (0.0 – 6.0)
Ulcer area (cm^2) median (p25-p75)	4.1 (2.0 – 12.7)	4.1 (2.0 – 12.6)

VAS, visual analog scale.

Most of the participants showed slight changes in the levels of this immunoglobulin across the treatment, sometimes decreasing, sometimes increasing. As for IgE (**Table 8**), during treatment, the levels were significantly elevated in eight participants (ALC16, C-C36, IMS35, JPF18, MPG17, NS07, PBL27, and WN37), although these already showed high pretreatment levels.

The results of the dot blot test are displayed in **Figure 3**. The participants' sera before (screening) and during treatment with the investigational product were challenged against the cryoprecipitate and thrombin-like enzyme, the main components of the bioproduct. None of the patients had a positive reaction neither before nor at the end of treatment to thrombin-like enzyme. In relation to cryoprecipitate, four participants were positive at the conclusion of treatment. Of these four, two were negative before and positive at the end, and two were already positive and remained positive at the end of the treatment.

To evaluate the safety of the product in relation to immunogenicity, we compared the data observed in nephelometry and dot blot with the clinical aspects of local AEs of greater relevance, such as enlargement of the ulcerated area, the opening of new ulcers, and peri-ulcer eczema. The opening of a new ulcer was not found to be related to the product, given that of the four participants who had this AE, only one presented a reaction in the dot blot to cryoprecipitate on the 10th visit. An increase in the ulcerated area occurred in three participants, and this AE was also unrelated to the use of the bioproduct, as none of the participants showed a change in the dot blot reaction. The appearance of peri-ulcer eczema occurred in two participants and possibly was not related to the bioproduct, since only one of the participants presented a positive dot blot reaction before treatment, thus remaining the same at the end. Two participants had a positive reaction on the dot blot at the 10th visit (E-S and VLA (**Figure 3**), although both had a good evolution of the healing process with a significant diminution of the ulcerated area or total healing.

Safety—Dose Confirmation

The minimum dose required to cover a maximum ulcer surface area of 60 cm^2 per participant was 0.1mL/cm^2. On average, 1.67 (SD 0.13) sealant kits were used per participant per dressing change during the follow-up period (a minimum of 1 kit and a maximum of 3 kits per participant per dressing change).

Secondary Outcomes

Preliminary Efficacy—Evaluation of the Healing Process

There was healing of 28 out of the 59 ulcers that were followed up until the final visit (47.5%). **Figure 4** displays the survival curve

TABLE 4 | List of local adverse events possibly related to the product.

EA possibly related to the product	Number of participants (n=31)n (%)	Number of AE* possibly related to the product (n=25) n (%)	Intensity of AE		
			Mildn (%)	Moderaten (%)	Severen (%)
Ulcer pain	08 (25.8)	13 (52.0)	01 (7.7)	11 (84.6)	01 (7.7)
Periulcerous maceration	04 (12.9)	04 (16.0)	03 (75.0)	01 (25.0)	0 (0.0)
Periulcerous itching	03 (9.7)	03 (12.0)	02 (66.6)	01 (33.3)	0 (0.0)
Critical colonization	02 (6.4)	02 (8.0)	01 (50.0)	01 (50.0)	0 (0.0)
Periulcerous eczema	01 (3.2)	01 (4.0)	0 (0.0)	01 (100.0)	0 (0.0)
Opening of a new ulcer	01 (3.2)	01 (4.0)	01 (100.0)	0 (0.0)	0 (0.0)
Increase in the ulcerated area	01 (3.2)	01 (4.0)	01 (100.0)	0 (0.0)	0 (0.0)

**The number of adverse events (AE) may differ from the number of participants with AE, as the same participant can have more than one episode of the same EA.*

TABLE 5 | Evaluation of the plasma concentration of Immunoglobulin M (IgM) in mg/dL of 30 participants[#] at T0, T1, T2 and T3.

Participants	T0	T1	T2	T3
ALC16	118	103	113	118
AMB03	128	110	130	*
BP26	55	50	42	51
C-A23	95	84	96	*
C-C36	89	91	87	88
CL12	74	94	83	69
CRS33	94	107	111	109
E-S01	106	86	86	100
EEV13	124	133	128	119
FAS05	40	38	42	**
HFS28	155	147	**	**
IMS35	58	55	55	54
J-S42	60	49	60	50
JPF18	109	107	84	80
JSF29	53	53	**	**
LS15	65	67	57	60
LOF29	39	41	42	42
MIV14	60	66	63	60
MPG17	110	103	104	100
NS07	84	80	93	85
NMA30	172	165	158	**
PBL27	116	106	111	**
SVR04	59	62	59	**
TAA10	83	84	76	82
VD10	173	173	176	168
VAR21	156	138	151	168
VAT34	84	85	89	80
VDR32	83	82	67	68
VLA11	64	79	79	72
WN37	73	89	90	*

T0 before treatment (screening); T1 (30 days), T2 (60 days) during treatment, and T3 (90 days) at the end of treatment.
**Participants who did not collected material for immunogenicity analyses at that visit.*
***Discontinued participants.*
IgM references values for adult individuals, 50 to 300 mg/dL.
[#]Mislaid of material collected for immunogenicity analyzes from all visits by one participant.

TABLE 6 | Evaluation of the plasma concentration of Immunoglobulin G (IgG) in mg/dL of 30 participants[#] at T0, T1, T2, and T3.

Participants	T0	T1	T2	T3
ALC16	1.289	1.234	1.199	1.227
AMB03	1.254	1.210	1.230	*
BP26	1.164	1.166	1.005	1.034
C-A23	1.248	1.301	1.211	*
C-C36	1.741	1.720	1.507	1.886
CL12	1.448	1.498	1.222	1.261
CRS33	1.380	1.320	1.396	1.369
E-S01	1.255	1.014	1.040	1.153
EEV13	1.094	1.185	1.072	1.025
FAS05	1.013	1.021	1.262	**
HFS28	1.136	1.105	**	**
IMS35	1.881	1.895	2.047	2.097
J-S42	1.781	1.663	1.552	1.743
JPF18	1.770	1.095	1.680	1.405
JSF29	1.423	1.531	**	**
LS15	1.423	1.420	1.213	1.338
LOF29	1.458	1.403	1.304	1.590
MIV14	1.429	1.354	1.198	897
MPG17	1.143	1.098	1.074	1.130
NS07	1.607	1.485	1.565	1.460
NMA30	1.371	1.541	1.388	**
PBL27	1.718	1.636	1.820	**
SVR04	1.312	1.181	1.203	**
TAA10	1.211	1.164	1.093	1.137
VD19	1.247	1.189	1.177	1.164
VAR21	1.407	1.120	1.248	1.354
VAT34	1.192	1.138	1.217	1.034
VDR32	1.664	1.641	1.698	1.680
VLA11	1.278	1.234	1.154	1.110
WN37	1.047	1.179	1.126	*

T0 before treatment (screening); T1 (30 days), T2 (60 days) during treatment, and T3 (90 days) at the end of treatment.
**Participants who did not collected material for immunogenicity analyses at that visit.*
***Discontinued participants.*
IgG references values for adult individuals, 490 to 1.140 mg/dL.
[#]Mislaid of material collected for immunogenicity analyzes from all visits by one participant.

in relation to healing. Note that after visit 2, it was already possible to start the healing process, but better results were achieved from visit 6. In addition to total healing, 13 out of 59 (22.0%) ulcers followed up until the final visit presented a reduction of ulcerated area of at least 50%. There was an improvement in the quality of the ulcerated bed when compared to the ulcers at the initial and final visits, with statistically significant differences. The quantity of exudation did not differ statistically between the initial and final visits. **Figure 5** has photographic records of some participants who have a good evolution of the healing process. **Supplementary Material 2** contains a table and graph with the evolution of the healing process of all participants followed up until to the end of the study.

TABLE 7 | Evaluation of the plasma concentration of Immunoglobulin A (IgA) in mg/dL of 30 participants[#] at T0, T1,T2, and T3.

Participants	Gender	T0	T1	T2	T3
ALC16	M	622	687	679	657
AMB03	M	434	409	434	*
BP26	M	370	384	333	332
C-A23	F	181	157	188	*
C-C36	M	385	416	402	431
CL12	F	244	276	216	214
CRS33	M	209	210	203	212
E-S01	F	732	500	531	516
EEV13	F	479	524	499	460
FAS05	M	224	215	247	**
HFS28	F	252	255	**	**
IMS35	F	425	423	423	415
J-S42	M	719	476	472	468
JPF18	M	<40	370	<40	<40
JSF29	M	253	267	**	**
LS15	M	472	469	460	533
LOF29	M	259	274	268	273
MIV14	F	406	411	409	330
MPG17	F	599	540	564	589
NS07	F	584	613	641	610
NMA30	F	313	343	313	**
PBL27	F	220	213	212	**
SVR04	F	267	249	242	**
TAA10	F	364	371	335	342
VD19	M	140	134	142	127
VAR21	F	532	445	483	513
VAT34	M	152	144	154	134
VDR32	M	266	295	253	252
VLA11	F	168	195	185	174
WN37	M	229	267	272	*

T0 before treatment (screening); T1 (30 days), T2 (60 days) during treatment, and T3 (90 days) at the end of treatment.
**Participants who did not collected material for immunogenicity analyses at that visit.*
***Discontinued participants.*
IgA references values for adult individuals, Male, 83.0 to 406.0 mg/dl; Female, 70.0 to 374.0 mg/dL.
[#]Mislaid of material collected for immunogenicity analyzes from all visits by one participant.

TABLE 8 | Evaluation of the plasma concentration of Immunoglobulin E (IgE) in mg/dL of 30 participants[#] at T0, T1,T2, and T3.

Participants	T0	T1	T2	T3
ALC16	1.751	1.803	1.682	2.178
AMB03	792	741	699	*
BP26	16.725	15.642	12.703	10.748
C-A23	7.998	6.123	5.904	*
C-C36	1.982	2.317	2.168	2.100
CL12	327	359	321	348
CRS33	937	811	572	822
E-S01	7.953	6.625	8.664	6.167
EEV13	111	115	76	94
FAS05	3.026	2.847	3.929	**
HFS28	1.393	1.400	**	**
IMS35	4.621	4.633	5.177	5.571
J-S42	29.758	15.186	14.265	15.187
JPF18	28	1.826	35	567
JSF29	2.931	3.374	**	**
LS15	1.375	1.494	3.227	261
LOF29	25	25	25	25
MIV14	921	686	474	364
MPG17	518	204	677	899
NS07	1.817	3.405	4.759	4.777
NMA30	1.742	1.306	954	**
PBL27	1.550	1.655	1.979	**
SVR04	209	239	315	**
TAA10	1.123	1.205	916	957
VD19	807	657	833	720
VAR21	173	177	143	207
VAT34	555	25	25	526
VDR32	1.994	1.743	1.635	1.684
VLA11	4.889	5.461	4.995	4.145
WN37	715	929	1.174	*

T0 before treatment (screening); T1 (30 days), T2 (60 days) during treatment, and T3 (90 days) at the end of treatment.
**Participants who did not collected material for immunogenicity analyses at that visit.*
***Discontinued participants.*
IgE references values for adult individuals, 1 to 183 UL/mL.
[#]Mislaid of material collected for immunogenicity analyzes from all visits by one participant.

DISCUSSION

According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use from European Medicines Agency [ICH guideline E8 (R1)—draft version 2019], "clinical studies should be designed, conducted, and analyzed according to sound scientific principles to achieve their objectives, and should be reported appropriately. The primary objective of any study should be clear and explicitly stated" (34).

Thus, the trials were based on phases. Phase I is characterized by the initial administration of a new experimental drug in humans. It is usually conducted in healthy volunteers to assess human pharmacology (pharmacokinetics and pharmacodynamics), but also initial safety and tolerability. Phase II has the main objective of exploring therapeutic efficacy in patients. At this phase, research can use a variety of study designs, including concurrent controls and comparisons with the initial state. Phase III is designed to confirm the preliminary evidence, accumulated in phase II, that a drug is safe and effective. Therefore, its main objective is to demonstrate or confirm the therapeutic benefits, and phase IV are all studies (other than routine surveillance) performed after drug approval by the regulatory agencies (35).

The Heterologous Fibrin Sealant (HFS), a uniquely Brazilian biopharmaceutical, was already been approved in preclinical tests carried out over 20 years ago by the CEVAP team (16, 17, 36, 37). It has also been academically approved for several clinical applications, including the treatment of venous ulcers (22, 23), although without the consent of the Brazilian National Health Surveillance Agency (ANVISA). In this case, the topical use in healthy individuals is not applicable, since there is a need for patients with chronic CVUs for testing. Thus, we opted for a clinical trial called phase I/II, non-comparative, under strict control measures, to assess the safety and immunogenicity, confirm the lowest dose used, and assess the preliminary efficacy of the product in a small number of volunteers who met the inclusion criteria.

The main outcomes investigated were local and systemic adverse events, both clinical and laboratory. Among the local AEs possibly related to the product, there was mainly pain in the ulcer region, maceration and itching of the peri ulcer skin, critical colonization, peri ulcerous eczema, opening of a new ulcer, and

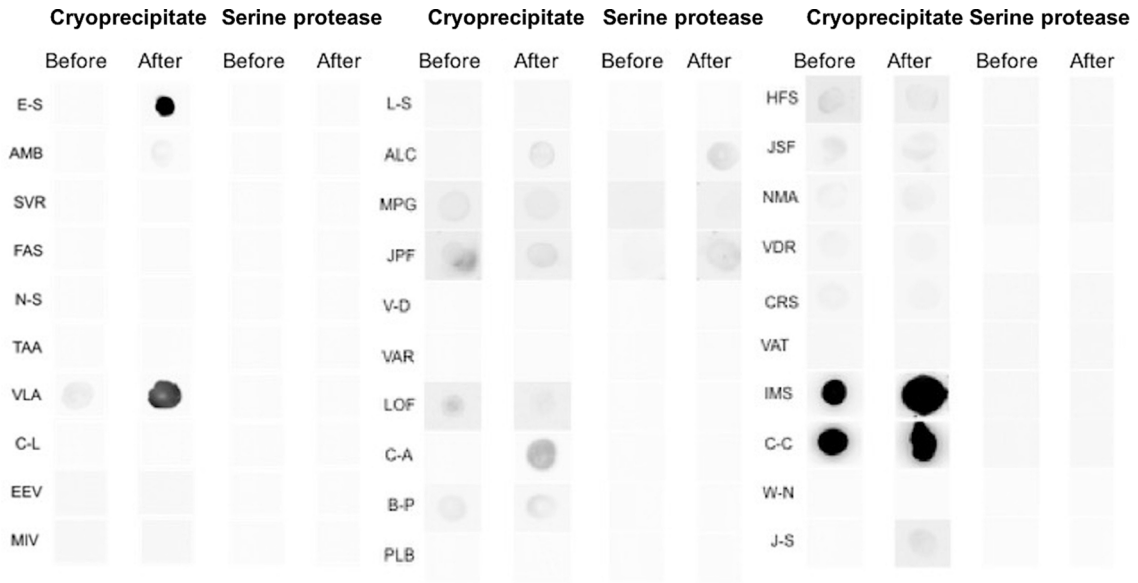

FIGURE 3 | Immunogenicity analysis using the secondary anti-IgE antibody (Abcam) followed by the streptavidin conjugate [HRP] (Abcam) to capture antibodies against cryoprecipitate and serine protease (thrombin like-enzyme) present in the serum of patients treated with heterologous fibrin sealant. The letters (left side of the image) correspond to the initials of the names of the study participants. The data are for 30 participants and not 31, as there was a mislaid of material collected for immunogenicity analysis from all visits by one participant.

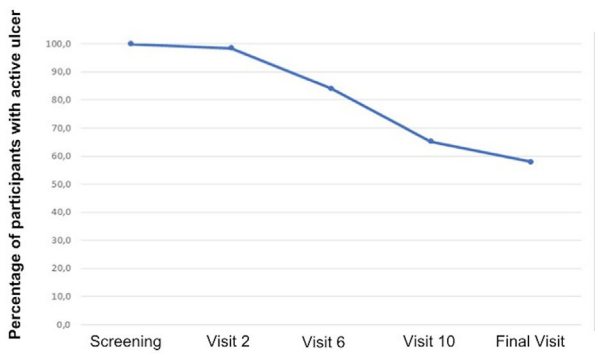

FIGURE 4 | Survival analysis in relation to healing.

an increase in the ulcerated area. Only one participant had pain classified as severe AE, while all others were classified as mild or moderate. It should be emphasized that no local AE has been described as definitely related to the investigational product.

To ascertain laboratory safety, particularly the possible AEs of the thrombin-like enzyme extracted from the venom of *Crotalus durissus terrificus*, the blood coagulation system of the participants was evaluated. According to the medical literature, envenomation caused by these snakes triggers important changes in blood coagulation through the consumption of coagulation factors, mainly fibrinogen and platelets (38–41). In the present study, there were no significant alterations in the levels of platelets, fibrinogen, or the erythrocyte sedimentation rate (ESR) compared to the evaluations made before, during, and at the end of treatment.

Furthermore, to assess the effect on blood coagulation, the APTT and PT of the participants were measured. The APTT is a subsidiary exam that evaluates the efficiency of the intrinsic coagulation pathway and the PT and its derivative international normalized index, also known as the international normalized ratio (INR). These are laboratory measures used to assess the extrinsic pathway of blood clotting. These hematological parameters remained stable and without significant changes when compared before, during, and at the end of treatment. The laboratory findings allowed us to conclude that no participant had a clinical manifestation of blood dyscrasia.

The product's laboratory safety was also assessed by triggering the acute phase reaction, which could be caused by the various proteins present in the product's components. This reaction was evaluated by alterations in the levels of white blood cells and C-reactive protein (CRP), a specific marker of acute phase reaction in humans (42–44). The observed results indicated that this reaction was not triggered. Therefore, it was possible to conclude that there was no systemic AE related to the product from the laboratory point of view.

The evaluation of immunogenicity was mainly based on the Guidelines of the European Medicines Agency—Science Medicine Health (45) and on the Guidance for Industry from the U.S. Department of Health and Human Services of the Food and Drug Administration (46). According to these guidelines, most biological and biotechnology-derived proteins induce an immune response. The immune response in these cases varies from individual to individual and involves several factors, including

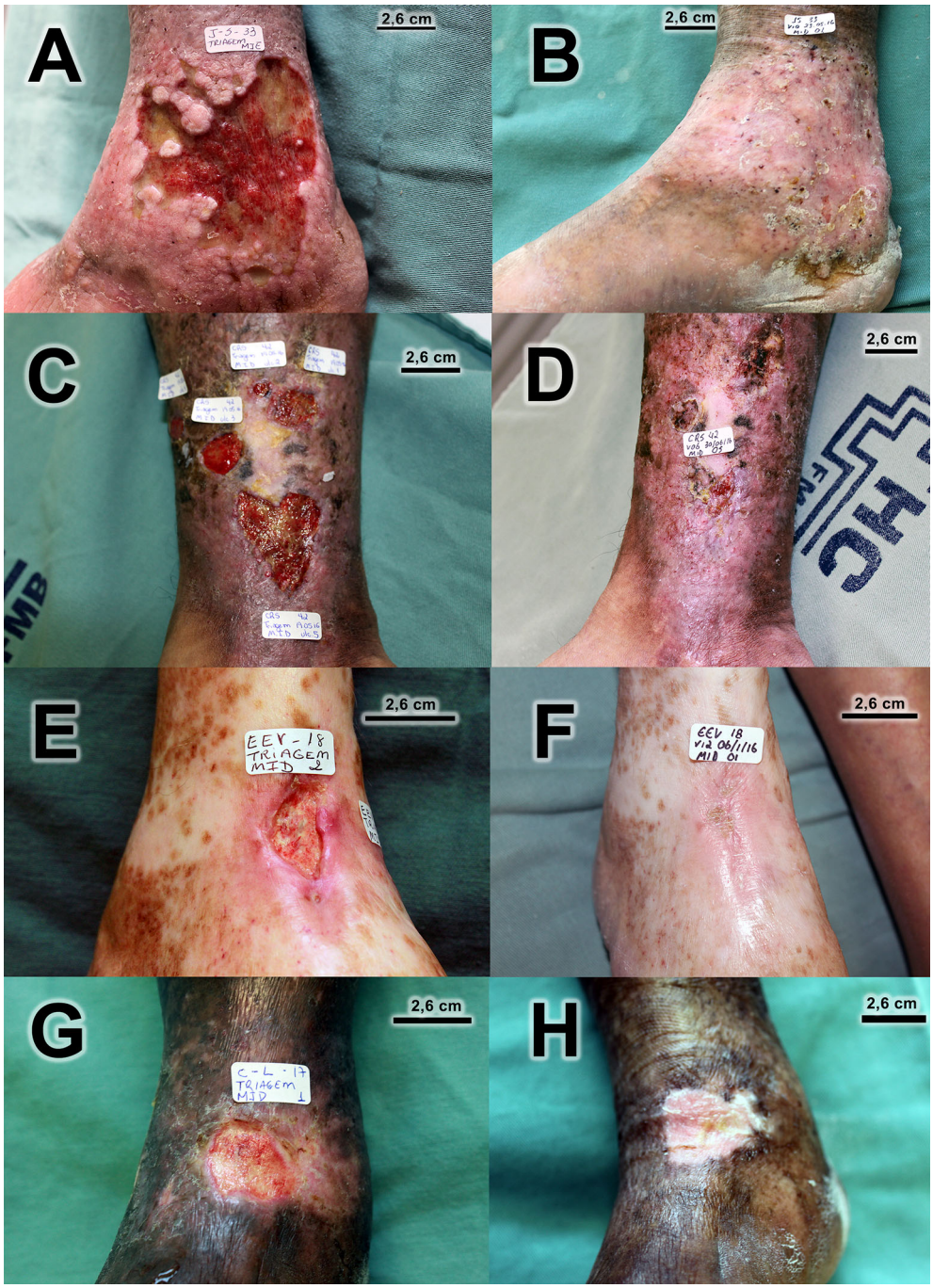

FIGURE 5 | Photographic records of venous ulcers of some participants who have a good evolution of the healing process. **(A)** Before treatment (ulcer area 51,83 cm^2)/**(B)** Complete healing at visit 10. **(C)** Before treatment (total ulcer area 19.67 cm^2)/**(D)** Only one ulcer did not heal completely at final visit. **(E)** Before treatment (ulcer area 3.48 cm^2)/**(F)** Complete healing at final visit. **(G)** Before treatment (ulcer area 4.89 cm^2)/**(H)** Complete healing at visit 10.

genetics, age, the disease to be treated, the proposed dosage, the route of administration, the presence of antibodies due to previous exposure to similar products, and the patient's own sensitivity (24, 47). The IgA, IgE, IgG, and IgM immunoglobulins were measured prior to and during treatment. The development of immunogenicity caused by products of biological origin may directly interfere with the efficacy and safety of the product under investigation and may even hinder the continuation of clinical trials. In this case, in addition to the detailed clinical analysis of the participants, one must seek, through biochemical methods, to produce neutralizing (IgM and IgG) and non-neutralizing (IgA and IgE) antibodies before and during treatment. Neutralizers can

cause a loss in the product's effectiveness by binding strongly to the active sites of the drug. Non-neutralizers, in general, trigger hypersensitivity reactions, although they may decrease the product's effectiveness indirectly. The latter, in most cases, is of the IgE class and may induce, due to hypersensitization, a reaction denominated as Coombs and Gell type I (48). This ranges from simple rhinitis, fever, asthma, and eczema to the severe form of the anaphylactic reaction (49–51).

In the present study, no patient presented a change in IgM levels before, during, or after treatment. Therefore, the investigational product did not interfere with the acute immune response. As for IgG and IgA, no participant showed significantly altered levels in relation to the reference values. Those levels above the reference values were found to be unrelated to any immunogenic reaction caused by the product under investigation because some dosages were already above and diminished throughout the study or remained high even before starting treatment.

Many participants in our study demonstrated altered total IgE levels in relation to the reference values. However, it should be emphasized that several patients possessed high levels of this antibody before starting treatment. To complement this evaluation, we performed dot blots to check for the presence of specific type E immunoglobulins; that is, an assay that can determine whether the change in the amount of IgE is directly related to the patient's exposure to the two main components of the product (52). In relation to cryoprecipitate, four participants were positive at the end of treatment. Of these four, two were negative before and positive at the end, and two were already positive and remained positive at the end of the treatment. Coincidentally, both also presented elevated levels of IgE through the nephelometry test before the start of treatment, and therefore, prior to exposure to the bioproduct. It should be emphasized that these patients, despite the alterations observed, had a good evolution of the healing process. Therefore, variations in serum IgE levels were not related to the investigational product. As for the thrombin-like enzyme, no patient had a positive reaction either before or at the end of treatment. In this case, the results suggest that this molecule was not able to sensitize the participants. It is important to note that other reasons may account for the elevated IgE levels in these participants, such as parasitic diseases, viral infections, immunological diseases, neoplasms, or hepatitis. Furthermore, factors such as sex, race, smoking status, season, and genetic potential can interfere with IgE synthesis (51).

Therefore, regarding the immunogenicity analyses, considering the four antibodies evaluated combined with anamnesis of the participants (background and history prior to the study) and the physical examinations performed during the visits, no participant presented clinical symptoms of hypersensitivity to the 12 applications of the product during the three months of treatment. It is concluded, therefore, that despite the application of the investigational product to the skin, which is not only the largest organ in the human body but also possesses greater capacity for hypersensitization (53), HFS did not show immunogenicity to their components.

Although the study was designed to primarily assess the safety of the product, we evaluated the healing outcomes for a preliminary analysis of efficacy. The results indicated a promising efficacy with an average application dose of 1.6 sealant kits per participant, per dressing change, in the proposed time period. There was healing of 47.5% of the ulcers, while 22% showed a significant reduction in the area, in addition to a qualitative improvement of the ulcerated bed of the unhealed ones. The healing capacity of the product will need to be confirmed through a phase III study that will be a multicentric, randomized controlled, and double-arm trial with a sample size sufficient to provide statistical power, resolvability, and reproducibility.

The fact that the study is of a non-randomized arm, without a comparative group, does not make it a limiting factor, since its main objective was to assess safety in relation to the dose chosen for the experimental product. As it is a bioproduct, an evaluation of the results of immunogenicity is indispensable, as the bioproducts are very immunogenic, which often prevents its continuous and long-term use, thus limiting its application. Immunogenicity was not verified in this study for at least 12 weeks.

Although it was possible to verify the assessed time, complete healing of 47.5% of chronic venous ulcers, a randomized, double-arm phase III study should be performed to corroborate the effectiveness. Likewise, some AEs may have occurred regardless of the investigational product due to the influence of other confounding factors, such as concomitant medications, the evolution of the disease, the care of the patients with the wounds, and genetic factors, including unknown factors (54). For this reason, there is a need to prove these results with a randomized controlled trial with a comparative group with standard treatment for CVUs.

It is important to emphasize that we evaluated fewer patients than registered in our protocol. The sample size was estimated considering examples of phase I/II clinical studies, which aim to study safety. In our original protocol, we recorded that it would be carried out in 40 participants, but as this study was a single-arm study and the main objective was safety, there was no formal calculation of the sample size. Therefore, when we reached the inclusion of 31, we concluded that there were enough data to finalize the study.

The biggest challenges of our study were as follows: first, to prospect, produce, and develop a new toxin-based bioproduct within good laboratory practices, then convince the regulatory agency and the ethics committee to authorize the conduct of the clinical trial; and second, to convince patients to join the study. As most of the candidate molecules found in animal toxins are present in very low amounts, isolation and production was a major challenge, and was only possible due to the development of the technical methodologies for the isolation and purification of the components used, allowing for its future registration and commercial scaling (12, 15, 24, 26).

CONCLUSIONS

The investigational product heterologous fibrin sealant (HFS) proved to be safe for the treatment of CVUs according to the proposed dosages. There were no systemic AEs related to the

product, whereas the few local AEs were mild to moderate in intensity. Regarding immunogenicity, it was observed that neither neutralizing (IgM and IgG) nor non-neutralizing antibodies (IgA and IgE) were produced or presented significant deviations. As for preliminary efficacy, there was total healing in 47.5% of the ulcers. For those that did not heal, there was a significant reduction in the area by 22%, in addition to an improvement in the quality of the ulcerated bed. Therefore, the investigated product was demonstrated to be safe and non-immunogenic, with promising efficacy for the treatment of CVUs in the proposed dosages. A multicentric phase III clinical trial will be needed to verify these findings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Commission on Ethics in Research (CONEP, Certificate of Presentation of Ethical Appreciation No. 37410814.4.0000.5411, v8, approved on 12/16/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LA, BB, and RF contributed to the conception and design of the study. LA, NM, and MC contributed to the study planning and design of the electronic form (Electronic Case Report Form—eCRF), which was developed specifically for this study. SB, MS, AL, GH, and MG contributed to the clinical data collection. LS was responsible for the immunobiological analyses. LA and BB performed the data analysis and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.627541/full#supplementary-material

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A Clot Twist: Extreme Variation in Coagulotoxicity Mechanisms in Mexican Neotropical Rattlesnake Venoms

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Rattlesnakes are a diverse clade of pit vipers (snake family Viperidae, subfamily Crotalinae) that consists of numerous medically significant species. We used validated *in vitro* assays measuring venom-induced clotting time and strength of any clots formed in human plasma and fibrinogen to assess the coagulotoxic activity of the four medically relevant Mexican rattlesnake species *Crotalus culminatus, C. mictlantecuhtli, C. molossus*, and *C. tzabcan*. We report the first evidence of true procoagulant activity by Neotropical rattlesnake venom in *Crotalus culminatus*. This species presented a strong ontogenetic coagulotoxicity dichotomy: neonates were strongly procoagulant *via* Factor X activation, whereas adults were pseudo-procoagulant in that they converted fibrinogen into weak, unstable fibrin clots that rapidly broke down, thereby likely contributing to net anticoagulation through fibrinogen depletion. The other species did not activate clotting factors or display an ontogenetic dichotomy, but depleted fibrinogen levels by cleaving fibrinogen either in a destructive (non-clotting) manner or *via* a pseudo-procoagulant mechanism. We also assessed the neutralization of these venoms by available antivenom and enzyme-inhibitors to provide knowledge for the design of evidence-based treatment strategies for envenomated patients. One of the most frequently used Mexican antivenoms (Bioclon Antivipmyn®) failed to neutralize the potent procoagulant toxic action of neonate *C. culminatus* venom, highlighting limitations in snakebite treatment for this species. However, the metalloprotease inhibitor Prinomastat substantially thwarted the procoagulant venom activity, while 2,3-dimercapto-1-propanesulfonic acid (DMPS) was much less effective. These results confirm that venom-induced Factor X activation (a procoagulant action) is driven by metalloproteases, while also suggesting Prinomastat as a more promising potential adjunct treatment than DMPS for this species (with the caveat that *in vivo* studies are necessary to confirm this potential clinical use). Conversely, the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) inhibited the direct fibrinogen cleaving actions of *C. mictlantecuhtli* venom, thereby revealing that

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the pseudo-procoagulant action is driven by kallikrein-type serine proteases. Thus, this differential ontogenetic variation in coagulotoxicity patterns poses intriguing questions. Our results underscore the need for further research into Mexican rattlesnake venom activity, and also highlights potential limitations of current antivenom treatments.

Keywords: rattlesnakes, venom, Mexico, blood, coagulotoxicity, snakebite

INTRODUCTION

Snakebite is a major global health crisis, with an estimated total of 94,000–138,000 fatalities and at least 400,000 cases of permanent disabilities per year. These numbers are well-recognized as gross-underestimates due to poor or non-existent epidemiological record keeping in the most affected regions (1, 2). At the root of such dismal statistics is a combination of factors such as rampant poverty, a lack of professional medical assistance in snakebite hotspots—leading to the time-wasting use of ineffective traditional "remedies"—and antivenoms which may be ineffective, inaccessible, or unaffordable (2–7).

Antivenom has long been a neglected or "orphan" drug due to the high costs of production, limited markets, and the fact that it is needed the most by those who can afford it the least (3, 5). The market limitations are due to venom being an extremely dynamic trait with extensive variations occurring between distantly related species, regional variations across the range of a widely distributed species, or even variations during the different life-stages of an individual snake. All these factors may dramatically limit the efficacy of an antivenom, thereby restricting the scope of its use (4, 8, 9).

Of particular concern for antivenom production and efficacy are wide-ranging, taxonomically complex clades such as rattlesnakes (genera *Crotalus* and *Sistrurus*), which are responsible for most snakebite envenoming cases in the United States (10–13) and a significant proportion throughout Latin America (10, 14, 15). Rattlesnakes are a highly diverse clade of pit vipers (Viperidae: Crotalinae) found throughout the Americas from southern Canada to northern Argentina (10). It is therefore unsurprising that they have received considerable research attention, ranking among the most studied snake clade worldwide for decades (16) and serving as model organisms for numerous works in several fields such as biogeography (17, 18), evolutionary biology (19, 20), and ethology (21). These snakes have adapted to a variety of ecosystems, from tallgrass prairies and deserts, through tropical and temperate forests, which resulted in great phenotypic and ecological diversity within the group (18, 22, 23).

Mexico harbors the highest diversity of rattlesnake species in the world (22, 24). Among the most iconic and medically significant rattlesnake species in Mexico are the Neotropical rattlesnakes: *Crotalus culminatus*, *C. ehecatl*, *C. mictlantecuhtli*, *C. simus*, and *C. tzabcan*. These species are part of the *Crotalus durissus* complex, which also includes the eponymous species *C. durissus* alongside *C. vegrandis* (22, 25, 26) and is in turn included in the *C. durissus* group with a sister clade comprising *C. basiliscus*, *C. molossus*, *C. ornatus*, and *C. totonacus* [(22, 27), **Figure 1**]. These medium- to large-bodied rattlesnakes range from the southwestern United States (*C. molossus*) to northern Argentina (*C. durissus*), where they are responsible for a considerable number of serious envenoming cases (14, 15, 28–32).

A large body of research has been conducted on venom activity and composition in the *C. durissus* group. The widespread presence of the neurotoxic phospholipase A_2 crotoxin in several species (33–37) places most Neotropical rattlesnakes (at least in early life stages) in the Type II venom category described by Mackessy (38). This class includes species possessing highly toxic venoms characterized by systemic neurotoxicity inducing rapid paralysis due to respiratory failure, rather than hemorrhagic symptoms (39–42). Conversely, phenotypes that are dominated by hemorrhagic and tissue-destroying snake venom metalloproteases (SVMPs) and generally devoid of neurotoxins are classified into the Type I category (38), which encompasses low-toxicity venoms inducing mostly cytotoxic and/or hemotoxic symptoms. However, the broad designation into Type I or Type II venoms does not fully account for factors such as ontogeny, prey specificity, intraspecific variation, and coagulotoxicity through differential biochemical pathways, and thus it is not reflective of actual biological diversity, which limits its categorical usefulness.

Neotropical rattlesnake venoms contain multiple toxins that disrupt hemostasis by targeting the blood clotting cascade, the concentration of which is often ontogenetic as well (35, 43–46). Research into coagulotoxicity produced by rattlesnake venoms has been largely focused upon anticoagulant toxins linked to the production of hemorrhagic shock through a combination of platelet inhibition, inhibition of activated clotting enzymes, depletion of fibrinogen levels, and degradation of the basement membrane of blood vessel walls leading to extravascular fluid loss (47–49). Fibrinogen depletion may occur in two ways, either *via* direct degradation by kallikrein-type serine proteases or metalloproteases, or through a pseudo-procoagulant action by 0005 kallikrein-type serine proteases where fibrinogen is converted to aberrant fibrin strands that form weak, transient clots that rapidly break down (50–54). Pseudo-procoagulant activity is distinguished from true procoagulant activity, i.e., the activation of clotting factors such as Factor X or prothrombin (55–59), by the nature of the fibrin clot formed. In pseudo-procoagulant venoms the direct action upon fibrinogen produces aberrant clots, while true procoagulant venoms generate endogenous thrombin which in turn produces well-ordered fibrin clots that contribute to the immobilization of prey through induction of stroke. In human victims, either scenario leads to venom-induced consumption coagulopathy

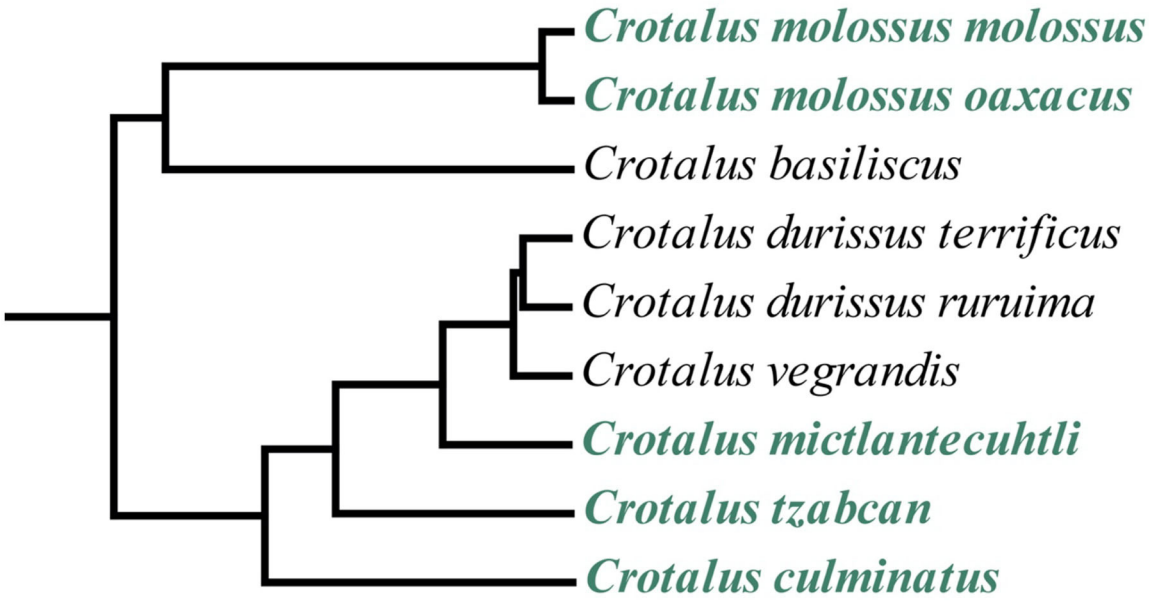

FIGURE 1 | Phylogenetic tree of the *Crotalus durissus* group from a parallel study (timetree.org) showing the relationships between the *C. durissus* complex (Neotropical rattlesnakes) and the *C. molossus* complex. Species analyzed in this study are shown in green. Not all members of the clade are represented in the tree.

(VICC), with extensive internal and external hemorrhage. Both SVMPs and snake venom serine proteases (SVSPs) are virtually ubiquitous across the rattlesnake clade, including Neotropical rattlesnakes (38).

In contrast to the well-documented anticoagulant effects, reports of true procoagulant activity in rattlesnake venom are scant and often inconclusive (60, 61), with the notable exception of *C. helleri* (62, 63). However, the paucity of data supporting the presence of true procoagulant toxins might have been influenced by intrinsic limitations in standard coagulotoxicity assays such as the procedure devised by Reid and Theakston (64), whereby Ca^{2+} and phospholipids are not added to citrated plasma prior to incubation with venom. As citration inactivates the clotting cascade by chelating ionized Ca^{2+}, it is essential to add Ca^{2+} back in to reproduce physiological conditions. Furthermore, since plasma alone lacks both activated platelets and activated/apoptotic endothelial cells (i.e., the physiological source of phospholipids), its phospholipid concentration is likely low (65). Therefore, while trace amounts of phospholipids are present in citrated plasma, such small concentrations are not reflective of normal physiological conditions and would be rapidly depleted. Many studies have indeed clearly documented that both cofactors significantly affect relative coagulotoxicity (50–53, 56–59, 66–76). However, despite this critical importance having been known for decades, assay designs in many snake venom coagulotoxicity studies have included Ca^{2+} but not phospholipids (77–93) or neither of the clotting cofactors (32, 94–104). This may dramatically skew the results, to the point that procoagulant activity might be missed entirely for venoms that are inactive in the absence of clotting cofactors or generate enzymes such as FXa which are themselves obligately dependent upon Ca^{2+} for activity.

Since *in vitro* coagulotoxicity assays for the Mexican members of the *C. durissus* complex have largely followed methodologies that did not reproduce physiological conditions (32, 44), true procoagulant venom phenotypes could have gone undiscovered in this lineage. This could hamper antivenom efficacy and symptomatic treatment alike, as both anticoagulant and procoagulant venoms result in a net anticoagulant effect in human victims and thus cannot be distinguished on the basis of symptomatology. Therefore, in this study we investigated the clinical implications and possible evolutionary characteristics of coagulotoxicity in four species of the *C. durissus* group from Mexico, with a particular focus upon elucidating the type of coagulotoxicity (i.e., anticoagulant, pseudo-procoagulant, or true procoagulant) caused by the venoms. We assessed venom-induced clotting times and clot strength on human plasma and fibrinogen, ensuring to include Ca^{2+} and phospholipids in the assays to replicate physiological conditions, and testing clotting factor dependency under controlled conditions. We also tested the neutralization of these venoms by Bioclon Antivipmyn®, one of the main antivenoms marketed in Mexico, which is produced using *Bothrops asper* and *Crotalus simus* venom. We then repeated the tests using the commercially available metalloprotease inhibitors 2,3-dimercapto-1-propanesulfonic acid (DMPS) and Prinomastat, which have been shown to neutralize SVMPs in other venomous snake species (105, 106). Our findings provide valuable information for clinicians and antivenom producers

regarding effective diagnosis and treatment of Neotropical rattlesnake envenoming in Mexico.

MATERIALS AND METHODS

Venom Selection and Preparation

All venom work was performed under University of Queensland Approval #IBC134BSBS2015. Our study included 25 venom samples from *C. culminatus* ($n = 15$), *C. mictlantecuhtli* ($n = 2$), and *C. tzabcan* ($n = 9$), from the venom bank of the laboratory at IBt, UNAM (Herpetario Cantil). The *C. mictlantecuhtli* samples were obtained from pooling the venoms of juvenile ($n = 5$) and adult ($n = 7$) individuals. **Table 2** details the age category and locality of origin of each snake. Three venom samples from *C. molossus* (1 *C. m. molossus* and 2 *C. m. oaxacus*) were taken from the Venom Evolution Lab long-term cryogenic collection. *One* mg of each venom was transferred into a 1.5 mL Eppendorf tube under sterile conditions. Subsequently, ddH_2O (double-distilled water) was added to the sample before vortexing for 5 s and centrifuging (4°C, 14,000 RCF; 10 min). The supernatant was then transferred to another 1.5 mL Eppendorf tube and the protein concentration determined in triplicate vortexing between replicates on a Nanodrop 2000 spectrophotometer at 280 nm (ThermoFisher Scientific). The resulting concentration values were used to obtain a final working stock of 1 mg/mL in 50% glycerol to prevent freezing at −20°C. Lastly, the samples were vortexed and aliquoted into 200 μL Eppendorf tubes for storage at −80°C until use. All venom samples were kept on ice throughout the process to avoid degradation.

Plasma and Fibrinogen Coagulation Assays

All human plasma work was performed under University of Queensland Biosafety Approval #IBC134BSBS2015 and Human Ethics Approval #2016000256. Healthy human plasma (3.2%, citrated Lots# A540020142331 and # A5400201137021, which were pooled together) was provided by the Australian Red Cross (44 Musk Street, Kelvin Grove, Queensland 4059). Plasma stocks were aliquoted into 1.5 mL Eppendorf tubes under sterile conditions before flash-freezing in liquid nitrogen and stored at −80°C until use. Human fibrinogen was purchased from Sigma Aldrich (St. Louis, Missouri, United States, catalog #F3879) and aliquoted into 1.5 mL Eppendorf tubes after reconstitution into a running buffer (150 mM NaCl + 50 mM TrisHCl in 1 L ddH_2O, pH 7.4) to a concentration of 4 mg/mL. The aliquots were flash-frozen in liquid nitrogen for 10 s and stored at −80°C until use.

Plasma and fibrinogen clotting times were measured on a Stago STA-R Max coagulation analyzer (Stago, Asniéres sur Seine, France) which determines clotting time *via* the time required for an oscillating magnetic ball inside a cuvette containing 250 μL solution to cease moving due to blockage caused by a clot. A detailed overview of the assays we performed is provided in **Table 1**. Prior to experimentation, a positive control for plasma was performed *via* an activated Partial Thromboplastin Time (aPTT) test as described by Lister et al. (107). A custom positive control assay was devised for fibrinogen whereby 50 μL 50% ddH_2O:glycerol, 25 μL of a 2:1 dilution of $CaCl_2$ + OK buffer, 50 μL phospholipids, and 75 μL human fibrinogen were incubated for 120 s before adding 50 μL thrombin (STA Liquid-FIB, Stago catalog # 00673) for a total volume of 250 μL. Negative controls for both plasma and fibrinogen were run by replacing the venom dilution with 50 μL 50% ddH_2O:glycerol.

TABLE 1 | Overview of coagulation assays performed on STA-R Max hemostasis analyzer.

Assay	Methodology
Venom-induced clotting time	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium (Stago catalog #00367) + 25 μL Owren-Koller (OK) buffer (Stago catalog #00360) + 50 μL phospholipids (Stago kit; catalog #00597) **Step 2:** 120 s incubation at 37°C + 75 μL human plasma/human fibrinogen
Calcium dependence	**Step 1**: 50 μL venom (100 μg/mL) + 75 μL OK buffer + 50 μL phospholipids **Step 2:** 120 s incubation at 37°C + 75 μL human plasma/human fibrinogen
Phospholipids dependence	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium + 75 μL OK buffer **Step 2:** 120 s incubation at 37°C + 75 μL human plasma/human fibrinogen
Antivenom	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium + 25 μL 2.5% antivenom + 50 μL phospholipids **Step 2:** 120 s incubation at 37°C + 75 μL human plasma/human fibrinogen
Prinomastat	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium + 25 μL 2 mM Prinomastat (Sigma-Aldrich, PZ0198-5MG) + 50 μL phospholipids **Step 2:** 120 s incubation at 37°C + 75 μL human plasma
DMPS	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium + 25 μL 2 mM/20 mM DMPS (ThermoFisher, U138044) + 50 μL phospholipids **Step 2:** 120 s/20 min incubation at 37°C + 75 μL human plasma
AEBSF	**Step 1:** 50 μL venom (100 μg/mL) + 50 μL 0.025 M calcium + 25 μL 2 mM AEBSF (Sigma-Aldrich, A8456-25MG) + 50 μL phospholipids

3.2% citrated plasma from cane toad (*Rhinella marina*) was aliquoted into 800 μL quantities, which were flash-frozen in liquid nitrogen, and stored at −80°C. This plasma was obtained under University of Queensland Animal Ethics Committee approval SBS/020/15/ARC.

Cofactor Dependence Assays

To test whether the (pseudo)procoagulant action of venoms requires specific cofactors, dependence tests were performed using the plasma protocols from 2.2 on six representative Neotropical rattlesnake venoms (fastest- and slowest-clotting samples on plasma per species, with the exception of second-slowest adult *C. culminatus*) whereby the samples were incubated with human plasma and fibrinogen in the absence of either Ca^{2+} or phospholipids. Additional tests were conducted in a non-plasma assay which allowed for the strict control of either co-factor (see section Blood Clotting Factor Activation Assay below).

Antivenom Neutralization and Inhibition Assays

One bottle of lyophilized Antivipmyn® antivenom serum [Instituto Bioclon, Calz. de Tlalpan 4691, Mexico City, Mexico; batch: B-6F-16, expiry date October 2010 and protein concentration of 13.7 mg $F(ab')_2$/mL] was diluted in 10 mL ddH_2O and centrifuged (3,900 RCF, 4°C, 10 min) to remove any potential particulates. Expired antivenoms were not a concern, as antivenoms have been shown to be stable over time, with powdered antivenoms shown to be particularly resilient but even liquid antivenoms have been shown to be active for at least 60 years (107–109). Subsequently, the antivenom mixture was filtered (0.45 μm) and aliquoted into 2 mL Eppendorf tubes in sterile conditions, then stored at +4°C until use. For testing in STAR-Max, the antivenom was diluted in OK buffer to a 2.5% concentration, as determined to be effective during preliminary testing against *C. mictlantecuhtli* (formerly *C. simus* from Veracruz, Mexico) due to the presence of venom from this species in the immunizing mixture. Eight-point dilution curves were run for six venoms incubated at eight different concentrations (μg/mL: 20, 10, 4, 1.66, 0.66, 0.25, 0.125, and 0.05).

To test for inhibition of venom metalloprotease activity on plasma, eight-point curves were run on two representative venoms whereby the metalloprotease inhibitors Prinomastat hydrochloride (catalog #PZ0198, Sigma Aldrich, St. Louis, Missouri, US) and 2,3-dimercapto-1-propanesulfonic acid (DMPS, catalog #D8016 Sigma Aldrich, St. Louis, Missouri, US) replaced OK buffer as reagents in separate assays. Prinomastat was solubilized in DMSO, diluted to a 10 mM concentration using ddH_2O, and subsequently stored at −80°C until use in STA-R Max. For this step, the inhibitor aliquots were thawed and pooled to a 900 μL total volume diluted into 3,600 μL OK buffer to dilute the concentration to 2 mM. DMPS was solubilized in DMSO and diluted in ddH_2O to a 20 mM concentration before storage at −80°C. Prinomastat and DMPS aliquots were covered in aluminum foil to prevent exposure to light and degradation. Antivenom and inhibitor testing were performed using pooled plasma batch # A540020103540). We repeated the original baseline values for all species to demonstrate congruence and the plotting of dilution curves for the species upon which antivenom and inhibitors were tested.

Inhibition of serine protease activity on fibrinogen in a representative venom was assessed by running an eight-point curve with the serine protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, catalog #A8456, Sigma Aldrich, St. Louis, Missouri, US) as a reagent in place of OK buffer. AEBSF was diluted with ddH_2O into 20 mM aliquots which were covered in aluminum foil and stored at −80°C until use. For testing in STA-R Max, a 20 min incubation step with AEBSF was included before addition of fibrinogen as per (52).

Thromboelastography

To assess the strength of venom-induced clots in plasma and fibrinogen, thromboelastography was performed on nine representative venoms using a Thromboelastogram® 5000 Hemostasis analyzer (Haemonetics®, Haemonetics Australia Pty Ltd., North Rdye, Sydney, Australia). The same ratio of reagents for STA-R Max assays was maintained for thromboestography. Briefly, 189 μL plasma (Label # A540020142331/A5400201137021) or fibrinogen (#Lot SLCC4502, #Lot SLBZ2294) were added to 72 μL $CaCl_2$ (25 mM solution), 72 μL phospholipids diluted in OK buffer, 20 μL OK buffer, and 7 μL venom (1 mg/mL). Thromboelastography for *C. molossus* ssp. samples was performed using pooled plasma batch # A540020103540 due to degradation of the original plasma stock during a COVID-19 lockdown period, with the repeating of the original baseline values to demonstrate congruence. For plasma, a spontaneous (i.e., negative) clotting control was run with 50% ddH_2O:glycerol in place of the venom, whereas 7 μL thrombin (STA Liquid FIB, Stago) or 7 μL bovine Factor Xa (Liquid Anti-Xa FXa, Stago) were used to run two independent positive controls. Only thrombin was used as a positive control for fibrinogen. Thromboelastography data were visualized on Adobe Photoshop.

Blood Clotting Factor Activation Assay

Venom-induced activation of coagulation Factor II (prothrombin) and Factor X (FX) for nine representative venoms was investigated using a Fluoroskan™ microplate fluorometer (ThermoFisher Scientific, 168 Third Avenue, Waltham, MA 02451, USA). This machine measures activation of clotting factors by monitoring cleavage of a specific substrate (and corresponding fluorescence emitted) by an activated enzyme. The following reagents were manually pipetted into each experimental well in 384-well plates: 10 μL phospholipids (STA CK Prest, Stago), 10 μL venom, 10 μL zymogen. To determine the activity of the venom directly on the substrate, the zymogen was replaced with 10 μL Fluoroskan running buffer without Ca^{2+} (150 mM NaCl + 50 mM Tris, pH 7.4) in venom control wells. Activated factors replaced zymogens in positive control wells. A blank control without zymogen or venom was also included. ES011 substrate (Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4- methyl coumarin) was diluted to a 2 μg/mL concentration in Fluoroskan running buffer with 10 mM Ca^{2+} (150 mM NaCl + 50 mM Tris in 1 L ddH_2O, + 10 mM Ca^{2+}, pH 7.4). Seventy microliters of the dilution were then dispensed into each well by the machine to enable factor activation. All zymogens were diluted in Fluoroskan running buffer without Ca^{2+} to a 10 μg/mL concentration. Venom concentration was 1 μg/mL in running buffer without Ca^{2+} for FX activation. The prothrombin assay required the venom and zymogen concentrations to be lowered to 0.1 and 1 μg/mL, respectively, for subsequent analysis purposes due to the otherwise excessively high activity of the thrombin control. Activation was measured as the percentage of activated factor for each venom compared to the positive control (i.e., active enzyme wells), which represented the 100% activation benchmark. To test for cofactor dependence in FX activation, the assay was repeated by incubating venom with both cofactors vs. without phospholipids vs. without Ca^{2+}.

1D Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Non-reduced 1D 12% SDS-PAGE was run in triplicate to assess the activity of selected venoms on prothrombin. Venom (0.2 μg) was reconstituted in ddH_2O and incubated at 37°C for 10 min with 2 μg prothrombin in a total volume of 7.5 μL. Negative (venom only; prothrombin only) and positive controls (thrombin only) were included in each gel. Then, 7.5 μL 2x laemmli dye (Bio-Rad Hercules, CA, USA) was added to each sample, resulting in a final volume of 15 μL. Lastly, the samples were stored at −20°C until use. Thirty milliliter of 12% resolving gel was prepared by pipetting 9.9 mL ddH_2O, 12.0 mL 30% Acrylamide mix (Bio-Rad, Hercules, CA, USA), 7.5 mL 1.5 Tris-glycine pH 8.8 (Tris- Sigma Aldrich, St. Louis, MO, USA; glycine- Sigma Aldrich, St. Louis, MO, USA), 300 μL 10% SDS (SDS- Sigma-Aldrich, St. Louis, MO, USA), 300 μL 10% Ammoniun persulfate (APS- Bio-Rad, Hercules, CA, USA), and 18 μL TEMED in a 50 mL falcon tube. Six milliliter 5% stacking gel was prepared by pipetting 4.2 mL ddH_2O, 990 μL 30% Acrylamide mix (Bio-Rad, Hercules, CA, USA), 750 μL 0.5 M Tris-glycine pH 6.8 (Tris- Sigma Aldrich, St. Louis, MO, USA; glycine- Sigma Aldrich, St. Louis, MO, USA), 60 μL 10% SDS (SDS- Sigma-Aldrich, St. Louis, MO, USA), 60 μL 10% APS (APS- Bio-Rad, Hercules, CA, USA), and 6 μL TEMED in a 15 mL falcon tube. Both gels were rested for 15 min to allow for polymerization before allocation into a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). 10x running buffer was prepared using the following recipe: 30 g Tris (Sigma Aldrich, St. Louis, MO, USA) + 144 g glycine (Sigma Aldrich, St. Louis, MO, USA) + 20 g SDS (Sigma Aldrich, St. Louis, MO, USA) diluted in 1 L ddH_2O. Subsequently, 100 mL 10x running buffer was diluted in 900 mL ddH_2O and poured into the electrophoresis chamber before manual loading of samples into the wells. A Dual Color protein standard (Bio-Rad, Hercules, CA, USA, range = 10–250 kD) was used as a ladder for molecular weight reference. Gels were run for 2.5 h at 120 V, then stained overnight with 1 g/L Coomassie colloidal brilliant blue G250 [34% methanol (VWR Chemicals, Tingalpa, QLD, Australia), 3% orthophosphoric acid (Merck, Darmstadt, Germany), 170 g/L ammonium sulfate (Bio-Rad, Hercules, CA, USA)] followed by destaining in ddH_2O.

Statistics

All tests were performed in quadruplicate ($n = 4$) bar the antivenom and inhibitor efficacy curves, which were run in triplicate ($n = 3$). Statistical analyses and graphing were performed in GraphPad PRISM v. 8.4.2. Cofactor dependence results were analyzed using repeated measures ANOVA *via* Dunnett's multiple comparisons test. This method allows for comparisons of each treatment (in our case, Ca^{2+}-devoid and phospholipids-devoid conditions) to a control (normal conditions with both cofactors present). A repeated-measures approach was chosen because all clotting tests (i.e., control vs. treatment conditions) were conducted on the same venom sample for each species. Correlation tests were performed using Spearman's rank-order correlation due to age being coded as an ordinal variable with four categories (1 = neonate, 2 = juvenile, 3 = young adult, 4 = adult). Normality was determined with four different tests (Shapiro-Wilk, Kolmogorov-Smirnov, Anderson-Darling, D'Agostino and Pearson) but only the Shapiro-Wilk results were used since n = 4 was too small for the other tests. Significance was set at $p = 0.05$.

RESULTS

Coagulotoxicity Assay

The venoms of neonate *C. culminatus* were strongly procoagulant, whereas adults appeared to have largely lost this trait (**Table 2**). Age of the animal and venom-induced clotting time were significantly correlated in *C. culminatus* for plasma ($r = 0.8506$, $p < 0.0001$) and fibrinogen ($r = 0.7423$, $p < 0.0001$). Both juvenile and adult *C. mictlantecuhtli* pools displayed short clotting times on plasma and especially fibrinogen, whereas greater individual variation was observed in *C. tzabcan* (**Table 2**).

The cofactor dependence results confirm that coagulotoxins in the venom of these rattlesnakes are strongly dependent on cofactors, particularly Ca^{2+} (**Tables 3**, **4**). Repeated-measures ANOVA yielded highly significant results regarding calcium dependence for all venoms on plasma, which was however markedly less pronounced for fibrinogen. Absence of phospholipids was not significant for one *C. tzabcan* sample and either *C. mictlantecuhtli* representatives (**Table 3**). Interestingly, all venoms clotted fibrinogen significantly faster in the absence of phospholipids than in normal conditions (**Table 4**). Relative co-factor dependence tests for zymogen activation by *C. culminatus* neonate were further investigated using completely controlled conditions in a non-plasma-based assay to eliminate the background fibrinogen-clotting effect (see section Blood Clotting Factor Activation Assay below).

Venom Neutralization by Antivenom and Inhibitors

Eight-point dilution curves of antivenom efficacy indicate that Antivipmyn® effectively counteracts the pseudo-procoagulant action of neonate *C. culminatus*, *C. tzabcan* (Solidaridad), and both *C. mictlantecuhtli* pools, with a noticeable spike in antivenom efficacy from a 1.66 μg/mL venom dilution onwards (**Figure 2**). Venoms from adult *C. culminatus* and *C. tzabcan* (Oxkutzcab) only weakly affected fibrinogen compared to the other four samples, facilitating nearly complete neutralization of pseudo-procoagulant activity by the antivenom. *C. mictlantecuhtli* venom was also markedly neutralized by the serine protease inhibitor AEBSF (148 ± 5.62 s, $n = 3$, figure not shown).

No detectable effect of Antivipmyn® was observed against neonate *C. culminatus* venom activity on plasma, and only marginal neutralization occurred against venom from an adult of the same species (**Figure 2**). Neonate *C. culminatus* venom-induced plasma clotting was instead greatly delayed by the metalloprotease inhibitor Prinomastat (particularly at low venom

TABLE 2 | Clotting times of human plasma and fibrinogen incubated with venoms from *C. culminatus*, *C. mictlantecuhtli*, and *C. tzabcan* specimens.

Species	Age	Locality	Mean clotting time (s) ± SD (plasma)	Mean clotting time (s) ± SD (fibrinogen)
C. culminatus	Neonate	Tlaltizapán, Morelos	11.625 ± 0.33	31.775 ± 0.45
C. culminatus	Neonate	Yautepec, Morelos	14.975 ± 0.20	59.25 ± 3.10
C. culminatus	Neonate	Puente de Ixtla, Morelos	15.175 ± 0.35	51.3 ± 4.95
C. culminatus	Neonate	Iguala, Guerrero	40.25 ± 0.19	58.1 ± 1.84
C. culminatus	Juvenile	Coahuayana, Michoacán	14.4 ± 0.21	190.3 ± 14.57
C. culminatus	Juvenile	Coahuayana, Michoacán	16.925 ± 0.20	233.3 ± 3.81
C. culminatus	Juvenile	Morelos	18.075 ± 0.41	30.35 ± 1.03
C. culminatus	Juvenile	Morelos	85.525 ± 2.13	106.625 ± 0.45
C. culminatus	Juvenile	Barranca Honda, Morelos	107.65 ± 8.68	144.9 ± 2.95
C. culminatus	Young adult	Coahuayana, Michoacán	19.6 ± 0.33	108.775 ± 1.46
C. culminatus	Young adult	Puebla, Puebla	90 ± 1.78	128.5 ± 0.93
C. culminatus	Adult	Barranca Honda, Morelos	120.175 ± 6.09	192.075 ± 1.53
C. culminatus	Adult	Tlaltizapán, Morelos	122.725 ± 4.27	189.575 ± 6.02
C. culminatus	Adult	Barranca Honda, Morelos	181.175 ± 7.99	260.825 ± 20.39
C. culminatus	Adult	Cruz Pintada, Tlaltitenango, Morelos	218.75 ± 0.73	801.675 ± 65.32
C. mictlantecuhtli	Juvenile (pool $N = 6$)	Veracruz	46.275 ± 1.11	37.1 ± 0.46
C. mictlantecuhtli	Adult (poo $N = 6$l)	Veracruz	48.825 ± 0.52	41.775 ± 1.30
C. tzabcan	Neonate	Dzibilchatún, Yucatán	161.3 ± 4.65	149.65 ± 9.26
C. tzabcan	Juvenile	Calakmul, Campeche	79.425 ± 1.26	77.975 ± 2.24
C. tzabcan	Juvenile	Mérida, Yucatán	242 ± 57.00	265.6 ± 21.35
C. tzabcan	Juvenile	Chetumal, Quintana Roo	230.525 ± 1.80	325.45 ± 4.95
C. tzabcan	Adult	Solidaridad, Quintana Roo	73.225 ± 2.53	83.675 ± 4.01
C. tzabcan	Adult	Chetuma, Quintana Roo	112.975 ± 2.17	109.875 ± 6.95
C. tzabcan	Adult	Mérida, Yucatán	211.525 ± 7.31	223.875 ± 5.39
C. tzabcan	Adult	Oxkutzcab, Yucatán	267.55 ± 1.75	902.175 ± 119.35

Plasma spontaneous control = 607 ± 23.39 s. Fibrinogen spontaneous control = 999 s.

TABLE 3 | Cofactor dependence tests for six representative venoms (*C. culminatus*, *C. tzabcan*, and *C. mictlantecuhtli*) incubated with human plasma.

Species	Locality	Normal	Phospholipid dependence (no phospholipids)	Ca^{2+} dependence (no Ca^{2+})
C. culminatus neonate	Tlaltizapán, Morelos	11.63 ± 0.33	22.10 ± 0.46***	54.975 ± 0.80***
C. culminatus adult	Barranca Honda, Morelos	181.20 ± 7.99	178.62 ± 4.77	404.32 ± 17.65**
C. tzabcan adult	Solidaridad, Quintana Roo	73.23 ± 2.53	96.35 ± 3.16***	164.77 ± 2.62***
C. tzabcan adult	Oxkutzcab, Yucatán	267.6 ± 1.75	333.47 ± 21.88*	999 ± 0***
C. mictlantecuhtli juvenile (pool)	Veracruz	46.29 ± 1.14	48.025 ± 3.36	79.325 ± 0.80***
C. mictlantecuhtli adult (pool)	Veracruz	48.83 ± 0.52	53.8 ± 3.84	84.95 ± 1.25***

*n = 4, values reported as mean ± SD. Statistical significance of cofactor-dependence tests compared to normal conditions (as inferred by Dunnett's multiple comparisons test following repeated-measures ANOVA) shown as follows: *p < 0.05, **p < 0.001, ***p < 0.0001. All values represent venom-induced clotting times (s).*

concentrations), whereas the adult individual was affected to a lesser degree. DMPS failed to neutralize either neonate or adult *C. culminatus* venom using the same assay as with Prinomastat (**Figure 3**). Furthermore, DMPS showed anticoagulant effects on plasma even in the absence of venom. Importantly, a different adult *C. culminatus* venom was used for antivenom + inhibitor tests and factor activation analysis (section Blood Clotting Factor Activation Assay) than the one used for clotting time assays and thromboelastography due to running out of the original stock. However, the results were congruent between the two venoms samples, which was consistent with both being from adult snakes from the same region.

Thromboelastography

Thromboelastography was conducted on plasma and fibrinogen as follows: **Figure 4** shows the human plasma thromboelastography traces for *C. culminatus, C. mictlantecuhtli,* and *C. tzabcan.* **Figure 5** shows the human plasma thromboelastography traces for the three *C. molossus* localities; **Figure 6** shows the human fibrinogen

TABLE 4 | Cofactor dependence tests for six representative venoms (*C. culminatus*, *C. tzabcan*, and *C. mictlantecuhtli*) incubated with human fibrinogen.

Species	Locality	Normal	Phospholipid dependence (no phospholipids)	Ca^{2+} dependence (no Ca^{2+})
C. culminatus neonate	Tlaltizapán, Morelos	33.75 ± 1.70	27.85 ± 0.17*	45.65 ± 0.34*
C. culminatus adult	Barranca Honda, Morelos	324.95 ± 15.73	190.20 ± 13.76*	764.60 ± 72.74*
C. tzabcan adult	Solidaridad, Quintana Roo	76.05 ± 2.88	39.43 ± 0.94*	120.50 ± 2.31**
C. tzabcan adult	Oxkutzcab, Yucatán	830.77 ± 52.91	333.47 ± 21.83*	999 ± 0*
C. mictlantecuhtli juvenile (pool)	Veracruz	34.88 ± 1.04	28.40 ± 0.33*	45.25 ± 0.64*
C. mictlantecuhtli adult (pool)	Veracruz	43.75 ± 1.60	33.25 ± 2.36*	61.75 ± 2.07*

*n = 4, values reported as mean ± SD. Statistical significance of cofactor-dependence tests compared to normal conditions (as inferred by Dunnett's multiple comparisons test following repeated-measures ANOVA) shown as follows: *p < 0.05, **p < 0.001, ***p<0.0001. All values represent venom-induced clotting times (s).*

thromboelastography traces results for *C. culminatus, C. mictlantecuhtli,* and *C. tzabcan*; **Figure 7** shows the human fibrinogen thromboelastography traces for the three *C. molossus* localities; **Figure 8** shows the human fibrinogenolytic effects for *C. tzabcan* and *C. molossus oaxacus*.

Thromboelastography on plasma (**Figures 4**, **5**) confirmed the marked procoagulant action of neonate *C. culminatus* venom on plasma on the STA-R Max assay, whereby a quick and strong clot was formed for the neonate, but not for the adult. The acceleration of clotting time and a strong, stable clot by the *C. culminatus* neonate venom is consistent with the activation of a clotting factor, which was specifically tested for in subsequent experiments (see section Blood Clotting Factor Activation Assay). None of the other venoms showed evidence of clotting factor activation in the plasma experiments (**Figures 4**, **5**).

Thromboelastography on fibrinogen to test for pseudo-procoagulant fibrin-clot formation (**Figures 6**, **7**) or destructive (non-clotting) fibrinogenolysis (**Figure 8**) also revealed sharp differences between age groups and species. The *C. culminatus* neonate retained as a background activity the basal pseudo-procoagulant activity widely present in rattlesnakes, but this trait was absent in the adult venoms. Intraspecific variation was evident in the *C. tzabcan* venoms, with one venom having pseudo-procoagulant activity upon fibrinogen whereas the other lacked this trait. Both neonate and adult *C. mictlantecuhtli* venoms displayed pseudo-procoagulant activity upon fibrinogen. This was not the case for *C. m. oaxacus*, while *C. m. molossus* showed only very slight activity in this regard. Further tests to see if *C. tzabcan* or *C. m. oaxacus* destructively cleaved fibrinogen revealed that while *C. tzabcan* did so only to a limited extent, *C. m. oaxacus* was extremely fibrinogenolytic, with the fibrinogen levels almost entirely depleted. Venoms were also tested on amphibian (cane toad) plasma, but none of them had any effect (data not shown).

Blood Clotting Factor Activation Assay

As the prior results indicated that neonate *C. culminatus* venom was activating a clotting factor, tests were undertaken to test for activation of FII (prothrombin), FVII, FIX, FX, FXI, and XII. Only Factor X returned a strong result (**Figure 9A**), with prothrombin only being activated at a trace level (**Figure 9B**) and none of the other factors affected (data not shown). Consistent with the dichotomy observed on other clotting tests, the adult *C. culminatus* was 20-fold less potent than the neonate in the activation of FX and displayed no meaningful activity upon prothrombin or any other clotting factor. FX activation by neonate *C. culminatus* venom proved to be highly dependent on both calcium and phospholipids (**Figure 10**), the absence of which nearly abolished any action of the venom on the zymogen. Thus, the cofactor dependence values on whole plasma for this venom in **Table 4** are artificially low due to the back-ground direct clotting of fibrinogen in a pseudo-procoagulant manner.

Intriguingly, *C. m. oaxacus* and *C. mictlantecuhtli* (juvenile pool) showed negative values. The [venom + substrate] controls were undertaken to provide the baseline activity of the venom in cleaving the substrate, with this amount to be subtracted from the results for the [venom + substrate + zymogen (FX or prothrombin)] experimental conditions. A negative value, whereby less fluorescence occurred for the [venom + substrate + zymogen] condition than for the [venom + substrate] indicates that in the [venom + substrate + zymogen] condition, less cleaving by the venom was observed than for the [venom + substrate] condition. This suggests that the venom was cleaving the substrate itself while simultaneously binding zymogen, resulting in less venom available to directly cleave the substrate when the zymogen was present. In addition, the interaction with the zymogen did not produce an active product from the cleaved zymogen. The ability to cleave the zymogen without yielding an active product could therefore represent a novel form of anticoagulation, as the zymogen would no longer be available to participate in the normal clotting cascade. This was evaluated experimentally with another fluorometric assay whereby *Pseudonaja textilis* venom, a well-known prothrombin activator (58), was incubated with intact zymogen and zymogen previously exposed to *C. m. oaxacus* venom for 1 h at 37°C. Activation in the *C. m. oaxacus*-treated zymogen was only 15% of that observed for the intact zymogen (**Figure 11**). Prothrombin degradation was further explored *via* gel electrophoresis [section 1D Polyacrylamide Gel Electrophoresis (SDS-PAGE)].

1D Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1D SDS-PAGE of venoms incubated with prothrombin revealed clear differences in action of toxins from different species on

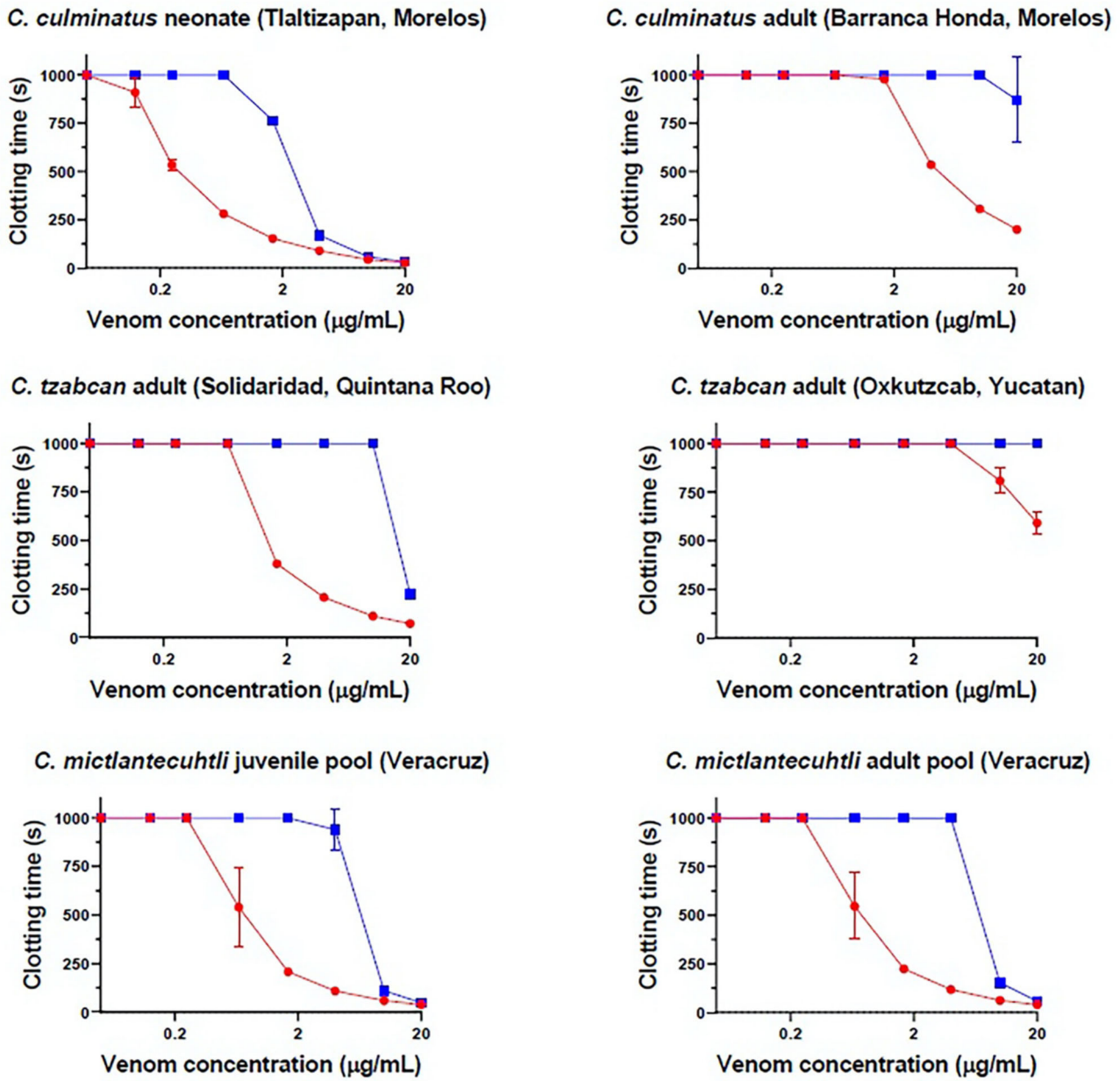

FIGURE 2 | Eight-point dilution curves for six representative venoms against Antivipmyn® antivenom (2.5%) on human fibrinogen, Red = venom only, blue = venom + antivenom. X-axis is displayed in log form. Negative control = 999 + / − 0 s.

this zymogen (**Figure 12**). In fact, *C. m. oaxacus* degraded prothrombin into several aberrant by-products spanning the region between the prothrombin and thrombin controls (72 and 36 kDa, respectively). On the other hand, *C. culminatus* (neonate) and *C. mictlantecuhtli* (pool of juveniles) affected the zymogen only weakly, with faint bands appearing in the 50–55 kDa region of the gel. A different neonate *C. culminatus* sample was used for this assay than in previous tests due to insufficient amount of venom remaining.

DISCUSSION

Synopsis

This study aimed to shed light on the evolutionary history and medical consequences of coagulotoxicity in a group of Mexican rattlesnakes of high clinical concern and evolutionary novelty. To this end, we assessed coagulotoxic venom activities in these snake venoms *via* multiple different assays to produce a robust set of results. We reproduced physiological conditions

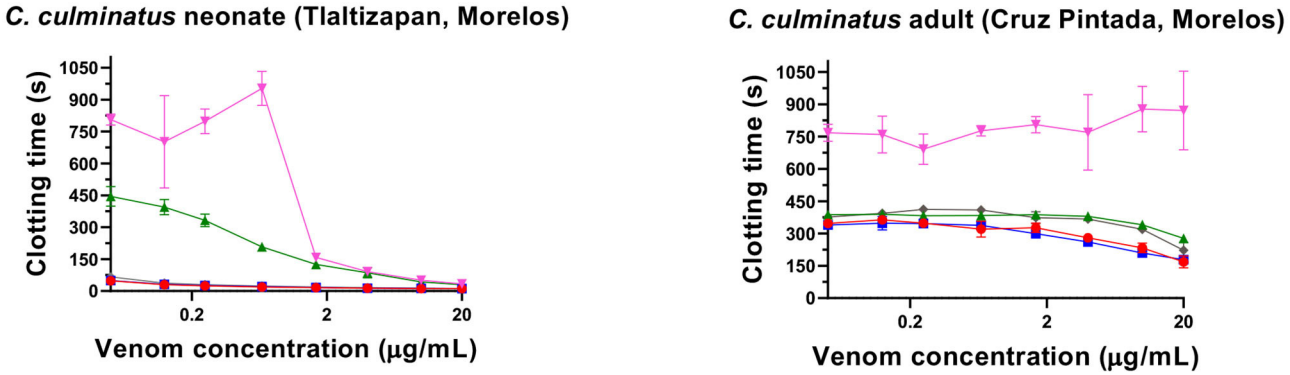

FIGURE 3 | Eight-point dilution curves for *C. culminatus* venom upon plasma (red lines for venom-only) against Antivipmyn® antivenom (blue lines), 2 mM Prinomastat hydrochloride with 2 min incubation (green lines), 2 mM DMPS with 2 min incubation (pink lines). Spontaneous clotting control = 444.87 ± 26.73 s. Prinomastat negative control = 450.02 ± 57.33 s. DMPS negative control = 626 ± 36.23 s.

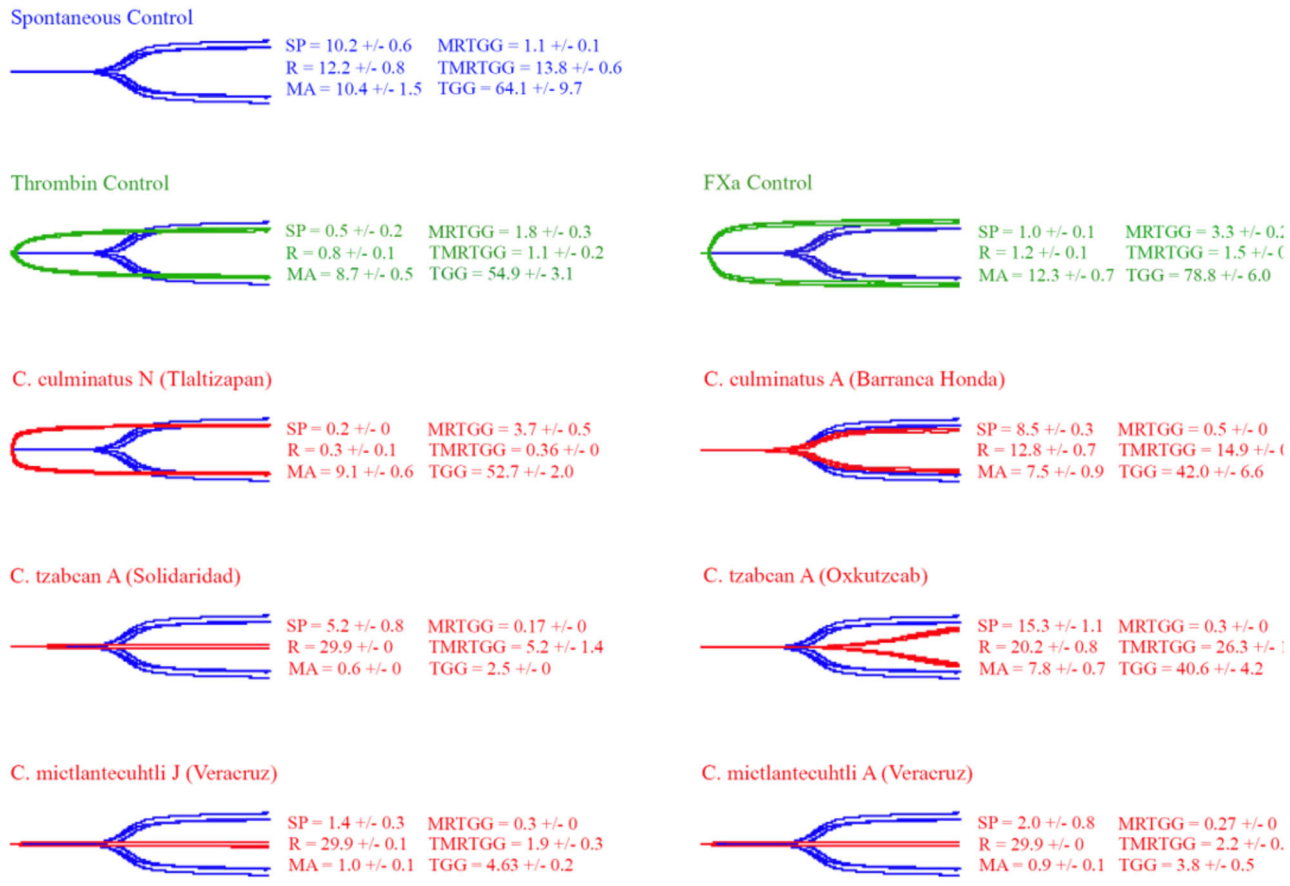

FIGURE 4 | Thromboelastography traces showing the coagulation pattern of human plasma incubated with venoms of six Neotropical rattlesnake respresentatives for neonates (N), juveniles (J), and adults (A). Venom traces are shown in red, spontaneous clotting control traces in blue, thrombin and Fxa control traces in green. SP, split point (min); R, time (min) to minimum detectable clot (2 mm): MA, maximum amplitude (mm); MRTGG, maximum rate of thrombus generation (dynes/cm^2); TMRTGG, time to maximum thrombus generation (min); TGG, total thrombus generation (dynes/cm^2). Results shown as $n = 4$ mean ± SD.

as best as possible to accurately characterize the venom effects. In doing so we revealed a previously unknown ontogenetic variation in *C. culminatus,* whereby neonates are potently procoagulant through the activation of Factor X, but adults are pseudo-procoagulant in that they cleaved fibrinogen into unstable, short-lived fibrin clots, thus contributing to a net anticoagulant state by depleting fibrinogen levels. The *C. culminatus* FX activation was shown to be biochemically

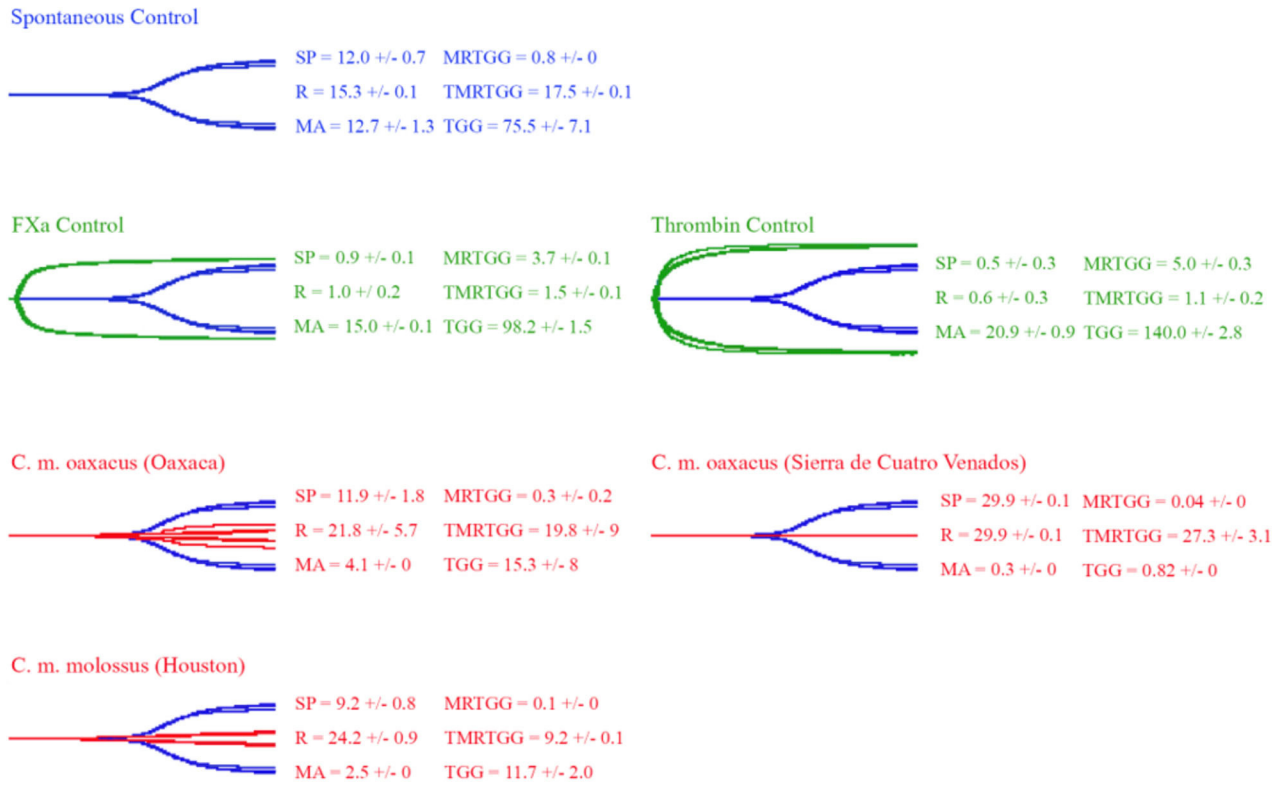

FIGURE 5 | Thromboelastography traces showing the coagulation pattern of human plasma incubated with venoms of three *C. molossus* specimens of two subspecies (*C. m. molossus* and *C. m. oaxacus*). Venom traces are shown in red, spontaneous clotting control traces in blue, thrombin and Fxa control traces in green. SP, split point (min); R, time (min) to minimum detectable clot (2 mm); MA, maximum amplitude (mm); MRTGG, maximum rate of thrombus generation (dynes/cm^2); TMRTGG, time to maximum thrombus generation (min); TGG, total thrombus generation (dynes/cm^2). Results shown as $n = 4$ mean ± SD.

extremely reliant upon calcium and phospholipids. These results reinforce what a dynamic trait venom is, as the other species depleted fibrinogen levels either by pseudo-procoagulant actions on fibrinogen or through destructive (non-clotting) cleaving.

Variations in Venom Biochemistry

Strikingly, we observed that venom from neonate *C. culminatus* clotted human plasma in our *in vitro* assay in 10–15 s, comparable to potently procoagulant snakes such as several Australian elapids (59, 76, 107, 110). We demonstrated that this activity was due to the activation of Factor X. The metalloprotease inhibitors Prinomastat and (to a much lesser degree) 2,3-dimercapto-1-propanesulfonic acid (DMPS) were effective in neutralizing the Factor X activation, revealing the activity to be driven by SVMP.

The unmistakably true procoagulant activity of *C. culminatus* venom was an unexpected finding in light of previous literature unanimously reporting a lack of any such trait in this species (32, 44). However, these studies did not include the clotting cofactors calcium or phospholipids in the assay conditions, which we show both clotting factors be critical through multiple assays in this study, and such cofactor dependence has long been documented in snake venoms (111). In addition to the venom activation of FX into FXa being obligately calcium-dependent, the bioactivity of the endogenous FXa which is produced by the venom is also obligately dependent upon calcium so even for venoms which are able to activate FX in the absence of calcium, their activity would be missed in assays which relied on protocol designs. The discrepancy between our results and previous literature is almost certainly due to the omission of clotting cofactors Ca^{2+} and phospholipids in prior research that relied upon the method developed in 1983 by Theakston and Reid (64), which did not include either clotting cofactor and has been largely followed with only minor modifications in toxicity studies of Mexican Neotropical rattlesnakes (32, 44, 112). Thus, calcium-obligate activities such as the Factor X activation discovered in this study would not be observable in assays lacking the clotting cofactors.

Such high levels of calcium dependence for procoagulant zymogen activation (Factor X or prothrombin) have been observed in other venomous snake lineages, including other pit vipers such as *Bothrops atrox* (113), true vipers of the genus *Echis* (56), Australian elapids (107) the genus *Atractaspis* within the Lamprophiidae family (57), and the colubrid genera *Dispholidus* and *Thelotornis* (74). In contrast, other genera are known to activate zymogens with much lower levels of calcium dependence, such as some species of *Echis* (56), and

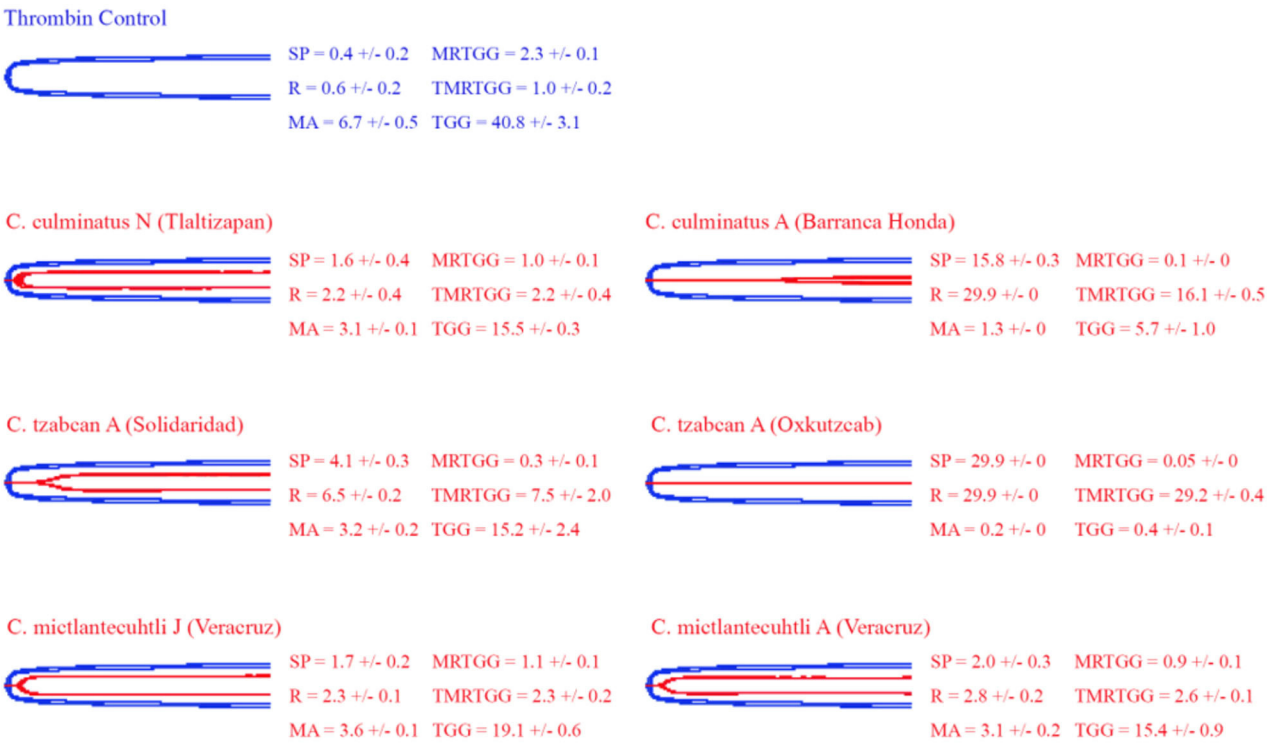

FIGURE 6 | Thromboelastography traces showing the coagulation pattern of human fibrinogen incubated with venoms of six Neotropical rattlesnake respresentatives for neonates (N), juveniles (J), and adults (A). Venom traces are shown in red, thrombin control traces in blue. SP, split point (min); R, time (min) to minimum detectable clot (2 mm); MA, maximum amplitude (mm); MRTGG, maximum rate of thrombus generation (dynes/cm^2); TMRTGG, time to maximum thrombus generation (min); TGG, total thrombus generation (dynes/cm^2). Results shown as $n = 4$ mean ± SD.

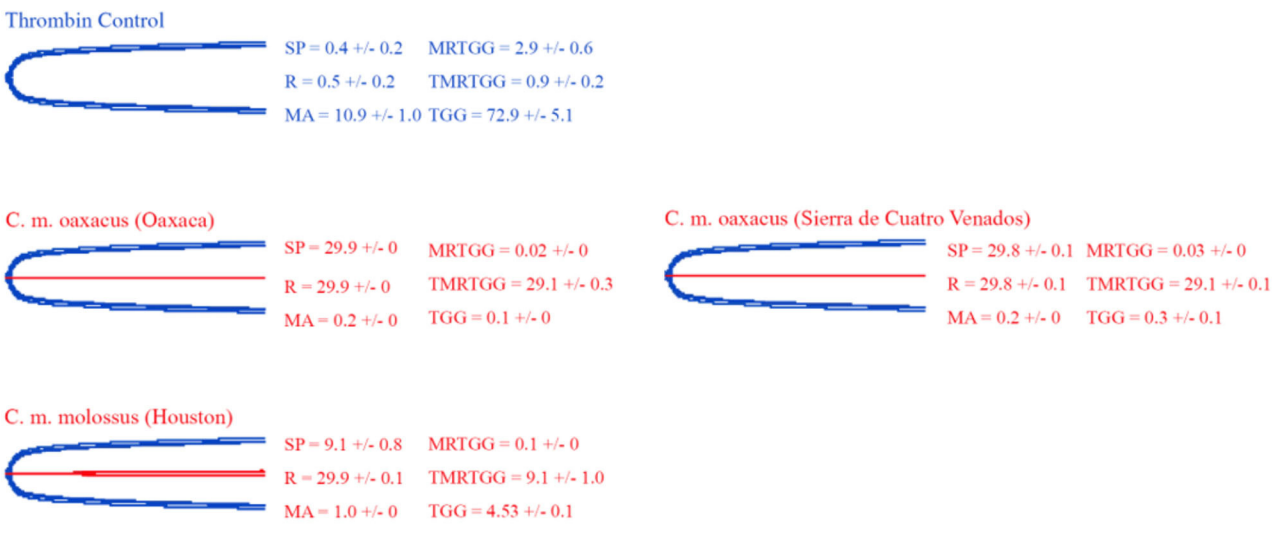

FIGURE 7 | Thromboelastography traces showing the coagulation pattern of human fibrinogen incubated with venoms of three *C. molossus* specimens of two subspecies (*C. m. molossus* and *C. m. oaxacus*). Venom traces are shown in red, thrombin control traces in blue. SP, split point (min); R, time (min) to minimum detectable clot (2 mm); MA, maximum amplitude (mm); MRTGG, maximum rate of thrombus generation (dynes/cm^2); TMRTGG, time to maximum thrombus generation (min); TGG, total thrombus generation (dynes/cm^2). Results shown as $n = 4$ mean ± SD.

the Australian elapid genera *Oxyuranus* and *Pseudonaja* (58, 59). Venom-induced FX activation by neonate *C. culminatus* was also highly dependent on phospholipids, which appear to be nearly as crucial as Ca^{2+}. This further highlights the importance for venom coagulotoxicity assays *in vitro* to include both cofactors so as to avoid skewing results.

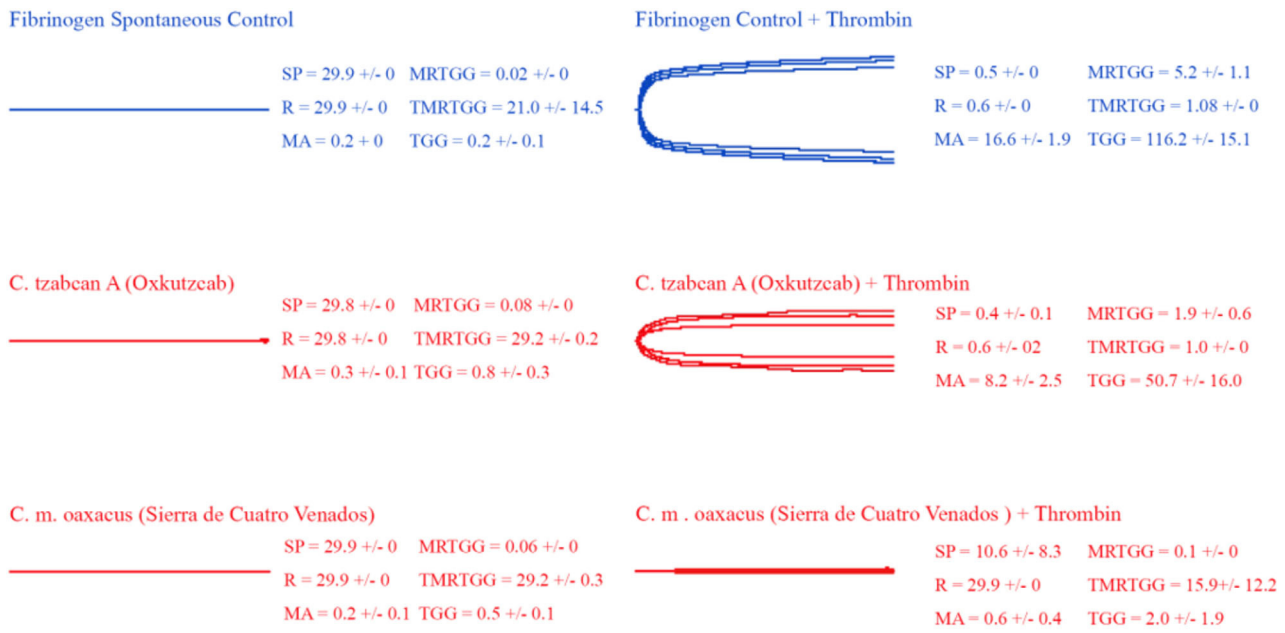

FIGURE 8 | Thromboelastography traces showing the fibrinogenolytic action of venoms from *C. tzabcan* (Oxkutzcab) and *C. m. oaxacus* (Sierra de Cuatro Venados). Venom traces are shown in red, spontaneous and thrombin control traces in blue. SP, split point (min); R, time (min) to minimum detectable clot (2 mm); MA, maximum amplitude (mm); MRTGG, maximum rate of thrombus generation (dynes/cm^2); TMRTGG, time to maximum thrombus generation (min); TGG, total thrombus generation (dynes/cm^2). Results shown as $n = 4$ mean ± SD.

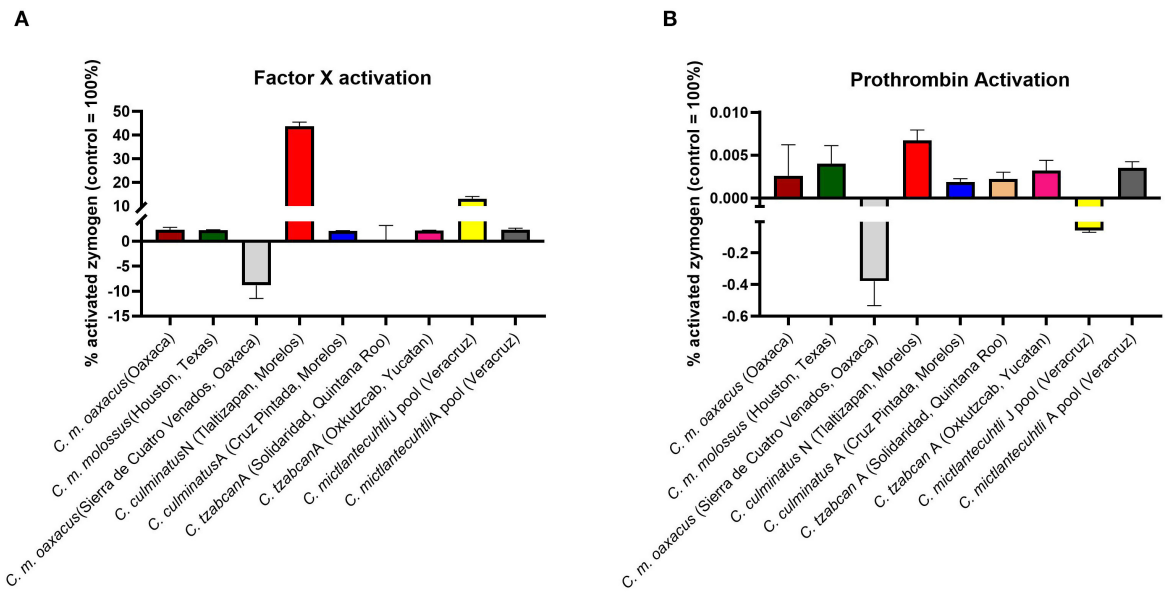

FIGURE 9 | Fluorometry graphs showing activation of FX **(A)** and prothrombin **(B)** by selected rattlesnake venoms. Activation is expressed as the relative percentage of zymogen converted to its active form against a benchmark positive control incubated with thrombin and FXa, respectively (i.e., 100% active enzyme). Note the difference in Y-axis between FX activation and prothrombin activation, indicating considerably greater potency for the former. Values are $n = 3$ mean ± SD.

The SVMP toxin class has been previously shown to be responsible for Factor X activation in a wide range of snakes, including the related pit viper genus *Bothrops* (113) and true vipers such as *Bitis worthingtoni* (76, 114). Thus, this trait either represents a remarkable case of functional convergence in the neofunctionalisation of an ancestral tissue-destroying metalloprotease or indicates that FX activation is an ancient trait that has been amplified on

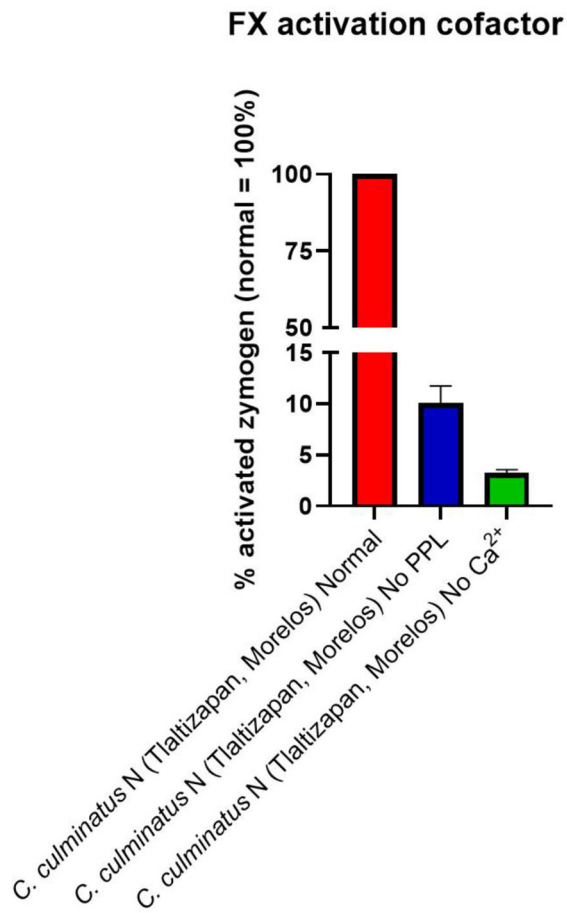

FIGURE 10 | Fluorometry graph showing activation of FX by venom from a neonate *C. culminatus* specimen with and without coagulation cofactors (Ca^{2+} and phospholipids). Activation is expressed as the relative percentage of zymogen converted to its active form, with the normal condition (i.e., both Ca^{2+} and phospholipidss present in the incubation) as the 100% benchmark. Values are $n = 3$ mean ± SD.

multiple convergent occasions but is only maintained at trace levels in most species. The answer to this question would require sequencing of the enzyme responsible for FX activation and reconstructing its molecular evolutionary history through the construction of a robustly supported molecular phylogenetic tree.

From a phylogenetic point of view, *C. culminatus* is consistently retrieved as an early divergence from the rest of the *C. durissus* complex (18, 26). Therefore, the true procoagulant venom phenotype observed in this species might have evolved independently or represented the ancestral state for this clade. The latter possibility was investigated by testing the venom of *C. molossus*, part of the sister clade to the *C. durissus* complex alongside *C. basiliscus*, *C. ornatus,* and *C. totonacus* (26, 27). However, our thromboelastography and factor activation results revealed only a weakly pseudo-procoagulant venom action for the nominate subspecies *C. m. molossus* and distinctly anticoagulant patterns for a *C. m. oaxacus* representative, which greatly degraded fibrinogen to a point where addition of thrombin was unable to form a clot. This is consistent with previous studies reporting high fibrin(ogen)olysis across the three subspecies of *C. molossus* (95, 115, 116) and does not support a procoagulant ancestral condition for the *C. durissus* group. Thus, this trait likely stems either from convergent amplifications of a basal FX-activating SVMP or convergent evolutions of neofunctionalised SVMPs in Viperidae.

The ability of the serine-protease inhibitor AEBSF to neutralize the pseudo-procoagulant activity of *C. mictlantecuhtli* venom demonstrated that this venom activity is driven by kallikrein-type serine proteases. The differential reliance upon Ca^{2+} extended to the pseudo-procoagulant actions on fibrinogen, with all the venoms acting notably more slowly (up to half as fast) in the absence of Ca^{2} (**Table 4**). The relative reliance upon phospholipids has also been shown to be a highly labile trait (66–73, 117). While the effect is less pronounced than for Ca^{2+}, it is still a significant variable, showing extreme variation within a genus or even within different geographic ranges of a single species (50–53, 56–59, 107). Notably, our cofactor dependence assay revealed a consistently significant acceleration of fibrinogen clotting in the absence of phospholipids. This phenomenon

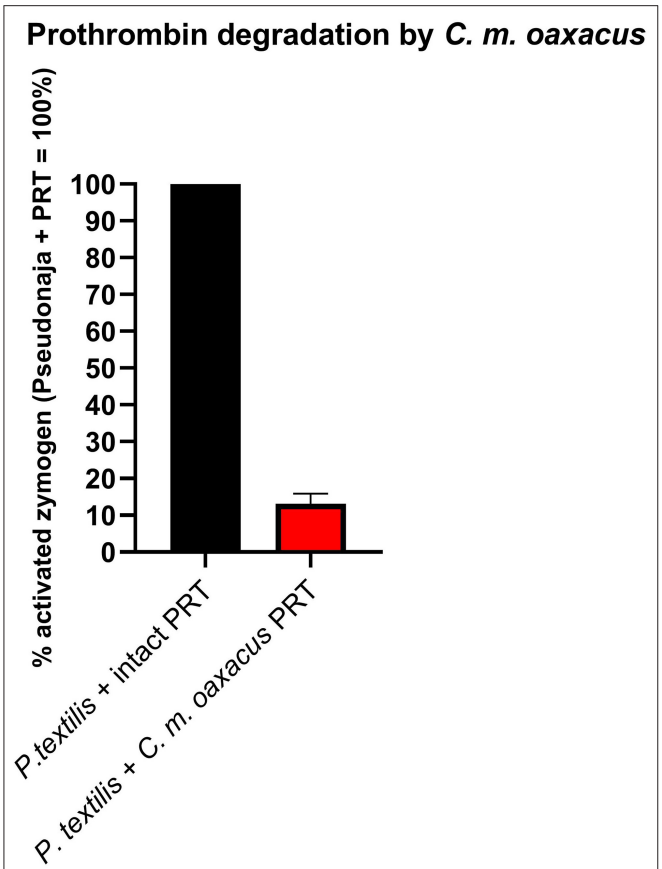

FIGURE 11 | Fluorometry graph showing degradation of prothrombin (PRT) by venom from *C. m. oaxacus* (Sierra de Cuatro Venados). Degradation is expressed as the difference in *Pseudonaja*-activated zymogen levels between intact zymogen (black) and zymogen previously incubated with *C. m. oaxacus* venom (red). Values are $n = 3$ mean ± SD.

was already observed in several Asian pitvipers of the genus *Trimeresurus* (52) and in the Australian elapid genus *Pseudonaja* (58). The biochemical dynamics underlying this pattern are unclear and warrant further research.

Another novel activity documented in this study was degradation of prothrombin by *C. m. oaxacus*, a phenomenon previously reported in several viper species (118, 119) but rarely in rattlesnakes (120). Such an activity would create a net anticoagulant state by depleting the amount of this endogenous clotting factor available for participation in the clotting cascade. This activity was first inferred from the negative values obtained in prothrombin activation tests, and then confirmed by two additional assays: first by incubating the venom with prothrombin, then adding a known prothrombin trigger, and comparing the results to the same trigger added to prothrombin that had not been exposed to *C. mo. oaxacus* venom; and secondly by an SDS-PAGE assay, whereby *C. m. oaxacus* produced several aberrant degradation by-products of higher molecular mass than thrombin. The net decrease in activity in the FX zymogen activation studies for *C. m oaxacus* is consistent with this species also degrading FX in addition to prothrombin. However, Factor X degradation was unable to be further examined due to running out of venom supplies. The fact that the venom produced the same negative values in the Flouroskan tests as was the case for prothrombin and with these negative values for prothrombin being confirmed by additional tests as indeed being reflective of degradation events, this is strongly suggestive of Factor X also being degraded by this venom. Future work to confirm this would involve assays such as were undertaken for prothrombin degradation in this study: (a) incubating the venom with Factor X, then adding a known Factor X trigger, and comparing the results to the same trigger added to Factor X that had not been exposed to *C. mo. oaxacus* venom; and (b) SDS-PAGE gels to ascertain relative cleavage products to determine if aberrant cleavage products were formed.

As discussed earlier, such stark individual variations are commonplace among rattlesnakes. *C. molossus* occurs throughout a vast range spanning from the southwestern US to southern Mexico, with blurred geographic and genetic boundaries among subspecies (10, 27). Our small sample size does not allow for documentation of subspecies- and population-level venom variability in this species, which therefore should be the subject of future research in order to elucidate to what extent venom variation reflects biogeographical and/or ecological drivers in the *C. molossus* complex.

Prey-Capture Evolutionary Implications

The procoagulant activation of zymogens into their active forms (e.g., FX into FXa; prothrombin into thrombin) in prey animals would result in rapid incapacitation due to stroke, induced by large blood clots. Interestingly, procoagulant venom activity *via* FX activation in *C. culminatus* appears to be an ontogenetic trait, with the shortest and longest clotting times for both plasma and fibrinogen observed in neonates and adults, respectively. This is corroborated by our thromboelastography and fluorometry results in terms of time to clot formation and FX zymogen activation. Ontogenetic shifts in venom composition and/or activity have been extensively documented in a variety of rattlesnake species and lineages (60, 112, 121–124), particularly with respect to a pattern of loss of crotoxin-like neurotoxic PLA_2s (Type II phenotype) in favor of hemorrhagic SVMPs (Type I phenotype) as the snake ages (38, 45, 46). This phenomenon is recurrent in the *C. durissus* complex (35, 43, 45). Such age-driven changes in venom composition are generally thought to stem from shifts in prey preference between juvenile and adult snakes (10, 121, 122), as seen in a variety of snakes ranging from Australian elapids (110) to lancehead pit vipers of the genus *Bothrops* (113, 125). However, our current knowledge—albeit fragmentary—points to *C. durissus, C. simus*, and *C. tzabcan* being rodent specialists throughout their life (10, 24, 126–129). While only scarce information is available for *C. culminatus*, reports indicate a rodent-centered diet as well (10, 23, 24, 130). This is supported by our thromboelastography results showing a strikingly potent procoagulant effect of *C. culminatus* venom on human plasma as opposed to no apparent activity at all on amphibian plasma, suggesting specialization for an endotherm-based diet. By contrast, the venoms of other vipers such as *Bitis worthingtoni* and several *Bothrops* representatives are known to

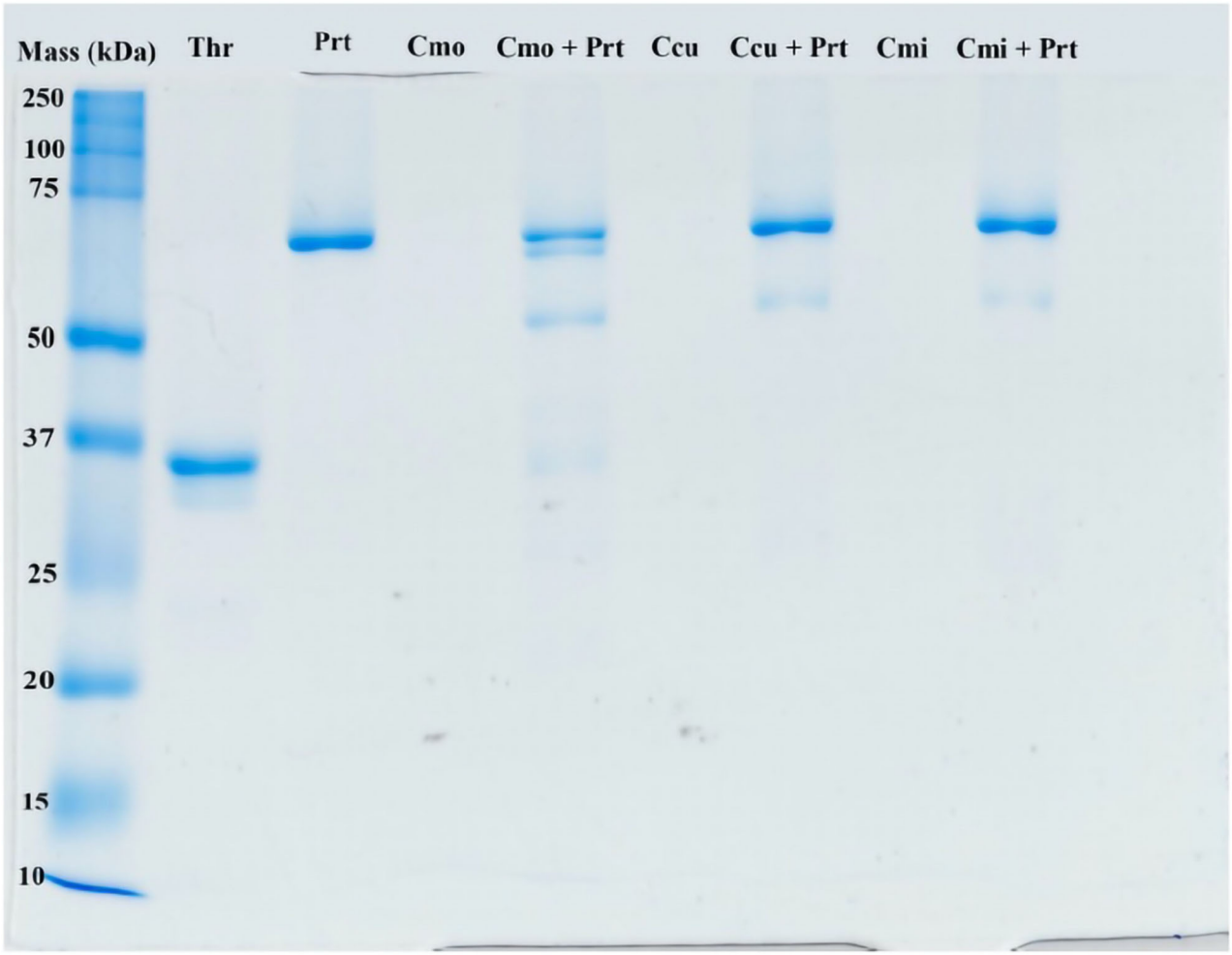

FIGURE 12 | Representative ID gel of selected rattlesnake venoms incubated with prothrombin to illustrate prothrombin products. The ladder on the far left shows molecular weights (kDA = kilodaltons). Thr, thrombin (positive control, i.e., typical final product of prothrombin cleavage); Prt, prothrombin; Cmo, *C. m oaxacus*; Ccu, *C. culminatus*; Cmi, *C. mictlantecuhtli*.

activate both mammalian and amphibian plasma, with potency showing a clear correlation with degree of specialization on amphibian prey (113, 114). The Factor X zymogen differs significantly in mammals compared to amphibians and diapsids (i.e., reptiles and birds). Future work should investigate the lineage-specific motifs that guide such differential activation. Testing of *C. culminatus* venom on reptile plasma (e.g., lizard) would be a logical follow-up to corroborate our findings, since this snake occurs in dry habitats at mid- to high elevations in southwestern Mexico where other reptiles abound (23, 130, 131).

It has been suggested that potent, fast-acting toxins possibly serve as a means for small-sized snakes to rapidly incapacitate prey using a substantially lower amount of venom than adults are able to inject (122, 126) and/or to quickly immobilize prey items (113, 122, 126). Intriguingly, while nearly all members of the *C. durissus* complex present variable quantities of crotoxin in their venom, *C. culminatus* lacks this neurotoxin entirely (35, 45). By contrast, this species possesses a significantly higher percentage of SVMPs than *C. tzabcan* and *C. simus*, with neonates and juveniles possessing metalloproteases not found in adults and vice versa (44, 45). It is therefore possible that highly procoagulant SVMPs in early-stage *C. culminatus* play a role akin to that of crotoxin-like neurotoxins in other members of the *C. durissus* complex and other rattlesnake lineages, as factor-activating SVMPs are known to induce rapid death by stroke in small-sized animals (132). Neonate and juvenile rattlesnakes require meals as early as possible to avoid starvation and support high rates of growth (10, 133). Thus, a highly potent toxic component in neonate rattlesnake venom may greatly improve prey-capture and survival into adulthood. Our results align with the observations of Margres et al. (134) in the equally non-neurotoxic species *C. adamanteus*, with higher venom toxicity in juveniles compared to adults. This indicates that such a pattern may be widespread among rattlesnakes beyond the simplistic Type I vs. Type II categorization, an intriguing possibility that invites further research.

While *C. culminatus* possesses a distinctly Type I venom phenotype (44, 45), the SVMP-driven procoagulant activity observed in this study might serve a functional role analogous to that of neurotoxic PLA_2 components in Type II venoms from juveniles of other species. Hence, a general classification such as the Type I vs. Type II dichotomy devised by Mackessy (38) might overlook peculiar toxic activities of venom in certain species and is therefore not reflective of the greater complexity present in biological reality. It must be noted, however, that SVMPs are considerably larger than crotoxin isoforms in terms of molecular weight, which might delay absorption *via* the bloodstream and/or lymphatic system, as documented for *C. simus* venom (135). Thus, further research on the ecology and natural history of this species alongside the pharmacokinetics of its venom is necessary in order to understand how procoagulant venom activity translates to a functional role for the animal.

The pseudo-procoagulant activity of the venoms also showed extreme taxon-specificity, being active on mammalian plasma but not amphibian plasma. The fibrinopeptide domain at which thrombin cleaves fibrinogen to form fibrin clots differs sharply in mammals vs. the homologous region of amphibians/diapsids (**Figure 13**). While the precise region at which the venoms cleave fibrinogen to form the unnatural fibrin clots has not been yet elucidated for these species, we observed a clear difference in clot strength between the thrombin-activated fibrinogen and that of the venom-activated fibrinogen (**Figure 3**). Much has been said in the literature about the inability of snake venom fibrionogenolytic enzymes to stabilize fibrin clots through the activation of Factor XIII, leading to weaker clots (136–142). However, this study (**Figure 3**) and previous research alike reported that venom-induced fibrin clots were still considerably weaker than thrombin-induced ones, even in the absence of FXIII, which is indicative of the venoms cleaving the fibrinogen differently relative to thrombin. Thus, snake venoms either cleave at a different region of the fibrinopeptide domain or at additional sites in the full-length fibrinogen chains to disrupt the latticework. Previous work on some species has revealed that some cleave only fibrinopeptide A, while others cleave only fibrinopeptide B, but with both at the same cleavage site as thrombin (143). However, cleaving at these sites should produce the same clot strengths as thrombin, yet they yield weaker clots. This suggests that if both fibrinopeptides are being cleaved at sites identical to those targeted by thrombin, yet produce weak, unstable, and short-lived clots, then the venoms are cleaving at additional sites, as would be the case for destructive (non-clotting) venoms. Such sites have been identified for some venoms (143, 144). Overall, however, this aspect of venom biochemistry is poorly researched. Future work should investigate whether the pseudo-procoagulant activity is mammal-specific by testing additional venoms on non-mammalian plasma. In addition, it is recommended to investigate the specific cleavage site to ascertain the differential nature of the cleavage between thrombin and the venoms.

Clinical Implications

As previously discussed, procoagulant activation of zymogens would rapidly incapacitate prey animals *via* thrombosis. Conversely, the venom is diluted into a much larger blood volume in human bite victims, which typically does not result in stroke, although this has been noted on occasion (145, 146). Instead, when venom is diluted throughout a large blood volume, venom-induced consumption coagulopathy (VICC) occurs *via* depletion of clotting factors following excessive activity of the coagulation cascade (147, 148). This net anticoagulant state can result in death *via* internal bleeding.

Our findings demonstrate that the FX-activating procoagulant action of neonate *C. culminatus* venom is not neutralized by Antivipmyn®, one of the most frequently used antivenom products in Mexico. A logical explanation is that Antivipmyn® does not include *C. culminatus* venom in its immunizing mixture, relying on venom from adult *C. simus* specimens instead (112). *C. simus* has been recently split into *C. ehecatl* and *C. mictlantecuhtli* throughout most of its Mexican range (26), which is likely to affect antivenom manufacturing in turn. To our knowledge, no snake antivenom is produced using venom from juvenile individuals, and this is due to practical constraints of lower venom yields from smaller snakes. Venom from this species complex lacks the metalloprotease-driven true procoagulant trait, being instead pseudo-procoagulant *via* kallikrein-type serine proteases as shown in this work and previous studies (32, 44, 149). The clinical effects of this toxic activity would be VICC *via* depletion of fibrinogen following formation of unstable fibrin clots by serine proteases, as reported for multiple other species (51, 52). This SVSP-based pseudo-procoagulant activity was drastically reduced by Antivipmyn® in our assay for all venoms possessing this activity (and by AEBSF as well in the case of *C. mictlantecuhtli*). Our results therefore confirm extensive cross-reactivity for Antivipmyn® against pseudo-procoagulant SVSPs in contrast to the failure against the neonate *C. culminatus* Factor X activation.

The BIRMEX® (Faboterápico polivalente antiviperino precio), antivenom, widely marketed in Mexico alongside Antivipmyn® to treat rattlesnake envenoming, has *C. basiliscus* and *Bothrops asper* as its main immunizing species (112, 150). Although this product displays a high degree of cross-reactivity across multiple rattlesnake species (32, 150), further testing is recommended to determine whether it is able to neutralize the true procoagulant activity found in *C. culminatus*. However, as the immunizing venom composition does not include this species, it is unlikely to produce a more promising result.

Our findings draw attention to the pivotal importance of which immunizing venoms are chosen for antivenom production, including the critical need to ascertain ontogenetic changes. Such antivenom issues have been noted for other genera such as *Pseudonaja* (Australian brown snakes), with juvenile venoms rich in neurotoxic three-finger peptides to specialize on lizard prey, and adult venom rich in the FactorXa:FactorVa toxin complex to prey upon mammals as well at later life stages (58, 59, 110, 151). Thus, the antivenom raised against adults performs poorly against neonates due to the pronounced differences in venom biochemistry.

Unlike antivenom, the commercially available metalloprotease inhibitor Prinomastat was able to suppress the procoagulant

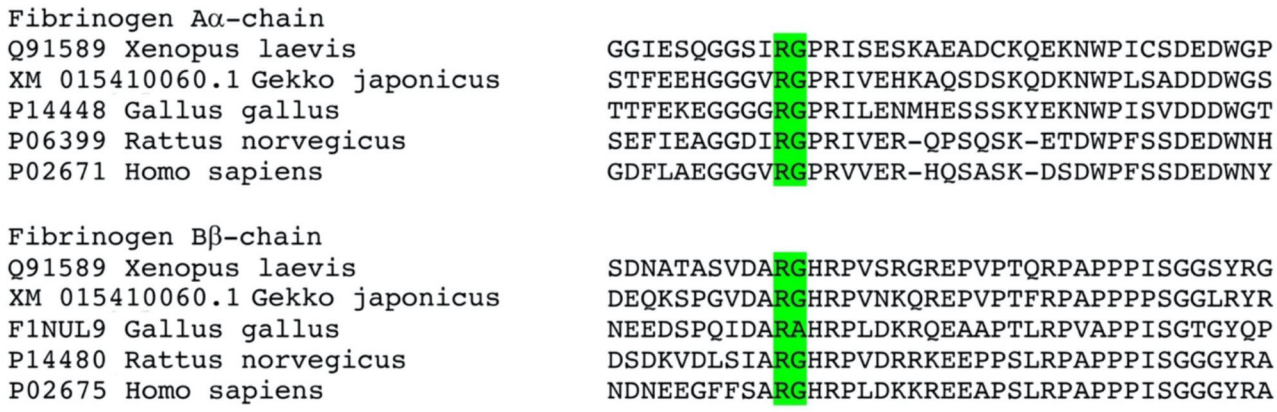

FIGURE 13 | Sequence alignment of the activation cleavage sites. Cleavage site of normal thrombin is shown in green, however the venom induced cleavage sites remain to be elucidated for these venoms. There are however clear sequence differences downstream of the known thrombin cleavage site that are distinct between mammals and amphibians/diapsids.

action of neonate *C. culminatus* venom. However, the inhibitor DMPS performed poorly compared to Prinomastat, indicating that Prinomastat has greater potential as a field-deployable, temperature-stable, first-aid measure. Further problematic for DMPS is its intrinsic anticoagulant action upon plasma (elevating spontaneous clotting times) that might exacerbate disruption of blood coagulation in a real-life VICC scenario. Such a marked divergence may be ascribed to the different action of the two molecules. DMPS is a metal chelator, commonly used to treat heavy metal poisoning (152, 153), that binds the Zn^{2+} ions required for SVMPs to function (106). In contrast, peptidomimetic hydroxamate-based inhibitors like Prinomastat directly inactivate the activity of metalloproteases by binding to their catalytic site in combination with chelation of Zn^{2+} (105, 154). Thus, it is possible that DMPS is slower acting and/or requires a higher concentration to effectively hamper SVMP activity compared to Prinomastat. This was corroborated by preliminary tests showing that DMPS at a 20 mM concentration incubated with venom for 20 min was more efficient in neutralizing venom effects than at 2 mM for 2 min. Such a prolonged venom-inhibitor proximity would be unlikely to occur in a dynamic system like the bloodstream. Thus, investigations into the efficacy of inhibitors should prioritize those which are fast acting. It should also be noted that SVMPs are a highly diverse toxin family consisting of three classes, each of which is characterized by different structures and active domains with important consequences for their toxic activity (132). Thus, it is possible that the metalloproteases found in *Echis* venom, which were shown to be neutralized by DMPS (106) and by ion chelators in general better than by peptidomimetic inhibitors (105) differ from those present in *C. culminatus* to an extent where cross-reactivity is poor for DMPS. However, the study that examined the suitability of DMPS for neutralizing *Echis* venoms (106) used different methodologies (e.g., a kinetic fluorogenic assay to assess the effect of DMPS and other chelators on plasma clotting and SVMP activity) and thus comparing the relative potency with the poor neutralization results obtained in this study is impossible. Future work should undertake head-to-head comparisons between Prinomastat and DMPS using the presently used methodology and with a larger species pool (including *Echis*) to ascertain if DMPS consistently performs less efficiently than Prinomastat. However, in another study DMPS was conspicuously unable to neutralize *Daboia russelii* venom (155), which exerts its powerfully procoagulant effect *via* SVMP-induced Factor X activation like the neonate *C. culminatus* venom in this study. This suggests two future hypotheses to test. First, that the SVMPs in the two venoms, and thus presumably the FX activators in other viper venoms such as *Bothrops*, share a common molecular ancestry, putatively all being P-IIId SVMPs, whereby two lectin peptides are covalently linked to the SVMP enzyme. This would in turn suggest that DMPS is unable to neutralize P-IIId SVMPs in general. The prior work on DMPS examined only *E. carinatus* and *E. ocellatus*, which are both PIIIa rich venoms but not P-IIId rich like *E. coloratus* and putatively *E. leucogaster* and *E. pyramidum leakeyi* (56). Thus, future work should test a broader diversity of *Echis* to determine the efficacy of DMPS in neutralizing venoms rich in P-IIId SVMPs.

In recent years, several studies have proposed the use of small molecule inhibitors as an adjunct treatment for snakebite envenoming, to be administered before or alongside antivenom (105, 106, 156, 157). Both Prinomastat and DMPS are already licensed and widely marketed worldwide and can be administered outside a hospital setting (even *via* oral ingestion for DMPS), facilitating their use in real-life envenoming situations. Taken together, our observations indicate that *C. culminatus* possesses a peculiar venom phenotype that hampers antivenom cross-reactivity with its closest relatives in Mexico and encourages the use of metalloprotease inhibitors as an adjunct treatment. However, it should be noted that, while small molecule inhibitors have shown considerable potential in countering symptoms of snakebite, their repurposing for use as an adjunct

treatment for envenomation will require *in vivo* investigations and clinical trials before regulatory authority approval.

From an epidemiological perspective, *C. culminatus* envenoming in humans is likely to occur regularly in the rural environments but is seldom documented due to poor epidemiology being a broad medical issue in such remote communities (Rebolledo, personal communication, January 2020), whereas *C. simus* (i.e., *C. mictlantecuhtli* + *C. ehecatl* + *C. simus*) is responsible for the majority of rattlesnake bite episodes in several Mexican states and Central American countries (14, 158). As populations centers spread into the remote areas occupied by *C. culminatus*, envenomations may increase in frequency. In addition, this species is sought after in the exotic pet trade and thus bites from captive *C. culminatus* individuals in the private reptile keeping sector may result in significant medical complications not neutralizable by available antivenoms, especially in countries where the species is not native. This species is therefore of potential clinical concern and we recommend further research on optimal treatments for its envenoming.

Conclusion

This study reports the first occurrence of true procoagulant venom activity in Mexican Neotropical rattlesnakes for the species *Crotalus culminatus*, especially in early life stages. This went largely undetected in previous studies due to the lack of Ca^{2+} and phospholipids in plasma clotting assays resulting in experimental conditions lacking physiological venom requirements for functional activity. The poor efficiency of one of the main Mexican antivenom products against this action highlights the need to include a wide array of snake species and life-stages in antivenom immunizing mixtures. The metalloprotease inhibitor Prinomastat however was highly effective in neutralizing the procoagulant venom activity in *C. culminatus*, further validating the use of small molecule inhibitors as adjunct treatment for snakebite despite DMPS performing poorly in comparison. Overall, we hope our results will contribute to the evidence-based design of clinical management strategies for rattlesnake envenoming in Mexico and emphasize the importance of natural history and evolutionary research on rattlesnakes and their venom.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human plasma was sourced from surplus supplies from the Australian Red Cross under Human Ethics Approval #2016000256. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by Rhinella marina plasma was obtained under the University of Queensland Animal Ethics approval SBS/019/14/ARC.

AUTHOR CONTRIBUTIONS

LS, CZ, AC, and CR performed the experiments. LS, AC, and CR analyzed the data. EN-C, MB-V, and AA provided the venoms. BF designed the study. LS, CZ, and BF wrote the manuscript. All authors proof-read and revised the manuscript prior to submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.612846/full#supplementary-material

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Association of a Network of Immunologic Response and Clinical Features With the Functional Recovery From Crotalinae Snakebite Envenoming

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Background: The immunologic pathways activated during snakebite envenoming (SBE) are poorly described, and their association with recovery is unclear. The immunologic response in SBE could inform a prognostic model to predict recovery. The purpose of this study was to develop pre- and post-antivenom prognostic models comprised of clinical features and immunologic cytokine data that are associated with recovery from SBE.

Materials and Methods: We performed a prospective cohort study in an academic medical center emergency department. We enrolled consecutive patients with Crotalinae SBE and obtained serum samples based on previously described criteria for the Surgical Critical Care Initiative (SC2i)(ClinicalTrials.gov Identifier: NCT02182180). We assessed a standard set of clinical variables and measured 35 unique cytokines using Luminex Cytokine 35-Plex Human Panel pre- and post-antivenom administration. The Patient-Specific Functional Scale (PSFS), a well-validated patient-reported outcome of functional recovery, was assessed at 0, 7, 14, 21 and 28 days and the area under the patient curve (PSFS AUPC) determined. We performed Bayesian Belief Network (BBN) modeling to represent relationships with a diagram composed of nodes and arcs. Each node represents a cytokine or clinical feature and each arc represents a joint-probability distribution (JPD).

Results: Twenty-eight SBE patients were enrolled. Preliminary results from 24 patients with clinical data, 9 patients with pre-antivenom and 11 patients with post-antivenom cytokine data are presented. The group was mostly female (82%) with a mean age of 38.1 (SD ± 9.8) years. In the pre-antivenom model, the variables most closely associated with the PSFS AUPC are predominantly clinical features. In the post-antivenom model, cytokines are more fully incorporated into the model. The variables most closely associated with the PSFS AUPC are age, antihistamines, white blood cell count (WBC),

HGF, CCL5 and VEGF. The most influential variables are age, antihistamines and EGF. Both the pre- and post-antivenom models perform well with AUCs of 0.87 and 0.90 respectively.

Discussion: Pre- and post-antivenom networks of cytokines and clinical features were associated with functional recovery measured by the PSFS AUPC over 28 days. With additional data, we can identify prognostic models using immunologic and clinical variables to predict recovery from SBE.

Keywords: snake bite, antivenin, cytokine, prognostic model, predictive modeling, chemokine, Patient Specific Functional Scale

BACKGROUND

Snakebite envenoming (SBE) is a neglected tropical disease of global significance that primarily impacts low resource areas in low and middle income countries (LMICs), and snake-specific antivenoms are the cornerstone of therapy (1–8). Snake antivenoms have been used for over 100 years and have well demonstrated efficacy, but there remain significant safety, cost, and access to treatment considerations that limit their consistent use (5, 9). Currently available United States (US) antivenoms have a remarkable safety profile as compared to previous SBE biologics (10–16). Globally the safety profile of antivenoms is more mixed, and the risk-benefit profile of their use varies (17–19). This creates a potential barrier to their use in non-life threatening SBE where administration will not impact mortality, but can impact recovery and potential permanent disability (20).

Despite positive cost-effectiveness assessments of snake antivenoms, the cost to individual SBE victims can be beyond the financial means of many of those affected (21–23). This creates another barrier to use if the need for treatment is unclear due to the uncertainty of the severity and anticipated clinical course of the SBE patient (24, 25). There is also little economic incentive to pharmaceutical companies to develop and overcome the regulatory hurdles in order to distribute antivenom to where it is most needed (22, 26–28). This fact, combined with the lack of healthcare infrastructure in these low resource areas with a high prevalence of SBE, results in a lack of a consistently replaceable antivenom supply (28, 29). The concern for wasting antivenom when not absolutely needed, is accompanied by the risk of undertreatment for patients that would otherwise benefit (12, 30, 31). The described barriers to antivenom use are particularly important as treatment is time dependent with worse outcomes when care is delayed (32–38).

The clinical courses of SBE patients are highly variable, even within a single envenoming species, which further contributes to uncertainty regarding both the value of administering antivenom and how much is necessary for an individual patient (39). Unfortunately, there is no validated and reliable method of predicting severity or recovery in SBE (40). Historically clinicians have had to rely primarily on their own clinical experience. This judgement has been augmented by treatment guidelines and severity scales, but these have not been evaluated as to whether they improve clinical outcomes (7, 41–43). This has led to wide practice variation in when and how much antivenom is administered even within the US where safety and access to antivenom are much less of a concern (44). A prognostic tool that could predict the severity of SBE would be valuable in overcoming the stated barriers to antivenom use and allow for therapy tailored to the individual in order to prevent both under- and over-treatment. Hence, the need for novel prognostic strategies persists.

Snake venom is a complex mixture of enzymatic and non-enzymatic proteins and peptides. In the sub-family Crotalinae, the species *Crotalus horridu*s and *Agkistrodon contortrix* are known to have a large proportion of their venom composed of secretory phospholipase A2 (sPLA2), snake venom metalloproteases (SVMPs), and serine protease toxins. These toxins are of particular biologic importance and contribute to the venom-induced tissue injury, coagulopathy, and systemic symptoms seen in these SBEs (45, 46). However, the host inflammatory response is felt to also play a significant role in the pathophysiology and clinical syndrome of SBE (47–50). Consequently, evaluating inflammatory markers such as serum cytokines and chemokines give us a potential technique to evaluate immunologic response in SBE and hold promise as a component of a comprehensive prognostic strategy (48). Advances in enzyme-linked immune-assays using fluorescent bead technology now allow for simpler, reliable and rapid determination of cytokine levels (51). Consequently, the role of cytokines in other disease states is being investigated in LMICs using this technology (52–54).

Clinical features alone have not shown adequate prognostic performance in SBE, despite having some predictive value (37, 38, 40, 55, 56). Combining clinical features with immunologic markers has been used to prognosticate outcomes in other disease states (57–60). An improved understanding of the immunologic response combined with available clinical features in SBE could inform a prognostic model to predict severity and recovery in SBE. The purpose of this study was to develop pre- and post-antivenom prognostic models comprised of clinical features and immunologic cytokine and chemokine data administration that are associated with recovery from SBE.

MATERIALS AND METHODS

Design

We performed a prospective cohort study at a single tertiary-care academic emergency department (ED) in the southeastern US in 2017 and 2019.

Participants

We enrolled consecutive patients with acute snakebite envenoming (SBE) based on the Surgical Critical Care Initiative-enVenomation Investigation Pilot to Expedite Recovery (SC2i - VIPER) based on the SC2i - Tissue and Data Acquisition Protocol (TDAP) (*ClinicalTrials.gov Identifier: NCT02182180*). As the parent protocol has been previously described, we provide additional methods specific to the SC2i-VIPER sub-study that are necessary for reproducibility (57). We enrolled patients age 27 - 80 years presenting within the first 24 hours with verified acute SBE. No venom levels were obtained for enrollment, as all local venomous snakes are of the sub-family Crotalinae. Local species include *Agkistrodon contortrix* (copperhead) and *Crotalus horridus* (timber rattlesnake). Regionally transferred patients were eligible with the addition of the following potential Crotalinae species: *Crotalus ademanteus* (eastern diamondback rattlesnake), *Sistrurus miliaris* (pygmy rattlesnake) and *Agkistrodon piscivorus* (cottonmouth snake). Species identification was not required for enrollment, however the majority of investigator-identified species were copperhead snakes which predominate throughout the region. Exclusion criteria were pregnancy, prisoners, or inability to obtain consent during their stay for any reason. Informed consent was provided by a patient or proxy and the Duke University Institutional Review Board approved the study.

Clinical Data

We assessed clinical variables as per SC2i protocol which includes static pre-existing variables such as sex, age, comorbidities and dynamic clinical variables such as laboratory results, medications, and vital signs. Clinical data were collected from patients and by review of the electronic health record using standard operating procedures.

Biological Sample Collection and Processing

Samples from SBE patients were collected as soon as possible following their enrollment, ideally before antivenom administration when possible, as well as after initial antivenom administration. Whole blood was collected and serum was isolated at the site for the SC2i Consortium Biorepository following consortium-wide standard operating procedures and Guidelines for Good Clinical Laboratory Practice (GGCLP), and then stored until use for these analyses. The methods of sample collection and preparation have been reported, but are briefly encapsulated here for clarity (61).

Sample Preparation

Frozen serum samples were thawed on wet ice for filtering. The samples could have a single freeze-thaw cycle. They were mixed and 400 uL were transferred to a 1.5 mL tube and centrifuged at 12,000g for 15 minutes at 4 degrees Celsius. A 350uL sample was transferred into a 0.65um filter tube (Millipore, Billerica, MA). They were centrifuged 12,000g for 30 minutes at 4 degrees Celsius. We then aliquoted 120uL of the filtered samples into clean tubes. We then flash froze samples in liquid nitrogen and stored in -80 degree Celsius freezer. The maximum time from freeze to thaw was less than one year.

After filtering, samples were thawed and a 1:50 dilution of samples were used. We measured levels of 35 unique cytokines using Luminex Cytokine 35-Plex Human Panel (Thermo Fisher Scientific, Waltham, MA) pre- and post-antivenom administration using standard cytokine preparation per kit instructions. A 1x wash preparation was created and used for all washes. Standard plates were prepared and 50ul of sample, standards, and incubation buffer were added to all wells and allowed to incubate for 2 hours. After washing, detection antibodies were added and incubated for one hour. After another wash, streptavidin-RPE was added and incubated for 30 minutes. These were washed again and beads were resuspended in wash buffer. For 35 plex analysis, we used Invitrogen Catalog# LHC6005M kits are from Thermo Fisher Scientific. The Comprehensive Human Cytokine Magnetic 35-Plex Panel provides reagents for the accurate, reproducible, and sensitive quantitation of human proteins including: EGF, Eotaxin, FGF-basic, G-CSF, GM-CSF, HGF, IFN-alpha, IFN-gamma, IL-1 beta, IL-1 alpha, IL-1RA, IL-2, IL-2R, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-17F, IL-22, CXCL10, CCL2, CXCL9, CCL3, CCL4, CCL5, TNF-alpha, VEGF.

Outcomes

The Patient-Specific Functional Scale (PSFS), a well-validated patient-reported outcome of functional recovery, was assessed at 0, 7, 14, 21 and 28 days. Individual patient PSFS recovery curves over time were assessed to determine the PSFS area under the patient curve (AUPC) over the course of 28 days. The median PSFS AUPC was determined. Patients with PSFS AUPC great than or equal to the median were considered to have good recovery and those less than the median defined poor recovery.

Data Analysis

For this preliminary analysis, we report baseline characteristics of all patients with available data. Continuous variables are summarized using mean, medians and interquartile range (IQR) as appropriate for the distribution of data.

To account for the small sample size with high dimensionality of the data set, we performed Bayesian Belief Network (BBN) modeling using a machine learning technique to build directed acyclic graphs that represent relationships with a diagram composed of nodes and arcs. Each node represents a cytokine, chemokine or clinical feature and each arc represents a joint-probability distribution (JPD). The JPDs were determined in a step-wise fashion over multiple iterations. We report associations of this cytokine/chemokine/clinical feature model with the patient's recovery (PSFS AUPC). We built the BBNs employing FasterAnalytics Version 7.0. A BBN starts with an observation, then calculates the relationships of various variables that are likely to impact that observation based on known probabilities (beliefs). BBNs are very useful for illustrating the differences in the immune systems of different patient groups since they are represented by acyclic directed graphs. These graphs can be overlaid on each other so that we can easily see

where there are differences in the cytokine, chemokine and clinical feature joint probability distributions. In this case, BBN's can 1) identify which relationships exist between variables 2) allow us to measure the magnitude of relationships (one variable on another) but also the number of relationships for any given variable 3) handle a large number of variables without large datasets 4) compare which relationships exist and how many relationships exist between the two groups of recovery (good and poor). These models also deal easily with missing data, which was anticipated in this cytokine data set.

For the Luminex 35-plex kit, we built four Bayesian Belief Networks (BBN) for preliminary modeling using a Minimum Descriptive Lengths (MDL) of 0.01. The MDL measures the trade-off between goodness-of-fit and complexity of the model. Models with lower MDLs have more complexity and are more likely to be overfit. For each time point (pre- and post-antivenom), we built one model on the full data set and then models using leave-one-out. For each timepoint, we looked at the models and found which variables were first-degree associates of recovery. We used these variables in final modeling. If a variable was missing for more than 20% of records, we dropped the variable for the models with that kit.

We used leave-one-out cross validation. We used the scores from each test case to build a receiver operating characteristic (ROC) and measure the area under the curve (AUC). We consider any AUC of 0.6 or above to be a good differentiator between good and poor recovery. We also provided statistics about the connectivity of the models.

RESULTS

Demographics

Twenty-eight snake envenoming patients were enrolled. Preliminary demographic data and PSFS AUPC is available for 24 patients with cytokine and chemokine data available from 9 pre-antivenom and 11 post-antivenom patients. The group was mostly female (82%) with a mean age of 38.1 (SD ± 9.8) years. **Table 1**.

TABLE 1 | Demographics and clinical features of enrolled patients.

	N = 24	N = 11
Age -Min, Median, Max	25, 45, 78	27, 35, 56
Age - 1stQ, Mean, 3rdQ,	33.5, 46.2, 56	30.5, 38.1, 46.5
Sex (% Female)	17 (70.1%)	9 (81.8%)
White	22 (91.6)	10 (90.1%)
Asian	2 (8.3%)	1 (9.1%)
Comorbidity (at least one)	11(45.8%)	5 (54.5%)
Immunosuppressed	1 (4.1%)	0

Recovery

The patients' functional recovery as measured by the PSFS AUPC, for the 24 patients with available data, had a median of 0.73 (range 0.43, 0.94) and is represented graphically in **Figure 1**.

Pre- and Post-Antivenom Cytokines

The pre- and post-antivenom cytokines and chemokines were compared in **Figure 2**. There were changes in log values pre- and post-antivenom with increases in the median of the log of basic fibroblast growth factor (FGFBasic)(1.74 to 2.97), interleukin-2 receptor (IL02R)(3.04 to 4.50) and regulated on activation, C-C motif ligand chemokine (CCL5)(0.28 to 0.41) and a decrease in human growth factor (HGF)(6.24 to 5.85).

The pre-antivenom individual cytokines and chemokines values were compared between good and poor functional recovery and are represented in **Figure 3**. In the pre-antivenom data, patients with poor recovery have higher log values of the eosinophil chemokine subfamily EOTAXIN (poor recovery median 4.35, good recovery median 3.99), HGF (6.44, 5.59), interleukin-1 receptor antagonist (IL01Ra) (3.98, 3.96), IL10 (1.90, 1.43), IL12 (4.47, 4.14), and C-C motif ligand 2 (CCL2) (6.06, 5.69). Patient with poor recovery had lower log values of CXCL10: C-X-C motif ligand 10 (1.39, 1.74), C-C motif ligand 4 (CCL4) (3.71, 4.40), CCL5 (7.26, 7.33), and vascular endothelial growth factor (VEGF) (0.26, 0.92). The post-antivenom individual cytokines and chemokines values were compared between good and poor functional recovery and are represented in **Figure 4**. Patients with poor recovery have higher values of endothelial growth factor (EGF) (poor recovery median

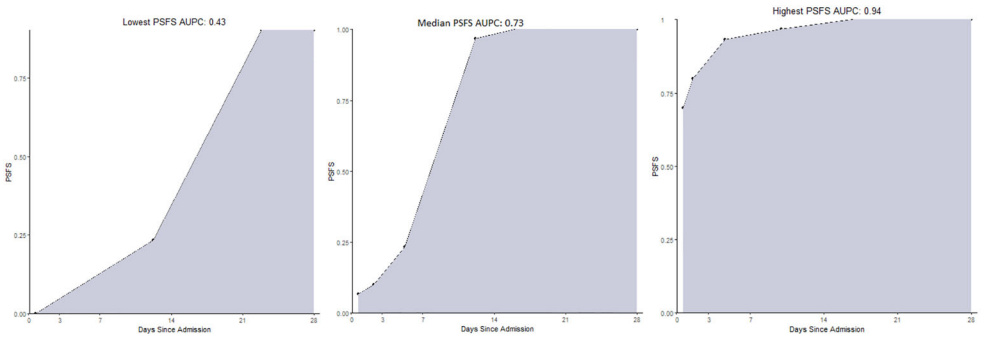

FIGURE 1 | The range of the Patient-Specific Functional Scale Area Under the Patient Curves at 28 days for 24 patients with available preliminary data. (examples lowest, median, highest). PSFS AUPC, Patient-Specific Functional Scale Area Under the Patient Curve.

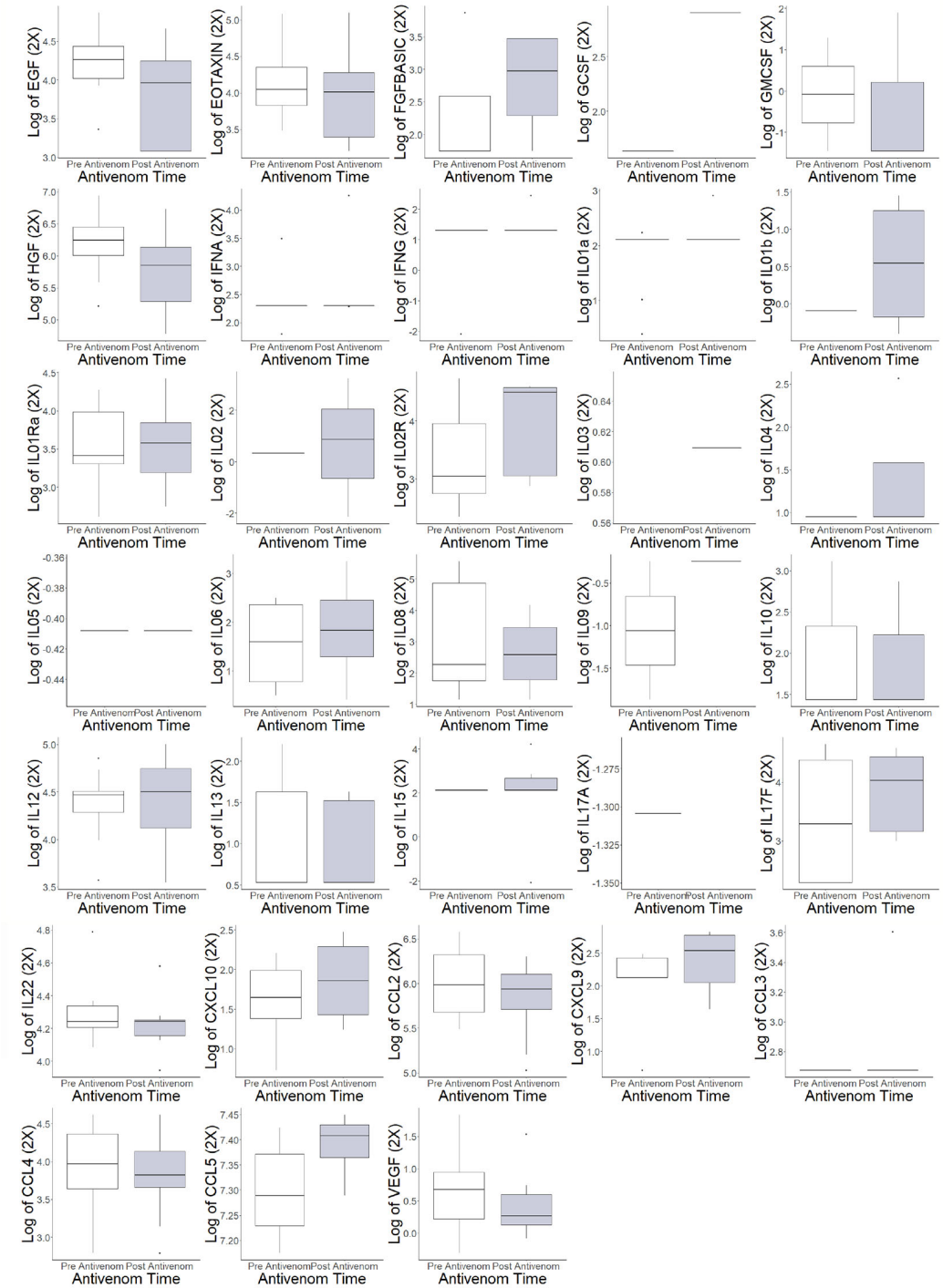

FIGURE 2 | Individual cytokine and chemokine values pre- and post-antivenom treatment. EGF: endothelial growth factor, EOTAXIN: eosinophil chemotactic proteins, *FGFBASIC: fibroblastic growth factor, basic, GCSF: granulocyte colony stimulating factor, GMCSF: ganulocyte-macrophage colony stimulating factor, *HGF: hepatocyte growth factor, IFNA: interferon alpha, IFNG: interferon gamma, IL01interleukin 1a, IL01b: interleukin 1b, IL01Ra: Interleukin 1 receptor antagonist, IL02: interleukin 2,* IL02R: interleukin 2 receptor, IL03: interleukin 3, IL04: interleukin 4, IL05: interleukin 5, IL06: interleukin 6, IL08: interleukin 8, IL09: interleukin 9, IL10: interleukin 10, IL12: interleukin 12, IL13: interleukin 13, IL15: interleukin 15, IL17A: interleukin 17A, IL17F: interleukin 17F, IL22: interleukin 22, CXCL10: C-X-C motif ligand 10, CCL2: C-C motif ligand 2, CXCL9: C-X-C motif ligand 9, CCL3: C-C motif ligand 3, CCL4: C-C motif ligand 4, *CCL5: C-C motif ligand 5, VEGF: vascular endothelial growth factor. *Difference in populations based on box plots.

4.25, good recovery median 3.08), HGF (6.09, 5.27), IL01Ra (3.75, 3.33), and IL12 (4.54, 4.34) with lower values of CXCL10 (1.62, 2.29), CCL2 (5.90, 6.05), and CCL4 (3.71, 3.98).

Prognostic Modeling Networks

In order to illustrate the effect antivenom has on the networks, the pre-and post-antivenom modeling networks are presented in **Figures 5** and **6**, respectively. In the pre-antivenom model, the variables most closely associated with the PSFS AUPC (i.e. recovery) are predominantly clinical features such as age, respiratory rate, CO2 (mostly bicarbonate) as measured by the basic metabolic panel, white blood cell count (WBC) and the need for antihistamines. These variables are also the most influential on the model, according to the Minimum Description Length (MDL) score. In the post-antivenom model, cytokines and chemokines are more fully incorporated into the model. The variables most closely associated with the PSFS AUPC are age, antihistamines, HGF, CCL5 and VEGF. The most influential variables are age, antihistamines and ECF. Within the limits of this small preliminary dataset, both the pre- and post-antivenom models perform well with AUCs of 0.87 and 0.90 respectively. Model stability was not assessed.

DISCUSSION

Using a preliminary data set from patients with SBE, we identified distinct pre- and post-antivenom patterns of clinical features combined with cytokines and chemokines that are associated with recovery. These findings are important as they demonstrate the feasibility of using this technique to develop a more predictive prognostic model than currently exists. Ultimately this could result in a clinical decision support tool capable of determining thresholds to treat with antivenom or other novel therapeutics and/or informing the need for escalating therapies. This individually tailored therapy approach holds the potential to most efficiently improve patient outcomes from SBE while preserving treatment for those likely to benefit.

The functional impact of SBE varies widely from patient to patient even within a given envenoming species. Specific SBE-related disabilities may impact different functions from work, to self-care, domestic life, social interactions, or civic life to name a few (62–64). By predicting and mitigating morbidity, we can maximize the economic, societal, and individual impact of SBE treatment. This is of particular importance in low-resourced areas of LMICs where the majority of SBE-associated negative consequences occur (5).

Our technique has already been used to enhance the care of other disease states such as infection and injury. In 2019, Gelbard et al. developed models using clinical data, cytokines, chemokines and growth factors that predicted severe sepsis and organ space infections following laparotomy for abdominal trauma (58). This modeling approach has expanded to combat trauma, where a predictive model composed of clinical features and immunologic biomarkers can accurately predict pneumonia in a predominantly blast-injured cohort of patients (59).

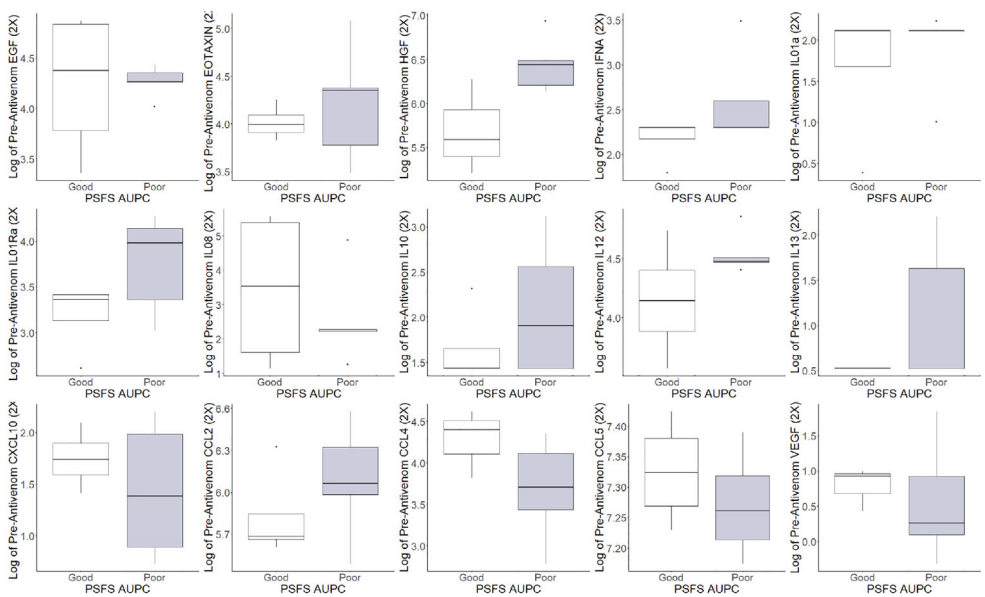

FIGURE 3 | Pre-antivenom cytokines and chemokines values of patients with good and poor recovery. EGF: endothelial growth factor, *EOTAXIN: eosinophil chemotactic proteins, *HGF: hepatocyte growth factor, IFNA: interferon alpha, IL01a: interleukin 1a, IL01Ra: Interleukin 1 receptor antagonist, IL08: interleukin 8, *IL10: interleukin 10, *IL12: interleukin 12, IL13: interleukin 13, *CXCL10: C-X-C motif ligand 10, *CCL2: C-C motif ligand 2, *CCL4: C-C motif ligand 4, *CCL5: C-C motif ligand 5, *VEGF: vascular endothelial growth factor. *Difference in populations based on box plots.

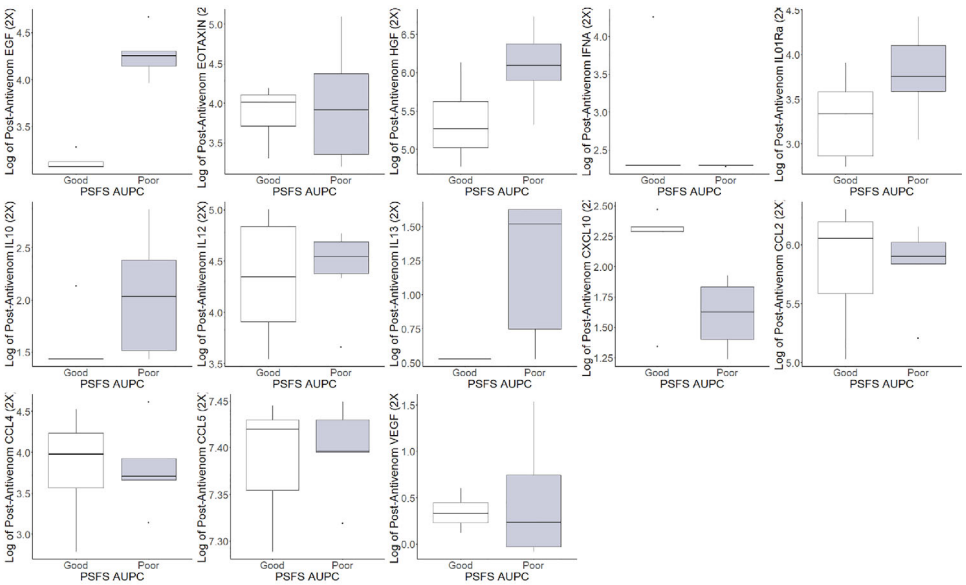

FIGURE 4 | Post-antivenom cytokine and chemokine values of patients with good and poor recovery. *EGF: endothelial growth factor, EOTAXIN: eosinophil chemotactic proteins, *HGF: hepatocyte growth factor, IFNA: interferon alpha, *IL01RA: Interleukin 1 receptor antagonist, IL10: interleukin 10, *IL12: interleukin 12, IL13: interleukin 13, *CXCL10: C-X-C motif ligand 10, *CCL2: C-C motif ligand 2, *CCL4: C-C motif ligand 4, CCL5: C-C motif ligand 5, VEGF: vascular endothelial growth factor. *Difference in populations based on box plots.

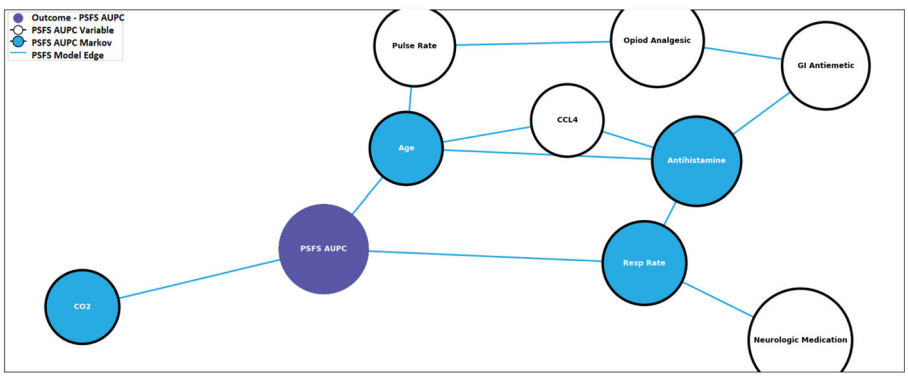

FIGURE 5 | Pre-antivenom prognostic model predictive of recovery. PSFS AUPC: Patient-Specific Functional Scale Area Under the Patient Curve, CCL4: C-C motif ligand 4, GI: gastro-intestinal, CO_2: carbon dioxide, Resp Rate: respiratory rate.

By expanding this technique to SBE, we have built on the prior work evaluating both the clinical features predicting severity and studies of the association of soluble biomarkers with severity (47, 48, 65, 66). In doing so, we are able to overcome some of the limitations of the initial forays into prognostication in SBE. For example, a multitude of basic clinical features have been assessed in predicting severe SBE (33, 34, 36, 37, 40, 43, 56, 67, 68). A number of these established clinical features, such as young age and time to care, are now known to increase the likelihood of a severe envenoming. However, none can adequately exclude severe SBE (40). Consequently, they have not been assessed as a tool to determine the need to treat or not to treat with antivenom. Additionally, the commonly applied laboratory testing have not even been demonstrated to confidently predict their own future trend (40, 56, 69). Finally, combinations of these clinical features and laboratory tests have been combined into a number of grading scales (42, 43). However, none of these have had comprehensive psychometric evaluations that assess their validity and reliability in predicting patient-centered outcomes.

Similarly, the associations of soluble immunologic biomarkers such as cytokines, chemokines and complement with acute findings of tissue injury are now being studied. In 2019, Ibiapina and colleagues evaluated the relationships between

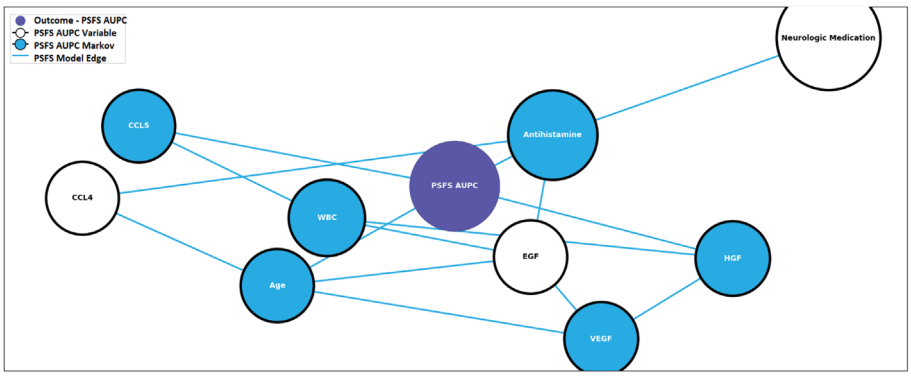

FIGURE 6 | Post-antivenom prognostic model predictive of recovery. PSFS AUPC: Patient-Specific Functional Scale Area Under the Patient Curve, CCL4: C-C motif ligand 4, CCL5: C-C motif ligand 5, ECG: endothelial growth factor, HGF: hepatocyte growth factor, VEGF: vascular endothelial growth factor, WBC: white blood cell count.

seventeen of these biomarkers and tissue injuries. Relationships were evaluated between biomarkers of healthy controls and patients with mild or severe tissue injury in *Bothrops asper* SBE. Important associations were established with individual biomarkers as well as classes of immune response polarizations such as Th-1,Th-2, and Th17. However, their results are limited by the lack of an established relationship between acute tissue findings they evaluated, such as swelling, blisters, or superficial necrosis, with important patient-centered outcomes such as functional recovery (48).

Our study also shows relationships with individual immunologic biomarkers and clinical outcomes. For example, Th-2 polarization-associated cytokines such as IL-10 and IL-13 were increased in patients with poor outcomes, as were the chemokines CXCL 10, and the growth factors EGF and HGF. Our preliminary data is not yet robust enough to reliably characterize the comprehensive nature of the immune response in SBE. However, for prognostication purposes, it appears that we can overcome prior limitations by using advanced machine learning models using clinical and immunologic laboratory features to accurately predict a well-validated, patient-centered, functional outcome.

In addition to prognostic model development, our data give us further insight into potential immunologic therapeutic targets in the sterile inflammatory disease state of SBE. For example, nonsteroidal anti-inflammatory drugs have been typically avoided in SBE due to their antiplatelet effects and the potential hemotoxicity of snake venom. However, the study of their beneficial impact on cytokine response and wound healing in trauma demonstrates the need to further study NSAIDs and immune-mediated outcomes in an appropriate SBE population (61, 70, 71). Ultimately, a comprehensive evaluation of individual biomarkers including their change over the natural history of SBE, their relationship with outcome, the impact of antivenom on their levels, their role in prognostic modeling, and the presence of existing therapeutics targeting the specific biomarker should all impact their candidacy as a viable therapeutic target.

Moreover, consistency of results will play an important role in this work. Interestingly, this study did not find a significant elevation of tumor necrosis factor alpha (TNF-alpha). Previous work has shown a relationship between increasing TNF and SBE (72–74). Our unanticipated finding may be due to the predominant species enrolled in this study, *Agkistrodon contortrix*, is known to typically have a less severe clinical course. However, this finding should be verified using other cytokine assays to better characterize its role compared to SBE from other species.

LIMITATIONS/FUTURE DIRECTIONS

Our preliminary findings are limited by the small sample size currently available for analysis. This resulted in some atypical demographic patterns such a female predominant study population; whereas, a similar SBE population in our geographic region is typically slightly male predominant (75). This also limits our ability to perform more nuanced analysis such as evaluating outcomes based on the total amount of antivenom administered. Despite this, the models do perform well and the anticipated additional data can be used to refine the models and assess model stability. In order to establish prognostic models that would be usable in clinical practice the immunologic evaluation relies on cytokine and chemokine data that can be easily obtainable in a timely fashion. A more comprehensive immunologic evaluation such as complement levels, flow cytometry, transcriptomics, and venom associated molecular patterns (VAMPS) would give a more global picture of the immunologic response to SBE than is available in this preliminary analysis. Likewise additional clinical data such as presenting severity grade or time from bite to presentation could be incorporated into the model.

This study is limited to only a few species of Crotalinae snakes and the predominant species is known to have a less severe clinical course than others. Yet, our data demonstrate a wide distribution of recovery as measured by PSFS AUPC over 28 days. The heterogeneity of the disease itself and the utility of our chosen outcome measure allows us to discriminate between patient's recovery and develop robust prognostic models. This technique can be easily applied to other Crotalinae snake species.

Lastly, as this is preliminary work, we have not used the models to determine thresholds to treat nor assessed clinical efficacy of implementing this application.

CONCLUSION

Pre- and post-antivenom networks of cytokines/chemokines and clinical features were associated with functional recovery measured by the PSFS AUPC over 28 days. With additional patient data, we can identify prognostic models using immunologic and clinical variables to predict recovery from snake envenoming.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Duke University School of Medicine Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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C5a-C5aR1 Axis Activation Drives Envenomation Immunopathology by the Snake *Naja annulifera*

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Systemic complement activation drives a plethora of pathological conditions, but its role in snake envenoming remains obscure. Here, we explored complement's contribution to the physiopathogenesis of *Naja annulifera* envenomation. We found that *N. annulifera* venom promoted the generation of C3a, C4a, C5a, and the soluble Terminal Complement Complex (sTCC) mediated by the action of snake venom metalloproteinases. *N. annulifera* venom also induced the release of lipid mediators and chemokines in a human whole-blood model. This release was complement-mediated, since C3/C3b and C5a Receptor 1 (C5aR1) inhibition mitigated the effects. In an experimental BALB/c mouse model of envenomation, *N. annulifera* venom promoted lipid mediator and chemokine production, neutrophil influx, and swelling at the injection site in a C5a-C5aR1 axis-dependent manner. *N. annulifera* venom induced systemic complementopathy and increased interleukin and chemokine production, leukocytosis, and acute lung injury (ALI). Inhibition of C5aR1 with the cyclic peptide antagonist PMX205 rescued mice from these systemic reactions and abrogated ALI development. These data reveal hitherto unrecognized roles for complement in envenomation physiopathogenesis, making complement an interesting therapeutic target in envenomation by *N. annulifera* and possibly by other snake venoms.

Keywords: ***Naja*** **snake venom, envenomation, complement system, C5a-C5aR1, complement inhibitors**

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INTRODUCTION

Complement activation is a crucial event influencing the development of innate and adaptive immune responses (1, 2). Once microbial associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) have been detected, complement can become activated through three intrinsic pathways, the alternative (AP), lectin (LP), and classical (CP) pathways, or through extrinsic pathways involving coagulation proteases, cathepsins, elastase, or snake venom metalloproteases and serine proteases (2, 3). All of these pathways converge at central events that culminate in the cleavage of C3, C4, and/or C5, leading to the generation of opsonins (C3b and C4b) and anaphylatoxins (C3a, C4a, and C5a) and assembly of the terminal complement complex (TCC;

C5b-9_n). Acting *via* various cell types and receptors, these products stimulate a number of inflammatory events, inducing mast cell degranulation (4), lipid mediator release (5–9), inflammasome assembly (10–12), chemotaxis (2), generation of ROS and NOS (13–15), and production of interleukins and chemokines (1). Although complement is essential to host defense and physiology, deficiencies or uncontrolled activation of complement components can be detrimental; complement components can promote excessive inflammation that culminates in tissue damage, organ dysfunction, permanent disabilities, and sometimes death. Such results have been observed in a myriad of inflammatory disorders (16–19), as well as in envenomation by snakes (20).

Snakebite envenoming constitutes a public health problem in tropical and subtropical countries of Africa, Asia, and Latin America. The clinical consequences of these accidents are diverse, including respiratory arrest, hemostatic disorders, bleeding, and tissue injury. Snakebites are responsible for more than 80,000 deaths per year and cause amputation or permanent disability in about 300,000 victims each year (20–23). Given the relevance of snakebite, the World Health Organization (WHO) has established a program to reduce snakebite envenoming-associated mortality and disabilities by 50% before the year 2030. This program includes incentives for studies of "next-generation" treatments (23), whose development will require optimal characterization of the molecular mechanisms involved in the physiopathogenesis of envenomation.

It is particularly notable that the snakebite envenoming process presents some of the same clinical features observed in certain complement-mediated inflammatory conditions, making this system an interesting therapeutic target. Several studies have shown that snake venom components can interact with complement proteins (3). By the last century, cobra venom factor (CVF), a C3b-like protein isolated from *Naja* venom had been fully characterized in terms of its complement-depleting activity. This venom has been shown to trigger AP activation in an exacerbated and uncontrolled manner in a number of experimental models (24–26). In addition, venoms from snakes of different genera, such as *Trimeresurus*, *Bothrops*, and *Micrurus*, have been shown to trigger complement activation in normal human serum (NHS) *in vitro*, leading to anaphylatoxin generation and soluble Terminal Complement Complex (sTCC) assembly. These events are in part associated with the action of snake venom metalloproteinases (SVMP) and snake venom serine proteinases (SVSP) on central complement components and regulators (27–33). In addition, other studies have demonstrated depletion of C3 and Factor B (34) and an increase in anaphylatoxins and sTCC plasma levels in envenomated patients, indicating complement activation (35, 36). Despite these experimental and clinical reports, the real impact of complement activation on the physiopathogenesis of envenomation by snakes remains unclear.

Recently, we have reported that *Naja annulifera* snake venom contains various potential complement activators, including CVF, SVMPs, SVSPs, and proteins containing mannose and N-acetylglucosamine residues. Furthermore, this cobra venom induces local reactions, characterized by swelling mediated by mast cell degranulation, release of lipid mediators and neutrophil infiltration, and systemic reactions characterized by an increase in plasma levels of C-C Motif Chemokine Ligand 2 (CCL2) and Interleukin 6 (IL-6), neutrophilia, monocytosis, and pulmonary damage (37). Considering these findings, we believe that *N. annulifera* venom exhibits interesting characteristics that make it useful for models to evaluate the impact of complement activation on envenomation physiopathogenesis.

Here, we have shown that *N. annulifera* venom triggers complement activation *in vitro* and *in vivo*, followed by the release of inflammatory mediators. By performing pharmacological interventions in a human whole-blood model of inflammation, we have also demonstrated that blockade of C3 or C5 cleavage and C5aR1 signaling inhibition reduce several inflammatory parameters associated with envenomation immunopathology. Furthermore, in various mouse models of envenomation, we have shown that C5a-C5aR1 axis activation is crucial for local and systemic inflammation and that changes induced by the venom, including pulmonary injury, can be abrogated by the use of PMX205, a C5aR1 antagonist. Thus, C5aR1 signaling seems to be an interesting therapeutic target in snakebite accidents involving *N. annulifera* or other snakes in which envenomation pathogenesis is driven by C5a-C5aR1 axis activation.

MATERIAL AND METHODS

N. annulifera Venom

N. annulifera snake venom (South Africa specimens) was purchased from LATOXAN Laboratory (Portes-les-Valence, France). The lyophilized venom was reconstituted in sterile saline at 5 mg/mL and stored at -80°C until use. The protein and endotoxin contents were quantified by using a BCA assay protein kit (Pierce) and PYROGENT™ Plus Gel Clot LAL Assay (Lonza, USA), respectively, according to manufacturers' recommendations. The endotoxin was present in the venom at a level below the assay's sensitivity (< 0.125 EU/mL).

Complement Therapeutics

PMX205 and P32 peptides were synthesized as previously described (38, 39). Cp40 was synthesized as described by Qu et al. (40). SB290157 was obtained from Cayman Chemical (Michigan, USA).

Ethics Statement

BALB/c male mice (18–22 g) were obtained from the Center for Animal Breeding of Butantan Institute. All procedures involving animals were carried out in accordance with the ethical principles for animal research adopted by the Brazilian Society of Animal Science and the National Brazilian Legislation n°.11.794/08. The protocols used in the present study were approved by the Institutional Animal Care and Use Committee of the Butantan Institute (protocol approval n° 5323120918). Experiments conducted with human samples were approved by

the Human Research Ethics Committee of the Municipal Health Secretary of São Paulo. Blood samples were obtained from healthy donors after informed consent (protocol approval n° 974.312 and 4.309.960).

In Vitro Experiments

Erythrocytes and Sera

Blood from normal sheep and rabbits was collected in an equal volume of Alsever solution (citrate, 114 mM; glucose, 27 mM; NaCl, 72 mM; pH 6.1) and maintained at 4°C. Human blood samples obtained from healthy donors were collected without anticoagulant and allowed to clot at 4°C for 4 h. The blood was then centrifuged at 400 x *g*, and the normal human serum (NHS) was collected and stored at -80°C until use.

Complement Assays

NHS samples (200 µL) were treated with crude *N. annulifera* venom (50 µg) or sterile saline and incubated for 30 min at 37°C. The anaphylatoxins and sTCC in these samples were then quantified using BD™ CBA Human Anaphylatoxin (BD Biosciences) and MicroVue SC5b-9 Plus Enzyme Immunoassay (Quidel) Kits, respectively. AP, CP, and LP activity assays were performed as described (41, 42).

Direct Cleavage of Human Complement Components

Samples of purified human C3, C4, and C5 (2.5 µg each) (CompTech, Inc) were incubated with *N. annulifera* venom (2.5 µg) for 30 min at 37°C in the presence or absence of 1,10-phenanthroline (1,10 Phe) (10 mM) or phenylmethylsulfonyl fluoride (PMSF) (10 mM) SVMP and SVSP inhibitors, respectively. The reactions were stopped by adding Ethylenediamine Tetraacetic Acid (EDTA) (15 mM), and the mixtures were subjected to electrophoresis on 10% Sodium Dodecyl Sulfate Polyacrylamide Gels (SDS-PAGE) (43) under reducing conditions, and the gels were silver-stained (40). In addition, the generation of the anaphylatoxins was quantified using the BD™ Cytometric Bead Array (CBA) Human Anaphylatoxin kit (BD Biosciences).

Ex-Vivo Human Whole-Blood Model

The ex-vivo human whole-blood model described by Mollness and colleagues (44) was used, with some modifications. Blood samples were collected by venipuncture into tubes containing 50 µg/mL of lepirudin (Refludan®, Colgene, USA), a thrombin-inhibitor anticoagulant, which does not interfere with complement activity. The whole-blood samples were treated with increasing concentrations of *N. annulifera* venom, ranging from 3.125 to 100 µg/mL, or with sterile saline and then incubated for 30 or 60 min at 37°C. Under these conditions, *N. annulifera* venom induces the production of inflammatory mediators but does not promote coagulation; however, at higher concentrations (50 and 100 µg/mL), *N. annulifera* venom is highly hemolytic. Considering that free hemoglobin may be highly inflammatory (45) and that there is no information about hemolysis on envenomation by *N. annulifera*, we chose 25 µg/mL for subsequent experiments, since this dose did not induce significant hemolysis (data not shown).

To assess complement's role in the inflammatory events promoted by *N. annulifera* venom, human whole blood was pretreated with either the compstatin analog Cp40 (C3/C3b inhibitor, 20 µM) (46), SB290157 (C3aR antagonist, 20 µM) (47), PMX205 (C5aR1 antagonist, 20 µM) (48) or P32 (C5aR2 agonist, 100 µM) (39) inhibitors/activator or with appropriate vehicle as control, *i.e.*, saline (Cp40 and P32), DMSO (SB290157), or 5% glucose (PMX205), for 5 min at room temperature. In addition, *N. annulifera* venom samples were incubated with 1,10 Phe (SVMP inhibitor, 15 mM) or vehicle (ethanol) for 15 min at room temperature. *N. annulifera* venom was then added to the blood samples, and the mixtures were incubated at 37°C for 30, 60, or 120 min under continuous agitation. Finally, the tubes were centrifuged at 405 × *g*, for 10 min at 4°C, the plasma was collected, and the samples were stored at -80°C for further quantification of inflammatory mediators.

In Vivo Experiments

Analysis of Systemic Complement Activity

BALB/c mice (n=6/group) were injected by the intraperitoneal (i.p.) route with a sublethal (56.5 µg) or lethal (94.2 µg) dose of *N. annulifera* venom to induce moderate or severe envenomation illness, respectively (37). As controls, mice were inoculated with sterile saline. After 30 min, all mice were euthanized with an overdose of two anesthetics (200 mg/kg ketamine, 20 mg/kg xylazine). The whole blood was collected by cardiac puncture and allow to clot at room temperature for 1 hr. The blood samples were then centrifuged at 1500 × *g* for 15 min at 4°C, and the sera obtained were stored at -80°C until analysis. Measurements of AP, CP, and LP serum activity were evaluated at level of C9 by using the Complement Pathway Mouse Assay ELISA kit (HycultBiotech) according to the manufacturer's instructions. The results were expressed as AP, CP, or LP activity [%].

Assessment of the C5a-C5aR1 Axis Contribution to *In Vivo* Reactions

Mice were treated by the subcutaneous (s.c.) route with PMX205 (C5aR1 antagonist) or vehicle (5% glucose solution) at 1 or 2 mg/kg b.wt. at 24 h or 1 h before the induction of local and systemic reactions.

Local Reactions

BALB/c mice (n=6/group) were each injected with 10 µg of *N. annulifera* venom (37), dissolved in a volume of 50 µL sterile saline, into the s.c. tissue of the plantar region of the left hind paw. The contralateral hind paw (control) was inoculated with 50 µL of sterile saline. The thickness of the hind paws was assessed with a caliper rule (Mitutoyo, Suzano-SP, Brazil; in increments of 0.01 mm) at various time points before injection (T0) and after 24 h, following inoculation with either venom or saline (Te). Increases in paw volume were expressed as a percentage (%), calculated according to the following formula: (Te-T0)/T0*100 (37). Mice were euthanized by anesthetic overdose (200 mg/kg ketamine, 20 mg/kg xylazine) at 20 or 60 min after venom inoculation, before collection of the s.c. tissue from hind paws for inflammatory mediator quantification.

Tissue samples were homogenized using a PT-10 Polytron homogenizer (Kinematica, Luzern, SWZ) in lysis buffer (NaCl, 200 mM; EDTA, 5 mM; Tris, 10 mM; glycerol, 10%; leupeptin, 1 μg/mL; aprotinin, 28 μg/mL; PMSF, 1 mM). The homogenized tissues were then centrifuged at 1500 × g for 15 min at 4°C. The supernatants were obtained, centrifuged again, filtered, and stored at -80°C until analyzed.

Systemic Reactions

The C5a-C5aR1 contribution to envenomation systemic reactions were scrutinized by sublethal and lethal experimental sets which represents moderate and severe envenomation conditions, respectively (37). In the moderate set mice were inoculated by the i.p. route with a sublethal *N. annulifera* venom dose (37) or with sterile saline and euthanized 1 h after venom injection with anesthetics overdose (200 mg/kg ketamine, 20 mg/kg xylazine) to blood samples obtention. Blood and lung samples from mice submitted injected with the venom lethal dose (37) were obtained 5 hours after venom inoculation which represents death moment or intense impairment of the animals this group. The animals that not died at 5 hours after venom injection were euthanized with anesthetics overdose (200 mg/kg ketamine, 20 mg/kg xylazine), since this period was determined as endpoint to this group. Blood samples were then obtained by cardiac puncture using EDTA (2.5 mg/mL) as an anticoagulant. Aliquots of these samples were used for systemic total and differential leukocyte counts, and other blood samples were centrifuged at 2800 × g at 4°C for 10 min. Plasma samples were stored at -80°C and used to measure inflammatory mediators.

After euthanasia, the lungs were extracted from the mice injected with the lethal *N. annulifera* venom dose, then fixed in 10% formaldehyde for 24 h. The pulmonary samples were then subjected to routine histologic fixation and stained with hematoxylin and eosin (HE). The tissue samples were examined under a light microscope for the presence of cellular/tissue changes, and a histopathological score was determined.

Quantification of Inflammatory Mediators

Human chemokines were detected by using a BD™ CBA Human Chemokine kit (BD Biosciences). The BD™ CBA Mouse Inflammation kit (BD Biosciences) was used to detect systemic chemokines and interleukins in the mouse plasma. Keratinocyte Chemokine (KC)/C-X-C motif chemokine ligand 1(CXCL1) was detected by using the LEGENDplex™ Mouse Anti-Virus response panel (Biolegend). Mouse myeloperoxidase (MPO) was quantified by using a MPO Mouse ELISA kit (HycultBiotech). leukotriene B4 (LTB_4), prostaglandin E2 (PGE_2), and thromboxane B2 (TXB_2) were quantified by the LTB4 Enzyme-Linked Immunosorbent Assay (ELISA), PGE2 Monoclonal, and TXB2 ELISA kits, all from Cayman Chemical. All assays were performed according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test for comparisons of the mean of two groups. One-way and two-way ANOVA, followed by Bonferroni's multiple comparison test, were applied to the results of the time and dose-response experiments. The statistical analyses were conducted using Graphpad Prism 5 software (La Jolla, California, USA). Differences were considered significant when $p \leq 0.05$.

RESULTS

N. annulifera venom acts on the human complement

In a previous study (37), we demonstrated the presence of several potential components in *N. annulifera* venom able to interact with the human complement system. To determine whether *N. annulifera* venom could interfere with the complement activity, NHS samples were incubated with the venom and then submitted to hemolytic (AP and CP) and C4b (LP) ELISA assays (41, 42). We found that cobra venom significantly reduced the activity of the three complement pathways (**Figures 1A–C**). In order to evaluate whether the reduction in complement activity was a result of activation or inhibition, since some animal venoms and secretions can contain complement inhibitors (3), we assessed anaphylatoxin generation and sTCC assembly. The results (**Figures 1D–G**) confirmed that venom promoted complement activation in NHS, as determine by the generation of C3a, C5a, and sTCC, and to a lesser extent C4a.

SVMP and SVSP Hydrolyze Human Complement Proteins

SVMP and SVSP present many substrates and actions on prey and human victims (49), making them potential complement activators. By incubating purified human complement proteins with *N. annulifera* venom and using specific inhibitors of metalloproteases and serine proteases, we observed that both classes of enzymes present in the venom were able to cleave C4 and C5, while C3 is cleaved only by SVMP (**Figures 2A–C**). In addition, C3, C4 and C5 cleavage by venom proteases was functional, since culminated in the C3a, C4a and C5a anaphylatoxins generation (**Figures 2D–F**).

N. annulifera Venom Induces Inflammation *In Vitro*

The *N. annulifera* venom inflammatory potential was tested in a human whole-blood *ex vivo* model in the presence of lepirudin, a thrombin inhibitor anticoagulant that does not interfere with complement activity (44, 45). Human blood samples were incubated with increasing *N. annulifera* venom concentrations (3.125 - 100 μg/mL) for 30 or 60 min at 37°C, and the production of inflammatory mediators was quantified. We found that *N. annulifera* venom triggered anaphylatoxin generation and sTCC assembly (**Supplementary Figure 1**); these processes were accompanied by the release of lipid mediators, including leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), and thromboxane A_2 (TXA_2) (**Supplementary Figure 2**). Furthermore, *N. annulifera* venom induced the production of the chemokines C-C motif chemokine ligand 2 (CCL2), CCL5 and C-X-C motif chemokine ligand 8 (CXCL8) (**Supplementary Figure 3**).

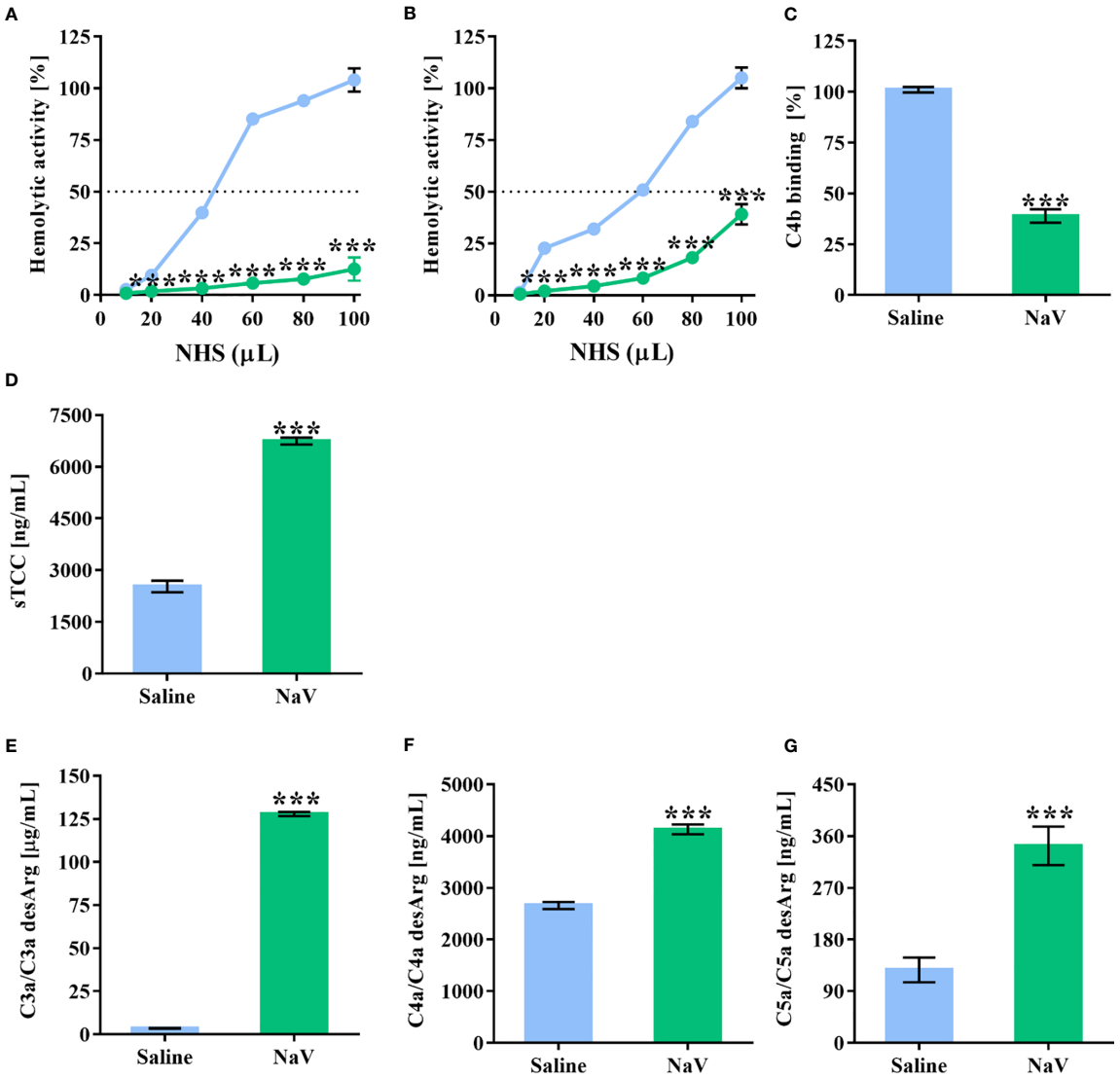

FIGURE 1 | *N. annulifera* venom (NaV) acts on the human complement system. NHS samples were incubated with NaV or sterile saline for 30 minutes at 37°C. Then, these samples were submitted to functional assays to determine NaV effects on AP **(A)**, LP **(B)** and CP **(C)** activity. In addition, C3a **(D)**, C4a **(E)** and C5a **(F)** anaphylatoxins generation, and sTCC **(G)** assembly were measured by ELISA. Data represent means ± SEM of five independent experiments from different NHS donors. ***p ≤ 0.001 (two-tailed *t*-test or two-way ANOVA, followed by Bonferroni post-test).

Inflammatory Events Induced by *N. annulifera* Venom *In Vitro* Are Complement-Mediated

To identify the contribution of complement to the inflammatory reactions promoted by *N. annulifera* venom, we characterized the effects of pharmacologic interventions on a human *ex vivo* whole-blood model. The compstatin analog peptide Cp40, a C3/C3b inhibitor (46), strongly reduced C3a (**Figure 3A**) and LTB_4 (**Figure 3D**) production and to a lesser extent that of PGE_2 (**Figure 3E**), CCL2 (**Figure 3G**), and CXCL8 (**Figure 3I**). In contrast, Cp40 failed to interfere with the generation of C5a or sTCC assembly (**Figures 3B, C**), suggesting that *N. annulifera* venom proteases actions upon C5 is responsible for it (**Figures 2C, F**). The incubation of *N. annulifera* venom with 1,10 Phe, a SVMP inhibitor, and further incubation with human whole blood resulted in the abrogation of C3a/C5a generation and sTCC formation (**Figures 3A–C**), suggesting an important role for SVMP in complement activation induced by *N. annulifera* venom in this *ex vivo* model. Since *N. annulifera* venom proteases can lead to complement activation, and Cp40 failed to control the generation of C5a or sTCC, we decided to evaluate the role of anaphylatoxin receptors in the inflammatory process induced by cobra venom.

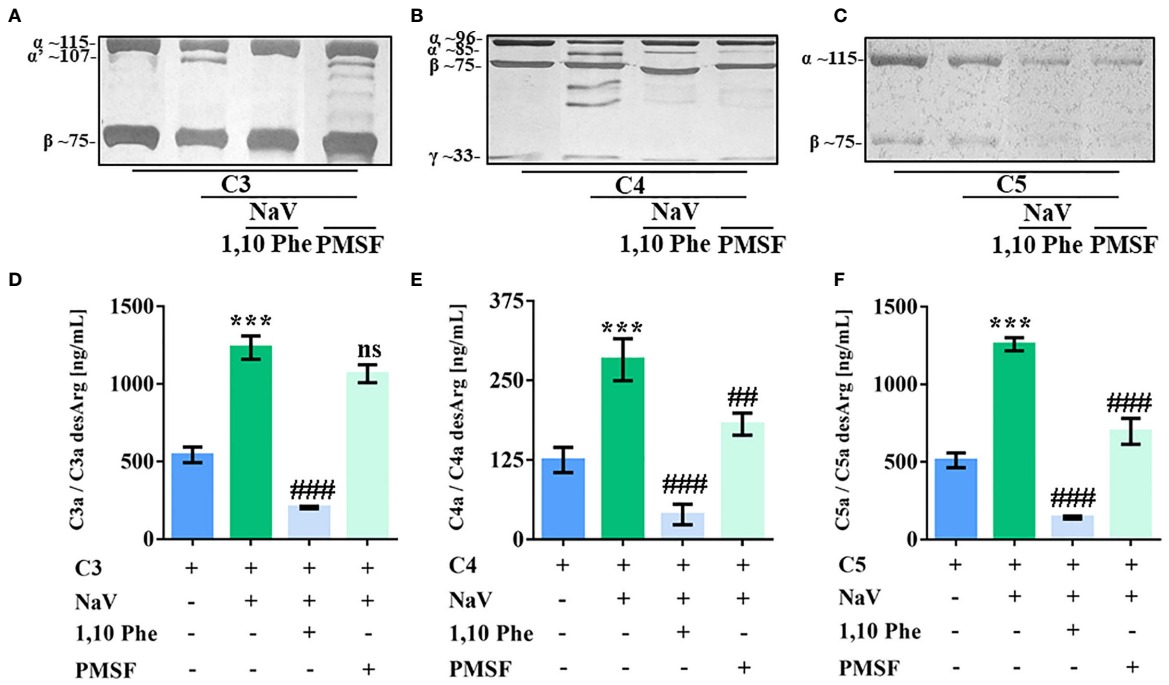

FIGURE 2 | SVMP and SVSP cleave human complement proteins. Human C3, C4 and C5 purified proteins (2.5 µg) were incubated with sterile saline or *N. annulifera* venom (NaV - 2.5 µg) with or without metallo- (10 mM) and serine-proteinases (10 mM) inhibitors for 30 minutes at 37°C. After this period, reactions were stopped and C3 **(A)**, C4 **(B)** and C5 **(C)** cleavage evaluated by SDS-PAGE and silver stanning. Images presenting in panels a-c were grouped and/or spliced (the original gels are presented as supplementary material – **Figure S5**). C3a **(D)**, C4a **(E)** and C5a **(F)** anaphylatoxins generation in these reactions were determined by CBA. Panels **(A–F)** represents five different experiments. Data are means ± SEM of three independent experiments ***$p \leq 0.001$ (two-tailed *t*-test). ### indicates a significant difference between complement purified proteins treated with *N. annulifera* venom + Vehicle and *N. annulifera* venom + Protease inhibitors. ##$p \leq 0.01$, ###$p \leq 0.001$. ns = non-significant.

Inhibition of C3a receptor (C3aR) by the antagonist SB290157 (47) resulted in a reduction in CXCL8 and CCL2 production (**Figures 3G, H**) and an increased release of lipid mediators (**Figures 3D–F**) and CCL5 (**Figures 3I**). In contrast, C5a receptor 1 (C5aR1) inhibition caused by the antagonist PMX205 (48) resulted in decreased levels of LTB_4, PGE_2, TXA_2, CXCL8, and CCL2 (**Figures 3D–H**).

C5a receptor 2 (C5aR2), a second receptor that binds C5a, has been described to act as an immune dampener to C5aR1 and TLR-4, regulating the production of inflammatory mediators (50). To test if C5aR2-mediated signaling could modulate the release of inflammatory markers in our model, a functionally selective agonist peptide (P32) (39) was used. P32 reduced CCL2 production to the same extent as did the other inhibitors (**Figure 3G**). On the other hand, C5aR2 stimulation by P32 potentiated LTB_4, PGE_2 (**Figures 3D, E**) and CCL5 release (**Figure 3I**).

Given that *N. annulifera* venom triggers complement activation by the extrinsic route and that the modulation of the C5a-C5aR1 axis interferes with the immunopathology associated with envenomation *in vitro*, we chose PMX205 for evaluating complement's contribution to local and systemic reactions in murine models of envenomation.

N. annulifera Venom Promotes Systemic Complementopathy

Previously, we showed that *N. annulifera* venom can induce systemic inflammatory reactions in mice (37). Nevertheless, action on the complement system in this scenario had not been evaluated. To examine this parameter, we injected groups of mice with sublethal or lethal (37) doses of venom by the intraperitoneal (i.p.) route; control animals were injected with sterile saline. Blood samples were obtained after 30 min, and complement activity was determined by functional assays at the level of C9 activation using mouse serum samples. Moderated and severe experimental envenoming induced CP and LP complementopathy at the same extent (**Figures 4B, C**). In both experimental groups, AP activity reduction was detected, however, in mice submitted to the lethal envenomation protocol the AP consumption was higher (**Figure 4A**).

C5a-C5aR1 Axis Modulation Reduces Local Reactions Induced by *N. annulifera* Venom

Groups of mice were treated with vehicle or PMX205 (1 mg/kg body weight [b.wt.] in 5% glucose solution) at 24 h and 1 h

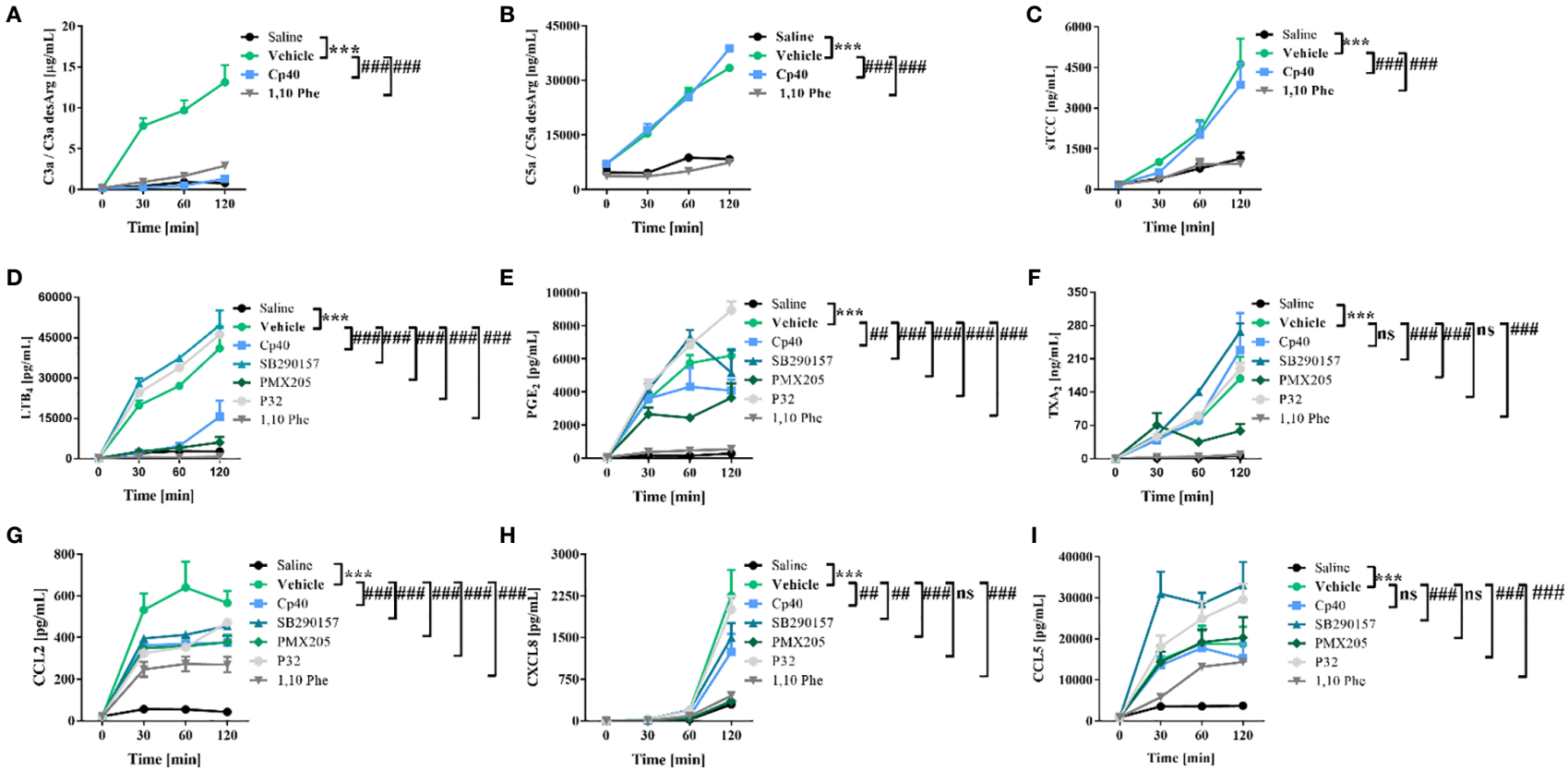

FIGURE 3 | Human complement activation by *N. annulifera* venom (NaV) results in the generation of chemokines and lipid mediators. Human whole-blood samples were incubated for 5 minutes, at room temperature, with Cp40 (C3 cleavage inhibitor), SB290157 (C3aR antagonist), PMX205 (C5aR1 antagonist), P32 (C5aR2 agonist) or 1,10 Phenanthroline (metalloproteinase inhibitor) inhibitors, or their respective vehicles. Then, all these samples were exposed to NaV or sterile saline during 30, 60 and 120 minutes, and complement activation products generation **(A–C)**, lipid mediators release **(D–F)** and chemokines upregulation **(G–I)** scrutinized by CBA or ELISA. Data are means ± SEM of six independent experiments with different whole-blood donors. ***$p \leq 0.001$ (two-tailed two-way ANOVA, followed by Bonferroni post-test). ### indicates a significant difference between *N. annulifera* venom + Vehicle and *N. annulifera* venom + Inhibitors. **(E, H)** ## means the comparison between samples exposed to the NaV + vehicle and NaV+ complement inhibitors in which statistical differences are $p \leq 0.01$. ns = non-significant.

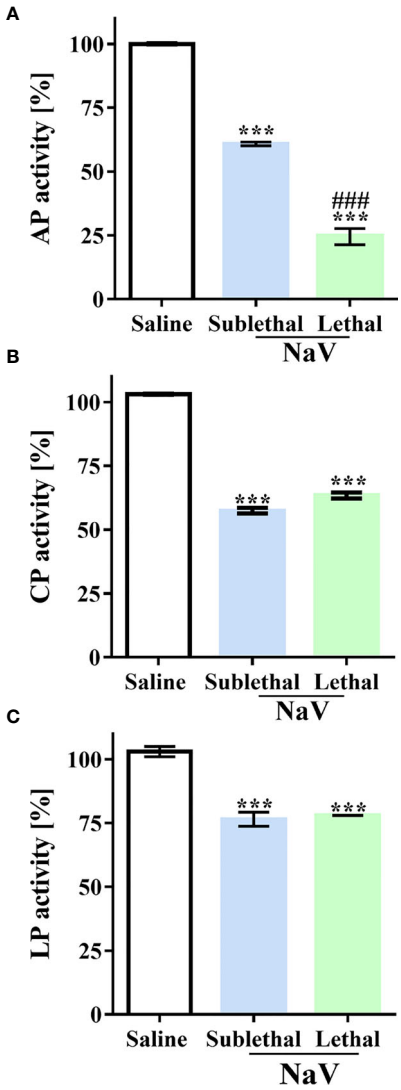

FIGURE 4 | *N. annulifera* venom (NaV) induces systemic complementopathy in the murine model. Serum samples were obtained from mice envenomated, i.p., with a sublethal or lethal NaV dose (n=6/venom dose) and AP **(A)**, LP **(B)** and CP **(C)** activity evaluated by functional assays. Data are means ± SEM of six independent experiments. ***$p \leq 0.001$ (two-tailed one-way ANOVA, followed by Bonferroni post-test). ### indicates significant difference between the sublethal and lethal venom doses.

before *N. annulifera* venom injection. The subcutaneous venom inoculation (10 μg) induced a rapid-onset edema in the hind paws of the mice, reaching a maximum peak at 20 min after injection (125% increase in paw volume, **Figure 5A**). The swelling persisted for several hours and disappeared within 24 h. Inhibition of C5aR1 signaling produced a significant decrease in the hind paw volume with time, mainly at edema peak (54% inhibition) (**Figure 5B**). Mechanistically, the edema reduction was characterized by a strong inhibition of LTB_4, PGE_2, and TXA_2 release (**Figures 5C–E**) at the edema peak.

N. annulifera venom injection also promoted KC (murine CXCL8 homolog) chemokine release, which was accompanied by a large neutrophil infiltration, as demonstrated by MPO quantification, that occurred in a C5a-C5aR1 axis-dependent manner, since receptor blockage reduced these inflammatory events (**Figures 5F, G**).

C5aR1 Blockage Abrogates Systemic Reactions Evoked by *N. annulifera* Venom

To demonstrate the contribution of complement to the systemic reactions induced by *N. annulifera* venom, we employed two sets of experiments. The first set was performed by injecting a sublethal dose of venom (56.5 μg; i.p.), which induces a clinical condition in mice characterized by apathy, bending of the column, a rough hair coat, dyspnea, and difficulty ambulating. This dose also promotes changes in some systemic parameters, including a decrease in circulating lymphocytes and an increase in neutrophils. It also promotes increases in IL-6 and CCL2 plasma levels. All the reactions induced by this dose reached a peak 1 h after venom inoculation, and the values returned to normal by 24 h (37). This dose was not able to induce death or organ damage during any evaluation period (37). Treatment with PMX205 (1 mg/kg b.wt.) was able to restore the normal percentage of circulating lymphocytes and decrease the number of neutrophils (**Figure 6A**). Furthermore, C5aR1 inhibition fully reduced IL-6 production and restored CCL2 to physiological levels (**Figures 6B, C**).

The second experimental set was performed by injecting mice with a dose of venom equivalent to the $1LD_{50}$ (94.2 μg, i.p.) (37). This treatment produced a more severe clinical condition than did the sublethal dose. Mice given the higher dose showed apathy, bending of the spinal column, a rough hair coat, dyspnea, and difficulty ambulating, and they died approximately 5 h after venom inoculation. Death was preceded by systemic inflammation, characterized by leukocytosis with lymphopenia, neutrophilia, and monocytosis (**Figures 7A–D**). Moreover, the LD_{50} venom dose induced higher levels of IL-6, CCL2, and TNF-α production than did the sublethal venom dose (**Supplementary Figures 4A–C**). In the lethal context, *N. annulifera* venom induced acute lung injury (ALI), with diffuse alveolar damage (DAD) featured by alveolar collapse, septal inflammation, and a thickening of the alveolar septum (**Figures 8A–D**). Use of PMX205 (2 mg/kg b.wt.) in these mice restored the blood cell parameters to physiological levels (**Figures 7A–D**). Strikingly, ALI was completely abrogated by PMX205 treatment, along with an increase in systemic levels of an anti-inflammatory cytokine, IL-10 (**Supplementary Figure 4D**). Nevertheless, in these lethal conditions, IL-6, Tumor Necrosis Factor α (TNF-α), and CCL2 production were still aggravated (**Supplementary Figures 4A–C**).

DISCUSSION

Current understanding indicates that snake venoms trigger complement activation in humans (27–36), but the overall

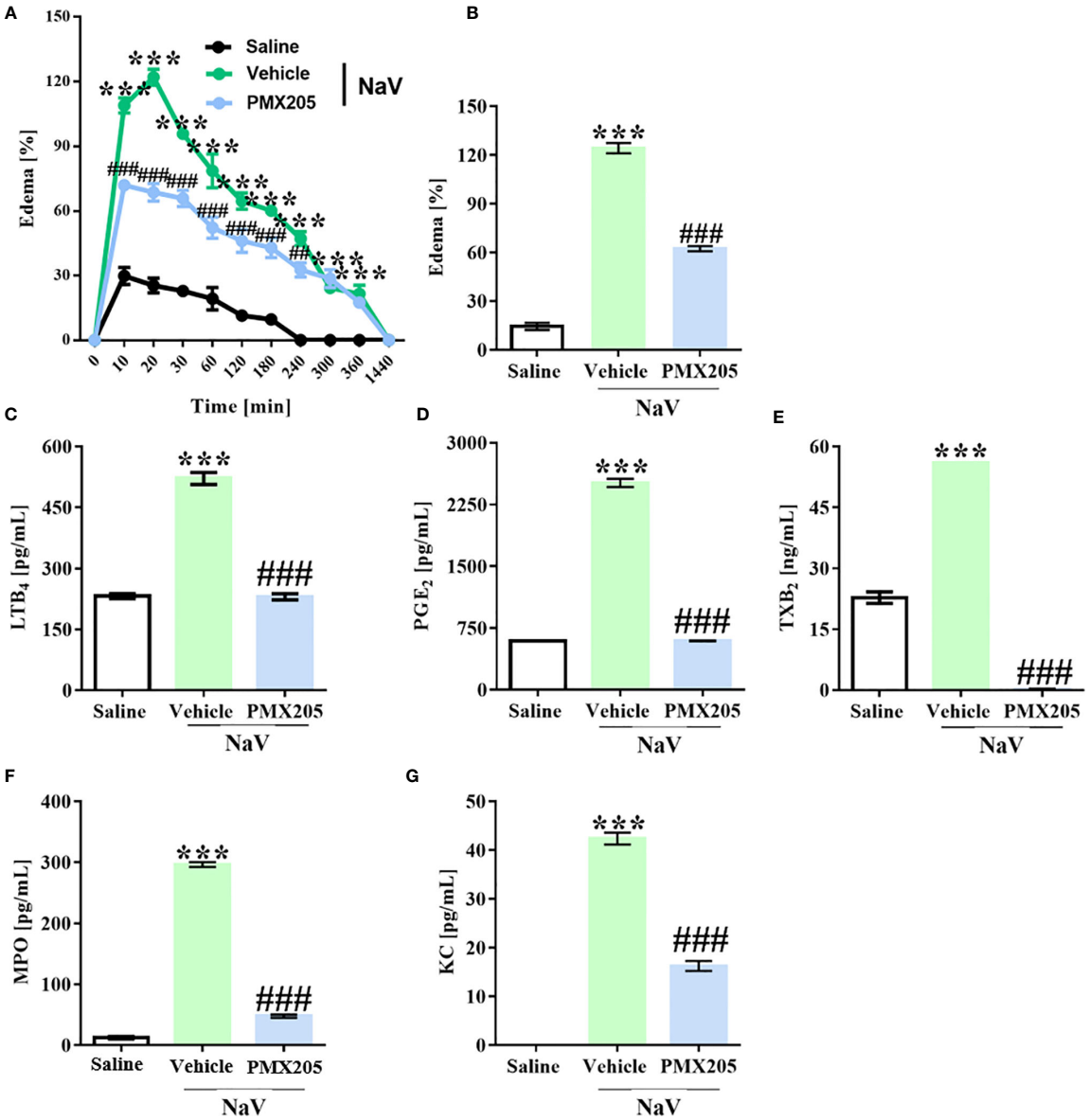

FIGURE 5 | *N. annulifera* venom (NaV) promotes C5a-C5aR1 axis-dependent edema. Mice (n=6/group) were pretreated with PMX205 (1 mg/kg), a C5aR1 inhibitor, or vehicle 24 and 1 hour before NaV injection. Following inhibitor administration, local reactions were induced by NaV inoculation into subcutaneous tissue from mice left hind paws and the swelling evaluated along the time with a caliper rule **(A, B)**. In addition, tissue samples were obtained along the time, homogenized, and then submitted to LTB_4 **(C)**, PGE_2 **(D)**, TXB_2 **(E)**, MPO **(F)** and KC **(G)** levels assessment by ELISA and CBA. Data are means ± SEM of six independent experiments. ***p ≤ 0.001 (two-tailed one-way ANOVA or two-way ANOVA, followed by Bonferroni post-test). ### indicates a significant difference between animals treated with *N. annulifera* venom + Vehicle and *N. annulifera* venom + PMX205. The ## symbol (240 minutes period) means the comparison between animals treated with the vehicle + *N. annulifera* venom and PMX205 + *N. annulifera* in which statistical differences are p ≤ 0.01.

molecular mechanisms induced by the envenomation have remained underexplored. Here, by coupling *in vitro* and *in vivo* approaches, we have determined that *N. annulifera* venom induces reduction in complement pathways activity, anaphylatoxins generation and sTCC assembly. These reactions were accompanied by lipid mediators release and production of chemokines and interleukins. C5a-C5aR1 axis signaling is the driver of these effects, since its modulation prevents local and systemic reactions induced by the venom and protects mice against an extensive ALI.

Complementopathy is a hallmark of a plethora of inflammatory, autoimmune, and degenerative conditions and is generally accompanied by an increase in the systemic levels of inflammatory mediators (51–56). A human whole-blood model of inflammation has been developed to study complement's role in various inflammatory events and

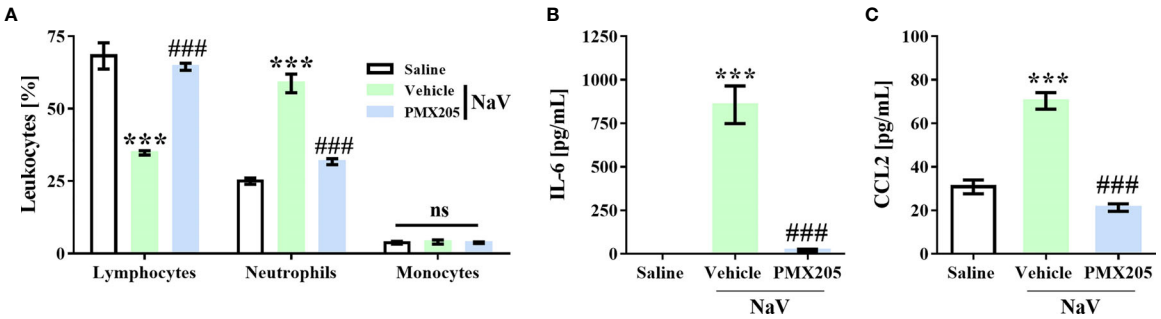

FIGURE 6 | C5a-C5aR1 signaling drives moderate systemic reactions induced by *N. annulifera* venom (NaV). Mice (n=6/group) were pretreated with PMX205 (1 mg/kg), a C5aR1 inhibitor, or vehicle 24 and 1 hour before NaV injection. Following inhibitor or vehicle administration, moderate systemic reactions were induced by the injection of NaV sublethal dose, *via* intraperitoneal route. One hour after envenomation, blood samples were obtained by cardiac puncture to determine hematological changes by blood smear **(A)**, and CCL2 **(B)** and IL-6 **(C)** systemic generation by ELISA and CBA. Data are means ± SEM of six independent experiments. ***p ≤ 0.001 (two-tailed t-test or two-way ANOVA, followed by Bonferroni post-test). ### indicates a significant difference between animals treated with NaV + Vehicle and NaV + PMX205. ns = non-significant.

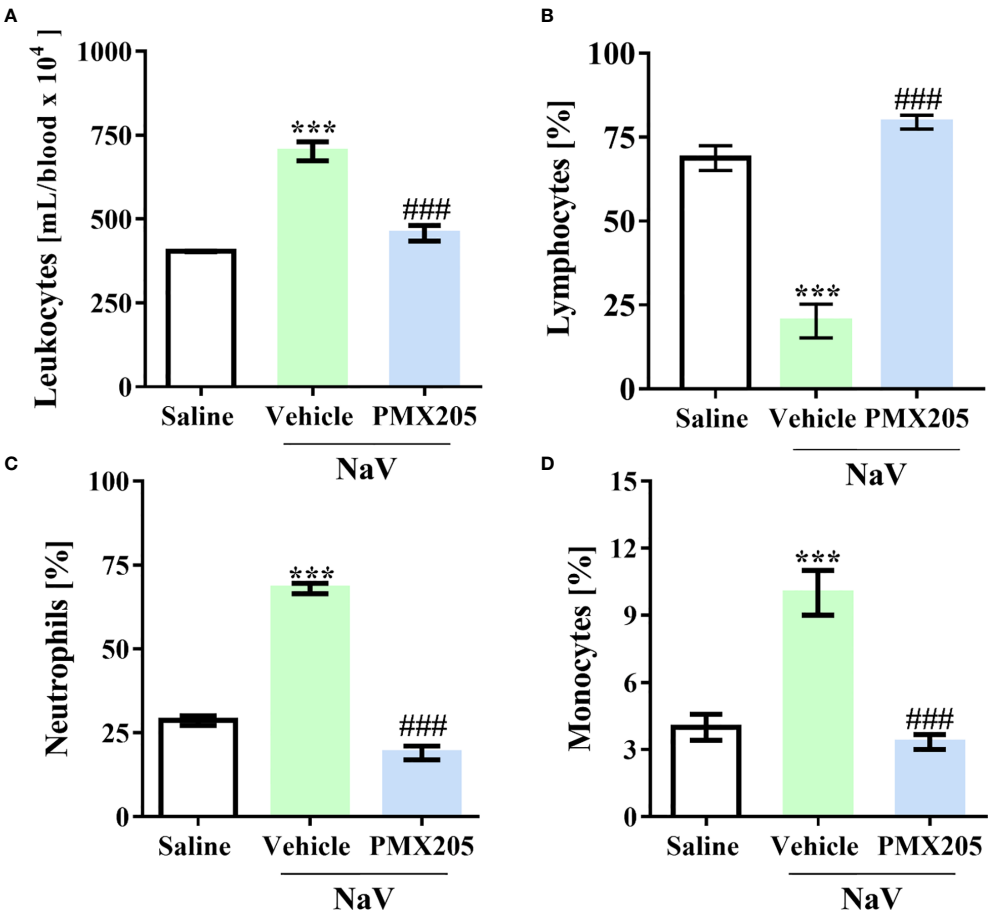

FIGURE 7 | C5a-C5aR1 activation cause hematological changes in severe experimental envenomation. Mice (n=6/group) were pretreated with PMX205 (2 mg/kg), a C5aR1 inhibitor, or vehicle 24 and 1 hour before *N. annulifera* venom (NaV) injection. Following inhibitor administration, severe systemic reactions were induced by the injection of NaV lethal dose, *via* intraperitoneal route. Five hours after envenomation, blood samples were obtained by cardiac puncture to determine leukocytosis **(A)**, by cell counting in a hemocytometer, and lymphopenia **(B)**, neutrophilia **(C)**, and monocytosis **(D)**, *via* blood smear analysis. Data are means ± SEM of six independent experiments. ***p ≤ 0.001 (two-tailed t-test or two-way ANOVA, followed by Bonferroni post-test). ### indicates a significant difference between animals treated with NaV + Vehicle and NaV + PMX205.

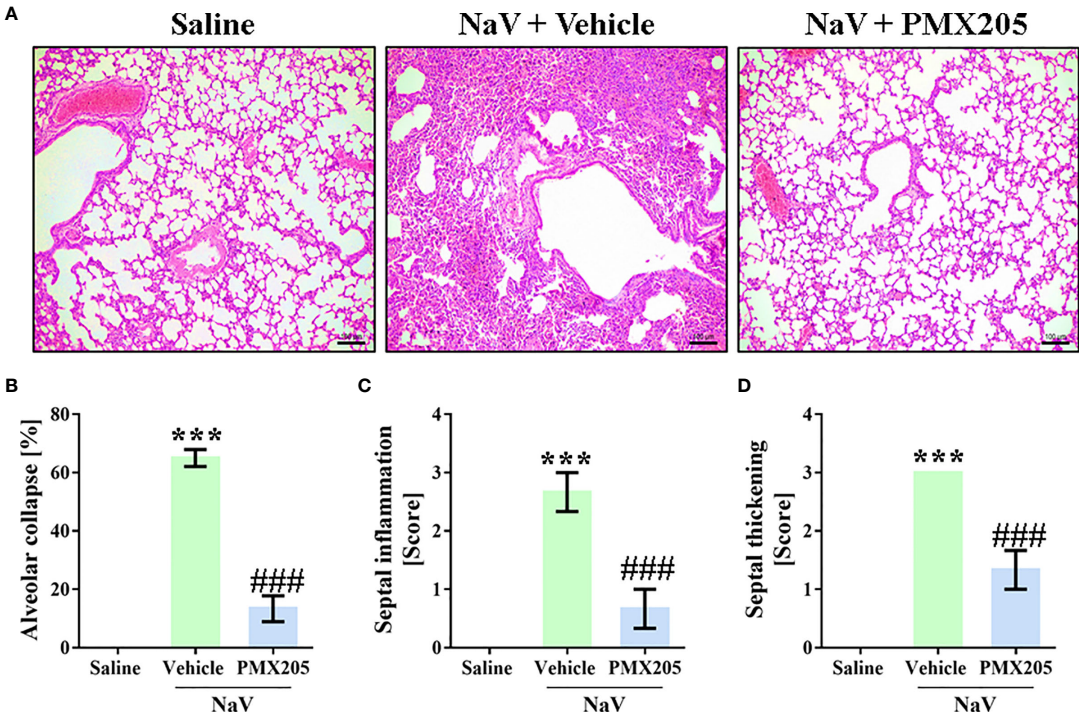

FIGURE 8 | C5aR1 signaling cause ALI development in severe experimental envenomation by *N. annulifera*. Mice (n=6/group) were pretreated with PMX205 (2 mg/kg), a C5aR1 inhibitor, or vehicle 24 and 1 hour before of *N. annulifera* venom (NaV) injection. Following inhibitor or vehicle administration, severe systemic reactions were induced by the injection of NaV lethal dose, via intraperitoneal route. Five hours after envenomation, the lungs were obtained, fixed, and submitted to histologic procedures **(A)** Results were expressed as area of alveolar collapse (%) **(B)** and histopathological scores **(C, D)**, i.e., 1- mild changes; 2- moderate changes; 3- severe/intense changes. Data are means ± SEM of six independent experiments. ***p ≤ 0.001 (two-tailed t-test or two-way ANOVA, followed by Bonferroni posttest). ### indicates a significant difference between animals treated with NaV + Vehicle and NaV + PMX205.

diseases, including sepsis (44) and hemolytic diseases (45). This experimental model permits the investigation of the role of complement in the complex inflammatory network, including all potential cellular and fluid-phase mediators present and able to interact simultaneously. We found that *N. annulifera* venom induced complement activation in the whole-blood model, with consequent release of pro-inflammatory mediators that included LTB_4, PGE_2, TXA_2, CCL2, and CXCL8. These findings are in line with the increased plasma levels of inflammatory markers observed in an *in vivo* model of envenomation, indicating that human envenomation by *N. annulifera* leads to uncontrolled inflammatory reactions and consequent development of ALI/ARDS (acute respiratory distress syndrome), a known cause of respiratory arrest and death, since patients with this inflammatory condition present augmented plasma and pulmonary levels of these pro-inflammatory mediators (57–60).

The imbalanced release of inflammatory mediators contributes significantly to envenomation pathology, since it promotes endothelial dysfunction, edema formation (61–65) pain, and tissue hypoxia, which can culminate in compartment syndrome (66–69). We have previously demonstrated that the use of eicosanoid inhibitors (37) reduces the edema induced by *N. annulifera* venom, but the underlying mechanisms have not been fully elucidated. Similar to observations in the *ex-vivo* human whole-blood model, injecting *N. annulifera* venom in the mouse subcutaneous tissue, we have demonstrated that C5a-C5aR1 axis activation was involved in the release of LTB_4, PGE_2, and TXA_2 into the hind paws; these mediators are likely to be responsible for the development of the extensive swelling promoted by the venom. LTB_4, PGE_2, and TXA_2 are lipid mediators that are mainly involved in vascular changes during the early stages of the inflammatory reaction (70). Nonetheless, if not controlled, the action of these mediators can be injurious, making them important players in various pathologies (70) such as in pulmonary edema and death promoted by *Tityus serrulatus* scorpion envenomation (71, 72).

Apart from swelling, *N. annulifera* venom promotes the infiltration of neutrophils into the venom inoculation site (37). C5a, LTB_4, and CXCL-1/IL-8 are powerful chemoattractants for neutrophils (73, 74) and they have been pointed to as orchestrators in various pathological conditions. In our experiments, pharmacological inhibition of C5aR1 resulted in the abrogation of neutrophil infiltration and a decreased production of LTB_4 and KC. Previously, we have shown that C5aR1 targeting by antibodies prevents the infiltration of neutrophils into the peritoneal cavity of mice injected with

Bothrops asper snake venom or its purified metalloprotease (75). However, whether the C5a-C5aR1-neutrophil triad in the snake envenomation context is destructive or protective remained to be explored.

An imbalance in the systemic levels of inflammatory mediators, including C3a, C5a, and sTCC, can potentially evolve to cause multiple organ dysfunction and death (17). Injection of BALB/c mice with a sublethal venom dose, which promotes a moderate envenomation state (37) has demonstrated that *N. annulifera* venom interfere in the complement activity, culminating in the generation of C5a, which binds to C5aR1 and promotes an increase in the number of circulating neutrophils as well as IL-6 and CCL2 systemic levels. Although systemic inflammation was induced by this dose, no tissue damage was detected in the evaluated organs (brain, lungs, heart, kidneys, liver, and spleen) (37); however, this assessment does not exclude changes at the physiological level, since C5a binding to C5aR1 leads to blood pressure alteration (76), electrophysiological changes (77), pain (78), and hemostatic disorders (17, 79, 80). Thus, studies targeting these physiological parameters need to be performed.

Among the consequences of the systemic and intrapulmonary complement activation is the development of ALI/ARDS (76, 81–85), a severe form of hypoxemic respiratory failure resulting from inflammatory insult to the lungs (53–55, 86, 87). One LD_{50} injection in mice produced a severe envenomation state, with a significant reduction of the complement activity, leukocytosis, neutrophilia, monocytosis, and strong systemic production of IL-6, CCL2, and TNF-α. Furthermore, this venom dose promoted extensive ALI and death. These changes induced in mice by injection of a lethal *N. annulifera* venom dose, coupled with the *in vitro* results we obtained from the human whole-blood model, suggest that the physiopathogenesis of envenomation by *N. annulifera* is similar to that occurring in sepsis and ALI/ARDS (17, 53–55, 86, 87). In addition, strong complementopathy and increased production of interleukins and chemokines induced by the *N. annulifera*'s venom lethal dose may be related with patients' poor prognosis, since these inflammatory events are risk factors to ALI/ARDS severity, multiorgan failure and death in sepsis and polytrauma (53, 88, 89).

The disruption of the C5aR1 signaling by the action of the PMX205 led to an increase of the anti-inflammatory IL-10

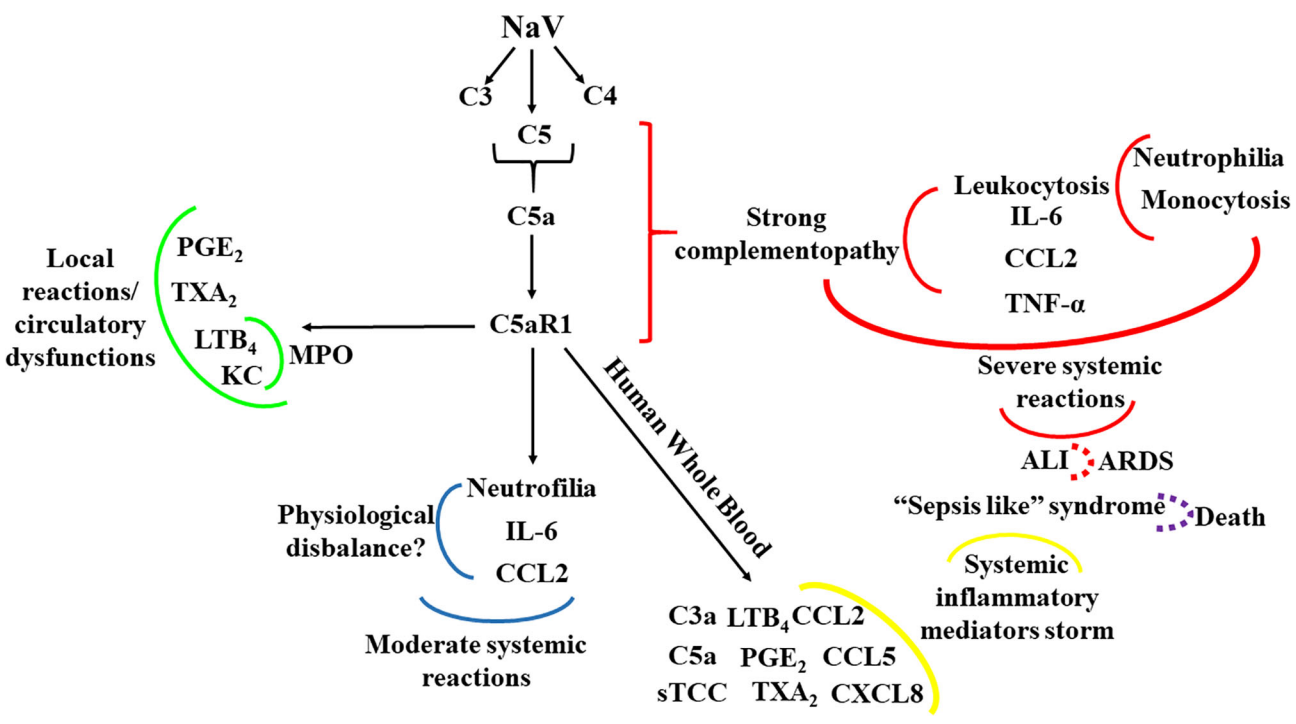

FIGURE 9 | Schematic representation of the contribution of the C5a-C5aR1 axis to local and systemic reactions induced by *N. annulifera* venom (NaV). NaV acts on complement proteins culminating in anaphylatoxins generation and sTCC assembly. C5aR1 engagement by C5a stimulates LTB_4, PGE_2 and TXB_2 generation, which cause endothelial dysfunctions promoting vascular leakage resulting in extensive edema. In addition, the release of LTB_4 and KC, triggered by C5aR1 signaling, may promote the attraction and activation of neutrophils into tissue, increasing MPO levels and potentiating local immunopathology (*green lines*). The systemic C5aR1 activation, stimulated by NaV sublethal dose injection, promotes moderate increase on CCL2 and IL-6 levels, as well in the neutrophil's percentage, which could lead envenomated animals to a several physiological disbalance (*blue lines*). By coupling human whole blood *ex vivo* (*yellow lines*) approaches and a severe experimental mouse model of envenomation, it was detected that NaV promotes a hyperacute inflammatory reaction featured by intense systemic inflammatory mediators' storm, induced by C5a-C5aR1 axis activation. This can evolve to a plethora of pathological conditions including ALI development (*red lines*) that can progress to ARDS (*red dotted lines*) and respiratory arrest, a common death cause in humans envenomated by *N. annulifera*. In addition, these inflammatory mediators can promote organ dysfunction featuring a sepsis like syndrome, which could be a death cause of the envenomated individuals (*purple dotted lines*).

cytokine systemic levels, rescued mice from the increase on circulating leukocytes and abrogated ALI development. Unfortunately, the blockage of C5aR1 activation and the increase in IL-10 levels were not followed by a decrease of IL-6, CCL2, or TNF-α in the plasma levels, and they could not control animals' death, induced by *N. annulifera* venom lethal dose, suggesting that perhaps other complement/inflammatory-mediated signaling pathways are acting in this context. By injecting a lethal venom dose, the physiological imbalance and tissue damage were stronger promoting high DAMPs release and amplifying complement activation. C3 is the most abundant circulating complement protein (90), and we must consider its involvement on cytokines release potentiation in lethal context, as well in the death, since *N. annulifera* venom presents on its composition various C3 activators, including CVF (37). In addition, through *in vivo* C5aR1 antagonism, C3aR activation can potentially stimulate the increase on systemic levels of inflammatory mediators (4, 11, 91–93) and promote pathological events, which can evolve to cause multiple organs dysfunction and death (53, 93–95). In addition, cobra venom also induces the formation of high amounts of sTCC, a complex with inflammatory and deleterious properties, which is a risk factor to multiple pathologies (17, 53, 96–100). Thus, by using other pharmacological tools to inhibit additional steps of the complement cascade, *in vivo*, our results may be expanded.

In recent decades, the contribution of complement to a plethora of inflammatory and degenerative diseases has been demonstrated and, in this context, various strategies to control complement activation have emerged. Complement inhibition, achieved by using eculizumab, a humanized monoclonal antibody that prevents C5 activation and improve diseases outcomes (19), has been used to treat autoinflammatory (*e.g.*, atypical hemolytic uremic syndrome and paroxysmal nocturnal hemoglobinuria) and autoimmune conditions (e.g., myasthenia gravis). Thus, considering that eculizumab is an FDA-approved medicine that is already used in the clinic and that *N. annulifera* venom induces a pathology mainly mediated by C5a, eculizumab may prove useful as a complementary treatment for this envenomation. In addition, PMX205 and Avacopan (CCX168), which are C5aR1 antagonists are also under clinical development for amyotrophic lateral sclerosis (38) and atypical uremic hemolytic syndrome (aUHS), anti-neutrophil cytoplasmic antibody associated (ANCA) vasculitis, and immunoglobulin A (IgA) nephropathy (19), respectively, thus also representing other potential therapeutic medicines for envenomation treatment.

In conclusion, we have shown here, for the first time, that activation of the C5a-C5aR1 axis is the main driver of the local and systemic reactions in envenomation by *N. annulifera*, a medically important snake on Sub-Saharan Africa. **Figure 9** summarizes the main findings of the current study, in which *in vitro* and *in vivo* models targeting C5a-C5aR1 signaling demonstrated that envenomation by *N. annulifera* is a harmful hyperacute inflammatory condition that predisposes individuals to circulatory dysfunctions and ALI/ARDS development. Thus, we postulate that modulation of the C5a-C5aR1 axis could improve clinical outcomes in envenomation by *N. annulifera* as well by other venomous animals in which the C5a-C5aR1 axis is activated during physiopathogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Research Ethics Committee of the Municipal Health Secretary of São Paulo. Blood samples were obtained from healthy donors after informed consent (protocol approval n° 974.312 and 4.309.960). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Butantan Institute (protocol approval n° 5323120918).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FS and DVT. Performed the experiments: FS and IV-B. Analyzed the data: FS, BC and DVT. Contributed with reagents/materials/analysis tools: DVT, BC, JL, ES, TMW. Wrote the paper: FS and DVT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.652242/full#supplementary-material

Supplementary Figure 1 | *N. annulifera* venom (NaV) induces complement activation in human whole blood. NaV (3.125 to 100 μg/mL) samples were incubated with human blood samples for 30 or 60 min at 37°C, and then anaphylatoxin generation **(A, B)** and sTCC assembly **(C)** were quantified by CBA and ELISA. Data are means ± SEM of three independent experiments. *** (different

venom concentrations *vs*. saline)/### (30 *vs*. 60 minutes) $p \leq 0.001$ (two-tailed two-way ANOVA, followed by Bonferroni post-test).

Supplementary Figure 2 | *N. annulifera* venom (NaV) triggers lipid mediators release in human whole blood. Human blood samples were incubated with NaV (3.125 to 100 µg/mL) during 30 or 60 min at 37°C. Leukotriene B4 (LTB4) **(A)**, prostaglandin E2 (PGE2) **(B)** and thromboxane A2 (TXA2) **(C)** production were analyzed by ELISA. Data are means ± SEM of three independent experiments. *** (different venom concentrations *vs*. saline)/### (30 *vs*. 60 minutes) $p \leq 0.001$ (two-tailed two-way ANOVA, followed by Bonferroni post-test).

Supplementary Figure 3 | *N. annulifera* venom (NaV) upregulates chemokines generation in human whole blood. Human blood samples were exposed to NaV (3.125 to 100 µg/mL) during 30 or 60 min at 37°C. Then, CCL2, CCL5 and CXCL8 chemokines release were accessed by CBA. Data are means ± SEM of three independent experiments with different whole blood donors. *** (different venom concentrations *vs*. saline)/### (30 *vs*. 60 minutes) $p \leq 0.001$ (two-tailed two-way ANOVA, followed by Bonferroni post-test).

Supplementary Figure 4 | Inhibition of C5a-C5aR1 signaling worsens production of inflammatory mediators in mice injected with a lethal dose of *N. annulifera* venom (NaV). Mice (n=6/group) were pretreated with PMX205 (2 mg/kg), a C5aR1 inhibitor, or vehicle 24 and 1 hour before NaV injection. Following the inhibitor administration, severe systemic reactions were induced by the injection of NaV lethal dose, *via* intraperitoneal route. Fiver hours after envenomation, blood samples were obtained to determine systemic increase on CCL2, IL-6, IL-10, TNF-α levels by ELISA and CBA. Data are means ± SEM of six independent experiments. *** (NaV + treatments *vs*. Saline)/### (NaV + Vehicle *vs*. NaV + PMX205). $p \leq 0.001$ (two-tailed t-test or two-way ANOVA, followed by Bonferroni post-test).

Supplementary Figure 5 | SDS=PAGE original gels from which **Figure 2** was organized.

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REVIEW
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A Quest for a Universal Plasma-Derived Antivenom Against All Elapid Neurotoxic Snake Venoms

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This review describes the research aimed at the development of universal antivenom against elapid neurotoxic snake venoms. The antivenoms produced in Thailand in the 1980s were of low potency, especially against the elapid venoms. This was thought to be due to the low immunogenicity of the α-neurotoxins, which are the most lethal toxins in these venoms. Comparisons of various α-neurotoxin conjugates and polymers, and also different immunological adjuvants, showed that the adjuvant used is the major determinant in the antibody response in horses. The potent Freund's adjuvant was not used due to its severe local side-effect in horses. Therefore, a novel immunization protocol termed 'low dose, low volume multi-site' was developed for use in horses. This immunization protocol has led to the production of highly potent monospecific antivenoms against several elapid and viperid venoms, and two potent polyspecific antivenoms, one against 4 neurotoxic and another against 3 hematotoxic venoms. The immunization protocol has also led to other improvements in antivenom production including: several fold increases in antiserum potency, a reduction in the time required to reach therapeutically useful antibody titers, a 90% reduction in the amount of venom used, and 100% of the horses responding to the immunization program. This development is partly responsible for significant decrease in the Thailand's annual snakebite death toll from a few dozens to mostly nil in recent years. Finally, a simple and novel immunization strategy, using a 'diverse toxin repertoire' composed of numerous elapid toxin fractions as immunogen, was proposed and tested. This immunization procedure has resulted in the successful production of a widely paraspecific antiserum against at least 36 neurotoxic venoms of 28 species encompassing 10 genera and from 20 countries on four continents, and possibly against all elapid venoms with α-neurotoxins as the lethal toxins. These results indicate that, with optimizations of the composition of the 'diverse toxin repertoire', the immunization scheme and antibody fractionation to increase the antivenom neutralizing potency, an effective universal antivenom against the neurotoxic elapid snakes of the world can be produced.

Keywords: universal antivenom, pan-specific antivenoms, elapid snakes, neurotoxic venoms, plasma-derived antivenoms, diverse toxin repertoire, immunization strategy, low dose low volume multi-site immunization

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INTRODUCTION

Snakebite envenomation is an important medical problem in many tropical countries (1). It has been estimated that snake bites are responsible for about 400 000 disabilities with 138,000 deaths annually (2).WHO has designated this problem as a Category A most neglected tropical disease and it has spearheaded efforts to reduce the deaths and disabilities inflicted by snakebites by half in 2030 (3, 4).

The most effective treatment for snakebite envenomation is the timely administration of safe and effective antivenom (AV). Currently available AVs are plasma-derived preparations (PDAVs) produced in large animals e.g. horses, sheep etc. Despite their demonstrated efficacy, current antivenoms have a number of drawbacks, including their low titer against relevant toxins of low immunogenicity. Thus they must be administered in large doses, which contribute to the high cost of treatment and the risk of adverse reactions. Furthermore, AVs are specific in that they are mostly effective against the venom(s) used in the immunization. Thus, despite the existence of cross-reactivity of antivenoms against some heterologous venoms, there are many instances where such cross-neutralization does not occur (5–7).

The immunological specificity makes it often necessary to identify the culprit snake before specific AV treatment. Also, with geographical variation within a given snake species, AV may be effective only against the venoms of certain snakes in specific countries or regions. Consequently, most AVs are produced in small volumes for use in a limited geographical area and thus the cost is high and often unaffordable to the snake bite victims which mostly reside in low-income countries (8). Another often cited drawback of PDAV is the heterologous source of plasma which could contribute to adverse reactions, such as immediate type hypersensitivity and serum sickness, in patients. However, when antivenoms are manufactured following good manufacturing practices (GMPs) and are composed by highly purified immunoglobulins or immunoglobulin fragments, their safety profile is adequate (1). Thus, there are several issues regarding the use of PDAV in the treatment of snakebite victims and attempts are being made to improve effectiveness, reduce the number of adverse reactions and develop cheaper alternatives (9).

Because of the shortcomings of the PDAV mentioned above, there is a growing interest in the development of 'new generation antivenoms' using new state-of-the-arts approaches (10, 11). Examples of these novel alternatives are: human monoclonal antibody with different types of antibody formats i.e. whole IgG, single-chain variable fragments (scFvs), antigen binding fragments (Fabs and $F(ab')_2$) (12), oligonucleotide aptamers (13), inhibitors of enzymatic toxins (14), inhibitors of phospholipases A_2 (15), inhibitors of snake venom proteases (16, 17), inhibitors of hyaluronidase (18), metal chelators (19–21) and neuronal acetylcholine receptor (nAChR) mimetics (22). All of these represent promising and interesting alternative therapeutic modalities to improve or replace PDAV, increase effectiveness, cause less adverse effects, and be cheaper to produce.

Whether the AV is plasma derived or synthetic, ideally it should be effective against the venom(s) used as immunogens and have an adequate safety profile. Moreover, the production cost, which includes the amount and cost of venom(s) and immunological adjuvant, should be low. The preparation of immunogen should be simple so that the production process can be easily carried out by manufacturers in developing countries. Importantly, the antivenom should be inexpensive and affordable to the snake bite victims. Furthermore, the AV should exhibit wide para-specific so as to be effective against other snake venoms producing a similar syndrome, and preferably against snakes in a wide geographic area or, even better, worldwide. If 'universal' AV can be produced and used in envenomations caused by numerous snakes, like the anti-rabies or anti-tetanus antitoxins, it can then be produced in large volumes, with the consequent reduction in production costs.

While the studies on these 'new generation AVs' are under active research, the production of conventional PDAV continues, and the products are currently being used to save countless lives worldwide. Moreover, some of the 'new generation AVs' may face hurdles due to the high cost and lack of information on venom and AV pharmacology, and the need to develop clinical trials to validate their use (23). Thus, the production of the 'new generation AVs' could take some time since none has progressed to clinical trials. Therefore, it is important that any feasible improvements to conventional PDAV should be explored and exploited. In the short term, it is relevant to point out that any simple improvements, quantitatively and/or qualitatively, to conventional PVAV production would be of immediate benefit to snakebite victims. Hence, the improvement of currently available antivenoms is a priority in the WHO strategy for reducing the impact of snakebite envenomation (3).

In this regard, one potentially fruitful adaptation to PDAV at present is to make possible the production of pan-specific or universal PDAV against the neurotoxic snakes. This is a line of research that we have carried out over the past several years and it constitutes the main topic of discussion of this review.

PREVIOUS PROBLEMS ENCOUNTERED IN THE PRODUCTION OF PDAV

AVs were first produced in Thailand by the Thai Red Cross Society at Queen Saovabha Memorial Institute (QSMI) in 1916, only 21 years after Albert Calmette's groundbreaking report in 1894. The production process had changed little even until the 1980s. AVs available then were of low potency (24) and were in short supply and sufficient for probably less than half of the demand in the country. This was due to several problems. Firstly, a low percentage of horses responded to the immunization, especially so for the horses injected with neurotoxic elapid venoms (less than 20% of them responded). Secondly, a long immunization period was needed to reach acceptable antibody titers (6-8 months). Finally, the immunization program usually required a large amount of venom(s). In some cases up to 150 mg venom per horse was required. These problems were encountered not only in Thailand but likely in other antivenom producers in Asia and elsewhere.

Immunogenicity of the Elapid Postsynaptic Neurotoxins and the Role of Immunological Adjuvant

The low potency of the anti-elapid antivenoms was thought to be due to the high toxicity and the low immunogenicity of the major lethal toxins of elapid venoms. These toxins are mostly α-neurotoxins, which are low molecular mass proteins of about 6-7 kDa (25). They bind quasi-irreversibly to the alpha subunits of the nicotinic acetylcholine receptor (nAChR) leading to blockage of neuro-muscular transmission at the muscle motor endplates (26). The high toxicity of the venoms limited the immunization doses previously believed to be required for a high antibody response (27). Thus, various attempts were made to detoxify the venom toxins. This has been done by chemical means such as treatment with formaldehyde (28), glutaraldehyde (17, 29), by iodination (30), and by physical means using X-irradiation (31), UV light (32) and gamma irradiation (33). It is relevant to note that the detoxification reactions invariably involve either modification of the 'active site' or otherwise alter the conformation of the toxins, thereby rendering them inactive. Consequently, owing to the modifications introduced in the structure of these toxins, the antibodies generated against these detoxified toxins usually failed to recognize and neutralize the native toxins leading to low potency of the antivenom. Furthermore, some of these detoxification reactions, e.g. glutaraldehyde polymerization (29) and iodination (34), are difficult to control and optimize especially when different toxins are involved, as occurs in the preparation of polyspecific antivenoms. Finally, immunization at high doses of (detoxified) venom could lead to immune tolerance (35, 36).

The low neutralizing potency of antivenoms was also thought to be due to the low molecular mass of elapid α-neurotoxins which might be associated with their low immunogenicity (37). Thus, various studies were made to conjugate the toxins to macromolecules or immunogenic carrier proteins e.g. bovine serum albumin (BSA) or tetanus toxoid. An example is the conjugation of toxins to cellulose particles that had been oxidized with sodium metaperiodate (SOC). These conjugates were found to increase anti-neurotoxin antibody titers 2.0-2.5 fold relative to the native toxin (38). However, these types of reactions (detoxification and conjugation/polymerization) on the venoms have not yet been applied to commercial antivenom production.

To identify the key factor(s) involved in the production of potent antivenoms, we carried out a study using nine immunogens prepared from the α-neurotoxin of a cobra venom (*Naja kaouthia* toxin 3, NK3). These immunogens included the crude venom, the purified toxin, various carbodiimide conjugates, and polymers obtained from controlled polymerization by glutaraldehyde or formaldehyde. These immunogens were tested in rats using Freund's adjuvants (29). It was shown that pure NK3 toxin elicited comparable specific antitoxin antibody titers as that of the crude venom which suggested that the toxin was immunogenic, and that 'antigenic competition' (39), if present, was not an important factor in the antibody response against the elapid toxins. The results also showed the absence of any immunosuppressive component in the crude venom (40) that could reduce the antibody response against the toxin. Thus the experiment clearly underscored that the elapid α-neurotoxins are capable of inducing a good antibody response in spite of the fact that they are of low molecular mass.

This conclusion is supported by our recent finding that the amino acid sequences of the α-neurotoxins contain T cell epitopes that are required for binding to major histocompatibility complex (MHC) class II proteins. The T cell epitope–MHC complex then interacts with a CD4+T cell receptor (TCR). The activated CD4+T cell then initiates a sequence of events leading to the production of toxin specific antibodies. The lack of T cell epitope abrogates activation of CD4+T cells and T cell dependent antibody responses (41, 42). In the case of the 71 residue α-neurotoxin from the cobra *Naja siamensis*, we used an online program "IEDB analysis resource" (43) to predict the T cell epitopes for human HLA (no information on horse MHC II was available in IEDB). The two T-cell epitopes are predicted to be in amino acids 1-9 (medium score epitope) and 28-36 (high score epitope). The fact that α-neurotoxins contain high score T cell epitopes and thus would be expected to be immunogenic, raised the question as to why antivenoms against the elapids are usually of low potency. There should be some other parameters employed in the immunization, i.e. dose, adjuvant, route of administration, volume, frequency (44), that contributed to the observed low potency.

Experiments on the immunogenicity of various derivatives of α-neurotoxins described above were carried out in rats using a variety of adjuvants including Freund's adjuvants. However, these adjuvants have been shown to cause granuloma and sterile abscesses at the site of immunogen injection (45–47). Consequently, their use in horses was discouraged (48). Thus, many antivenom producers use other adjuvants (bentonite, squelene/Aracel A, aluminum salts, sodium alginate etc.) in their antivenom production. The Thai Red Cross at Queen Saovabha Memorial Institute (QSMI) used bentonite as the adjuvant in horses.

We therefore carried out a comparative study on some of the *N. kaouthia* toxin immunogens using three different adjuvants (IFA, bentonite and squalene/Arlacel A). The results showed that only IFA gave a good specific antitoxin antibody response (49). Thus, it was concluded that the low antibody response normally observed in PDAV production in horses was mainly due to the ineffective adjuvant used and not necessarily to the low immunogenicity of the toxins. Since co-administration of the immunogen with an effective adjuvant is an essential requirement in antibody production (36, 41), it is critical that the most effective adjuvant is used in the horse. Therefore, to improve the effectiveness of PDAV production, it was necessary either to find new and better adjuvants, or to find a way to use CFA/IFA safely in horses to avoid the adverse reactions previously observed. The ineffectiveness of the therapeutic antivenoms available combined with a severe shortage in Thailand at the time created a critical situation that led us to choose Freund's adjuvants since they have an excellent record of immunostimulatory effect as compared to other adjuvants (50–52).

Complete Freund Adjuvant (CFA) is a water-in-oil suspension containing purified light paraffin oil and mannide monooleate, a

surfactant, as emulsifier. It also contains heat-killed dried *Mycobacterium tuberculosis*. The Incomplete Freund Adjuvant (IFA) contains the same ingredients but without the Mycobacterium. With CFA, the hydrophilic and amphipathic snake toxins reside in the aqueous phase, which keeps them in their native conformation (53). The water-in-oil preparation serves as a depot for slow and continuous release of the venom antigens from the injection site for prolonged stimulation of antibody producing cells. It also protects the immunogen from rapid proteolytic degradation and elimination, resulting in the production of high levels of antibody by the host. The mineral oil component of the antigen adjuvant emulsion serves as a vehicle for antigen transport throughout the lymphatic system to immune effector cells and promotes interaction with antigen-presenting cells like dendritic cells. In CFA, the mycobacterial cell wall contains lipoprotein, lipomannans and lipoarabinomannans that interact with Toll-like receptor-2 (TLR-2), as well as TLR-4 and TLR-6 (54–57). This provides immunostimulation by recruiting, activating, and enhancing differentiation of the cells of the immune system (58). It has been shown that CFA and IFA have served with unsurpassed record in the stimulation and production of high titers, high affinity and high avidity antibodies (50, 51) and are useful for low molecular weight antigens (59). Moreover, these adjuvants can be produced in large volume with high consistency and inexpensively. Thus, an immunization protocol that allows the safe use of CFA/IFA must be found.

The Use of Freund's Adjuvants in Horses and the 'Low Dose, Low Volume Multi-Site' Immunization Protocol

After extensive inquiries and discussions with some PDAV producers, it was concluded that the severe adverse reactions in horses resulting from CFA injection were due to the injection of a large volume of immunogen emulsified in CFA at one single anatomical site (47, 60–62). The inflammation at the large injection area inevitably caused skin rupture that, under non-aseptic conditions, caused severe infection that in some cases could result in death. It was hypothesized that the lesions produced by CFA could be reduced or eliminated by minimizing both the total injection volume and the volume injected at each site.

A simple and novel immunization protocol termed 'low dose, low volume, multisite' immunization was therefore proposed and tested in horses (63, 64). This immunization protocol involves subcutaneous injection of the CFA emulsified immunogen in small volumes (50-200 µl/site) carrying a very low venom dose (about 1-2 mg of venom in total/horse) around the neck at approximately 20 sites. Because of the low volume injected at each site, the local reaction is mild, and when the preparation of immunogen and the injection were performed aseptically, no infection or skin rupture occurred (63, 64).The injection is made subcutaneously (2-3 mm depth from the skin surface) in the epidermis where the dendritic cells, the most potent antigen presenting cells, are located (65). Furthermore, the injections are made around the neck area of the horse where the majority of the lymph nodes are situated. This ensures maximum exposure of the immunogen to the lymphocyte traffic. The low volume used at each of the 20 injection sites also increases the total exposed surface area of the droplets containing the immunogen by 2.4 times when compared to injection of the total volume at one single site, assuming the droplets are spherical. This results in increased exposure of the immunogen to the stellar shape dendritic cells. The simple immunization procedure can be easily carried out (taking about 2-3 minutes to inject 20 sites per horse) using slightly modified tuberculin syringes (**Figure 1**). Using this procedure the reaction at the injection sites was mild or absent (63, 64). This protocol was included and recommended in the WHO Guidelines for antivenom production and control (66).

This immunization protocol has allowed the safe use of Freund's adjuvants. It has been repeatedly shown to induce high specific antibody titers in horses (41, 67–69).

It should be noted that the very low venom/toxin dose used for the immunization not only reduces the cost because of the lower amount of venom used but, more importantly, also stimulates the production of high affinity antitoxin antibody thus increasing PDAV potency (67–69).

This novel immunization protocol has resulted in the production of highly potent antiserum (2-4 fold increase in potency) against *N. kaouthia* venom (63). It reduces the time required for a horse to reach maximum antibody titers to about 6 to 8 weeks instead of several months. It reduced the amount of venom immunogens to just 10% of what was previously used; and it increased the percentage of responder horses from about 60% to 100% (70). These improvements have resulted in vast increase in antivenoms production by QSMI (70) with enough surpluses for export to neighboring countries. Furthermore, this development is partly responsible for the decrease in the country's annual snakebite death from a few dozens to mostly nil in recent years (Snakebite in Thailand, Annual Epidemiological Surveillance Report, Ministry of Public Health).

The introduction of this novel immunization protocol has also resulted in the successful production of two potent polyspecific antivenoms. Based on a 'Syndromic strategy' (9), one polyspecific antivenom is against three neurotoxic venoms: *N. kaouthia* "Thai monocellate cobra", *Bungarus fasciatus* "Banded krait" and *Ophiophagus hannah* "The King cobra" (64) while another is against three hematotoxic venoms: *Cryptelytrops albolabris* "White lipped pit viper", *Calloselasma rhodostoma* "Malayan pit viper", and *Daboia siamensis* "Russell's viper" (69). These polyspecific antivenoms are currently produced commercially by The Thai Red Cross at QSMI using the 'low dose, low volume multi-site' CFA immunization protocol. Recently, the polyspecific anti-neurotoxic antivenom produced by QSMI also includes the venom of *Bungarus candidus* "the Malayan krait".

PARASPECIFICITY OF PDAV AND THE PRODUCTION OF PAN-SPECIFIC ANTIVENOM AGAINST NEUROTOXIC VENOMS

The production of poly specific AVs using the "low dose, low volume, multisite" immunization protocol has led to improvements in treatment due to reduced production costs

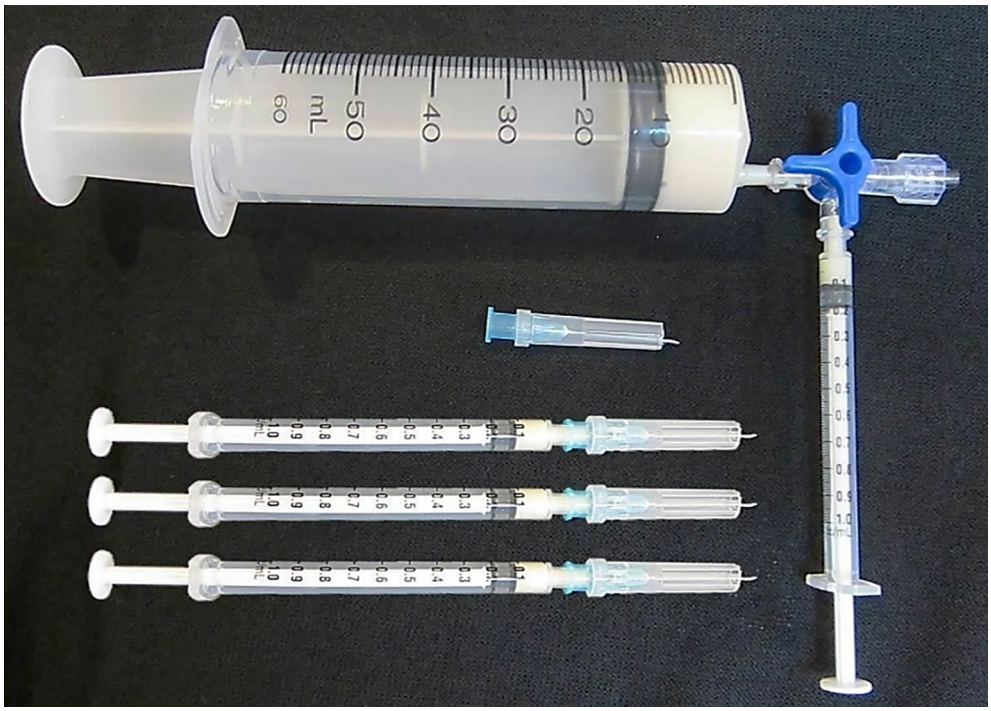

FIGURE 1 | The modified tuberculin syringes used to deliver 0.1 ml of Freund's adjuvant emulsified immunogen into the horse subcutaneous site at the depth of 2-3 mm from the skin surface according to the 'low dose, low volume multi-site' immunization. Please see details in (66).

and increased effectiveness of antivenoms. Interestingly, these two polyspecific AVs have been shown to significantly cross-neutralize various medically important hematotoxic and neurotoxic venoms of snakes distributed in Southeast Asian and South Asian countries (71–74). Moreover, it has been observed that many other polyspecific AVs offer immunochemical cross reactivity with heterologous venoms from various species (6, 75–80).

It would be highly desirable if a PDAV can be produced to cover dozens of related venoms which are medically important to people in several countries or regions. Such a 'pan-specific' PDAV should be very useful to a large number of snakebite victims. However, the upper limit of venoms used as immunogens for polyspecific antivenom production is only about 5-6 venoms. When higher number of venoms are used in the immunization, lower antibody titers against some or all the venoms are obtained.

A SIMPLE AND NOVEL IMMUNIZATION STRATEGY USING A 'DIVERSE TOXIN REPERTOIRE' AS IMMUNOGEN AND THE PRODUCTION OF PAN-SPECIFIC ANTIVENOM AGAINST ELAPID SNAKE VENOMS

From the above discussion, it was noted that while some of the monospecific antivenoms exhibited no or very narrow cross reactivity (5–7, 81), polyspecific antivenoms prepared from various laboratories showed wide paraspecificity (71–80). It seemed that the number of heterologous venoms neutralized by a polyspecific antivenom is greater than the sum of heterologous venoms neutralized by the antivenoms prepared separately as monospecific antivenoms. These observations suggested that the numerous antibodies in a polyspecific antivenom somehow act cooperatively to cross neutralize heterologous toxins, resulting in wider paraspecificity of the antivenom. This can happen if two or more heterologous antibodies bind, even weakly, to a target toxin and together enable cross-linking to form lattice and neutralization which otherwise could not occur. This would be a 'positive cooperative' effect of the heterologous antibodies against a heterologous toxin. If this conjecture is true, it should be possible to prepare wider paraspecific antivenoms by increasing the number of venoms used in the immunization.

With the aim of producing a pan-specific PDAV against elapid venoms of Asia, we therefore proposed and tested a simple and novel immunization protocol using a 'diverse toxin repertoire' consisting of several neurotoxic venoms as immunogen (82). The 'diverse toxin repertoire' was obtained from toxin fractions of 12 neurotoxic venoms of Asian origin. These toxin fractions were prepared by ultrafiltration of the venoms to remove toxicologically-irrelevant high molecular mass and highly immunogenic venom proteins. The fractions were individually shown to contain the lethal toxins (α-neurotoxins and β-neurotoxins) and total lethal activity of

the venoms. The mixture of these toxin fractions was used to immunize horses at very low doses (about 12 μg of each toxin fraction) using the 'low dose, low volume multisite' protocols (63). It was found that the horse antiserum could neutralize 11 homologous and 16 heterologous neurotoxic venoms from elapids of Asian and some African countries (82). Thus the pan-specific PDAV could offer broad cross neutralization of venoms from different and geographically separated snakes and could benefit a large number of snakebite victims. The rationale of the novel immunization strategy was discussed previously (83). The result of this study is a proof of concept of the 'diverse toxin repertoire' immunization strategy in the production of pan-specific antivenom against neurotoxic venoms. It also indicates that it should be possible to produce a universal PDAV against the elapid snakes of the world.

A QUEST FOR UNIVERSAL PDAV AGAINST ALL THE NEUROTOXIC ELAPID SNAKES

To further test the concept of the 'diverse toxin repertoire' immunization strategy, we assayed the ability of the pan-specific PDAV to inhibit the venoms of a variety of elapids from different continents. It was shown that the pan-specific PDAV could effectively neutralize at least 36 neurotoxic venoms of 10 genera and from 4 continents including sea snakes from both Australia and the Arabian Sea (83).

These results suggest that universal antivenom against all elapid snakes is possible if the 'diverse toxin repertoire' is modified to include a few more neurotoxic venoms. The bases for our idea are as follows.

a). Most of the elapid venoms contain α–neurotoxins and some also contain the highly lethal β-neurotoxins. For simplicity, the discussion will be confined only to the α–neurotoxins. All of the elapid α–neurotoxins are highly lethal and are responsible for most of the deaths caused by a large number of elapid species. They have high amino acid sequence homology with one another and all share the same mechanism of toxicity in that they bind specifically to the α–subunits of nAchR at the motor endplate in the neuromuscular junction (26, 84, 85). Thus all these toxins are structural and functional homologs. These toxins, although previously believed to be poorly immunogenic, are in fact able to induce high affinity neutralizing antibody (41, 67). This is supported by the fact that they all contain high score T helper epitopes in their molecular sequences as discussed above.

b). The horse antibody repertoire is vast and far exceeds the epitope repertoire of all the world's elapid α-neurotoxins. Thus the horse is capable of producing specific antibodies against any elapid α-neurotoxin. This conclusion is based on the following information and calculation.

The Repertoire of the Elapid α–Neurotoxin Epitopes

Given their small molecular size and constraints imposed with the formation of a biologically active conformation, it is likely that each α–neurotoxin contains a relatively small number of dominant epitopes on its surface, with each epitope made up of about 12 amino acid residues (86, 87). The average accessible surface area of an epitope is about 846.59 ± 278.87 sq Å (88). The total accessible surface area of the 71 amino acid residue α-neurotoxin of *N. siamensis*, venom (PDB 1CTX; alpha-cobratoxin from *Naja siamensis*) is calculated to be about 5,206 sq. Å using a program described by Ribeiro et al. (89). The number of non-overlapping epitopes on the α-neurotoxin surface is therefore about 6 epitopes per toxin.

The family Elapidae comprises 382 species (www.reptiledatabase.org). Assuming that each of these shows three geographic variations regarding their α-neurotoxins structure, this will give 1,146 (382x3) α-neurotoxins amino acid sequences. If each elapid produces an average of three different α-neurotoxin isoforms (83), this will give a total of 3,438 elapid neurotoxin isoforms. If each isoform has six non-overlapping epitopes, a total of about 2.06 $x10^4$ elapid α-neurotoxin epitopes would exist in nature. This number is probably overestimated since some of the epitopes from homologous toxins are conserved and similar for structural and functional reasons. However, suffice is to say that the total number of different epitopes of the world elapid α-neurotoxins is finite and in the range of tens of thousands.

The Horse Antibody Repertoire

On the opposite end of the antigen-antibody interaction is the antibody paratope. The diversity of the antibody paratopes generated spontaneously in a large animal, e.g., human and horse, is enormous. Due to the random immunoglobulin genes rearrangement, it has been estimated that 10^{15}-10^{18} of naïve antibody specificities could be generated (90, 91). In another study, it was estimated that the total potential repertoire in human is immense at 10^{26} different antibody specificities (92). However, with new immature B cells being produced at the rate of about 10^9 per day and the number of circulating peripheral naïve mature B-cells at any one time is about 10^9 (93), a repertoire size of naïve antibodies in human is thought to be about 10^{12} specificities (94). Moreover, this repertoire of naïve antibodies is expanded exponentially by somatic hypermutation after antigen encounter (95). This number is thought to be adequate to handle about 1400 potential pathogenic species thought to be infectious to humans (96).

From the above calculation, the total number of elapid α-neurotoxin epitopes is about 2.06 $x10^4$. Therefore, the naive antibody sequences present each day in the horse should be enough to recognize and bind to all the epitopes of elapid α-neurotoxins of the world.

c) It is likely, therefore, that when a horse is immunized with the 'diverse toxin repertoire' from venoms of numerous snakes, there will be enough B-cells with antibody paratopes/specificity generated against this repertoire. Furthermore, these antibodies should include a large number of those capable of cross reacting with other heterologous toxins. That this is most likely the case is supported

by our previous results (83). When the horse was immunized with the toxin fractions of 12 Asian elapids of only 6 species and 2 genera (*Naja* and *Bungarus*), the antiserum was shown to neutralize 36 venoms of 28 species and 10 genera from 4 continents.

It should be mentioned that among the 37 elapid venoms tested, only the heterologous venom of *Dendroaspis angusticeps* was not neutralized by the pan-specific antivenom (83). The lethal toxins of this venom have not been identified but are thought to act synergistically (97, 98). Thus, if the toxin fractions of this mamba together with other selected WHO Category 1 elapid venoms (66) from various continents are included in the immunization mix, it is most likely that a PDAV with paraspecificity against all elapid neurotoxic venoms can be produced.

A universal PDAV against neurotoxic snake venoms would be analogous to the equine anti-rabies and anti-tetanus sera, in the sense that it could be used in a wide geographical range. Using the facilities already available in most antivenom producers, the production could be implemented within a relatively short time and without additional investment. It could be produced in large volume and, with the economy of scale, it could be produced at relatively low cost and be affordable to low income snake bite victims of the world. Last but not least, the availability of a universal PDAV eliminates the need for species identification of the culprit elapids.

ADVANTAGE AND CHALLENGES OF THE 'DIVERSE TOXIN REPERTOIRE' IMMUNIZATION STRATEGY

One advantage of the 'diverse toxin repertoire' immunization strategy is that it generates unprecedented wide paraspecificity against at least three dozen elapid venoms and possibly against all elapid venoms having α-neurotoxins as lethal components. Moreover, the procedures involved are very simple. For example, the preparation of the toxin fractions, the immunization and the antibody fractionation can be readily carried out using the existing facilities of most current antivenom producers. Ultrafiltration can be used to purify the neurotoxic fractions because the requirement is to obtain a mixture of lethal toxins with all its isoforms, rather than any single purified toxin. The fractionation process of antibody IgG or F(ab')2 can be carried out using the equipment for routine PDAV production in antivenom manufacturing laboratories. Furthermore, the production time should be shorter than that required to produce several monospecific or polyspecific PDAVs. The cost of producing one universal antivenom might be lower than that for several polyspecific antivenoms. However, detailed analysis on the cost of all the production steps is necessary to make a valid comparison.

It should be mentioned that although a polyspecific PDAV can neutralize many venoms, its potency (ED50 or Effective Dose50) against different neurotoxic venoms may vary and thus different dosages of the PDAV may be required for treatment of envenomation by different elapids. However, this is quite normal in the treatment of snakebite envenomation because the antivenom dose administered to the patient depends on the severity of the case, which largely depends on the amount of venom delivered by the snake. This in turn depends on many parameters; for example, the sizes of the snake and of the victim, the toxicity of the venom, the site of the bite, the time lapse between the bite and the treatment, etc. Thus protocols are developed to establish the dose that needs to be administered.

However, it is likely that the paraspecific potency of the PDAV against some heterologous venom(s) may be low and may pose a clinical problem. In these cases, the neutralizing potency against these venoms can be improved by modifying the immunization schemes and/or by antibody fractionation in the following ways. First, during the immunization, the heterologous venoms that are poorly neutralized could be included in the 'diverse toxin repertoire' immunogen and thus serve as homologous venom antigens so as to increase the antibody titers against them. The neutralizing potency against some venom can also be enhanced by booster injections with the toxin fractions of only these poorly neutralized venoms. These immunization schemes have been shown to work well in the production of the polyspecific antivenoms in Thailand. Second, during antibody fractionation, the neat horse serum can be fractionated by salt precipitation to obtain about 2 fold increases in neutralizing potency (99). Subjecting this refined globulin fraction to α-neurotoxin affinity chromatography could result in a further 10 to 12 fold increase in potency (100). Thus, a 20 fold increase in neutralizing potency could be achieved by antibody fractionation. The combination of optimized immunization and antibody fractionation will result in substantial increases in the neutralizing potency of the antivenom over that of the antiserum.

With these modifications and optimization, it is likely that universal PDAV against elapid neurotoxic venoms can be produced and used for the treatment of envenomation by elapid snakes.

CONCLUSION

Plasma-derived antivenom (PDAV) is still the mainstay of the current therapies for snakebite victims. There are some drawbacks to PDAV and this has led to attempts to produce 'new generation' antivenoms. However, this is likely to take some time until these new therapies reach the clinical trial stage. Consequently, improvements to PDAVs that lead to the production of universal PDAV against the world elapid venoms would be of immediate benefit. The production of a pan-specific PDAV against at least three dozen neurotoxic venoms from four continents has been achieved through a simple and novel immunization strategy using a 'diverse toxin repertoire' as immunogens. The 'diverse toxin repertoire' was made up of toxin fractions of numerous elapid venoms. The strategy has resulted in unparalleled wide paraspecificity. With careful selection of toxin fractions of elapid venoms to serve as immunogens together with an optimized immunization scheme and antibody fractionation, it is most likely that a universal PDAV with high neutralizing potencies against elapid venoms can be produced. Such a PDAV is analogous to the anti-rabies and

anti-tetanus antitoxins that are produced for use worldwide. Universal PDAVs can be produced in large volume which, with the economy of scale, should be more affordable to poor snakebite victims in many parts of the developing world and save numerous lives before 'new generation' antivenoms become available.

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The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Snakebite Envenoming Diagnosis and Diagnostics

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Snakebite envenoming is predominantly an occupational disease of the rural tropics, causing death or permanent disability to hundreds of thousands of victims annually. The diagnosis of snakebite envenoming is commonly based on a combination of patient history and a syndromic approach. However, the availability of auxiliary diagnostic tests at the disposal of the clinicians vary from country to country, and the level of experience within snakebite diagnosis and intervention may be quite different for clinicians from different hospitals. As such, achieving timely diagnosis, and thus treatment, is a challenge faced by treating personnel around the globe. For years, much effort has gone into developing novel diagnostics to support diagnosis of snakebite victims, especially in rural areas of the tropics. Gaining access to affordable and rapid diagnostics could potentially facilitate more favorable patient outcomes due to early and appropriate treatment. This review aims to highlight regional differences in epidemiology and clinical snakebite management on a global scale, including an overview of the past and ongoing research efforts within snakebite diagnostics. Finally, the review is rounded off with a discussion on design considerations and potential benefits of novel snakebite diagnostics.

Keywords: envenoming, clinical toxinology, diagnosis, diagnostics, ophidism, snakebite management, syndromic approach

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INTRODUCTION

Every year, people lose their livelihoods, limbs, and lives to a disease that is as neglected as it is ancient: Snakebite envenoming. The exact burden of snakebite envenoming is notoriously difficult to assess, because data on envenoming prevalence are scarce, and the available data points are often inaccurate or not representative for broader geographical areas (1–4). Nonetheless, studies suggest that mortality due to snakebite envenoming may exceed 125,000 deaths per year globally, while the number of people suffering permanent sequelae may be around 400,000, and the toll of associated disability-adjusted life years might add up to a total of over 6 million (4–8). To make matters worse, snakebite envenoming is both a disease mainly affecting the poor and a disease that leads to further impoverishment (7, 9–12). In spite of the immense burden of snakebite envenoming on victims, their families, and local communities, the disease remains largely neglected and has historically only received few resources, and limited efforts have gone into the development of better treatments and diagnostics (13).

Once diagnosed, the mainstay treatment of severe envenoming is antivenom in combination with auxiliary treatment (7, 14, 15). Monovalent antivenoms can be used when the species of the offending snake is known, while polyvalent antivenoms are useful in cases where the snake species has not been identified. However, the ability of polyvalent antivenoms to neutralize a broad range of venoms might come at the cost of decreased efficacy, as the relative proportion of antibodies in a polyvalent antivenom that targets toxins of a specific snake venom is often not as great as the proportion of antibodies in a monovalent antivenom targeting the same venom toxins. Therefore, it can become necessary to administer a greater dose of a polyvalent antivenom than of a monovalent antivenom in order to treat a given envenoming (7, 16). Increasing the dose can, in turn, affect the cost of treatment and the risk of developing adverse reactions to the antivenom (7). Unfortunately, polyvalent antivenoms are favored in many places either due to the lack of monovalent antivenoms or due to the difficulty in choosing which monovalent antivenom to administer in the absence of reliable information on the perpetrating snake species (17).

In addition to enabling the administration of monovalent antivenoms, where available, identifying the offending snake species or the type of venom might enable clinicians to predict and prepare for the development of clinical manifestations. To aid clinicians in this task, there is a common and deceptively simple categorization of elapid venoms as being primarily neurotoxic and viperid venoms as being primarily cytotoxic and/or hemotoxic (here understood as toxicity directed toward blood and the cardiovascular system, including hemostasis) (7). While this simplification does represent a general trend, it can cause clinical misinterpretation and there are several important exceptions to the rule. For example, many major Australian elapid snakes commonly cause coagulopathy, often without evident neurotoxicity, while some important viperids cause minimal cytotoxicity, yet important neurotoxicity (18). Similarly, bites from some elapid species of the cobra genus, particularly spitting cobras, can cause strong cytotoxic symptoms without causing neurotoxicity (19), which may be confused with viper envenomings. Matters are further complicated by the fact that snake venom composition can vary within genera and even within species due to ontogeny and geographical distribution (20–23). As such, the variability of clinical manifestations of envenoming and the time courses of their development complicate the treatment of snakebite (7). Thus, while snakebite is generally well-handled in many areas, room for innovation and improvement still exists. Encouragingly, recent years have seen a renewed interest in such innovations and improvements, with much research being published not only on novel treatment modalities (e.g. recombinant antivenoms and small molecule inhibitors) but also on novel diagnostics (e.g. enzyme-linked immunosorbent assays, lateral flow assays, impedimetric immunoassays, infrared imaging, and polymerase chain reaction-based methods) [see **Table 1** and (104–108)]. In time, some of these diagnostic tools may enter the clinic, where they could be utilized to obtain valuable information, such as the identity of the perpetrating snake species or genus, allowing use of monovalent antivenoms, or quantitative measures of the degree of envenoming. Additionally, if implemented in rural settings, diagnostic kits may guide treatment decision for less experienced clinicians, enabling proper management of snakebite victims at rural facilities. This information might support clinical management of snakebite envenoming and epidemiological studies of relevance to antivenom development, resource management, and advocacy for increased attention to snakebite.

COMMONLY ADOPTED APPROACHES FOR DIAGNOSIS OF SNAKEBITE ENVENOMING

A basic diagnosis of snakebite envenoming requires a thorough patient history, targeted examination, and appropriate laboratory investigations (109). Taking a detailed history includes asking about the circumstances of the bite (e.g. geography, time of the incident, activity, and number of bites), details of the snake (if seen, brought, or photographed), clinical manifestations of envenoming (including time of onset), first aid applied, and past medical history (e.g. allergies, prior snakebites, relevant medications, and pre-existing medical conditions) (109). Laboratory investigations almost always include an evaluation of the blood clotting profile to screen for venom-induced coagulopathies. In its simplest form, a blood clotting test can be carried out in the form of a 20 minute whole blood clotting test (20WBCT). If more sophisticated equipment is available, it is common to run repeated tests of the International Normalized Ratio (INR) of blood clotting, activated partial thromboplastine time (aPTT), D-dimer, and/or fibrinogen degradation products (FDP), supplemented by hemograms and potentially also by electrocardiograms. Acute falls in hemoglobin and hematocrit values may indicate internal bleeding, and a drop in fibrinogen levels might be indicative of coagulopathy (7, 110, 111). Blood samples are usually also screened for creatine kinase (CK) levels, electrolytes, urea, nitrogen/creatinine, which together with urinalysis (hematuria, proteinuria, urea levels, and urine output) can be used to assess venom-induced rhabdomyolysis and associated complications, such as myoglobinuric renal failure or polyuria, oliguria, or anuria (110, 111). See **Table 2** for a list of these laboratory investigations. Based on the patient history and laboratory tests, trained toxinologists may be able to infer the offending snake species, and this can in turn guide the choice of treatment.

Diagnostic algorithms summarize much of the knowledge required to diagnose snakebites. They have been developed for some settings and regions to provide support for doctors and other healthcare workers tasked with frontline management of patients with suspected envenoming. Here, it is acknowledged that frontline staffs will often have limited training in managing envenoming and limited rapid access to clinical toxinology expertise to guide their important treatment decisions in the

TABLE 1 | Overview of snakebite diagnostics capable of differentiating snake venoms.

Type	Subtype	Abs	Area	Snake(s) targeted	LoD	Assay duration	Tested on patient samples?	Sample matrix	Notes	Year	Ref.
Immuno-assay	Immuno-diffusion	Equine		Cobra spp. (possibly *O. hannah*)	1:100,000,000 dilution		Patients: 1 (case study)	Tissue homogenate		1957	(24)
	Immuno-diffusion	?	Australia	*A. antarcticus*, *N. scutatus*, *P. porphyriacus*, *P. textilis*	?	>3 hours	?	Serum exudate (guineapig)	Only abstract available	1967	(25)
	Agglutination test	Caprine & equine	California	*C. v. helleri*		>2 hours	Patients: 16	Serum		1968	(26)
	Immuno-diffusion	Leporine	Africa	*B. arietans*, *C. maculatus*, *E. carinatus*, *N. haje*, *N. melanoleuca*, *N. nigricollis*		48 hours (immuno-difussion) & >1 hour (immuno-electrophoresis)	Patients: 101	Wound aspirates, blister fluids, sera, urine	Sensitivity: 39.6% (40/101)	1974	(27)
	RIA		Australia	*N. scutatus*, *P. textilis*	<10 ng	>24 hours	Patients: 2 (also tested on rabbit serum)	Serum, sample buffer		1974	(28)
	RIA		Australia	*N. scutatus*, *P. textilis*		>24 hours	Patients: 3	Tissue samples, fluids		1975	(29)
	ELISA	Leporine & equine IgGs	Multiple	*B. arietans*, *C. maculatus*, *E. carinatus*	1-5 ng/mL	O/N incubation	No – Tested in rodents	Serum (human and rat)	Cross-reactivity to the following species tested: *A. rhodostoma, B. gabonica, C. adamanteus, E. schistosa, N. haje, N. naja, N. nigricollis, O. scutellatus, V. berus*	1977	(30)
	RIA	Leporine	Australia	*N. scutatus*		O/N incubation	Patients: 3	Urine, serum, clothing, tissue samples		1977	(31, 32)
	RIA	Leporine IgG	Australia	*A. antarcticus*, *A. superba*, *N. scutatus*, *O. scutellatus*, *P. australis*, *P. porphyriacus*, *P. textilis*	0.1-0.4 ng/mL	>20 hours	Unpublished	Tissue samples		1978	(33)
	ELISA	Leporine IgG		?	0.5-2 ng/mL	30-90 minutes	?	?	Only abstract available	1980	(34)
	Enzyme immunoassay	Leporine IgG	Australia	*Acantophis* spp., *Notechis* spp., *Oxyuranus* spp., *Pseudoechis* spp., *Pseudonaja* spp.	5-15 ng/mL	20-40 minutes	Patients: 43	Whole blood, urine, wound swab		1982	(35)
	ELISA	?	?	?	1 ng/mL	>3 hours	No - Tested in rabbits	Blood, urine, exudate	Only abstract available	1983	(36)
	ELISA	Leporine IgG	?	*A. rhodostoma*, *N. naja*	7.8-15.6 ng/mL	35-45 minutes	Yes	Serum	Only abstract available	1983	(37)

(Continued)

TABLE 1 | Continued

Type	Subtype	Abs	Area	Snake(s) targeted	LoD	Assay duration	Tested on patient samples?	Sample matrix	Notes	Year	Ref.
	ELISA	Leporine IgG	USA	*A. contortrix*, *C. atrox*, *C. scutulatus*	0.1-.01 µg/mL	O/N incubation	No - Tested in animals	Sero-sanguineous fluid, blood, urine, peritoneal fluid, pleural fluid, lung, kidney	Cross-reactions with venoms of other snakes were extensive at higher concentrations	1984	(38)
	ELISA	Equine	Myanmar	*D. russelii*	10 ng/mL	O/N incubation	Yes	Serum	No cross-reactivity found to *B. fasciatus, N. naja, O. hannah, T. gramineus*	1984	(39)
	ELISA	Leporine	Thailand	*N. kaouthia*	2 ng/well		Yes	Serum		1986	(40)
	Enzyme-linked coagulation assay	Murine monoclonal IgG		*D. russelii*	2-10 pg/mL		No			1987	(41)
	ELISA		Nigeria	*B. arietans*, *C. maculatus*, *E. carinatus*		O/N incubation	Patients: 31	Blood, serum, urine, sputum, bite site aspirates		1987	(42)
	ELISA		Philippines	*N. n. philippinensis*		O/N incubation	Patients: 1 (postmortem)	Blood		1987	(43)
	ELISA	Leporine IgG	Thailand	*C. rhodostoma*, *D. russelii*, *N. kaouthia*, *T. albolabris*	10-20 ng/mL	O/N incubation	Patients: 251	Serum		1987	(44)
	RIA	Murine monoclonal Abs	Thailand	*D. russelii*	4-20 ng/mL depending on matrix	O/N incubation	Patients: 4	Serum, urine	Known to cross-react with cobra venom.	1987	(45)
	ELISA	Equine	Europe	*V. ammodytes*	<1 ng/mL	<20 minutes	No - Tested in rabbits	Blood	Specificity mentioned as being a problem.	1988	(46)
	ELISA	?	Asia	*A. b. blomhoffii*	5.4 ng/well	?	No - Tested in mice	Serum	Only abstract available. No cross-reactivity to *R. t. tigrinus* venom.	1988	(47)
	ELISA	Leporine IgG	Brazil	*B. jararaca*	14.6 ng/mL	O/N incubation	No – Tested in mice	Serum	Tested for cross-reactivity to *Bothrops* spp., *Crotalus* spp., *Lachesis* spp., and *Tityus serrulatus* venom.	1990	(48)
	ELISA	?	?	*A. b. blomhoffii*	?	?	No - Tested in rats & rabbits	Serum	Only abstract available	1990	(49)
	Agglutination test	Leporine IgG	Thailand	*B. fasciatus*, *C. rhodostoma*, *D. russelii*, *N. kaouthia*, *N. n. siamensis*, *O. hannah*, *T. albolabris*	0.16-1.2 µg/mL	40 minutes	Serum samples: 59 Wound swabs: 26	Serum, wound swabs	Sensitivity of 52.5%. Tested for hook effect and interference from sample matrices.	1991	(50)
	ELISA	Equine F $(ab')_2$	Brazil	*C. d. terrificus*	1-3 pg/mL	O/N incubation	No – Tested in mice	Serum (mice), sample buffer		1991	(51)
	ELISA	Leporine IgG	Myanmar	*D. russelii*	10 ng/mL	O/N incubation	Patients: 311 Controls: 118	Serum	Specificity 88% (14 false positives from 118 negatives). Tested for	1991	(52)

(Continued)

TABLE 1 | Continued

Type	Subtype	Abs	Area	Snake(s) targeted	LoD	Assay duration	Tested on patient samples?	Sample matrix	Notes	Year	Ref.
									cross-reactivity to *B. fasciatus*, *N. kaouthia*, *O. hannah*, *T. erythrurus*.		
	ELISA	Equine F $(ab')_2$	Europe	*Vipera* spp.	2-7 ng/mL (depending on sample matrix)	>4.5 hours	Yes	Blood, serum, urine	Tested for cross-reactivity to *B. jararaca* and *C. d. terrificus*.	1992	(53–56)
	ELISA	Leporine IgG	Australia	*A. antarcticus*, *N. scutatus*, *O. scutellatus*, *P. australis*, *P. textilis.*	2.5 ng/mL	O/N incubation	Unpublished	Sample buffer		1992	(57)
		Leporine IgG	Southern Thailand	*C. rhodostoma*	5 ng/mL	50 minutes	No	Sample buffer	Cross-reactivity to 26 venoms tested.	1992	(58)
	Agglutination test	Leporine IgG	Thailand	*B. fasciatus*, *C. rhodostoma*, *D. russellii*, *N. kaouthia*, *O. hannah*, *T. albolabris*	2-635 ng/mL	60-120 minutes	Serum samples: 59 Wound swabs: 26	Serum, wound swab	Sensitivity of 81.3% for serum samples and 61.5% for wound swabs. Cross reactivity at concentrations at least 62 times higher.	1993	(59)
	ELISA	Leporine IgG	Brazil	*B. alternatus*, *B. atrox*, *B. jararaca*, *B.jararacussu*, *B. moojeni*, *B. neuwedi*, *C. d. terrificus*, *C. d. collineatus*, *L. muta*	<0.01-0.1 µg/mL	O/N incubation	No	Sample buffer, serum (non-envenomed humans)		1993	(60)
	ELISA	Leporine IgG	Brazil	*B. atrox*, *L. m. muta*	20 ng/mL	2 hours	Yes	Serum	Also tested in mice.	1993	(61)
	Fluorogenic ELISA	?		*D. russelii*	0.1 pg/mL	?	?	?	Only abstract available. Shown to cross-react with several other venoms.	1993	(62)
	ELISA		Papua New Guinea	*P. papuanus*		O/N incubation	Patients: 9	Serum, urine, wound aspirates		1994	(63)
	ELISA	Leporine IgG	Tunisia	*E. pyramidum*	<10 ng/mL	O/N	Yes	Serum		1994	(64)
	ELISA		North America	*Agkistrodon* spp.	2 ng/mL	>5 hours	No – Tested in rabbits	Serum		1994	(65)
	ELISA		Myanmar	*O. hannah*	<20 ng/mL		Patients: 2 (case studies)	Serum		1995	(66)
	ELISA & RIA	Ovine Fab	Europe	*Vipera* spp.	0.8 ng/mL (ELISA) & 2 ng/mL (RIA)	>3 hours (ELISA) & O/N incubation (RIA)	Yes	Plasma, urine		1996	(67)
	ELISA	Leporine F $(ab')_2$	India	*B. caerulus*, *D. russelii*,	1 ng/mL	30 min	Patients: 27	Blood, serum, urine, wound swab	Only abstract available	1996	(68)

(Continued)

TABLE 1 | Continued

Type	Subtype	Abs	Area	Snake(s) targeted	LoD	Assay duration	Tested on patient samples?	Sample matrix	Notes	Year	Ref.
				E. carinatus, *N. naja*							
	ELISA	Equine F $(ab')_2$	Martinique	*B. lanceolatus*		3 hours	Patients: 40 Controls: 120	Serum	Sensitivity 46%, specificity 88%.	1997	(69)
	ELISA & agglutination assay	Equine	Central America	*Micrurus* spp.	0.3 mg/mL (agglutination assay) & 4 ng/mL (ELISA)	>5 minutes (agglutination test) & O/N incubation (ELISA)	No – Tested in rabbits and mice	Serum, plasma		1997	(70)
	ELISA	Caprine & leporine IgG	India	*Bungarus* spp., *Daboia* spp., *Echis* spp., *Naja* spp.	0.1 ng/mL	>5 hours	Yes (postmortem only)	Tissue samples		1999	(71)
	ELISA	?	Taiwan	Cobra spp.	0.5 ng/mL	6 hours	?	Calf serum and human urine	Only abstract available	2002	(72)
	Optical immunoassay	Leporine IgG	Asia	*B. multicinctus*	2.5-10 ng/mL	25 minutes	No – Tested in mice	Blood, tissue samples	Cross-reactivity to 11 venoms and toxins tested.	2002	(73)
	Agglutination test	Equine	Venezuela	*Bothrops* spp., *Crotalus* spp.	167 µg/mL	10 minutes	No	Sample buffer	LoD unit uncertain.	2004	(74)
	Optical immunoassay	Leporine IgG	Vietnam	*C. rhodostoma*, *N. kaouthia*, *O. hannah*, *T. albolabris*	0.2-0.8 ng/mL depending on the venom and sample matrix	40 minutes	Patients: 83 Samples: 125	Serum, urine, wound exudate		2004	(75)
	ELISA	Avian IgY & leporine IgG	India	*N. naja*	0.1-300 ng	O/N incubation	Patients: 12 (live) Patients: 7 (postmortem)	Skin, blood, cerebrospinal fluid		2006	(76)
	ELISA	Leporine IgG	India	*B. caeruleus*, *N. naja*		O/N incubation	Samples: 22 (postmortem)	Skin, blood		2007	(77)
	Immuno-flourescence			*N. kaouthia*	5–10 ng/mL	3 hours	No	Sample buffer		2008	(78)
	ELISA	Leporine IgG	Australasia	*Oxyuranus* spp.	0.15 ng/mL	O/N incubation	Patients: 17	Serum	Also tested in rat serum and for cross-reactivity with Australian snake venoms.	2010	(79)
	ELISA	Leporine IgG	Colombia	*L. acrochorda*	3.9 ng/mL		No	Sample buffer	Specificity 100%.	2012	(80)
	ELISA	Leporine IgG	Egypt	*N. haje*, *N. nigricollis*, *W. aegyptia*	<10 ng/well	O/N incubation	No	Sample buffer	Avidities of 2.5-2.8 depending on the venom	2013	(81)
	LFA	Avian	Taiwan	*N. atra*	5 ng/mL	20 minutes	Patients: 88 (34 cobra and 54 non-cobra)	Serum	Sensitivity 83.3%, specificity 100%.	2014	(82)
	LFA	Equine & leporine IgG	India	*Daboia* spp., *Naja* spp.	0.1 ng/mL	10 minutes	No - Tested in mice	Plasma		2016	(83)
	ELISA	Leporine IgG	India	*Bungarus* spp., *Daboia* spp.,	1 ng/mL	20-25 minutes	No - Tested in mice	Sample buffer		2017	(84)

(Continued)

TABLE 1 | Continued

Type	Subtype	Abs	Area	Snake(s) targeted	LoD	Assay duration	Tested on patient samples?	Sample matrix	Notes	Year	Ref.
				Echis spp., *Naja* spp.							
	ELISA & LFA	Equine	Taiwan	Neurotoxic vs hemorrhagic venom	LoD of 5-50 ng/mL (LFA) & LoQ of 0.39-0.78 ng/mL (ELISA)	10-15 minutes	Patients: 21	Serum	Sensitivity and specificity of 100% for neurotoxic venoms. Sensitivity of 36.4% for hemorrhagic venoms.	2018	(85)
	Impedimetric immunoassay	Equine	Brazil	*Bothrops* spp.	0.27 ug/mL	>25 minutes?	No	Sample buffer	Tested for cross-reactivity to *C. d. terrificus* and *M. leminiscatus*.	2018	(86)
	ELISA	Leporine	Sri Lanka	*B. caeruleus*, *D. russelii*, *H. hypnale*, *N. naja*	0.19-1.56 ng/mL (depending on the venom)	>2 hours	Patients: 19 Controls: 20	Serum	Quantitative. Cross-reactivity between the venoms was tested.	2020	(87)
	LFA	Avian & equine	South-East Asia	*Daboia* spp., *Naja* spp.	10 ng/mL (*in vitro*)	25-30 minutes	Samples: 5	Serum		2020	(88)
	LFA	Leporine & equine	Asia & Africa	*Naja* spp.	5-10 ng/mL for Asian cobras and <500 ng/mL for African cobras	>20 minutes	No	Serum (fetal bovine)	Based on (82).	2020	(89)
Molecular biology	PCR	N/A	Thailand	*N. kaouthia*		>2 hours	No - Tested on mice	Wound swabs (mice)	Also tested on venom from *B. fasciatus, C. rhadostma, D. russelii, O. Hannah.*	2001	(90)
	PCR	N/A	Thailand	*B. fasciatus*, *C. rhodostoma*, *D. siamensis*, *Hydrophiinae* spp., *Naja* spp., *O. Hannah*, *Trimeresurus* spp.	0.025 ng/mL	>67 minutes	No	Saliva (snake)		2015	(91)
	PCR	N/A	Nepal	*Bungarus* spp., *Naja* spp., *O. hannah*, *O. moticola*, *Trimeresurus* spp.		O/N incubation	Patients: 565	Wound swab	Specificity 100%.	2016	(92)
Misc.	Enzymatic activity assay	N/A	Sri Lanka & Australia	*B. caeruleus*, *D. russelii*, *H. hypnale*, *N. naja*, *P. porphyriacus*			Patients: 108	Serum		2014	(93)
	Infrared thermography	N/A	Brazil	*B. moojeni*, *C. d. terrificus*, *B. jararaca*		>15 minutes	Patients: 8			2017	(94)
	Enzymatic activity assay & ELISA	N/A	Australia	Elapid spp.	0.1-0.2 ng/mL (ELISA)		Patients: 115 Controls: 80	Serum		2018	(95)

Many studies did not report on the duration of the diagnostic procedure. In such cases, assay duration was reported in this table as "> total incubation time", e.g. ">3 hours" for an assay with an incubation time of 3 hours. For other unreported values, the corresponding fields were left empty. In some cases, only the abstracts of the studies were available to us, and in these cases, values not reported in the abstract have been marked "?". Studies describing the detection of venom-specific antibodies in snakebite victims [e.g. (96–98)] or the detection of toxins or toxin activities for the purpose of venom characterization rather than diagnosis [e.g. (99–103)] were not included in this table. Abs, antibodies; ELISA, enzyme-linked immunosorbent assay; F(ab')$_2$, fragment antigen binding 2; IgG, immunoglobulin G; LFA, lateral flow assay; O/N, overnight; PCR, polymerase chain reaction; RIA, radioimmunoassay.

TABLE 2 | Examples of auxilliary tests that are frequently performed for suspected snakebite victims.

Auxilliary tests	
Type	**Subtype**
Hemograms	Platelet count
	Blood count (hemoglobin, white cell count, absolute lymphocyte count)
	Examination of blood film for evidence of intravascular hemolysis (schistocytes, spherocytes, etc.)
Clotting profile	Fibrinogen level
	Prothrombin time/INR of blood clotting
	Activated partial thromboplastine time (aPTT)
	D-dimer/fibrinogen degradation products (FDP)
Serum biochemistry	Electrolytes
	Bilirubin
	Liver function tests
	Creatine kinase (CK; CPK)
Urinalysis	Hematuria
	Myoglobinuria
Renal function	Serum creatinine
	Urea
	Glomerular filtration rate
	Urine output (polyuria, oliguria, anuria)
Electrocardiagram	

critical early hours after a bite. The purpose of such diagnostic algorithms is to synthesize and distill the knowledge and experience of experts in clinical toxinology into a readily and rapidly accessible format to guide less experienced health professionals toward optimal care of bitten and envenomed patients. Formally assessing the effectiveness of diagnostic and treatment algorithms for envenoming is a challenge, with no clear published research available. However, experience in countries such as Australia (first world) and Myanmar (developing world) appears to indicate that diagnostic algorithms developed for snakebite, individualized for each country or region, are both widely used and accepted. In Myanmar, snakebite diagnostic algorithms were developed by a team of health professionals through a series of drafts, tested by frontline healthcare workers, and a final version was adopted and rolled out nationally by the Ministry of Health. Feedback from frontline healthcare workers in Myanmar was strongly positive. Diagnostic algorithms do not replace expertise in clinical toxinology, but can be an important part of an optimal care pathway. However, it must always be acknowledged in such algorithms that they are merely a guide and cannot cater for every possible clinical scenario and presentation.

As previously mentioned, it is also a common procedure to ask whether the patient saw the snake, and if so, what it looked like (112). However, using the victim's description of the snake is often not, in isolation, a reliable diagnostic method for identifying the snake, although for some snakes in some countries (e.g. Russell's viper in Myanmar) it may be reasonably reliable. In some cases, the biting snake is not seen, and even if it has been spotted, the victim's description can potentially be misleading (113). While it may be easier to identify the snake if it is brought to the hospital for identification, even in these cases, misidentification can occur, and in some communities, there is an unwillingness among hospital staff to inspect or handle the snake (114). As an example, hump-nosed pit vipers (*H. hypnale*) are often misidentified as saw-scaled vipers (*E. carinatus*) in India, resulting in administration of ineffective antivenom (115). Even if the snake has been positively identified by an expert herpetologist, the clinical presentation of the patient is pivotal, as different specimens of venomous snakes (e.g. from different regions) can cause different clinical envenoming syndromes (109, 116). Another caveat of this approach is the inherent risk of attempting to capture or kill the snake, which can lead to further envenoming of the victim or a helper attempting to catch the snake; however, if a snake has already been killed, this is a potentially valuable diagnostic aid. Hospital staff should be encouraged to examine and preserve all such presented snakes, as this can allow retrospective studies clearly defining medically relevant species for a particular region. In most settings, 70% ethanol is an appropriate preservative for dead snakes, immersing the entire snake and injecting the preserving fluid into the body cavity. It is critically important that preserved snakes are adequately labeled and that preservation methods that will not lead to deterioration of the label (in the preserving fluid) are employed, so that subsequent examination can unequivocally link the dead snake to a particular snakebite patient.

SNAKEBITE DIAGNOSIS IN AUSTRALIA

In Australia, snakebites are diagnosed based on patient history and laboratory investigations, as described above. In combination with neurological assessment, it is possible to identify most severe envenoming cases within 12 hours of the bite (117), after which patients with confirmed or suspected envenoming can be discharged if clinical findings and laboratory test results indicate no envenoming has occurred. In rare cases, envenoming – in particular by death adders – does not manifest itself before 24 hours post-bite, though it is unclear whether such late-presenting envenomings can progress to severe or life-threatening envenomings in subsequent hours.

Precise epidemiological data on snakebites in Australia are not available; however, one estimate suggests between 500 and 3,000 snakebites occur annually (118), while another study reported 6,123 hospital admissions due to contact with venomous snakes in a period from August 2001 to May 2013, or an average of about 500 cases per year (119). Snakebite envenoming in Australia is not common, but can be severe with an average of 2.2 deaths annually in the past 15 years with out-of-hospital cardiac arrest being the most common cause (120, 121). Most medically significant snakebites can be attributed to five terrestrial snake groups: Brown snakes (*Pseudonaja* spp.), members of the tiger snake group (*Notechis scutatus, Tropidechis carinatus, Austrelaps* spp., and *Hoplocephalus* spp.), black snakes and mulga snakes (*Pseudechis* spp.), taipans (*Oxyuranus* spp.), and death adders (*Acanthophis* spp.) (18, 109, 121). Some of these snakes can easily be confused by people without experience in identifying snakes. For example, a snakebite victim may report to have been bitten by "a brown snake", which could belong to any number of

species, e.g. the eastern brown snake (*Pseudonaja textilis*) or the king brown snake (*Pseudoechis australis*) (see **Figures 1A, B**), the clinical significance and treatment of which would differ. Diagnostic algorithms can aid clinicians in determining the snake species most likely to have caused the bite, as exemplified in **Figure 2** (109, 116).

Major local effects, such as hemorrhagic blebs and necrosis after snakebites, are rare in Australia and minimal for the brown snakes that cause most cases of snakebite. Nonetheless, some species may cause at least moderate local swelling, and local bruising can uncommonly occur following bites by those species causing defibrination coagulopathy (116). Systemic effects vary depending on species and may include neurotoxic flaccid descending paralysis, systemic myolysis, defibrination coagulopathy, anticoagulant coagulopathy, acute kidney injury (AKI), sudden collapse, cardiac collapse/arrest, anaphylaxis, and microangiopathic hemolytic anemia (MAHA) (109, 116). Death adders, taipans, tiger snakes, and the rough scaled snake commonly cause neurotoxicity; however, tiger snakes, the rough scaled snake, and taipans can also cause myotoxicity. Black snakes and mulga snakes cause myotoxicity and anticoagulant coagulopathy, while defibrination coagulopathy (referred to by some authors as "venom-induced consumption coagulopathy", "VICC") is frequent for brown snakes, tiger snakes, rough scaled snake, broad headed snakes (*Hoplocephalus* spp.), and taipans (116). Defibrination coagulopathy can be diagnosed based on an elevated INR of blood clotting and aPTT and grossly elevated D-dimer; the latter may be the first evidence of developing coagulopathy, before any changes in INR of blood clotting and aPTT occur (109). In case of anticoagulant coagulopathy, fibrinogen and degradation products are at normal levels, and aPTT and possibly INR of blood clotting can be prolonged/elevated, whereas defibrination coagulopathy leads to decreased or undetectable levels of fibrinogen and elevated levels of degradation products, both D-dimer and FDP (109, 116). Typically, symptoms of coagulopathy are seen early, sometimes upon arrival to the emergency department, while neurotoxicity and myotoxicity take hours to develop with CK levels peaking between 24-48 hours after the bite (116).

If severe envenoming is diagnosed, diagnostic algorithms in conjunction with the Seqirus (formerly Commonwealth Serum Labs, CSL) Snake Venom Detection Kit (SVDK) can help determine which snake venom immunotype is involved (109). The SVDK is a non-laboratory, rapid, freeze-dried, immunoassay kit, developed for Australian and some Papua New Guinea snake venoms, that uses bite site swabs, or alternatively a urine sample, to detect the venom immunotype. The SVDK is widely distributed and available in Australia, but its usage has declined, in part because of concerns over accuracy and reliability. The reliability of the SVDK is debated due to a high risk of false positives when the SVDK is inappropriately tested on non-envenomed patients, the occurrence of false negatives with envenomed patients (121), and the presence of a hook effect (also known as prozone effect – an effect which describes how the measured analyte concentration can decrease even as the actual concentration increases) (122). The propensity for false positives has proven especially problematic, as the SVDK has frequently been used inappropriately for all suspected snakebites, sometimes as a screening tool – a function that it was not designed for and is not suitable for. One study suggests that false negatives are often a result of operator errors (123), and perhaps for this reason, there is now an annual quality assurance process for all laboratories using the SVDK to minimize the likelihood of operator errors. Antivenom is available at 750 hospitals across Australia, and if an immunotype can be determined *via* the SVDK or otherwise, the appropriate "monovalent" antivenom can be selected as treatment (109). In addition to the five terrestrial "monovalent" antivenoms, a polyvalent antivenom against the five snake groups is also available (121). If the diagnostic algorithms and the SVDK results are in conflict, then either polyvalent antivenom or an appropriate mixture of two "monovalent" antivenoms should be used, but the large volume of antivenom needed, particularly if using polyvalent, represents a potential increased risk of adverse reactions (7, 109). If a clinician is in doubt when handling suspected or confirmed snakebite cases, then advice may be sought *via* the antivenom producer (Seqirus, Melbourne) or through the Clinical Toxinology service (Women's & Children's Hospital, Adelaide) (109).

From 2005 to 2015, the median dose of antivenom administered to Australian snakebite victims has decreased from four vials to one vial, with debated implications for treatment (121, 124). Meanwhile, the median time to first antivenom administration has remained unchanged at 4.3 hours (121), despite increasing evidence of a more favorable outcome when antivenom is administered early (125, 126). This lack of change in time to antivenom administration might be because Australia covers a large landmass, with many areas being remote from major health services, making delays in treating snakebites more likely to occur, particularly in remote sites, where antivenom is not stocked and aeromedical retrieval is required. Competing demands on aeromedical retrieval services can exacerbate delays. Additionally, many dangerously venomous snakes in Australia only cause envenoming in a minority of cases. As prophylactive antivenom administration can negatively affect the patient, antivenom should not be administered until it is certain that the patient has been envenomed; this can necessitate further delays in antivenom administration as symptom development is monitored. For these reasons, it seems unlikely that the time to antivenom administration will improve significantly in Australia.

SNAKEBITE DIAGNOSIS IN ASIA

The epidemiology of snakebite envenoming differs across Asia as a result of high inter- and intra-species diversity and varying population density of venomous snakes. The impact of snakebite is relatively high in many countries in South- and Southeast Asia, where the overall estimated mortality rate is 1.05 to 5.42 deaths per 100,000 people (4). This includes the Philippines, Thailand,

FIGURE 1 | Comparison of venomous snakes with similar names and/or appearance and/or clinical syndromes. Visual comparison of two Australian snakes with similar names and appearances: **(A)** A king brown snake (*Pseudonaja textilis*), which belongs to the black snake genus, and **(B)** an eastern brown snake (*Pseudonaja textilis*), which belongs to the brown snake genus. Visual comparison of two venomous snake species from Brazil: **(C)** *Bothrops* sp. and **(D)** *Lachesis* sp. Species from these genera can appear similar to those not trained in snake identification, can cause similar clinical manifestations, and are both locally known as 'surucucu' in certain parts of Brazil. Visual comparison of **(E)** a puff adder (*Bitis arietans*) and **(F)** a horned viper (*Cerastes cerastes*), the venoms of which can cause similar clinical manifestations. **Figures 1A, C, D** copyright © Prof. Julian White, **Figure 1B** copyright © of Prof. Sean Bush, **1E, 1F** were found on WikiMedia Commons are are copyright © of the user 4028mdk09 and the user Broobas, respectively.

Vietnam, Laos, Cambodia, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka, and India, where envenomings are predominantly incurred from the following snakes: Cobras (*Naja* spp.), kraits (*Bungarus* spp.), Russell's vipers (*Daboia* spp.), saw-scaled vipers (*Echis* spp.), Malayan pit viper (*Calloselasma rhodostoma*), hump-nosed pit vipers (*Hypnale* spp.), and green pit vipers (*Trimeresurus* spp.) (114, 127–129). In Japan, Korea, Hong Kong, Taiwan, and Indonesia, most envenomings are caused

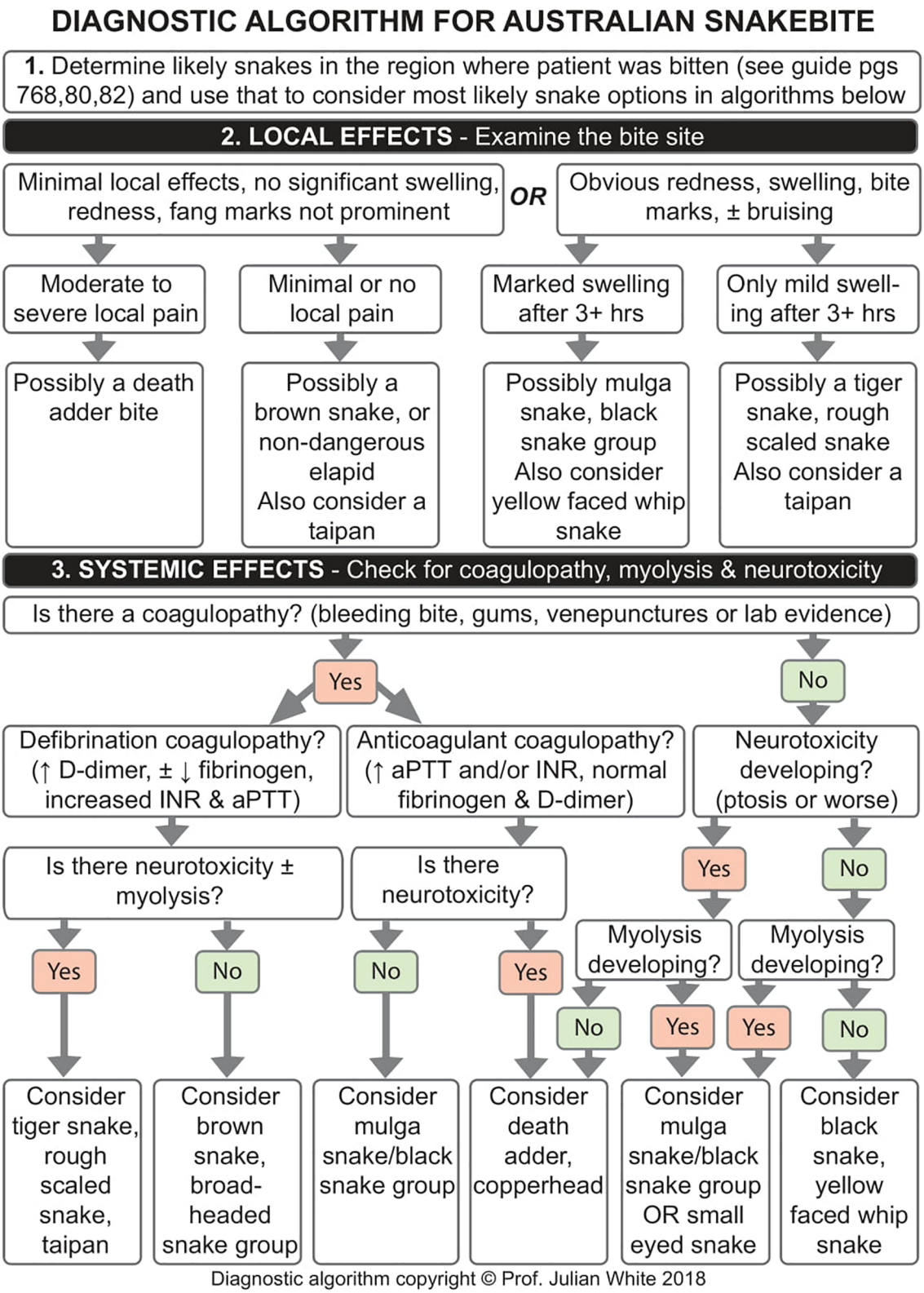

FIGURE 2 | Diagnostic algorithm for snakebite envenoming in South Australia. Algorithm copyright © Prof. Julian White.

by pit vipers (subfamily: *Crotalinae*), which might be associated with lower mortality rates (130–134). Data on the epidemiology of snakebite in Central-, West-, and North Asia, including Russia and the Middle East, are limited, but estimates suggest that the rates are low compared to the subtropical and tropical regions of South- and Southeast Asia (4). Similarly, data on the epidemiology in China are limited, with one study suggesting that mortality rates in East Asia, including China, range from 0.033 to 0.347 per 100,000 people (4).

The majority of physicians in South- and Southeast Asia rely on the circumstances of the bite and clinical manifestations to diagnose the victim (135). Syndromic diagnostic tools and algorithms are available for Southeast Asia in general (SEARO guide (136)) and for some countries in particular (e.g. Myanmar (9, 137, 138)). Similar to Australia, a thorough patient history can be helpful in identifying the type of snake involved in the accident. E.g. if a victim has been bitten in a house during the night and has developed paralysis, the culprit is more likely to be a krait (*Bungarus* spp.), while a bite sustained from a venomous snake in a tree might suggest a green pit viper (*Trimeresurus* spp.) (135, 139, 140). Systemic signs of envenoming can also be helpful in clinical practice, as the venom of most species in South- and Southeast Asia are mainly toxic to either neuromuscular or hemostatic systems. Neurotoxicity is often related to bites by cobras, king cobras, and kraits, while hemotoxicity usually indicates envenoming by a true viper (subfamily: *Viperinae*) or pit viper, although in rare cases it may indicate envenoming by a colubrid, such as a red-necked keelback (*Rhabdophis subminiatus*) or a tiger keelback (*R. tigrinus*) (139). It can sometimes be difficult to differentiate between neurotoxic envenoming by cobras and kraits based on clinical signs. However, krait bites are often associated with delayed onset and prolonged total period of paralysis, while cobra bites are often associated with significant local evidence of envenoming (136, 141). Behavioral differences of the snakes might further elucidate the matter, as krait bites primarily occur at night, while cobra bites are much more likely to occur during the day (135, 140). When documenting the clinical manifestations of envenoming, some clinicians use a standardized questionnaire based on national snakebite management guidelines to support the diagnostic process (142). A systematic syndromic approach combined with a scoring system based on clinical manifestations has been proposed to assist clinicians in identifying the offending snake species (143), but sufficient data on envenoming profiles to create such systems are lacking for many species throughout South- and Southeast Asia (137, 143).

India has more snakebites and snakebite-related deaths per year than any other country in the world (135, 144). It is home to 52 venomous snake species, out of which the Russell's viper (*D. russelii*), the common krait (*B. Caeruleus*), the Indian cobra (*N. naja*), and the saw-scaled viper (*E. Carinatus*), known as the "Big Four", are considered the most medically important. Both polyvalent and monovalent antivenoms are available in India, but they do not cover all venomous species. Furthermore, there is a need for a standardized quality control process for manufacturing of snakebite antivenoms to ensure that they are safe and effective (145). National guidelines for management of snakebites in India do exist and some states have developed their own protocols. However, in many cases, these protocols are not followed strictly, leading to misdiagnosis and inappropriate management (146). As an example, hump-nosed pit vipers (*H. hypnale*) are often misidentified as saw-scaled vipers (*E. carinatus*), resulting in administration of ineffective antivenom (141). Furthermore, some doctors and hospitals are unwilling to manage snakebite victims, causing potentially critical treatment delays (147). A diagnostic tool for identification of the offending snake species combined with a coordinated approach to ensure that healthcare workers across India have adequate knowledge, skills, and confidence to manage snakebite patients could potentially reduce this problem.

Snakebite envenoming remains an important health issue in many regions of Asia, especially throughout South- and Southeast Asia, where incidence and mortality rates are among the highest in the world. The high species diversity complicates clinical management, although this problem is alleviated somewhat by the widespread use of polyvalent antivenoms. While these polyvalent antivenoms are convenient for physicians, they may arguably be disadvantageous for overall patient outcomes, as they can be a disincentive for quality epidemiologic and clinical envenoming studies. Uncertainty about the offending snake species may result in masking of "new" envenoming syndromes, thereby hampering the inclusion of new species into antivenom immunization protocols. The continuing absence of *Hypnale* spp. from the immunizing mix for Indian polyvalent antivenoms (148–150) can be mentioned as an example of this.

SNAKEBITE DIAGNOSIS IN THE UNITED STATES AND CANADA

In the United States (US) and Canada, around 6,500 people suffer from snakebites annually, resulting in 5-6 deaths (5, 151, 152). The US has about 26 indigenous venomous snake species, where rattlesnakes (*Crotalus* spp.), moccasins (*Agkistrodon* spp.), and pygmy rattlesnakes (*Sistrurus* spp.), all of which belong to the pit viper (*Crotalinae)* subfamily, are the main genera implicated in snakebites. Coral snakes (*Micrurus* spp.) are also present in a limited southern distribution but do not account for many bites (153), with an estimated 70-80 annual cases reported to the American Association of Poison Control Centers. In Canada, rattlesnakes are the only medically relevant snake species, and with a very limited distribution, the risk of snakebite is relatively small (154).

Pit vipers are the most prolific group of snakes involved in snakebite accidents in the US and Canada; therefore, when managing a snakebite patient, it is important to keep in mind that less than 10 percent of pit viper bites are dry bites (155, 156). Pit viper venom typically contains hemotoxins causing direct or indirect lysis of fibrinogen, thrombocytopenia, and vascular endothelial damage (157, 158), thereby emphasizing the

importance of carefully monitoring the patient's blood coagulation profile through laboratory tests. Furthermore, the presence of Mojave toxin in Mojave rattlesnake (*C. scutulatus*) and southern pacific rattlesnake (*C. helleri*) venoms causes potentially severe systemic neurotoxicity, including cranial neuropathy and flaccid paralysis. Severe neurotoxic clinical manifestations, when present, are a relevant diagnostic indicator (159–161). It is recommended to perform laboratory tests of the patient every 6-8 hours and twice prior to discharging the patient in order to follow the progression of the envenoming (110).

Coral snake envenoming generally causes only mild local effects, while the systemic manifestations can include euphoria, lethargy, nausea, vomiting, excessive salivation, ptosis, dyspnea, convulsions, abnormal reflexes, and motor weakness or paralysis leading to respiratory paralysis, which is lethal in absence of clinical intervention (162–165). In case of a coral snake envenoming, serum creatine kinase activity may rise, and myoglobin may be detected in the urine (164, 166), but coagulopathy is not a feature (165–168). The observation time in the hospital depends on the severity of the envenoming, the age of patient, and the location of the bite wound, ranging from at least 8 hours to 12-24 hours for mild envenomings, where repeated laboratory evaluations are advised (110). The marked visual appearance and clinical presentation of coral snake envenomings in the US make coral snake envenomings easy to distinguish from pit viper envenomings. The genus-specific antivenom, Pfizer Antivenin, has been available for the treatment of coral snake envenoming but is currently in very short supply, resulting in rationing (162, 169), though the recent recommencement of production should alleviate this shortage.

Like several other countries, the US also has a treatment algorithm: The unified treatment algorithm, published in 2011, with the purpose of streamlining the management and diagnosis of snakebites in the US (110). However, since the algorithm was published, a new antivenom has become available, and the algorithm has not yet been updated accordingly. As Canada does not have indigenous snake species that are different from those in the US, it is likely that this algorithm is applicable to assess snakebite cases in Canada as well.

Clinicians in the US will often factor in information provided by the victim or bystanders about the identity of the snake. A study comparing the snake identifications of expert herpetologists with those of snakebite victims, witnesses, and healthcare providers in southern parts of the US found that 40% of the specimens identified as copperheads (*A. contortrix*) were actually cottonmouths (*A. piscivorous*), with juvenile snakes being particularly difficult to identify, leading to confusion (170). While other species were less frequently confused, it might be problematic that (possibly erroneous) snake identifications are used by poison control centers when recommending treatment (170). Although pit viper bites in the US are treated with polyvalent antivenom (CroFab or AnaVip) when required, misidentification of pit vipers might still negatively impact treatment. For example, AnaVip, which is based on equine $F(ab')_2$ antibodies, has proven more efficient in treatment of late onset and recurrent coagulopathy than CroFab, which is based on ovine Fab antibodies (171). This difference in efficacy versus coagulopathies might be related to the different half-lives of Fab and $F(ab')_2$ antivenoms (171). Both CroFab and AnaVip are recommended for treatment of rattlesnake envenoming in North America, but AnaVip has not received FDA-approval for treatment of bites by cottonmouths and copperheads. Conversely, CroFab works well for treatment of copperhead (*A. contortrix)* bites, by decreasing limb disability subsequent to bites (172) and being associated with fewer patients using opioids to treat pain related to the envenoming (173). It has additionally been demonstrated that early administration of CroFab for copperhead bites results in faster limb recovery than does late administration (174). Thus, in cases of copperhead envenomings, it might be especially beneficial to rapidly identify the culprit species so the optimal polyvalent antivenom can be administered early on.

SNAKEBITE DIAGNOSIS IN LATIN AMERICA

In Latin America and the Caribbean islands, 80,000-129,000 snakebite envenomings occur each year, leading to an estimated 540-2,300 deaths (4). Throughout the Latin American countries, bites from lanceheads (*Bothrops* spp.) are the most prevalent. Rattlesnakes (*Crotalus* spp.), bushmasters (*Lachesis* spp.), and Coral snakes (*Micrurus* spp.) are also present, but especially the latter two are far less common causes of snakebites (175, 176). In Central America, snakebites are also caused by moccasins (*Agkistrodon* spp.), jumping pit vipers (*Atropoides* spp.), palm pit vipers (*Bothriechis* spp.), montane pit vipers (*Cerrophidion* spp.), and hog-nosed pit vipers (*Porthidium* spp.). The venoms of pit vipers indigenous to Central America can be treated with polyvalent antivenom (175). The clinical utilization of polyvalent antivenom makes diagnosis at a species or even genus level less important, as noted earlier for Asia and the US and Canada. However, it is important to determine which family (viper, elapid, or other) the perpetrating snake species belongs to, whether an envenoming has taken place, and the severity of the envenoming (175). For South American countries, both polyvalent and genus-specific antivenoms are available (111, 176–179).

Several Latin American countries have protocols for diagnosis and treatment of snakebite envenoming, describing the use of the syndromic approach and the laboratory investigations mentioned in **Table 2** (111, 175, 176, 178–180). Several of these protocols mention coagulation time as a commonly investigated parameter for early detection of a pit viper envenoming (111, 175, 176, 178–180). Often in pit viper envenomings, the 20WBCT is positive (no clot at 20 minutes), while for elapids it remains negative (normal clot at 20 mins) (165–168). In Mexico, where rattlesnakes are the predominant genus, the Lee-White clotting time (LWCT) is utilized to determine the presence of coagulation disorders, which can in

turn give an indication of the urgency of commencing treatment (180). LWCT is fundamentally similar to the 20WBCT described earlier, with the only difference being that the LWCT is observed once per minute after an initial incubation time of five minutes (181). The effectiveness of LWCT was assessed in Brazil for its sensitivity toward detecting coagulopathy in lancehead envenomings and was considered a valuable tool in evaluating the need for antivenom therapy (181).

Pit viper envenomings may cause both local and systemic effects, but there are two distinct patterns. Most Central and South American pit vipers cause moderate to severe local effects and coagulopathy, often with hemorrhagic features. The exception is rattlesnakes, which are more likely to cause major systemic effects including neurotoxicity, rhabdomyolysis, and coagulopathy, while only causing mild local effects. Local effects following pit viper bite, with the exception of rattlesnakes, may include edema, severe local pain, swelling, local hemorrhage, inflammatory erythema, lymphangitis, bleeding from the bite wound, blistering, ecchymosis, tissue necrosis, and secondary infections (7, 111, 175, 176, 178–180, 182–185). Systemic effects may include early syncope, confusion, transient loss of vision or darkening of vision, hypotension, shock, renal damage, cardiac tachyarrhythmia or bradyarrhythmia, coagulopathy, and systemic hemorrhage (7, 111, 175, 176, 178–180, 182, 183, 185). With the knowledge of which snakes induce which clinical manifestations, the syndromic approach works well and is widely used. However, the approach requires thorough knowledge of the different venomous snakes (7, 186) and relies on the presence of polyvalent antivenoms targeting the venoms of one or multiple genera.

The similarity between the local effects of lancehead species and bushmaster species makes differentiating the two a challenging task, which can be further complicated by the fact that, in some regions, both genera are known locally as "surucucu" (see **Figures 1C, D**). However, the vagomimetic effects, sometimes induced by bushmaster venom on the gastrointestinal system, may cause diarrhoea, thus indicating the most likely genus of the culprit snake. Although this can be a strong indicator, the lack of such effects does not exclude the presence of a lachetic envenoming (151, 176), nor do their existence confirm it.

Unlike bites from lanceheads and bushmasters, many rattlesnake bites are more easily recognized by the neurotoxic effects that they can inflict. South American rattlesnakes (*C. durissus*) generally do not cause severe local manifestations but instead induce neurotoxicity resulting in neuromuscular paralysis (183, 184, 187), caused by neurotoxic crotamines and crotoxins present in the venoms. Envenomings by South American rattlesnakes often lead to mild to severe neurotoxic manifestations in the patient, which are clinical hallmarks that may guide the physician toward a correct diagnostic assessment (111, 159–161, 176, 178, 180). However, it has been reported that envenomings by juvenile South American rattlesnakes can result in coagulopathy as the main systemic manifestation, instead of neurotoxicity, which may lead to misdiagnosis and administration of wrong antivenom (188).

Coral snake envenomings are associated with very different clinical manifestations, such as local paresthesias, vomiting, muscle paralysis including paralysis of respiratory muscles, ptosis, ophthalmoplegia, diplopia, and late manifestations including secondary renal damage and respiratory failure (111, 168, 175, 176, 178–180, 183, 184). Although clinical manifestations may overlap with those of some rattlesnakes, the recognizable color schemes of coral snakes make a strong case for coral snake envenoming. Coral snakes found in most of the Pan-American countries are visually very distinct from pit vipers. However, nonvenomous snake species mimicking the venomous coral snakes exist (e.g. milk snakes: *Lampropeltis triangulum*). These are difficult to distinguish by a non-professional, but guidelines based on the color scheme of the snakes can be found that aid in the differentiation (162, 175, 189).

Snake biodiversity varies significantly throughout Latin America, from Argentina inhabited by three medically important snake genera (*Bothrops*, *Crotalus*, and *Micrurus*) to the plethora of medically important species found in Mexico and the Central American countries (190). This shift in indigenous snake species greatly impacts the diagnostic approach, where the severe local effects of lancehead envenomings become a specific indicator in Argentina (111), but is easily confused for a lachetic envenoming in Brazil (176). Polyvalent antivenoms alleviate the dependence on successful determination of the species of the culprit snake by simply requiring successful assessment of the snake family involved. However, as discussed previously, there may be disadvantages to being restricted to polyvalent antivenoms, and different polyvalent antivenoms may perform differently in a given clinical case.

SNAKEBITE DIAGNOSIS IN AFRICA

The extent of the snakebite problem in Africa is difficult to assess due to the scarcity of epidemiological data (182). However, of all the African regions affected, snakebite is most commonly observed in sub-Saharan Africa, where an estimated 90,000-420,000 envenomings occur annually, resulting in 3,000-32,000 deaths (5). In comparison, an estimated number of 3,000-80,000 bites occur in North Africa and the Middle East combined, leading to 4,000-8,000 deaths annually (5). To the best of our knowledge, a combined mortality rate for all of Africa has not been recorded, but it has been estimated that some of the populations most vulnerable to snakebite worldwide are found in Africa (191). The snakes that are responsible for the majority of bites and are associated with serious or life-threatening envenomings are saw-scaled vipers (*Echis* spp.), large African adders or vipers (*Bitis* spp.), spitting or cytotoxic cobras (*Naja* spp.), neurotoxic cobras (*Naja* spp.), and mambas (*Dendroaspis* spp.) (192). In addition to the potency of the snake venoms themselves, factors potentially contributing to the high mortality rate may include scarcity of antivenoms (partially due to the high cost of antivenoms relative to personal income levels), low quality, inappropriate, or counterfeit antivenoms, suboptimal health services, difficulties with quick access to health centers,

and insufficient training in clinical snakebite management, including a lack of diagnostic training and/or tests (2, 193–196).

In many African cases, appropriate clinical management of snakebite patients requires identification of the distinctive clinical syndrome based on epidemiological, clinical, and laboratory data (e.g. 20WBCT), and consequently the syndromic approach is often recommended (15). Researchers and clinicians have sought to objectively quantify the severity of snakebite envenoming to minimize confusion due to the ambiguity of the definitions offered by current guidelines (197). In Southern Africa, five main clinical syndromes of snakebite envenoming are recognized and often these guide diagnosis: Local pain and progressive swelling (cytotoxicity), progressive paralysis (neurotoxicity), incoagulable blood (hemotoxicity), moderate to marked local swelling (associated with otherwise neurotoxic bites), and mild to moderate swelling, with negligible or absent systemic effects (neurotoxicity and cytotoxicity) (15). However, with the syndromic approach, it is possible to misidentify snake species due to the similarity between symptoms that develop following envenoming from different types of snakes. For instance, mixed hemorrhagic and cytotoxic symptoms develop following envenoming caused by saw-scaled vipers, puff adders (*Bitis arietans*), and horned desert vipers (*Cerastes cerastes*) (see **Figures 1E, F**) (192).

Several polyvalent and a few monovalent antivenoms have been marketed for the treatment of envenomings caused by African snake species, but the antivenoms are not necessarily equally appropriate for the treatment of bites from a given genus or species, in spite of being marketed as such (198–201). The antivenoms are also not evenly distributed throughout the continent, and some areas have been plagued by antivenom shortages (2, 193, 196, 202). It might therefore be expected that the disparity in the availability and types of antivenoms in Africa is reflected by a variability in the demands for diagnosis. However, to be diagnosed or treated, the patient must make their way to either a health center or a properly trained clinician, which may often be difficult or result in long delays. A study published in 2015 estimated that about 29% of the population in Africa are geographically marginalized from emergency medical care and live more than two hours from the nearest public hospital (203). The same study found that only 16 of 48 countries have more than 80% of their population living within two hours' travel time of emergency hospital care (203). Thus, it is no surprise that many snakebite victims in rural communities resort to seeking out traditional healers, rather than trained physicians (195). This trend is also observed outside of Africa, when looking at other rural parts of the world that are heavily burdened by snakebite (204–209). This delay in receiving proper medical care will, in most cases, worsen the symptoms and thus increase the likelihood of a poorer clinical outcome (206, 210).

For diagnosis, management, and treatment of snakebite victims in Africa to improve, it will be essential to address the knowledge gap between the health institutions, rural communities, and their local traditional healers (206). One strategy to approach this is *via* outreach and education programs promoting snakebite prevention and first aid (211). Such programs could even include traditional healers in an attempt to utilize their status as authority figures (211), rather than attempting to fight strongly-held community cultural beliefs. Also, increased availability of mobile phones with inbuilt cameras could facilitate the involvement of (distantly located) expert herpetologists in snake species identification without the need to capture or kill the snake (206, 212).

SNAKEBITE DIAGNOSIS IN EUROPE

Snakebite incidents are a relatively rare occurrence in Europe with an incidence rate of 1.06 bites per 100,000 people and about 4 deaths annually (213). Contrary to what some believe, snakebites from species indigenous to Europe can cause severe envenoming and require immediate medical attention. All significantly venomous snakes in Europe belong to the *Viperinae* subfamily, with the common European adder (*Vipera berus*), European asps (*V. aspis*), and common sand adder (*V. ammodytes*) being responsible for the largest proportion of severe envenomings (213, 214).

Many areas of Europe are inhabited by only one species of venomous snake, especially in Northern and Central Europe (213, 214). If diagnosis is necessary in areas with more than one species, it is usually based on witness statements, a picture of the culprit snake, or the snake itself brought by the victim (215–217). In severe cases, the presence of neurotoxicity can be an indication that the envenoming was caused by either a common sand adder or a European asp, as these two species are the most common causes of neurotoxicity due to envenoming by indigenous European snakes. Additionally, because these species have disjunct distributions, neurotoxicity can help pinpoint exactly which species caused the bite (213, 218). However, the absence of neurotoxicity does not exclude European asp bites, as most subpopulations do not possess neurotoxic venom (219). Neurotoxic clinical features have also occasionally been reported after envenoming by the common European adder, but this has been limited to a few geographical areas in Eastern Europe and has mostly been caused by the subspecies known as the Bosnian viper (*V. berus bosniensis*) (216, 217, 220). For this reason, in most of Europe, elaborate laboratory tests for diagnosis of the culprit snake species is a low priority. However, laboratory tests are used to assess the severity of envenoming, and thereby the need for antivenom (214, 221). Clinical manifestations monitored include hypotension, neurologic or gastrointestinal symptoms, edema, and leukocytosis. A full overview of clinical manifestations is given elsewhere (222).

Snakes inject a variable amount of venom and dry bites can occur (223–226). Victims are normally admitted for observation for 24 hours to monitor possible symptom progression (225, 227). Despite the impracticality of using clinical signs for diagnosing the species involved in most European snakebites, the severity of the symptoms and signs can be used to determine the need of antivenom administration in moderate to severe envenomings. A grading system for assessing the severity of an

envenoming has been proposed based on data on the appearance of clinical manifestations from common European adder and European asp cases and has been used as a guideline in research and in certain clinical settings (53, 221, 225, 228).

Despite the close phylogenetic relationship between *Vipera* spp., inter- and intraspecific venom variability might occur, both with regard to the toxins present and their individual abundances, which, in turn, may affect antivenom efficacy (213, 229–231). However, available monospecific antivenoms may still show cross-reactivity between venoms, and studies have shown that antivenom raised against venom from one species can, in some cases, have clinical efficacy against venoms from other vipers indigenous to Europe (214, 215, 232).

Bites by exotic snakes are not as prevalent as those by indigenous species. However, they are still the cause of a few severe bites around Europe, mostly affecting amateur snake keepers (233–235). In these cases, rapid identification of the responsible species is important as it can help predict clinical manifestations and aid symptomatic treatment. As the snake is not endemic to the country, clinicians will usually rely on statements from witnesses for identification, and required antivenom should be sourced as soon as possible as it might not be stocked in the given country (236) (exotic antivenom banks exist in a few countries, e.g. the Netherlands and Germany).

POTENTIAL BENEFITS OF NOVEL SNAKEBITE DIAGNOSTICS

Studies find that early treatment of Australian and North American snakebite victims is linked to faster recovery and shorter time to hospital discharge (126, 174). In a similar vein of inquiry, it was established that delays in treatment increase the risk of acute kidney injury in snakebite victims in Myanmar and the risk of acute renal failure and the overall severity of envenoming in snakebite victims in Brazil (237–239). One of the studies also found that patients who developed acute renal failure required more antivenom and were hospitalized for a longer period of time than those who did not (238). These studies point to the unsurprising conclusion that delays in treatment often negatively impact patient outcome, which in turn can result in prolonged hospitalization time and increased resource consumption at the treatment facility. It thus seems plausible that improved diagnostics might enable rapid diagnosis and thereby facilitate early and correct treatment, as well as improved patient outcomes. This is backed by a recent study of 742 snakebite patients in Sri Lanka, which argues that delays in antivenom administration reflect an absence of diagnostics for early detection of envenoming, and that such diagnostics are required for improved, early treatment with antivenom (240). Novel diagnostics will likely have the greatest impact in areas where transportation to the treatment facility and antivenom availability are not limiting factors, areas with many different indigenous snake species that are visually difficult to discern, areas where monovalent antivenoms are available, and areas with medical or paramedical personnel with limited training in clinical management of snakebite envenoming.

In addition to their utility in supporting clinicians in diagnosing snakebite patients and choosing the correct antivenom on a case-by-case basis, novel snakebite diagnostics could also be of interest on a grander scale. They could enable epidemiologists to map patterns of snakebite incidence. In turn, knowing which snake species are responsible for the majority of bites in an area can help authorities manage their resources, when deciding which antivenoms to procure in which quantities, and where to deploy them within a healthcare system (241). Improved diagnostics might also inform the design of novel antivenoms, and they could become indispensable tools for clinical trials of future generations of antivenoms, and later (if adopted as companion diagnostics) in clinical snakebite management. Based on the potential use cases and benefits listed above, it is perhaps hardly surprising that researchers and physicians have indicated the need for improved diagnosis of snakebite victims for decades (7, 83, 240, 242–248).

SNAKEBITE DIAGNOSTICS REPORTED IN THE LITERATURE

Several diagnostic assays have been developed to meet the demand for improved diagnosis of snakebite victims. The diagnostics rely on techniques varying from immunoassays (typically ELISAs), over enzymatic activity assays, to forensic genetic methods (see **Table 1**). These studies demonstrate that snakebite envenoming can be diagnosed using various technologies, and they showcase the development of snakebite diagnostics throughout the past six decades. As evident from **Table 1**, there has been a gradual shift in the preferred methodologies from radioimmunoassays and agglutination tests over the ever-popular ELISA format, toward an increased focus on LFAs and more diverse non-immunological methods. As a reflection of this technological progression, the experimental diagnostics reported in literature have become faster over time, although interestingly, their limits of detection do not seem to have improved significantly. One hypothesis explaining this could be that, while faster immunoassays have been developed, the antibodies at the core of these assays are essentially unchanged, with most still being derived from horses, rodents, and lagomorphs (see **Table 1**).

Many of the earliest reported diagnostic tests for snakebites were developed for first-world countries, with Australia being prominently featured (see **Table 1**). However, this trend has changed, and snakebite diagnostics have now been developed for countries all over the world. As an example, in Brazil, an ELISA-based diagnostic tool has been utilized experimentally to aid differential diagnosis on a genus level (176). Similar assays have been developed that make it possible to evaluate the effectiveness of the antivenom administered to neutralize the venom (176). More recent examples of innovation within snakebite diagnostics in Brazil include an impedimetric immunosensor based on electrochemical impedance spectroscopy (86) and the use of

infrared thermography (94). Meanwhile in Asia, Hung et al. developed a sandwich-type enzyme-linked immunosorbent assay (ELISA) capable of detecting Taiwan cobra (*N. atra*) venom in biological samples with a detection limit of 1 ng/mL (72). The same group later developed an immunochromatographic strip to detect Taiwan cobra venom in patient serum in only 20 minutes (82), while a different group similarly developed an ELISA and an immunochromatographic strip for diagnosis of snake species in Taiwan (85). A number of other molecular diagnostic PCR-based tests for stratifying venom from Asian snake species have also been reported. However, these tests typically take at least 3-4 hours to complete and have lower specificity compared to immunoassays (90–92, 249). Generally, issues with cross-reactivity of the tests toward several species remains a problem for rapid diagnosis of snakebite envenoming, and many reported rapid diagnostic methods are not reliable enough for clinical use and can only be used for research purposes (30, 40, 71, 73, 76, 77).

Although the studies referenced above clearly demonstrate that snakebite diagnostics can be developed for the stratification of many snake species and using many methods, to the best of our knowledge, the SVDK is the only snakebite diagnostic to have been adopted in the clinical setting. The success of the SVDK in Australia may reflect the preference there for using monovalent antivenoms, unlike many other countries, which rely on polyvalent antivenoms. This reliance could create a barrier for adoption of venom detection tests. Generally speaking, the reason for the low adoption rate for novel diagnostic assays is not entirely clear, but a variety of explanations of both technical, financial, and implementational nature are likely to be part of the underlying cause (250). The antivenom market is notoriously financially unstable in many regions (196), and if this is any indication, it leaves little financial incentive for marketing snakebite diagnostics. To exacerbate the problem, snakebite diagnostics are perhaps above all else needed by clinicians in remote healthcare facilities with no training in clinical snakebite management. A lack of education in snakebite management among the users of future diagnostics might complicate the implementation of the diagnostics. Even if these and other financial and implementational challenges can be surmounted, a number of technical pitfalls still exist that one needs to be aware of. Below follows a discussion of some of these pitfalls and design considerations that developers of snakebite diagnostics should take into account to avoid them.

DESIGN CONSIDERATIONS FOR SNAKEBITE DIAGNOSTICS

When developing a diagnostic for a Neglected Tropical Disease, one of the most important factors to consider is affordability. The association between snakebite envenoming and poverty greatly affects the availability of treatment (2, 7, 11, 12, 251, 252), and this link between affordability and availability is likely to also exist for diagnostics. Affordability may place restrictions on the types of equipment required to use the diagnostic, especially at small, remote treatment facilities, where access to electricity can be unreliable, and for point-of-care testing. Point-of-care testing additionally requires greater user-friendliness, as the person carrying out the test may have received only limited or no training in its use. For these reasons, a PCR with a low limit of detection and a requirement for specialized laboratory equipment and knowhow, such as that developed by Supikamolseni et al. (91), and a user-friendly lateral flow assay with a higher limit of detection, such as that developed by Liu et al. (85), may be differentially suited for use at centralized treatment facilities and point-of-care settings, respectively. However, with the implementation of different types of PCR [see e.g (253–255)], it will likely be possible to make fast, user-friendly, PCR-based diagnostics for point-of-care testing in the future.

The sample matrix and sampling method should also be considered and as far as possible be adapted to the intended use case. In a healthcare facility, it may be convenient to use blood samples for diagnostics, as it is a standard procedure to take blood samples from snakebite patients for use in the existing laboratory diagnosis (192, 256). However, in point-of-care use cases, wound swabs and exudates may be more readily available. While the sampling method affects user-friendliness, the sample matrix may affect the technical specifications of the diagnostic, as different sample types are likely to contain different concentrations of the analyte at different time points, as well as different concentrations of interfering substances (i.e. substances that alter the detected concentration of the analyte). For example, blood samples have notoriously complex compositions compared to e.g. urine samples, and this increases the risk of blood samples containing interferants. Conversely, the collection of blood samples at healthcare facilities is a highly standardized procedure, unlike the collection of wound swab samples, which may additionally be affected by subjection of the bite wound to inappropriate first aid methods or other forms of tampering. Being collected from the surface of the body, wound swab samples may not be representative of the amount of venom actually delivered into the body of a victim, although they may still provide valuable information about the type of snake involved. As demonstrated in **Table 1**, diagnostics have been developed and tested on various different sample matrices, including blood (and as derivatives hereof: Plasma and serum), urine, tissue samples, wound exudate, and wound swabs. Preferences for the sample matrix vary, with some researchers placing more emphasis on standardization and how well the venom content in the sample type reflects the venom content at the active sites in the body, while others emphasize user-friendliness and a low risk of interference from other sample components. Perhaps to account for the advantages and disadvantages of the various sample types, some assays, e.g. the SVDK from Australia, function with multiple different sample matrices (257).

An additional factor to consider is the time required to use the diagnostic. Because snakebite envenoming is acute in nature, with some toxins exerting their effects within minutes, it would likely be beneficial for a diagnostic device intended for clinical

use to function on a timescale of minutes rather than hours. Conversely, for forensic and purely epidemiological studies, rapid assay time may not be a requirement. Therefore, time-consuming diagnostics, such as ELISAs with overnight incubations, may be as well-suited for retrospective diagnosis as more rapid assays. Furthermore, it is advantageous for the diagnostic tool to be stable over a wide range of temperatures and environmental conditions, as the provision of cold-storage may be problematic in some areas of the world (16, 241).

Important technical parameters, which are not specific to snakebite, also need to be considered, including specificity, sensitivity, and positive predictive value. Low specificity (i.e. the number of true negatives divided by the total number of individuals not suffering from a condition) leads to false positives, as demonstrated in a study by Ho et al. (40), where the researchers set up an ELISA to study snakebites in rural Thailand. Here, non-specific reactions of ELISA reagents led to a false positive rate of up to 75% (40). A study by Isbister et al. demonstrated how low sensitivity (i.e. the number of true positives divided by the total number of individuals with the condition) of the 20WBCT for Russell's viper envenoming led to a high rate of false negatives, which in some cases resulted in delayed antivenom administration (258). If the sensitivity, specificity, and disease prevalence are known, they can be used to calculate the positive predictive value, using the formula:

$$Positive\ \ predictive\ \ value = \frac{sensitivity\ \cdot\ prevalence}{sensitivity\ \cdot\ prevalence\ +\ (1-\ \ specificity)\cdot(1-prevalence)}$$

The positive predictive value is an indication of how likely patients with positive test results are to truly suffer from a condition (e.g. snakebite envenoming sustained by a cobra). Unfortunately, although Ho et al. argued the importance of reporting these measurements of assay performance already in 1986 (40), very few studies involving snakebite diagnostics contain these values, and some diagnostics are not even tested on patient samples (see **Table 1**). The absence of positive predictive values for snakebite diagnostics in the literature may be a reflection of the lack of available data on disease prevalence. As more epidemiological data hopefully becomes available, it may become easier to evaluate the potential of novel diagnostics by using the positive predictive value as a performance measurement.

Additional technical parameters of importance include limit of detection (LoD), quantitativity, and limit of quantitation (LoQ). In the literature, snakebite diagnostics have been reported with limits of detection (i.e. the lowest concentration of a substance that can be distinguished from the absence of the substance) ranging from 0.1 pg/mL to 0.3 mg/mL (see **Table 1**). The limit of detection required depends on the pharmaco-/toxicokinetics of the analyte. For example, if the analyte has a short half-life and a high volume of distribution, the limit of detection in a blood sample will need to be much lower than for an analyte with a long half-life and low volume of distribution. The influence of analyte kinetics is also relevant when discussing quantitative diagnostics. Quantitative diagnostics are interesting, because they can provide information not only about the presence of an analyte but also about its abundance. For quantitative assays, in addition to establishing the limit of quantitation (the lowest amount of analyte that can be quantitatively measured with a certain precision and accuracy), it may also be important to establish threshold values. For example, if the analyte measured is a biomarker of kidney injury, which is also found in low amounts in healthy individuals, it is important to determine a threshold value to distinguish patients with normal amounts of biomarker from patients with abnormal amounts. If the analyte is a snake venom toxin, it might be relevant to determine several thresholds corresponding to commonly used categorizations of "mild", "moderate", and "severe" envenomings, which correspond to different treatment strategies. Quantitative diagnostics could potentially also be used to evaluate the effectiveness with which antivenoms sequester toxins, by monitoring unbound toxins in blood samples from patients. This could make quantitative diagnostics useful tools for antivenom performance evaluation and monitoring of patients' disease progression/envenoming grade alike. A low level of free toxins might mislead non-toxinologist clinicians into believing the patient can be discharged, but due to a depot effect and a mismatch between the toxicokinetics of the venom and the pharmacokinetics of the antivenom, symptoms can recur (259). Potentially, if patients are kept under observation, quantitative diagnostics will enable clinicians to detect an increase in free toxin levels, before symptoms recur, and prepare accordingly. However, the Achilles' heel of diagnostics relying on toxin detection may be the underlying assumption that the toxin concentration is measurable in a readily available sample, and that this concentration is always reflective of the toxin concentration at the site where the toxin exerts its effects. While some studies have found correlations between venom antigen concentration in patient samples and certain clinical manifestations of envenoming, others have found the opposite (52, 53, 69, 260–262). This dichotomy underlines the complexity of the relationship between toxin concentration and distribution over time (263). The matter is complicated further for both toxin and non-toxin analytes, if the influence of preexisting morbidities on the analyte's kinetics is factored in. For instance, diseases such as chronic kidney disease may alter the clearance or even the baseline concentration of an analyte (if the analyte is a naturally occurring biomarker, see (264) for examples). The disparity between measured analyte concentration and signs of envenoming might be alleviated by using samples of the affected tissues instead of more distant tissues, e.g. by using a muscle biopsy instead of using a wound swab, if one is trying to assess myotoxicity. However, for clinical (as opposed to forensic) samples, this strategy could be highly problematic, as the risks involved for the envenomed patient may outweigh the potential benefits. If, in spite of these challenges, any meaningful thresholds can be established for quantitative diagnostics, it will be important that assay precision and accuracy are high in the surrounding range. It should also be determined in which range there is a linear correlation between actual and measured analyte concentration, and whether the assay is affected by the hook effect.

As evidenced by **Table 1**, many snakebite diagnostics have been developed using immunological methods, often using equine, leporine, or murine IgGs. For diagnostic immunoassays, it may be worth considering the format and origin of the antibodies used. While format and origin are key decisions that greatly affect antivenom utility (177), these antibody properties may be somewhat less influential in diagnostics, because the diagnostic antibodies are not injected, thus rendering their pharmacokinetics and pharmacodynamics irrelevant. However, it is still possible that endogenous factors in a patient sample (e.g. anti-idiotypic antibodies, other heterophilic antibodies, or factors in a blood sample) can react with heterologous antibodies on a diagnostic test, thereby causing a high background signal, which needs to be accounted for (40). In addition to establishing standard curves and subtracting such background signals, less laborious options are available. For example, it might be possible to use a different sample type that does not contain the problematic factors or to filter the problematic factors out of the originally selected sample type, especially if their sizes are very different from that of the analyte [see e.g (265, 266)]. Alternatively, an excess of unspecific antibody could be added to outcompete the diagnostic antibodies for unspecific binding to the interfering factors. The antibody format should also be considered, as different antibody formats have different avidities and different options for chemical modifications, such as linkage to dyes and tags or attachment to surfaces or larger particles (267, 268).

It is also important to consider which information is of most use for treatment. Several parameters exist that, if measured, could provide useful information to the treating physician. For example, measurement of biomarkers for the development of clinical manifestations is already used to help physicians identify and predict pathologies such as coagulopathies, rhabdomyolysis, and acute kidney injury (7, 109, 256). Detection of snake venom toxins and/or identification of snake species is also of interest, as knowing the snake species or type of venom injected into the victim can aid in deciding which antivenom (if any) is appropriate, as well as it might help predict later clinical manifestations. It may be relevant to distinguish which snakes are of greatest interest to discern from an epidemiological and a clinical viewpoint, respectively. For example, snake stratification at a species level may be very valuable in epidemiological studies, as it can uncover neglected species, which should be included in future antivenoms. Conversely, stratification at the species level may be entirely irrelevant from a clinical perspective, if no species-specific antivenoms are available. As such, the taxonomic level at which snakes should be differentiated depends on the intended usage of the diagnostic, and for diagnostics intended to support clinicians in deciding on treatment, it will depend on the treatments available in that area. Whichever analyte is chosen, it will inform subsequent interference studies (i.e. studies that determine which, if any, substances from patient samples interfere with the measured analyte concentration). E.g., if the diagnostic measures the concentration of a cobra cytotoxin in order to diagnose a patient with cytotoxic cobra envenoming, it should be investigated whether the diagnostic also reacts with cytotoxins from the venoms of other snakes found in the same area. Additionally, in this example it should be tested whether antibiotics (which are sometimes administered to fight infections at the bitesite), prophylactically administered antivenom, or other factors found in the sample matrix (e.g. anti-idiotypic antibodies as mentioned above or medications used to treat preexisting morbidities) can interfere with analyte measurement, e.g. by potentiating the enzymatic activities of snake venom toxins (269). Several of the studies listed in **Table 1** describe investigations of the potential for cross-reactivity with other snake venoms, with some studies having screened multiple venoms, and others only a few, but none of the studies report a broader, systematic screening for interferents.

Ultimately, the design of a novel diagnostic will be fraught with compromises, as developers will have to weigh the pros and cons of diagnostic technologies for different applications. Comparatively slow and sensitive ELISAs may be ideal for coroners and researchers attempting to retrospectively identify the type of venom that caused a patient's death, while rapid and user-friendly, albeit potentially less sensitive, LFAs may be preferable to first-responders trying to decide on appropriate first aid (or maybe, in the future, to decide on whether to use first-line-of-defense drugs, such as varespladib or batimastat). The most desirable properties of a diagnostic will thus always be determined by its intended usage, and it is unlikely that there will be a one-size-fits-all solution to developing snake venom diagnostics. Rather, multiple technologies are likely to find use in various applications.

TABLE 3 | Overview of clinically commonly used diagnostic methods for snakebite envenoming in different parts of the world.

	Syndromic approach	Visual identification of the snake, if available	Patient history	20WBCT	Lab. tests	Immuno-assays
Australia	✓	✓	✓	(✓)	✓	✓
Africa	✓	✓	✓	✓	(✓)	
Asia	✓	✓	✓	✓	(✓)	
Europe	✓	✓	✓		✓	
North America	✓	✓	✓	(✓)	✓	
Latin America	✓	✓	✓	✓	(✓)	

Diagnostic methods can vary between and within countries in these regions, and tick marks in parentheses indicate that the method is infrequently used or only used in relatively few areas.

Novel diagnostic tools for snakebite envenoming and snake identification do not have to come in the form of bioassays. Recently, an alternative was proposed with the suggestion that apps capable of recognizing photos of biting snakes and/or matching syndromes to snakes could empower healthcare providers and facilitate better treatment (212). Additionally, improved diagnosis of snakebite victims will likely depend on other initiatives in addition to novel diagnostics. For instance, studies from multiple different countries indicate that misidentification of snakes occurs, and in some cases prompts inadequate treatment (112, 115, 170, 270–273). Several authorities have therefore indicated the need for improved education and training of healthcare workers (7, 9, 244, 252, 274–277), a sentiment that is echoed in the World Health Organization's 2019 strategy for snakebite envenoming (211).

CONCLUSION

Snakebite envenoming has long been neglected, and the lack of care is – in the words of Williams et al. – a cruel anachronism (278). This neglect affects education, treatment, and diagnosis, none of which have received the attention or resources they deserve. However, with the reinstatement of snakebite envenoming on the World Health Organization's list of top-prioritized Neglected Tropical Diseases in 2017, snakebite is garnering more attention, funding, and resources. This constitutes an excellent opportunity for scientists, physicians, and other stakeholders (many of whom have been working tirelessly for decades to alleviate the burden of snakebite envenoming) to critically revisit both current practices and new efforts within clinical snakebite management. In this relation, it is important to remember the symbiotic relationship between treatment and diagnostics. Treatments should only be administered when so indicated by differential diagnosis, and the pertinence of diagnostics depends on the available treatments. Currently, the most widely used method of diagnosis of snakebite envenoming is the syndromic approach (see **Table 3**). This approach can be highly effective, when the treating physician possesses sufficient knowledge on snakes and has been properly trained in providing correct differential diagnosis. Unfortunately, not all physicians possess this knowledge and expertise, and in some regions, utilization of the syndromic approach is challenging. If novel diagnostics could be implemented in such clinical settings, they could support the standardization of snakebite diagnosis. In combination with improved training of healthcare workers, this could in turn further improve standardization of treatment. Additional benefits could be reaped by using diagnostics to improve our knowledge of prevalence and inform the design of antivenoms and resource management.

AUTHOR CONTRIBUTIONS

AL, CK, and JJ conceptualized the manuscript. AH, CK, JJ, RF, SF, and SD prepared the original draft. All authors contributed to the article and approved the submitted version.

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Immunological Responses to Envenomation

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Venoms are complex mixtures of toxic compounds delivered by bite or sting. In humans, the consequences of envenomation range from self-limiting to lethal. Critical host defence against envenomation comprises innate and adaptive immune strategies targeted towards venom detection, neutralisation, detoxification, and symptom resolution. In some instances, venoms mediate immune dysregulation that contributes to symptom severity. This review details the involvement of immune cell subtypes and mediators, particularly of the dermis, in host resistance and venom-induced immunopathology. We further discuss established venom-associated immunopathology, including allergy and systemic inflammation, and investigate Irukandji syndrome as a potential systemic inflammatory response. Finally, this review characterises venom-derived compounds as a source of immune modulating drugs for treatment of disease.

Keywords: venom, detoxification, innate immunity, adaptive immunity, immunopathology, Irukandji syndrome, venom allergy, systemic inflammation

INTRODUCTION

Venoms are complex mixtures of proteins, peptides, biogenic amines, and salts produced by a diverse range of animals for predation, protection, and competition (1–4). In humans, needle-like stinging apparatuses inject venom compounds into dermal-epidermal junctions, capillary vessels, and skeletal muscle fibres (5). The consequences of envenomation range from innocuous to lethal (6, 7).

As the initial site of venom's interaction with the immune system, the dermis provides protection through physical, chemical, and cellular defence mechanisms (8, 9). Prominent defenders in the dermal immune network include keratinocytes, endothelial cells, and tissue-resident and infiltrating immune cells for fast and non-specific responses (innate) and acquired long term protection (adaptive) (8, 9). The primary role of these cells is host defence. However, venom-mediated immune dysregulation can contribute to envenomation severity (10). Accordingly, this review discusses both the protective and pathological responses of barrier cells and the immune system towards venom compounds.

INNATE RESPONSES TO ENVENOMATION

Defence against envenomation requires an acute response achieved by the body's innate immune system. Innate mechanisms comprise barrier and cellular defences for immediate but non-specific resistance to foreign bodies (such as venom compounds), injuries, and pathogens. Physical barriers (skin and mucosal membranes) and secretions (chemical substances and enzymes) along with resident and infiltrating immune cells provide readily available protection without requiring prior exposure to the damaging compounds (11). Instead, sentinel and scavenger cells express receptors that sense evolutionarily conserved structures common to microbes, cellular stress, and harmful substances (12).

A wide diversity of innate signalling receptor and response types is responsible for efficient detection and neutralisation/elimination of various host threats (12). The detection of danger or stress signals initiates proinflammatory events. Broadly, these include the production of cytokines and chemokines for immune cell recruitment/activation, the release of antimicrobial peptides that directly kill pathogens, the phagocytosis and destruction of foreign particles and microbes, the generation of reactive oxygen species (ROS), reactive oxygen intermediates, and reactive nitrogen intermediates, and the release of enzymes with potent protein degrading and microbicidal properties (11).

Regulated innate effector functions are also critical for tissue repair and homeostasis (13). In addition, the presentation of foreign macromolecules, required for the establishment of acquired (adaptive) immune responses, is achieved by innate antigen-presenting cells (APC), including dendritic cells (DCs), monocytes (MNCs), and macrophages (MΦ) (11). Likewise, plasma proteins, including those of the complement system (an ancient protein defensive system), promote inflammation or directly kill pathogens (14).

Detection of venom compounds by innate mechanisms initiates inflammatory reactions critical to host protection, venom detoxification, and ultimately the resolution of symptoms (15, 16). Participation by the epidermis, endothelium, neutrophils, MNCs, MΦs, mast cells, and soluble effector mediators increases host resistance to the damaging events of bites and stings. Yet, as discussed below, many venom constituents can augment the activity of these components leading to venom-induced, immune-mediated host damage.

Epidermis (Keratinocytes)

The epidermis, the outermost layer of the skin, comprises 95% keratinocytes arranged in four layers (17). Tight junctions formed by keratinocyte-derived proteins provide a physical barrier from the external environment and structural support for Langerhans cells (epidermal-resident DCs), melanocytes, Merkel cells (tactile epithelial cells), and sensory neurons (18). Keratinocytes serve important sentinel and proinflammatory functions, where cross-talk between keratinocytes and cells of the dermal-epidermal junction direct immune cell function and maturation during both initial and late phases of inflammation (19). Like cells of the immune system, keratinocytes express cytokine receptors and pattern-recognition receptors (PRRs), enabling the detection of pathogen-, damage-, and venom-associated molecular patterns (PAMPs, DAMPs, and VAMPS) (20, 21). Activation of keratinocyte proinflammatory genes, such as by venom compounds, initiates the synthesis and release of cytokines, nitric oxide (NO), and alarmins, stimulating resident immune cells and attracting immune cell infiltration (20, 21).

To counteract this defensive barrier, many animal venoms contain matrix metalloproteinase (MMP) and L-Amino acid oxidase (LAAO) enzymes (22–25). Venom-derived MMPs and LAAOs can induce keratinocyte cell death by autophagy, apoptosis, or necrosis (22–25). Proteolytic degradation of the dermis facilitates access of venom-derived toxins to the circulation, lymphatics, and target organs for prey/predator immobilisation (22–25). In some instances, the induction of apoptosis stimulates the overexpression of endogenous MMPs, indirectly triggering tissue destruction (26). For example, brown recluse spider (*Loxosceles rufescens*) bites cause significant dermonecrotic effects, systemic inflammation, and potentially death in children (26). Interestingly, the molecular mechanism underpinning the initiation of cutaneous necrosis (a common reaction in loxoscelism) involves keratinocyte-derived enzymes (26). Induction of apoptosis by *Loxosceles* sphingomyelinase D, the main component of *Loxosceles* venom, stimulates the expression/activation of secreted and membrane-bound MMP-2 and MMP-9 in keratinocyte cultures (26). It has been shown that the augmented expression of MMPs has a role in the necrotic skin lesions associated with *L. rufescens* envenoming (26). Hence, tetracycline has shown protective effect against venom-induced cell death by inhibiting the activation of MMP proenzyme precursors and MMP enzymatic activity (26).

Endothelium

Throughout the vascular system, endothelial cells (ECs) line the interior surface of blood and lymphatic vessels (27). Although once considered bystanders in the inflammatory process, ECs can dictate inflammatory responses under homeostatic and pathophysiological settings (28). As a primary point of contact for bloodborne pathogens and other host assaults, including toxins, ECs play an important sentinel role (29–32). Expression of numerous PRRs, including toll-like receptors (TLRs) and receptors for tumour necrosis factor (TNF) and interleukin (IL)-1β, enables the intravascular detection of harmful compounds, the activation of proinflammatory genes, and the alteration of the microenvironment (29–32). ECs also express major histocompatibility complex (MHC) molecules, classes I and II, and costimulatory molecules, such as the CD40 ligand (CD40L) that allows intravascular antigen presentation and EC-mediated activation of effector memory T cells (T_{EM}) (33). However, ECs principally modulate immune function by directing leukocyte trafficking and distribution (34–36). Leukocyte tethering, rolling, and extravasation occurs in response to highly selective expression of cell adhesion molecules (CAMs), such as intercellular adhesion molecule-1 (ICAM-1) and selectins, on the apical surface of ECs. CAMs are essential for the homing and migration of immune cells towards secondary lymphoid tissue and inflammatory foci (34–36). The

powerful influence of ECs on immune function has led to hypothesise that immune dysregulation, such as seen in systemic inflammation, might be partially mediated by the endothelium (37). As venom compounds from different species can modulate EC function (described below), this may have important implications in venom-induced systemic inflammation or allergy.

Venom from different species can induce EC perturbations, including distortions in cellular function, morphology, cytoskeletal organisation, and cell viability (38–41). Collectively these actions alter vascular permeability and blood vessel stability (38–41). Additionally, snake and spider venoms are highly proinflammatory in EC cultures, commonly provoking the secretion of IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL2), and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES/CCL5) (42). Together, these events modify the extracellular environment and leukocyte activity in local and systemic compartments, which may have important implications for the pathology of some envenomations (41, 42). For instance, though neutrophil depletion abrogates *Loxosceles* venom-induced necrotic lesions, neutrophils are not a direct target of *Loxosceles* venom-derived toxins (43). While neutrophils are likely the proximal cause of inflammation and tissue destruction, direct exposure to venom does not provoke this response (43). Instead, research has shown that the venom contains EC agonists that elicit dysregulated activation and cellular damage (43). *Loxosceles* venom strongly stimulates EC-secretion of IL-8, a potent neutrophil chemoattractant, and low-level surface expression of E-selectin (43). Researchers have noticed an unusual activation response in neutrophils to these venom-mediated proinflammatory signals (43). Specifically, neutrophils adhere to venom-stimulated ECs *via* selectin-mediated tethering in a time- and dose-dependent manner, yet without transmigration (43). In culture, these sequestered leukocytes rapidly increase intracellular Ca^{2+} levels and release primary and secondary granules containing the lytic enzymes responsible for tissue degradation (43). Accordingly, the initiation of *Loxosceles* necrotic lesions appears to be dependent upon toxin-mediated EC responses (43). These findings, further to work by Paixão-Cavalcante and colleagues, suggest a role for immune-targeted (in addition to toxin-targeted) therapeutic strategies for envenomation (26).

Mononuclear Phagocytic System

MNCs and MΦs form a crucial phagocytic component of innate immunity. Both MNCs and MΦs are highly migratory, enabling tissue surveillance, antigen capture, and migration to draining lymph nodes for antigen presentation to adaptive immune cells (44, 45). As such, they function primarily as sentinel phagocytes and regulators of immunity (46). MNCs and MΦs secrete a wide range of cytokines and chemokines that modulate immune cell function and are potent mediators of neutrophil recruitment (47).

Many animal venoms can modulate the metabolism and function of MNCs and MΦs. For example, *Crotalus durissus terrificus* venom (CDTV) significantly inhibits the trafficking and phagocytic capacity of rat peritoneal-resident, thioglycollate-elicited, and *Mycobacterium bovis* strain bacille Calmette Guérin (BCG)-activated MΦs, without affecting cell viability at 2 h, 4 days, or 7 days post intraperitoneal administration (48). In contrast to these immunosuppressive effects, CDTV can enhance the production of hydrogen peroxide (H_2O_2) and NO from phorbol 12-myristate 13-acetate-stimulated resident, elicited, and activated MΦs (48). Further, CDTV-treatment augments cellular metabolism *ex vivo*. Extracted peritoneal cells showed upregulated glucose and glutamine usage and increased maximal activity of hexokinase, glucose-6-phosphate dehydrogenase, citrate synthase, and phosphate-dependent glutaminase (48). These venom-mediated actions result in amplified MΦs candidacidal activity and decreased phagocytosis potential (48).

Comparably, venom from the pit viper, *Bothrops alternatus* (BAV), stimulates increased production of superoxide anion (O_2^-) from isolated thioglycollate-elicited MΦs (49). Again, BAV-treatment showed a limited impact on MΦ viability, as evaluated by Trypan blue exclusion, and did not interfere with MΦ's adhesion or detachment capacity up to 100 μg/mL BAV (49). Pretreatment with the protein kinase C inhibitor, staurosporine (14 nM/mL), suppressed O_2^- production and phagocytosis, suggesting the involvement of a PKC-dependent signalling pathway (49). However, unlike CDTV, Setubal et al. observed increased MΦ complement receptor (CR3)-mediated phagocytosis following incubation with BAV (49). Phagocytosis of serum-opsonised zymosan particles was significantly higher in venom-stimulated MΦs compared to vehicle control (49). It was hypothesised that increased phagocytic activity and excessive release of superoxide might be involved in the local tissue destruction caused by *B. alternatus* snakebite (49).

Studies using human MNCs have revealed the potent proinflammatory properties of different venom compounds. For example, venom from the *Androctonus crassicauda* scorpion induces IL-12p40 mRNA expression and protein secretion from purified MNCs (50). However, venom exposure also produced concentration- and time-dependent cytotoxicity, as evidenced by significant LDH release in MNC cultures (50). Further examples include a C-type lectin (BjcuL) isolated from *Bothrops jararacussu* snake venom that induces TNF production from resting $CD14^+$ cells without stimulating proliferation (51). Phospholipase D from *Loxosceles laeta* spider venom promotes MNC migration in THP-1 cell cultures and cytokine release from skin fibroblasts (52). *Bothrops* snake venoms provoke the release of proinflammatory mediators, prostaglandin E_2 (PGE_2), macrophage inflammatory protein 1-alpha (MIP-1α/CCL3), and IL-1β, and induces activation of NF-κB in human MNCs (53). Given the immunostimulatory role of MNCs and MΦs on immune function, these data demonstrate the capacity of venom to induce systemic inflammatory responses.

Contrasting this research, Khemili et al. examined the immunosuppressive potential of ion channel modulators from scorpion venom using murine MΦs (54). Voltage-gated potassium channels (K_V) play a crucial role in calcium signalling and immune cell excitability (54). In the resting

state, murine MΦs predominantly express the $K_V1.5$ subunit of the $K_V1.5/K_V1.3$ heterotetrameric complex (54). Innate activation, including LPS stimulation, induces $K_V1.3$ overexpression (54). Using non-cytotoxic concentrations of *Androctonus australis hector* (Aah) venom, the authors observed a voltage-independent inhibition of K_V current amplitude in LPS-activated (M1) MΦs (54). On the contrary, venom perfusion did not significantly decrease K_V current amplitude in resting cells (54). These results suggest the presence of an ion channel blocker with a higher affinity for the $K_V1.3$ subunit, abundant on the cell surface of activated MΦs (54). However, as indicated by the authors, the downstream functional consequences of MΦ ion channel modulation requires further examination (54).

Additional immunomodulatory functions, such as TLR inhibition, have been identified using synthetic venom components (55). TLR signalling is a critical element in innate detection and MΦ activation (56). Many animal venoms contain VAMPs that strongly provoke immune stimulation *via* TLR engagement (56). Contrasting this, recombinant rhodostomin (Rn), a snake venom-derived disintegrin, exhibits potent TLR2 inhibition against lipopeptide-stimulated THP-1 cells (55). Incubation with Rn suppresses TNF, IL-1β, and IL-8 release and IκB degradation from Pam3CysSerLys4-activated cells (a TLR1/TLR2 agonist), in a dose-dependent manner (55). In THP-1 cell cultures, Rn reverses the phosphorylation of focal adhesion kinase downstream kinases, thereby inhibiting signal transduction (55). In the caecal ligation and puncture (CLP) model of sepsis, Rn significantly suppresses CLP-induced TNF, IL-6, and MCP-1 production and reduces animal mortality (55). Histology has also revealed that Rn significantly alleviates CLP-induced tissue-damage (55). Studies such as these highlight venom as a source of compounds for drug discovery.

Granulocytes (Neutrophils)

Neutrophils are the most abundant leukocyte, constituting 40-75% of circulating white blood cells (WBC) (57). Derived from pluripotent stem cells in the bone marrow, a segmented nucleus of three to five lobes and the presence of secretory vesicles/granules characterise mature cells (58). Although short-lived, estimates range from hours to several days, they are the first phagocyte recruited and mobilised from the bone marrow or periphery to the infection/injury (59, 60). Upon arrival, these granulocytes directly destroy pathogens, inactivate toxins, and mount inflammatory responses through oxidative and non-oxidative pathways (59, 60). Like other immune cells, neutrophils are prolific producers of cytokines and chemokines and can mount robust proinflammatory responses (61).

For non-infectious/sterile challenges, such as envenomation, exocytosis of granules/secretory vesicles releases up to 700 defensive proteins into the extracellular milieu (58). These proteins include defensins, serine proteases, neutrophil elastase, proteinase 3, cathepsin G, cytokines, and chemokines, some of which inactivate venom components through proteolytic degradation (62). An additional neutrophil defensive strategy, critical during envenomation, is the neutrophil extracellular trap or "NET". NET formation (NETosis) occurs through programmed self-destruction, whereby the release of nuclear DNA forms a sticky "net" of extracellular fibres, containing the dissemination of toxins, bacteria, and pathogens (63, 64). However, whether neutrophils protect against or promote venom injury is disputed. Certainly, the participation of neutrophils in venom-associated pathologies, such as dermonecrosis, has been well documented (26, 43). Nevertheless, neutrophilic functions, including toxin trapping and inactivation, provide critical defence against systemic injury and death (65). Additionally, neutrophil clearance of necrotic tissue is essential for muscle regeneration following snakebite (16).

Snake venom, such as from *Echis carinatus*, induces NETosis and ROS generation in a time- and dose-dependent manner in animal models and cell cultures (65). While these neutrophilic-defensive actions hinder venom's systemic dissemination, dense NET accumulation can block blood vessels, resulting in localised tissue damage and impeding antivenom's efficacy (65). Unfortunately, though research shows that co-treatment with DNase 1 prevents tail injury in *E. carinatus* experimentally envenomed rodents, mortality is significantly higher among these mice (65). Interestingly, follow-up work by Stackowicz et al. determined that localised tissue damage is neutrophil independent (66). Despite verifying that DNase-treatment does indeed reduce tail injury at the expense of survival, the study reported similar occurrences in both neutrophil-sufficient and deficient settings (66). These data suggest that extracellular DNA from multiple dying cell types, including neutrophils, mediate capillary obstruction following envenomation.

Regardless of DNA source, toxin retention inhibits systemic injury to the detriment of the localised compartments (66). NET formation and capillary obstruction can lead to severe consequences, such as amputation, which has devastating implications for victims' lives (67). Accordingly, there is an urgent need for effective therapeutics that minimise tissue necrosis and facilitate antivenom efficacy. However, given that neutrophil participation is critical in tissue repair post-envenomation, neutrophil-targeting therapies may be counterproductive (16). Hence, further research is required.

Granulocytes (Mast Cells)

Mast cells (MCs) are long-lived, tissue-resident effector cells derived from a myeloid lineage and matured under the influence of stem cell factor and cytokines (68). MCs are positioned near entry points of mucosal, epithelial, and sub-endothelial connective tissue to provide innate defence and perform a wide range of physiological functions that maintain tissue homeostasis (68). MCs induce killing and assist in the clearance of parasites and pathogens. For venom/toxin defence, sequestering and neutralisation occur (69). Expression of multiple PRRs on the cell surface enables rapid detection and response to immune challenges, including venom toxins. Activation of PRRs induces *de novo* synthesis of cytokines, chemokines, and eicosanoids to attract and stimulate other effector cells (70). A classic feature of MCs are weaponised granules, containing preformed toxic inflammatory mediators, including enzymes (tryptase, chymase, and carboxypeptidase A3), amines (histamine and heparin), and cytokines (TNF). MC activation, mediated by immunoglobulin E (IgE)-bound

FcεRI, causes rapid degranulation potentially inducing a systemic proinflammatory response (71, 72).

Despite the widely recognised role of MCs in allergy and anaphylactic shock, animal models have provided evidence of MCs' protective function against envenomation (73, 74). As reviewed by Galli et al., functional MCs enhance the survival of mice challenged with sub-lethal doses of snake (*Atractaspis engaddensis; Daboia russelii)*, Gila monster *(Heloderma suspectum)*, European honey bee *(Apis mellifera)*, and scorpion (*Leiurus quinquestriatus hebraeus; Centruroides exilicauda)* venoms (75). The significantly higher mortality among MC-deficient mice has been attributed to the dysregulation of serine proteases (carboxypeptidase A3 and mast cell protease 4), which degrade peptides, and heparin and histamine (69, 75–77). In healthy individuals, the release of heparin and histamine can neutralise the effects of venom-derived toxins (69, 71). Adversely, the release of these amines provokes dangerous allergic symptoms in hypersensitive individuals, particularly in response to Hymenopteran venom (the venom of bees, wasps, and ants) (70).

Chemical Mediators

The immune network is vast and highly complex. Intercellular communication across the network requires small soluble protein effectors, known as cytokines (78). Cytokines (interferons, interleukins, chemokines, and growth factors) are secreted by cells to instruct and regulate the immune system's activity for protection against injury, infection, and disease (78). Biological functions include cellular activation, proliferation, differentiation, growth, and immune regulation (78). Further, as chemoattractant proteins, chemokines exert their effects *via* cell recruitment, migration, and adhesion (79). Like hormones, cytokines have autocrine, paracrine, or endocrine functions for localised or systemic effects (78). Broadly, they elicit either pro or anti-inflammatory action (80, 81). The reality, of course, is more complicated as many cytokines exhibit pleiotropic effects that are dependent on cellular source, target receptor, and the stage of the inflammatory process (80, 81). Additionally, immune cells adapt to the overall profile of the cytokine milieu they encounter (80, 81).

The expression and release of these potent chemical mediators are tightly regulated (82, 83). Nevertheless, infection, cancer, injury, disease (such as autoimmunity), and medical interventions (including drugs and organ transplant) can provoke dysregulation in cytokine levels resulting in devastating pathophysiological effects. Unchecked, cytokines and other proinflammatory mediators cause severe tissue destruction, systemic pathology, multiple organ failure, and potentially death (82, 83). Existing literature extensively describes diverse pathophysiology induced by dysregulated inflammatory mediators. These include cytokines (IL-1β, IL-6, TNF, IFN-γ, IL-10, IL-12, and GM-CSF) and chemokines (IL-8, MCP-1, eotaxin/CCL11, IP-10/CXCL10, MDC/CCL22, MIP-1α, and TARC/CCL17), as well as bradykinin, eicosanoids (prostaglandins and leukotrienes), cyclooxygenases, NO, and histamine (84). Dysregulation of these mediators is associated with inflammatory and neuropathic pain (85, 86), tissue destruction (87), systemic inflammation (88–90), autoimmunity, and allergic reactions (91). Unsurprisingly, the same proteins are detected in the serum of victims of envenomation, where pain and systemic injury occur (92, 93). Notably, similar secretion profiles are also present in experimentally envenomed animals and cell cultures (92). Additionally, venoms can have detrimental effects on platelet function and components of the complement system (94, 95). In particular, snake venoms can trigger critical pathologies, such as venom-induced consumption, thrombocytopenia, and hemorrhage (94, 95).

Cytokines and their respective receptors represent important immunotherapeutic targets for numerous conditions (96). Accordingly, it might seem plausible that targeting proinflammatory cytokines, chemokines, and small molecules (or their receptors) similarly represents novel therapeutic avenues for certain envenomations. However, research in this area is still in its infancy, and to date, studies have described both beneficial and detrimental outcomes of immunosuppression during experimental envenomation. For example, the detection of snake and bee venom toxins by NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome, triggers immune cell activation, potent IL-1β secretion, and neutrophil influx (15). Interestingly, Palm and Medzhitov showed that although inflammasome inhibition, such as seen in caspase-1-deficient mice, successfully inhibited cytokine release and leukocyte influx, it unexpectedly resulted in a higher susceptibility of the mice to the noxious effects of venoms, including mortality (15). Conversely, Zoccal et al. determined that using a hexapeptide ligand for the MΦ scavenger receptor (CD36) protected mice against a lethal dose of *T. serrulatus* scorpion venom through decreased production of IL-1β, IL-6, TNF, CCL3, and PGE2, and restrained lung inflammation (97). While reduced IL-1β secretion and neutrophil influx was observed in both models, together, these data demonstrate the importance of innate immunodetection in the defence against bites and stings.

Adaptive Responses to Envenomation

The immune system's adaptive arm is predominantly comprised of B cells and T cells. The primary effector function of B cells is the generation of antibodies (immunoglobulins; Ig) for humoral defence (98). T cell effector functions are produced by a range of subsets, including cytotoxic ($CD8^+$) T cells and helper ($CD4^+$) T cells (TH) cytotoxic ($CD8^+$) and helper ($CD4^+$) lymphocytes. Cytotoxic $CD8^+$ T cells protect against intracellular pathogens and suppress infectious disease and tumour growth, while $CD4^+$ T cells maintain homeostasis and shape proinflammatory and regulatory immune responses (99).

Bites, stings, and intentional venom inoculation stimulate the generation of venom protein-specific antibodies (100, 101). Antibody-mediated neutralisation effectively counteracts venom activity (102). However, a primary B cell response is slow (requiring days to weeks to become fully active), while defence against rapid venom action requires an immediate response (103). As an alternative to host antibodies, antivenom, produced in large mammals and purified for

medical purposes, can provide passive immunity to victims of life-threatening envenomation (104–106).

The following provides a simplified overview of a primary (thymus-dependent) humoral response towards envenomation. Following bite or sting, APCs, such as DCs, MNCs and MΦs, capture and process venom proteins at the site of injury, promoting maturation (100, 101). Matured APCs migrate to secondary lymphoid tissue to present venom antigen to naïve T_H cells *via* membrane-bound peptide-MHC II protein complexes (100, 101). In lymph nodes, engagement of a T cell receptor (TCR) with cognate peptide-MHC molecule initiates T_H activation (signal 1) (107). Critical secondary signals, required for complete T cell activation, are provided by APCs. APCs, especially DCs, highly express ligands (including CD80 and CD86) for T cell co-stimulatory molecules, such as CD28 (signal 2) (108). Next, APC-derived and circulating cytokines (as well as autocrine IL-2) induce T cell proliferation and differentiation (signal 3). For extracellular immune challenges, such as envenomation, $CD4^+$ T cells acquire a T_H2 phenotype with effector functions that include B cell activation (100, 101).

During a primary antibody response, B cells require multiple stimulatory signals. The first occurs when a B cell receptor (BCR) encounters its specific soluble or membrane-bound epitope (100, 101). The internalised antigen is processed and displayed on the B cell surface as a peptide-MHC complex for T_H presentation (100, 101). TCR binding triggers upregulation of co-stimulatory ligands, such as CD40L, and the production of proinflammatory cytokines, including IL-4 (107). CD40L engagement with B cell CD40 mediates the recruitment of intracellular adaptor proteins essential for propagating downstream signalling (107). Additionally, cytokines secreted by primed T_H2 cells provide B cells with accessory stimulation for the early (proliferation and clonal expansion) and later (differentiation, antibody production, and isotype switching) stages of B cell activation (109). Proliferating B cells form germinal centres (GCs) where memory B and antibody-secreting plasma cells develop. Here, B cells also undergo somatic hypermutation and isotype switching (IgM and IgD to IgG, IgE, or IgA) to generate high-affinity antibodies for robust immune responses (110).

Yet, critical though they may be, adaptive responses can also produce severe pathology (74). For example, IgE isotype switching following venom challenge can, in a percentage of hypersensitive individuals, lead to fatal allergic reactions (discussed below) (74). In addition to allergy, dysregulation of adaptive responses and loss of self-tolerance stimulate destructive auto-reactivity (111). As such, lymphocytes (T cells in particular) are a target for therapeutic modulation (111). Serendipitously, venoms can contain ligands for T cell ion channels and receptors, able to modulate immune function with high specificity (described below) (112–121).

VENOM-INDUCED IMMUNOPATHOLOGY

Cell-specific venom-mediated immune dysregulation is described above. The following sections discuss modes of immunopathology, including venom-induced allergic reaction and systemic inflammation.

Venom Allergy

Despite a notorious reputation for venomous snakes, spiders, and jellyfish, Australia's largest proportion of venom-related fatalities occur due to anaphylactic events (122). Reflecting a global trend, honey bee (*A. mellifera*) stings are a significant contributor to venom injury in Australia, representing 16.3% of anaphylactic fatalities between 1997 and 2013 (123).

Venom from stinging Hymenopterans is commonly associated with allergic reactions worldwide (123). While most sting responses are localised and self-limiting, fatality can occur due to immune-mediated respiratory and/or cardiovascular failure (124). In these incidences, systemic reactions (SR) are predominantly mediated by IgE-mechanisms; however, dose-dependent IgE-independent responses are also possible (7).

Among venom-sensitised individuals, SR's develop in 0.3% to 8.9% of cases (124, 125). Accordingly, Hymenopteran major allergens (antigens that bind IgE in greater than 50% of venom-sensitive individuals) have been well-characterised (7). For honey bee venom, these hypersensitivity-inducing proteins include phospholipase A2, hyaluronidase, acid phosphatase, and dipeptidylpeptidase (124). In vespid venom (wasp and yellow jacket), Antigen 5 and phospholipase A1 are the recognised major allergens (124).

Classic IgE-mediated allergic disease begins with a sensitisation process. Keratinocytes and resident immune cells detect damage induced by noxious substances, such as venom-derived compounds, stimulating the release of alarmins, cytokines (IL-4, IL-5, and IL-13), and other proinflammatory mediators required for antibody production (126, 127). DCs capture and process antigen for presentation to naïve T cells in draining lymph nodes, triggering events eventuating in plasma cell IgE antibody production (126, 127). Elevated IgE is a normal physiological response following a bite or sting and is not necessarily predictive of disease (69, 75, 124). Nevertheless, in some individuals, systemic IgE levels remain elevated longer term and can trigger SR, including anaphylactic shock, after multiple stings (7).

The symptoms of immediate (Type-1) allergic reactions occur during secondary antigen challenges. When IgE encounters its cognate antigen, crosslinking of FcεR1 on MCs and basophils in mucosal and epithelial tissues provoke activation and degranulation (128–130). Preformed inflammatory mediators, including histamine and proteases, are rapidly released from granules into the extracellular environment (128–130).

Histamine is chiefly responsible for the clinical consequences of Type-1 allergic reactions (131, 132). Histamine's protective functions include toxin binding and deactivation (69, 75). In allergic disease, histamine (acting upon H_1 and H_2 receptors) causes smooth muscle contraction, constriction of airways, swelling of the epiglottis, and increased vascular permeability. These events lead to dangerously low blood pressure, oedema and potentially death (131, 132). Further to this, proinflammatory genes, stimulated during the initial phase, induces *de novo* synthesis of the leukotrienes (particularly

LTB_4), cytokines, and chemokines responsible for the late phase (or delayed-type) symptoms (133, 134). These mediators are potent inducers of cell activation, migration, and the influx of lymphocytes and neutrophils (128, 134). The incidence and severity of biphasic anaphylaxis are highly variable, and fatalities can occur, necessitating continued patient observation following the resolution of initial symptoms (132, 133, 135).

The acute nature of fatal anaphylactic shock means death is more likely to occur in the home (87% of cases) than in the hospital (122, 123). Adrenaline autoinjectors (AAI) are an essential first-line treatment; however, death may still occur despite prompt administration (123, 136). Additional therapies include H_1 and H_2 antihistamines to counter the pathophysiological effects mediated through these receptors and critical supportive care (137, 138). For individuals with verified IgE-mediated allergy, venom immunotherapy (VIT) may generate a lifesaving tolerance to known allergens (139).

The pathogenic role of MCs and IgE-mediated granule release is well established (74). However, it has been postulated that allergy may be a barrier function disease in which cellular damage and perturbations of the epithelium and endothelium induce excessive proinflammatory responses from the resident immune cells (140). If this hypothesis is correct, the therapeutic modulation of these cells may correct the imbalanced proinflammatory response but this hypothesis has not been investigated for venom-associated allergy.

Hypersensitivity to Marine Stings

In Australia, contact with venomous marine animals and plants accounts for 9% of venom-related hospitalisations (122). The phylum *Cnidaria* (classes Hydrozoa, Scyphozoa, and Cubozoa) comprises approximately 10,000 jellyfish species distributed throughout the world (138, 141). Of these, ~1% are medically relevant (142). Jellyfish stings typically trigger local or large local responses, manifesting as pain, swelling, and erythema, but are usually not life-threatening (143, 144). However, severe delayed cutaneous reaction, allergy, and anaphylactic shock can occur (145–147).

It may not be surprising then that in 1902 the unexpected discovery of anaphylaxis by physiologists Charles Richet and Paul Portier involved marine venom from the Portuguese man-of-war (*Physalia physalis*) and sea anemone (148, 149). When attempting to immunize dogs against harmful venom effects, Richet and Portier found that rather than confer protection (phylaxis), a subsequent venom challenge resulted in death. Although allergy was yet to be characterised, the experiments recognised immune involvement and the term "anaphylaxis" (against protection) was coined. This discovery later won Richet the 1913 Nobel Prize in Physiology or Medicine (148, 149).

Jellyfish envenomation is the most common marine sting type, impacting fishers, surfers, and sea bathers globally. An estimated 150 million stings occur annually, with peak incidence coinciding with blooming (or swarming) seasons during warmer months (148). A characteristic feature of the phylum *Cnidaria* is specialised stinging organelles, known as nematocysts. Located within cnidocytes, nematocysts are explosive capsule organelles containing coiled, barbed and threadlike tubules coated in venom (150). High-velocity capsule release, triggered by physicochemical stimuli, causes inversion of tubules into harpoon-like threads able to puncture and penetrate prey and predators (151, 152). Through nematocysts, jellyfish venom, containing pore-forming compounds, metalloproteases, serine proteases, and phospholipases, is injected into the victim, causing paralysis of prey and in humans dysregulation of immune function, cardiac function, respiratory function, and potentially fatal outcomes (153).

Although jellyfish venom can cause severe immediate-phase and delayed-type allergic reactions, the causative allergens are mostly unknown. *Chironex yamaguchii* is the box jellyfish species responsible for 78% of reported stings in Japan (144). Recently, the N-linked glycoprotein, CqTX-A (a hemolytic toxin), was identified as a major allergen from this venom (144). While the underlying mechanism has yet to be elucidated, this finding has important implications as CqTX-A shares significant sequence homology with other lethal pore-forming jellyfish proteins, specifically, CfTX-1 and CfTX-2 (*Chironex fleckeri*), CrTX-A (*Carybdea rastoni*), CaTX-A (*Carybdea alata*) and CqTX-A (*Chiropsalmus quadrigatus Haeckel*) (154–157).

For Hydrozoa (which includes Portuguese man-of-war), Scyphozoa (true jellyfish), and Cubozoa (box jellyfish), nematocysts are located on the tentacles, oral arms, and in some instances, the bell of the jellyfish (158, 159). Contact with tentacles can result in inoculation from potentially millions of nematocysts (160). Application of acetic acid and careful removal of tentacles from the victim's skin prevents the further discharge of unfired nematocysts but does not deactivate the already injected toxins (161). Once stung, venom distribution occurs *via* capillaries and the lymphatic system to target organs, while the barbed tubules remain embedded in the skin until clearance (149, 160). Tubules are allergenic scaffolds comprising carbohydrates, proteins, chitin, and mini-collagen (148, 149, 162–164). As such, it has been suggested that impaired clearance, especially of chitin, may contribute to more severe outcomes of envenomation and hypersensitivity (149).

Beyond stings, there are recently described cases of severe allergic reactions to edible jellyfish consumption (147). It has also been reported that jellyfish stings, particularly among surfers, lead to sensitisation of foods containing gamma-glutamic acid, such as fermented soybean (165, 166). Collectively, these data highlight the antigenic properties within jellyfish nematocysts and tissue, in addition to their venom-derived destructive potential.

Systemic Inflammation

Systemic inflammation, including cytokine release syndrome (CRS), is a life-threatening immune condition triggered in response to endotoxemia, severe viral infections (including influenza), and immunotherapies (167–169). Clinical manifestations can include fever, nausea, tachycardia, dyspnea, headache, muscle and joint pain, and in severe cases, neurotoxicity, pulmonary oedema, respiratory failure, and death (167). Certain envenomations can similarly provoke a systemic inflammatory response, most notably is scorpionism (10).

Scorpion envenomation is another leading cause of venom-associated morbidity and mortality, affecting more than one million individuals per annum (6). Although most stings produce only local symptoms (81% of cases), envenoming by dangerous species can initiate a surge of endogenous neurotransmitters, adrenaline and noradrenaline, resulting in an autonomic storm and severe systemic effects (6, 10). Additionally, venom-derived toxins induce spontaneous acetylcholine (Ach) release from peripheral nerves, responsible for the life-threatening cardiac dysfunction seen in severe cases (170). Interestingly, these mediators are also implicated in the box jellyfish pathology, Irukandji syndrome (171).

Along with intense acute pain and distress, severe scorpion envenoming (grade III stings) produces complex pathophysiology in victims (172). Like CRS, symptoms can include respiratory distress, cardiac dysfunction, pulmonary oedema, multiple organ failure, and potentially death, especially among children and the elderly (173). These clinical consequences are principally mediated by neurotoxic peptides, able to cause hyperexcitability of the autonomic nervous system through Na^+, K^+, Ca^+ or Cl^- ion channel modulation (172). Ion channel modulation is also implicated in the development of pulmonary oedema, a symptom present in many fatal sting cases (174). Research led by Comellas et al. observed decreased lung fluid clearance in *Tityus serrulatus* envenomed rats, postulating venom-induced impairment of Na^+/K^+ ATPase in alveolar epithelial cells as the mechanism (174).

Beyond neurotoxic effect, the immune network plays a role in significant scorpion envenomations (175). Immune participation in SR is multifactorial, involving direct antigenic activation and indirect stimulation *via* the neuroendocrine-immune axis (176). In addition to neurotransmitters, stings induce a rapid release of proinflammatory mediators. Elevated IFN-γ, IL-1β, IL-6, IL-8, IL-10, and TNF have been detected in the plasma of sting patients and animal models of scorpionism (10). Scorpion venom also provokes hypersensitivity mediators, particularly histamine (177). Blockade of the histamine H1 receptor has shown to be protective in *Androctonus australis hector* envenomed mice (177). Specifically, pretreatment with hydroxyzine (H1 receptor antagonist) reduced immune cell infiltrate and oedema in the brain and spinal cord and diminished levels of circulating proinflammatory cytokines (177). Further, scorpion toxins activate components of the complement system, including the generation of anaphylatoxins, which are potent chemotactic proteins (10). As further evidence of immunological involvement, heightened dermal reactions are reported in individuals predisposed to scorpion venom, such as seen in delayed-type hypersensitivity reactions (10).

For direct immunological activation, the most extensively studied species is the Brazilian scorpion, *T. serrulatus* (10). Whole *T. serrulatus* venom (TsV) and select purified toxins are potent stimulators of innate immune cells, including MΦs (56). *In vitro* assays have revealed that surface receptors TLR2, TLR4, CD14, and CD36 recognise TsV compounds, triggering cellular activation and production of cytokines and lipid mediators (56). Engagement of CD14 and co-receptor TLR4 promotes NF-κB and AP-1 signalling pathways and transcription of potent proinflammatory genes, including IL-1β (10). Consequently, TsV stimulates cytokine release from innate immune cells in a time- and dose-dependent manner, independent from cytotoxic effect (10). In addition, NF-κB signalling regulates cyclooxygenase-2 (COX-2) expression and the secretion of eicosanoid, PGE_2 (178). PGE_2 is a lipid mediator with pleiotropic roles in the initiation and resolution of inflammation, particularly inflammatory pain (179). Among its diverse biological functions, PGE_2 activates IL-1β, MCP-1, and IL-6 pathways *via* prostaglandin EP4 receptor signalling (179). Accordingly, IL-1β and its receptor (IL-1R) are strongly suppressed by EP4 antagonism (179). IL-1β and IL-1R are potential therapeutic targets for multiple inflammatory diseases, including scorpion envenomation (180). As such, inhibition of the COX-2/PGE_2/EP4 pathway has shown a cardiopulmonary protective effect in envenomed mice (97, 170).

The eicosanoid leukotriene B4 (LTB_4) is also upregulated in cell culture and animal plasma following treatment with whole TsV or purified toxins (178). A study by Zoccal et al. demonstrated that activation of the class B scavenger receptor, CD36, directs eicosanoid metabolism towards LTB_4 *via* a 5-lipoxygenase (5-LOX)/peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway, opposing the events of TLR and CD14 receptor signalling (97). CD14 and TLR4 appear to be critical for TsV-induced cytokine and eicosanoid secretion (180). Further work by Zoccal and colleagues showed that $CD14^{-/-}$ mice fail to produce significant levels of PGE_2 or IL-1β post-TsV envenomation (180). In addition, $CD36^{obl/obl}$ mice secrete increased levels of PGE_2 and IL-1β post-TsV envenomation but do not produce LTB_4 (180). Critically, LTB_4 synthesis suppresses IL-1β maturation and secretion and the associated animal mortality (178). CD36, therefore, represents a novel therapeutic target for severe scorpion envenomation (180).

Mouse models of TsV envenomation produce autonomic dysfunction that is similar to clinically observed symptoms (170). A lethal inoculation of TsV induces sweating, ocular and nasal secretions, lethargy, and convulsions in mice, preceding cardiovascular disturbances and death (170). Observed hyperglycemia and neutrophilia are also consistent with sting patients (170). The neurotransmitters adrenaline and ACh, responsible for sympathetic and parasympathetic symptoms, respectively, are elevated in peripheral blood as well as in cardiac tissue in response to TsV (170). Treatment with atropine, a muscarinic receptor antagonist, but not propranolol, prevented venom-induced cardiovascular alterations, which are a leading cause of death in severe scorpionism (170). Curiously, despite showing systemic elevation of adrenaline, the study did not investigate the effect of an alpha-adrenergic blocking agent, such as prazosin (170).

In parallel to excessive ACh, lethal TsV envenomation stimulates the systemic and cardiac secretion of PGE_2 and IL-1β. Reis et al. have recently proposed IL-1R as a neuro-immune link responsible for innate heart inflammation and TsV-induced heart failure (170). Research by their group demonstrated that

TsV co-administered with PGE_2 enhanced IL-1β and ACh release from cardio fibroblasts, an effect which was blocked by an EP receptor antagonist. In contrast, IL-1R silencing repressed PGE_2, IL-1β and ACh levels and rescued mice from fatal TsV administration (170). As such, the study determined that PGE_2 amplifies IL-1β release, which upon binding IL-1R potentiates upregulation of PGE_2 and PGE_2-dependent ACh release post TsV envenomation (170).

Existing scorpion sting management comprises specific antiserum and symptomatic treatment, such as pain and low dose anti-inflammatory medications (6). Grade III stings and stings in children younger than 15 require intensive care (6). Polymorphism within scorpion venom-derived proteins is geographically varied, impeding the manufacture of a standardised antivenom (6). Variability in toxin immunogenicity further limits the usefulness and cost-effectiveness of antivenom production (6). Accordingly, some experts challenge the use of antiserum therapy due to insufficient neutralising capacity and the additional shock risk associated with poorly purified serum (6, 181).

Recently, success has been reported using novel immune-based therapies in animal models of TsV envenomation. In 2019, Zoccal et al. showed that the experimental peptide, EP80317 (a CD36 ligand), protected C57BL/6 mice against a lethal dose of TsV (97). Indeed, the therapeutic administration of EP80317 at 0.5 h and 2 h post-envenomation provided complete protection against a lethal dose of venom. Lymphocytes and neutrophils in the bronchoalveolar lavage fluid were significantly lower in the treatment group than venom alone. Accordingly, cAMP concentrations and proinflammatory cytokines (IL-1β, IL-6, TNF, and CCL3) were also considerably decreased (97).

Although promising, the estimated time and cost of developing a new drug and bringing it to market is 10 – 15 years and hundreds of millions of dollars (182). Conversely, drug repurposing circumvents the requirement for lengthy and expensive preclinical development. A recent *in vivo* study from the same group found that therapeutic administration with high dose dexamethasone (DEX) (5 mg/kg) improved TsV-induced cardiac dysfunction and reduced mortality after a fatal venom dose (170). The study showed that early treatment (15 min and 1 h post-inoculation) strongly suppressed PGE_2 and IL-1β release in tissues, abrogating systemic ACh and IL1R-mediated/ACh-induced cardiac dysfunction (170).

In reviewing the effects of jellyfish venom on the immune system, Tibballs et al. highlighted similarities between the clinical features of Irukandji syndrome and scorpion envenomation (149). While the mechanisms underpinning the pathology of Irukandji syndrome have remained unresolved for decades, they may likewise involve both autonomic and inflammatory pathways. If so, immune-based therapies may also prove beneficial in severe box jellyfish envenomation and warrant further investigation.

Irukandji Syndrome

While most jellyfish stings do not require medical attention, several species found in tropical waters constitute a public health threat (141). In Australia, the box jellyfish *Chironex fleckeri* and *Carukia barnesi* are of particular medical relevance. A high *C. fleckeri* venom dose can cause rapid and fatal cardiac arrest (183). In contrast, the smaller box jellyfish, *C. barnesi*, induces an extremely painful systemic pathology known as Irukandji syndrome (IS) (184–186).

C. barnesi was the first confirmed causative agent of IS after its namesake, Dr Jack Barnes, famously subjected himself, his nine-year-old son, and a local lifeguard to intentional envenoming in 1961 (187). Yet, due in part to the elusive nature of this highly venomous jellyfish, research over the years has failed to unravel the mechanisms behind the distinctive syndromic illness (184, 188). *C. barnesi* are small and transparent, with the medusal bell measuring ~20 mm wide (**Figure 1**) (184, 189). As with other carybdeids, *C. barnesi* have a single tentacle per pedalium (184, 189). Both bell and tentacles are covered with nematocysts, comprising distinct venom composition (190).

A retrospective case study of 128 marine sting presentations to Cairns Base Hospital revealed a wide variation of symptom severity among individuals (186). Of the 39 patients with skin scrapings consistent with *C. barnesi* nematocysts, some experienced only minor symptoms, while in others, envenomation proved fatal (186). Typically, an IS presentation includes a mild local reaction followed by a characteristic incubation period of five to 60 min before the onset of systemic effects (**Figure 2**) (191, 192). Pain in the abdomen, chest, lower back, limbs, and joints, is severe, often intractable to opioids and accompanied by extreme distress and agitation (193, 194). In parallel, the manifestation of tachycardia, hypertension, diaphoresis, dyspnea, and in some instances, priapism may occur (193, 194). In severe cases, life-threatening complications, such as cardiomyopathy and cardiogenic shock, can arise (193). Due to the significant cardiac dysfunction associated with *C. barnesi* envenomation, cardiogenic pulmonary oedema may develop (195, 196). Tragically, venom-induced intracerebral hemorrhage resulted in the death of two individuals in 2002 (192, 197).

An "Irukandji" antivenom is unavailable, and, as *C. barnesi* is only one of several causative species, an antivenom is unlikely to be produced (198). Therefore, treatment of severe envenomation is heavily reliant upon opioid-based pain management and symptomatic supportive care, with a mean expected hospital stay of 1.6 days (186). The clinical manifestations of IS have been attributed to excessive catecholamine release, such as seen in pheochromocytoma, scorpionism, or funnel-web spider envenomation (149, 198, 199). As such, IS has been described as "a painful hypercatecholaminergic condition" (193). Accordingly, individuals at particular risk of fatal outcomes are those with pre-existing cardiovascular pathologies, potentially making the use of alpha/beta-adrenergic blocking agents prohibitive (200).

Supporting this hypothesis, adrenaline and noradrenaline have been transiently detected in the plasma of *C. barnesi* experimentally envenomed piglets (200). Peak catecholamine release was observed 10 min post intravenous (IV) venom administration, coinciding with the onset of systemic and pulmonary hypertension and tachycardia (200). Plasma

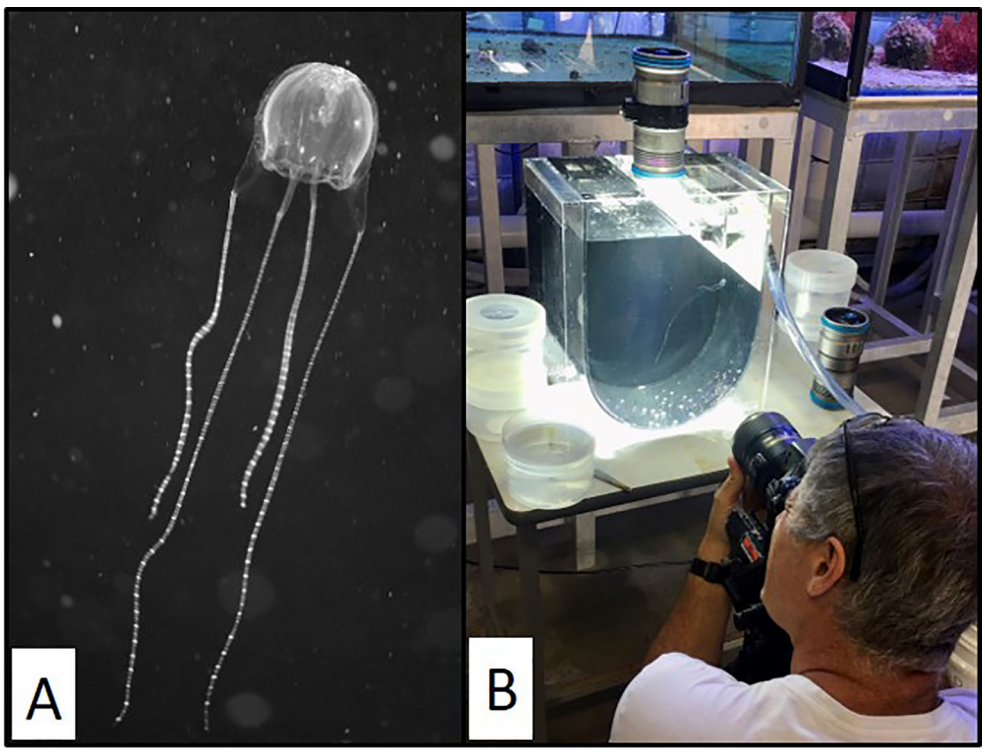

FIGURE 1 | Image of Carukia barnesi jellyfish. Images showing **(A)** close up and **(B)** relative size of adult *C. barnesi* jellyfish. Prof Jamie Seymour pictured. Photos were taken by **(A)** Jamie Seymour (JCU, Cairns, Australia) and **(B)** Rachael Ryan (JCU, Cairns, Australia).

catecholamines remained elevated in envenomed animals until 60 min but declined to non-significant levels within 2 h (200). Pretreatment with 1 μmol/L tetrodotoxin (TTX) attenuated tachycardia responses from rat and guinea-pig isolated right atria but did not significantly alter venom-induced contraction of rat mesenteric small arteries (200). These data suggest the presence of a presynaptic neuronal voltage-gated sodium channel agonist within the venom, as well as the presence of a TTX-insensitive vasoconstrictor (149, 198–200). While a physiological stress response towards IV administration of any toxin may similarly stimulate adrenaline and noradrenaline release, the authors reported the uniqueness of the reaction compared to other box jellyfish venom (200).

Research by Ramasamy et al. found pretreatment with prazosin (50 μg/kg) partially reduced tachycardia in *C. barnesi* envenomed rats, further supporting the role of endogenous catecholamines in the pathogenesis of IS (198). However, the residual pulse pressure observed in the study suggested the contribution of factors besides catecholamines (198). Furthermore, the result was not reproduced by Winkel et al. with 0.3 μM prazosin pretreatment, possibly due to dose- or time-dependent factors that were not clearly stated in either study (198, 200). Unfortunately, as both cardiovascular studies required the use of anesthetised animals, euthanised after 2 h, the critical evaluation of later time points was not possible (198). Yet, in sting victims, symptoms can remain for days, potentially requiring intensive care (186).

Regardless, in line with these findings, current clinical guidelines recommend magnesium sulphate ($MgSO_4$) therapy to attenuate pain and suppress excessive catecholamine release in severe IS (201). Its success in doing so has generated divided opinions (202). The results of a randomised trial completed in 2012, and reviewed in 2017, were unable to confirm the ability of $MgSO_4$ infusion to reduce opioid requirement (202, 203). Both studies reported varied success from the 39 patients, ultimately showing no significant benefit from $MgSO_4$ therapy (202, 203).

Akin to scorpion envenomation, the symptoms of IS cannot be wholly attributed to sympathetic hyperstimulation (10). Also akin to scorpionism, generalised IS symptoms resemble those of CRS. Interestingly, $MgSO_4$ potently suppresses MNC-mediated cytokine production following TLR stimulation (204). $MgSO_4$ increases IκBα levels in MNCs, thereby decreasing NF-κB nuclear translocation and its activity (204). Accordingly, the ability of $MgSO_4$ to inhibit pain in some sting patients could in part be due to a dampened immune response, although this theory has not been investigated.

Presently, neither catecholamines nor inflammatory mediators have been measured in *C. barnesi* sting patients. Recently, a study by Staedtke et al. proposed an intriguing link between "cytokine storm" and "catecholamine storm" in systemic inflammatory response syndrome (SIRS) and capillary leak syndrome, which may apply to venom-induced systemic inflammation (205, 206). This study showed that adrenaline

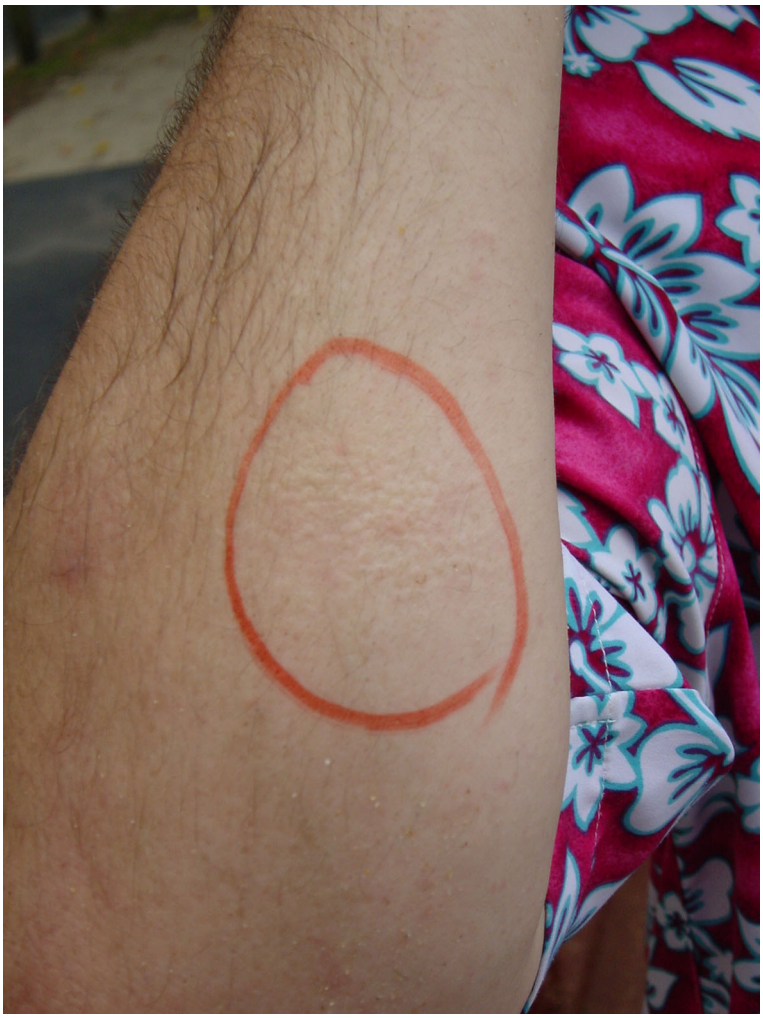

FIGURE 2 | Local response to *C. barnesi* envenomation. Image showing a typical dermal reaction on the arm following a sting from a *C. barnesi* jellyfish. The red marker indicates the sting site. Photo by Jamie Seymour (JCU, Cairns, Australia).

contributes to the positive feed-forward cytokine dysregulation seen in CRS (206). Encouragingly, the blockade of α_1-adrenergic receptors (also expressed on immune cells) or the inhibition of tyrosine hydroxylase (required for catecholamine biosynthesis) by prazosin or metyrosine hindered the self-amplifying proinflammatory loop *in vitro* and *in vivo* (206).

Specifically, CRS, induced by humanised CD19 CAR-T cells in mice engrafted with a leukemia cell line, caused excessive levels of adrenaline, noradrenaline, and myeloid-derived cytokines (IL-6, KC, MCP-1, and TNF) in the plasma of animals with high tumour burdens (206). Consequently, higher mortality was observed among these mice. In contrast, pretreatment with metyrosine or prazosin lowered circulating catecholamines and cytokines, improving survival (206). In mouse peritoneal MΦs, LPS-stimulation induced the release of catecholamines and proinflammatory cytokines, IL-6, KC, MCP-1, and TNF (206). Cytokine and catecholamine secretion was markedly enhanced in LPS/adrenaline co-cultures (206). Conversely, reduced MΦ catecholamine production significantly reduced IL-6, KC, MCP-1, and TNF levels (206). Although this model is unrelated to envenomation, it suggests that immune stimuli, such as venom-induced TLR activation, initiates a proinflammatory response that is enhanced by the presence of catecholamines. The dual stimulatory signals create a positive feed-forward loop, resulting in cytokine amplification. Collectively, these studies suggest a therapeutic potential of prazosin for severe IS, except where contraindicated, and warrant the investigation of plasma cytokines in sting patients.

However, while such research supports the therapeutic inhibition of catecholamines in envenomation, understanding their possible protective function has not been investigated. For example, it is known that catecholamine release promotes alveolar fluid clearance (174). In scorpionism, a rapid catecholamine surge following a dangerous sting is reasoned to increase alveolar fluid reabsorption, protecting the lungs from venom-induced flooding (174). Therefore, the resolution of the

surge may decrease the ability of the lungs to clear venom-induced oedema, ultimately progressing toward fatal pulmonary oedema (174). Accordingly, animal models of IS should thoroughly scrutinise both the benefits and limitations of these mediators.

Finally, given the immune system's propensity toward hyper-responsiveness and allergic reaction to jellyfish venom, the overlap in IS and CRS generalised symptoms, and the recently described link between CRS and catecholamine storm, immune involvement in the pathology is plausible. Nevertheless, to date, no *C. barnesi* immunology-based research has been published.

THE IMMUNOSUPPRESSIVE POTENTIAL OF VENOM-DERIVED MOLECULES

Despite the health burden of human envenomation, venom-immune interactions have been exploited in traditional medicine for centuries (207). More recently, research groups throughout the world have demonstrated the *in vitro* and *in vivo* efficacy of whole venom and venom-derived compounds in ameliorating a wide range of autoimmune symptoms (112, 208–211).

Presently, the most promising drug leads belong to the class of ion channel modulators. Ion channels, particularly calcium-activated and voltage-gated potassium channels, are attractive therapeutic targets for autoimmune diseases. Firstly, ion channels, such as the Shaker-related voltage-gated $K_V1.3$ and Ca^{2+}– dependent KCa3.1, regulate Ca^{2+} signalling in activated immune cells, allowing cell depolarisation and maintenance of membrane potential. Intracellular Ca^{2+} levels dictate T cell activation, proliferation, metabolism and cytokine production (212). Secondly, unique ion channel dimers are differentially expressed in various tissues, including immune cell subsets, permitting cell type and subset-specific blockade (213, 214). For example, activated effector memory T cells (T_{EM}), B cells and MΦs, known mediators in the pathogenesis of various autoimmune diseases, preferentially upregulate $K_V1.3$ (215–217). In contrast, naïve (T_n) and central memory cells (T_{CM}) express KCa3.1 ion channels, allowing for channel-specific inhibition (218). Finally, inhibition of Ca^{2+} influx *via* ion channel blockade allows targeted and reversible immune modulation, rather than complete T cell suppression, as induced by T cell Ca^{2+} modulating drugs, including calcineurin inhibitors and steroids.

Venom from snakes, spiders, scorpions, cone snails, and sea anemones comprise a diverse range of peptide and small molecule ion channel blockers that exhibit selectivity at picomolar concentrations (219). Blockade of lymphocyte ion channels using venom-derived compounds has therapeutic effects in animal models of rheumatoid arthritis (RA), asthma, multiple sclerosis (MS), delayed-type hypersensitivity, and allograft rejection (113, 208, 219–224). Notably, a selective peptide blocker, *Stichodactyla helianthus* toxin (ShK), from sea anemone venom, and anuroctoxin, a peptidyl toxin isolated from *Buthus sindicus* scorpion venom, have been shown to specifically target $K_V1.3$ channels with high affinity, preventing Ca^{2+} influx and thereby inhibiting T_{EM} activation, proliferation and cytokine production (113, 115, 225–227).

Structural studies centred on the selectivity of peptide ion channel blockers have revealed that specificity is due to single amino acid effects rather than *en bloc* backbone structure (228). Thus, venom-derived peptides may act as promising drug scaffolds, notably because disulphide bonds encode robust biological stability (229). This has important implications for drug development, as synthetic manipulation may improve drug activity or remove toxicity from the natural peptide blueprint. For example, ShK(L5), a synthetic analog of ShK, contains an N-terminal L-phosphotyrosine extension and shows higher selectivity than the native peptide for $K_V1.3$ channels over the neuronal ion channel $K_V1.1$ (113).

Aside from ion channel blockade, venom-derived components have demonstrated potent *in vitro* and *in vivo* anti-inflammatory activity through the cholinergic anti-inflammatory pathway *via* an alpha7 nicotinic acetylcholine receptor antagonist (114). Venom-derived peptides, such as the α-neurotoxin from the Thailand cobra, are potent nicotinic receptor antagonists (218). In a rodent RA model, Cobratoxin-treatment reduced expression of the pro-inflammatory cytokines IL-1β, IL-2, and TNFα, resulting in decreased paw sensitivity and joint destruction (230).

Other neurotoxins, such as the principal toxin (NTX) from *Naja atra* venom (NNAV), have shown therapeutic effects in animal models of adjunctive arthritis, RA, Systemic Lupus Erythematosus (SLE), and nephropathy (209, 211). Additionally, NTX-treatment prolonged skin allograft survival in rats and inhibited cell-mediated immune responses in a dose-dependent manner through decreased Th1-type cytokines (IL-2 and IFN-γ). Although low NTX concentrations were cytotoxic, heat-treatment reduced NTX toxicity without reducing its immunosuppressive activity (211). In another study, orally administered NTX suppressed murine T cell proliferation, specifically Th17 and $CD8^+$ T cell activity, increasing NK cell and B cell proliferation in a dose-dependent manner (209).

Venom from the honey bee has been used for centuries in traditional medicine to treat chronic inflammatory diseases due to its reported anti-inflammatory activity (207). Investigations into the mechanism of action of honey bee venom and its major components, melittin and phospholipase A2, have confirmed a protective effect in animal models of asthma and RA (207, 231). The polarisation of T cells towards a Th2 phenotype is associated with allergies and chronic inflammatory diseases (232). An essential driving factor in lineage determination is cytokine expression. It has been shown that melittin inhibits LPS-induced inflammation by binding to the C- terminus of the NF-κB p50 subunit, thus preventing translocation into the nucleus and transcription of pro-inflammatory cytokines, including TNF (233–235). Moreover, treatment with whole honey bee venom polarised T cells towards a Th1 phenotype by inducing T-bet and IFN-γ in $CD4^+$ T cells (210). Conversely, PLA_2, an enzyme found within the venom of multiple species, including the western honey bee, can hydrolyze membrane

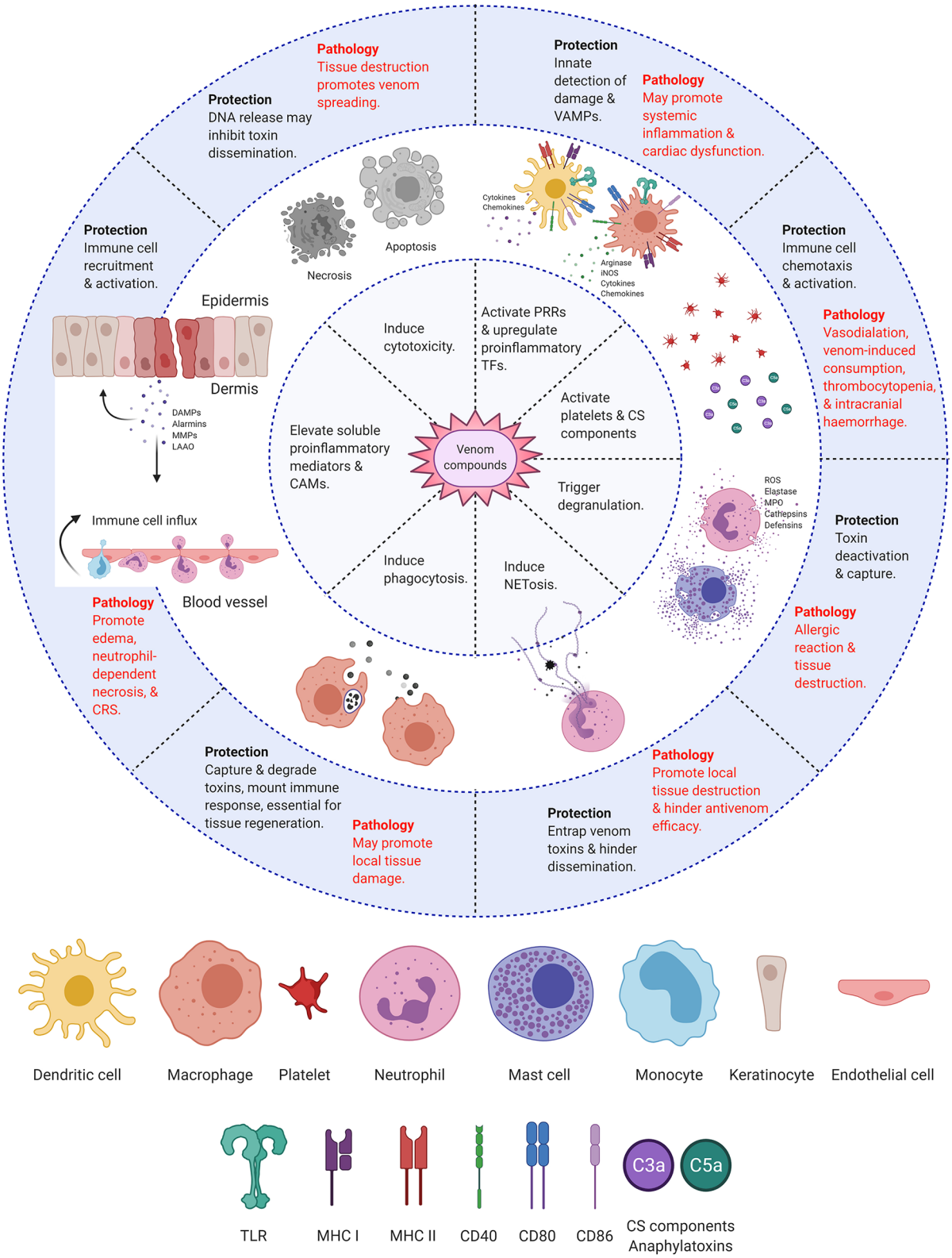

FIGURE 3 | Immunological responses to envenomation. Diagram summarizing the protective and pathological responses of the host's immune system towards venom compounds. Created with BioRender.com.

phospholipids and induce Th2 cytokine responses through the activation of ST2, a component of the IL-33 receptor on innate immune cells (236).

Other known venom immune modulators include tick salivary protein (Salp) 15 from *Ixodes scapularis* and spermine. Salp15 binds the CD4 co-receptor, MHC-II, inhibiting TCR ligation and T cell activation by misaligning CD4 with the TCR complex (237). Spermine, an acylpolyamine found in snake and spider venom, suppresses mitogen-induced activation and proliferation of PBMCs by inhibiting LAF-1 protein expression, involved in RNA remodelling (238).

Collectively, these studies highlight the potential of venom-derived molecules to modulate immune cells as unmodified venom-derived compounds or as scaffolds for drug development. Venom-derived compounds induce immune suppression using diverse modes of action. Thus, screening venom for its immunosuppressive and immune-activating potential may result in new immunomodulatory drugs and the discovery of new biological pathways.

CONCLUSION

Conferring protection against venom's potentially lethal action requires rapid immune recognition and response. Extensive research focuses on the degree to which immune responses themselves contribute to the severity of envenomation (**Figure 3**). However, there is disagreement regarding whether the body's defensive reactions are helpful or harmful. Perhaps the most significant cause of division lies in the difficulty of distinguishing the actual venom-induced symptoms from immune-induced pathology. The classic inflammation markers (heat, pain, redness, swelling, and loss of function) are typical biological responses to envenomation across many species. Therefore, determining which symptoms are treatable using immunological approaches requires further research.

Nevertheless, venom's ability to modulate immune activity has two therapeutic implications. Firstly, continued research could inform improved treatment strategies for fatal bites and stings. Secondly, as venom is a rich source of specific and potent biomodulators, exploring venom-immune interactions may lead to discovering novel pathways/receptors or the development of venom-derived immunomodulatory drugs.

AUTHOR CONTRIBUTIONS

Writing—original draft preparation, RR. Writing—review and editing, RR, JM, MI, JS, AL, and JL. Supervision, JM, MI, and JL. Funding acquisition, JM. All authors contributed to the article and approved the submitted version.

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REVIEW
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Crotalus Durissus Ruruima: Current Knowledge on Natural History, Medical Importance, and Clinical Toxinology

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Crotalus durissus ruruima is a rattlesnake subspecies mainly found in Roraima, the northernmost state of Brazil. Envenomings caused by this subspecies lead to severe clinical manifestations (e.g. respiratory muscle paralysis, rhabdomyolysis, and acute renal failure) that can lead to the victim's death. In this review, we comprehensively describe *C. d. ruruima* biology and the challenges this subspecies poses for human health, including morphology, distribution, epidemiology, venom cocktail, clinical envenoming, and the current and future specific treatment of envenomings by this snake. Moreover, this review presents maps of the distribution of the snake subspecies and evidence that this species is responsible for some of the most severe envenomings in the country and causes the highest lethality rates. Finally, we also discuss the efficacy of the Brazilian horse-derived antivenoms to treat *C. d. ruruima* envenomings in Roraima state.

Keywords: *Crotalus durissus*, *Crotalus durissus ruruima*, rattlesnake, snakebite, envenoming, venom, antivenom

CROTALUS DURISSUS RURUIMA: NATURAL HISTORY

The species *Crotalus durissus* is widely distributed in South America and occurs sporadically from Colombia to Argentina. It includes 11 subspecies (*C. d. durissus, C. d. cascavella, C. d. collilineatus, C. d. cumanensis, C d. marajoensis, C. d. maricelae, C. d. ruruima, C. d. terrificus C. d. trigonicus, C. d. unicolor and C. d. vegrandis*) (1). The highly uneven distribution of *C. durissus* in South America, which includes open habitats to the north and south of the Amazon rainforest as well as

open relictual formations, adds considerable interest to phylogeographic studies of this complex species (1). In Brazil, this rattlesnake species (*C. durissus*) presents six subspecies (*C. d. durissus*, *C. d. cascavella, C. d. collilineatus, C. d. marajoensis*, *C. d. ruruima* and *C. d. terrificus*) (2). The subspecies *C. d. ruruima* was described by Hoge in 1966 (3), based on specimens collected at Mount Roraima in Venezuela (**Figure 1**). Among the six Brazilian rattlesnakes, the *C. d. ruruima* is one of the most intriguing subspecies due to its restricted distribution (limited to the northern area of the state of Roraima state in Brazil and southern Venezuela) and the unique biochemical and pharmacological properties of its venom (5, 6).

Geographic Distribution

C. d. ruruima occurs in open areas of savannas (*lavrados*) in the state of Roraima (Brazil) and on the Mount Roraima slopes and Mount Cariman-Peru in the state of Bolívar (Venezuela) (7) (**Figures 2B, C, G, H**). It may also occur in adjacent territory of Guyana (7). In Roraima, *C. d. ruruima* has been recorded at the edge of forests near the municipality of Rorainópolis, in Viruá National Park in Caracaraí, in Caubi near the Mucajaí River, Apiaú, Taiano, Fazenda Salvamento, Maloca Boqueirão, Maloca da Mangueira in Alto Alegre, Vila Serra Grande-Cantá, Maracá Island, and to the west of Tepequém in Amajari. In the open regions (working land), it has been recorded close to Boa Vista city on the 7th Infantry base, Bom Intento, Monte Cristo, Passarão, Campo Alegre, Igarapé Garrafa, and Igarapé Carrapato, and follows the open formations from Bonfim to Normandia and the far north of Pacaraima and Uiramutã.

C. d. ruruima is restricted to the open areas of the savannas, which is the expected behavior for this species whose life history is related to these plant formations (8). However, it has already been recorded in the Apiaú region probably by the intense deforestation that provided many open areas that the rattlesnake colonized. This type of population dispersion for *C. durissus* has already been recorded in Rio de Janeiro for subspecies *C. d. terrificus* (9).

Taxonomy

C. d. ruruima was recognized by Peter & Orejas-Miranda (1970) (10) and Cunha & Nascimento (1980) (11) as a valid taxon. The geographically close subspecies, *C. d. dryinus* and *C. d. trigonicus*, were synonymized with *C. d. ruruima*. *C. d. trigonicus*, described by Harris & Simmons (1978) (12), was synonymized with *C. d. dryinus* by Abuys (1987) (13) and subsequently, following the recommendations of Cunha and Nascimento (1980) (11), with *C. d. ruruima* (14). Vanzolini & Caleffo (2002) (8) examined a paratype of *C. d. dryinus* and agreed with Rubio (1998) (14). Campbell and Lamar in 2004 (7) considered the validity of *C. d. trigonicus* questionable. Costa and Bérnils (2018) did not consider *C. d. trigonicus*, and indicated only *C. d. ruruima* for Roraima (2).

Morphology

The name of the genus *Crotalus* derives from the Greek word "Krotalon", which means "rattle" or "castanets" in reference to the characteristic appendix at the end of the tail of these snakes (7) (**Figure 2A**). The rattlesnake belongs to the Viperidae family and Crotalinae subfamily, which is characterized by presenting species with a loreal pit, vertical pupils and keeled dorsal scales (7). Similar to the other subspecies of *C. durissus*, *C. d. ruruima* has a distinct pair of dark stripes on the neck and a triangular head, which is distinguishable from the neck (7).

C. d. ruruima presents 167-170 ventral scales in males and 174-177 in females, and 25-29 subcaudal scales in males and 21-23 in females (3). In relation to the coloring pattern, the paravertebral bands are distinctly marked with white and lighter scales in the center, with the same light center pattern and white outer edges in the head markings (3). The dorsal diamonds are less contrasted than in *C. d. durissus* and sharply surrounded with white, thus approaching the pattern of the specimens of *C. d. terrificus* from the southern end of Brazil. Some specimens have a tendency to have the obliterated pattern of *C. d. vegrandis*.

Size

The total body length of *C. d. ruruima* varies between 315 mm and 1345 mm, and the average length is 839 mm (15). The smallest male and female presented 368 mm and 315 mm, respectively, and the largest male and female presented 1250.9 mm and 1115 mm, respectively (15). Female specimens tend to be slightly larger, presenting a mean rostro-cloacal length of 630.9 mm (n=22), while males presented 627.3 mm (n=41) (15). However, sexual dimorphism was not statistically evident in *C. d. ruruima*; thus the analysis of a greater number of specimens is necessary for a better understanding of possible differences in body length (15). Generally, in species in which male individuals are larger than female, male-to-female combat occurs (16), and this behavior has already been witnessed in populations of *C. durissus* in southeastern Brazil (17, 18).

Habitat and Activity

Rattlesnakes of the species *C. durissus* are characteristic of open areas (savannas), while it can also occur in areas that have been altered by human activity (pastures and crops) and forest edges (9, 15, 19–23). In Roraima, this snake is present in the open areas of savannas, woods edges, areas modified by the crop plantings (e.g. corn and soybean), and acacia (*Acacia* sp.) plantations and pastures (15, 19, 22).

The rattlesnakes present greater activity during the night twilight period, and are relatively inactive at the beginning of the day (6 to 8 am), at this time usually going from areas such as firebreaks to their nests (15). During the afternoon, they usually do not present activity; thus, a large part of the specimens are observed in this period inside their burrows (15). During the twilight-night period, these rattlesnakes present greater activity between 7 pm and midnight, with their activity peak between 9 pm and 10 pm (15).

When they are not hunting during the day, they remain inside their burrows and among grasses and shrubs (15), probably because these microhabitats favor thermoregulation and also contribute to the protection from possible predators (20). In this situation, where the snake is partially covered by vegetation, its visual detection becomes more difficult (24). Thermoregulation

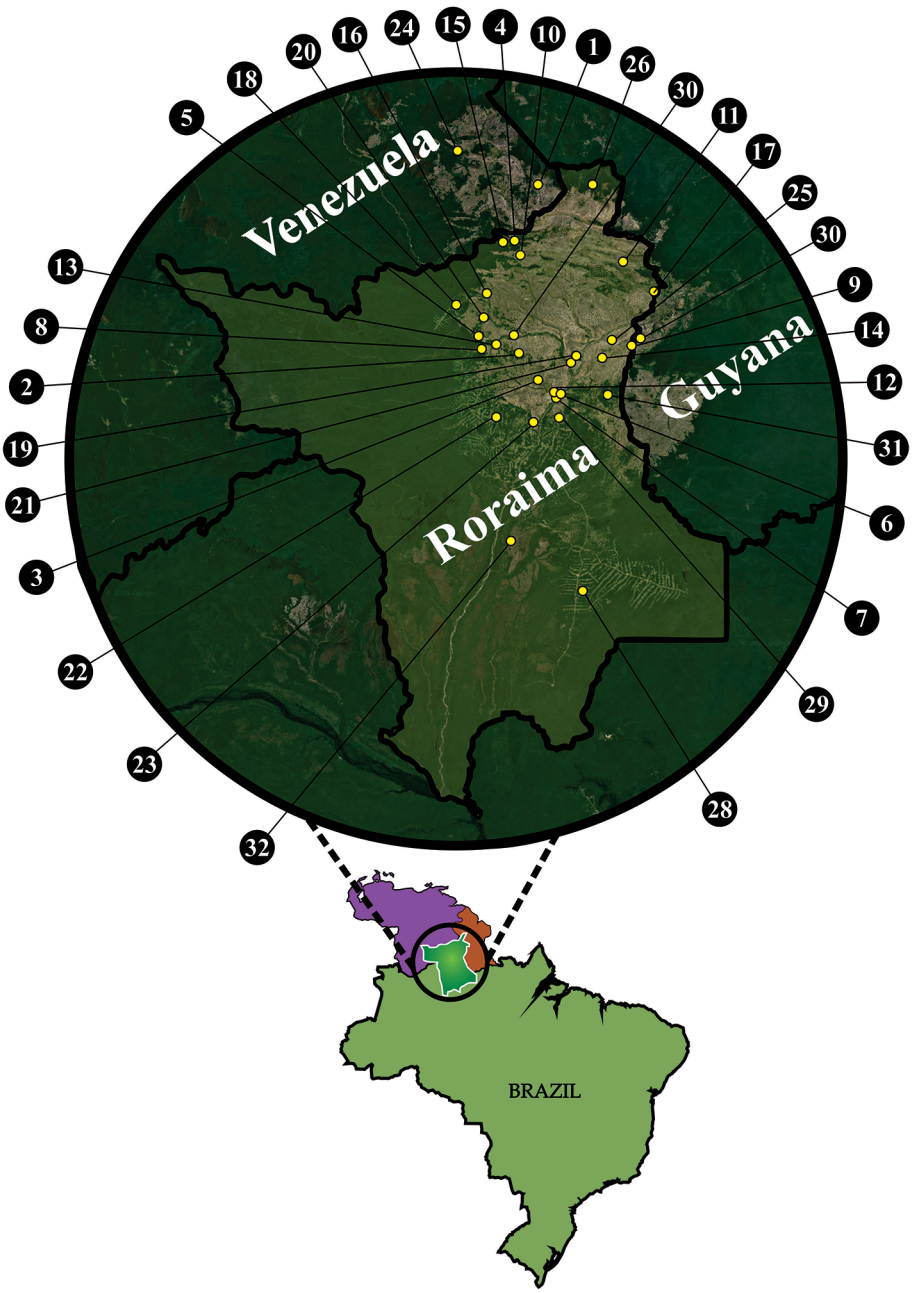

FIGURE 1 | Map with records of *Crotalus durissus ruruima* in Brazil (BR), Venezuela (VE), and Guyana (GU): 1 - Paulo Camp., Mount Roraima, VE (5°00'N;60° 52'W) (21); 2 - Taiano, Alto Alegre, RR, BR (3°15'N;61°04'W) (21); 3 - Bom Intento Farm, Boa Vista, RR, BR (2°58'N;60°52'W) (14); 4 -Pacaraima, RR, BR (4° 25'N; 61°08'W) (21); 5 - Maracá Island, RR, BR (03°25'N; 61°29'W) (18); 6 - Boa Vista, RR, BR (02°49'N;60°39'W) (21); 7 - 7° BIS, Boa Vista, RR, BR (02° 47'N; 60°41'W) (21); 8 - Maloca Mangueira, Alto Alegre, RR, BR (03°18'N;61°27'W) (21); 9 - Bonfim, RR, BR (03°21'N;59°49'W) (21); 10 - Surumu River, RR, BR (04°16'N;61°03'W) (21); 11 - Maloca Boqueirão, Alto Alegre, RR, BR (04°12'N; 59°59'W) (21); 12 - Monte Cristo Farm, Boa Vista, RR, BR (02°51'N; 60° 42'W) (21); 13 - Salvamento Farm, Alto Alegre, RR, BR (03°20'N; 61°18'W) (21); 14 - Igarapé Garrafa, Boa Vista, RR, BR (03°12'N; 60°12'W) (21); 15 - Sorocaima, RR, BR (04°25'N; 61°11'W) (21); 16 - Três Corações, RR, BR (03°52' N; 61° 24' W) (this study); 17 - Normandia, RR, BR (03°51'N, 59°35'W) (14); 18 - Tepequém, RR, BR (03°45'N; 61°43'W) (21); 19 - Campo Alegre, Boa Vista, RR, BR (3°16'31.7"N 60°31'27.0"W) (14); 20 - Amajari, RR, BR (03°37'N; 61° 26'W) (21); 21 - Passarão, Boa Vista, RR (03°11'N;60°35'W) (14); 22 - Apiaú, Alto Alegre, RR, BR (02°35'N;61°18'W) (14); 23 - Mucajaí, RR, BR (02°32'N;60° 55'W) (14); 24 - Carimán-Paru, Gran Sabana, VE (0521N; 6142W) (4); 25 - Bonfim, RR, BR (03°23'N; 60°06'W) (14); 26 – Uiramutã, RR, BR (4°60', 60.18'W) (21); 27 - Colônia Coronel Mota em Taiano, Alto Alegre, RR, BR (03 °26'N, 61.07'W) (21); 28 - Rorainópolis, RR, BR (0°46'27.7"N 60°24'13.6"W) (21); 29 - Vila Serra Grande, Cantá (this study), RR, BR (2°34'47''N 60°38'57''W); 30 – Lethem, GU (3°22'59''N 59°48'17''W) (14); 31 - São Francisco Village, Bonfim, RR, BR (2° 48'58''N 60°08'34''W) (this study); 32 - Viruá National Park – Caracaraí, RR, BR (1°17'39.3"N 61°09'04.6"W) (21). Georeferencing was made with QGis software and the final figure was prepared using CorelDraw.

constitutes the main diurnal activity of nocturnal Viperidae snakes (20, 24), which may explain most cases of snakebite in Roraima state, since this period is also that of human activity in the area (25, 26).

Diet and Foraging Activity

C. durissus is terrestrial and hunts mainly by waiting for rodents (20, 21, 27) (**Figure 2F**). Small mammals, mainly rodents, make up most of the diet of *C. durissus* in the southeastern (27, 28) and central regions (29) of Brazil, though lizards (*Ameiva ameiva*) are also consumed in a smaller proportion. Birds have also been recorded in the diet of northeastern populations (30) (**Figure 2D**). Indeed, rodents were the main findings in the stomach contents of *C. d. ruruima* and, less frequently, lizards (*Tropidurus hispidus*) (15). Unlike other populations of *C. durissus*, anurans (*Leptodactylus macrosternum*) were found in *C. d. ruruima* stomachs (31) (**Figure 2E**), which may be related to differences observed by some authors (6) in the venom of this subspecies.

In relation to the hunting strategy, just as in the populations of other subspecies (20, 23), *C. d. ruruima* hunts by waiting on the ground, seeking preys that exhibit surface activity (rodents, lizards e.g., *T. hispidus* and the amphibians e.g., *L. macrosternum*) (15). In the burrows where they usually shelter, there have been cases in which 5 or 7 rattlesnakes were found occupying the same burrow (15).

Seasonality

During the year, *C. d. ruruima* shows greater activity from August to February (15), coinciding with warmer and drier days, which have an average temperature of 27 °C and average humidity of 71.2%.

Reproduction

C. durissus is a viviparous species, giving birth to an average of 11 young in southeastern Brazil and between 12 and 33 in northeastern Brazil (32). The populations of the southeast present long vitellogenesis, beginning in March, with gestation between October and January and the birth of the offspring between January and March (33, 34). Male individuals can perform combat ritual (17, 18).

There is little information regarding aspects of reproduction of *C. d. ruruima* (15). Males are more active throughout the year with peaks in the months of August to November, while females are more frequent in the months of December to the end of January (15). Juveniles are more frequent during the months of March to May (15), during the rainy period in Roraima (March to June) (35), which probably corresponds to the birth period in offspring. Two pregnant females were recorded during the month of January, one has the total body length of 580 nm with seven embryos and the other of 730 mm with nine.

Defensive Behavior

When a human approaches, the rattlesnake (*C. durissus*) most often flees but may turn the head and anterior region of the body in the direction of the observer, while shaking the rattle of the tail and curling itself up (21). Usually, this snake only shakes its rattle when a person comes very close to it (about a meter or less) (21). Sawaya et al. also studied the defensive behavior of *C. durissus* in the Cerrado area of southeastern Brazil. When handled, it was observed that this snake can shake the rattle of the tail, strike a bite, perform cloacal discharge, open its mouth, struggle, bite, squirt liquid from the cloacal gland in the form of jets, and flatten and rotate the body. The secretion from the cloacal gland of *C. durissus* has a strong smell and can cause nausea and burning in the mouth and eyes, indicating that this can be an important defensive tactic against predators (21).

Genetics

Several molecular analyses, which included different lineages of *C. durissus*, have generated strong support for monophyly. This includes the lineages of *C. durissus*, *C. simus*, *C. tzabcan* (southern lineages), and *C. culminatus*, together with two endemic species recently described for Central America: *C. mictlantecuhtli* and *C. ehecatl* (northern lineages) (1, 36–40).

All subspecies of *C. durissus* that occur in Brazil are sister groups to *C. d. vergrandis* and *C. d. cumanensis* from Venezuela (40). However, *C. d. ruruima*, together with *C. d. durissus* from Guyana (previously assigned as *C. d. dryinas*) form an arrangement clearly separated from the other subspecies found in Brazil. Furthermore, comparative genomic hybridization involving ancient and advanced lineages of snakes has shown that *C. d. ruruima* and *C. d. terrificus* (Brazilian subspecies) possess different landscapes of repetitive sequences in their genomes, that are likely associated with particular differentiation processes at species level (41). This highlights that the real diversity of the Neotropical rattlesnake complex is currently underestimated and it is yet to be fully investigated. As such, this would require comparative analyses encompassing all extant subspecies of South American *C. durissus* lineages.

CROTALUS DURISSUS RURUIMA: VENOM COMPOSITION

Snake venomics enables the qualitative and quantitative understanding of venoms of different species (42). There are few studies comparing the venomics of Brazilian rattlesnake subspecies (5, 43, 44). Interestingly, while originated from the same species (*i.e. C. durissus*), the Brazilian rattlesnake subspecies present significant differences in venom composition (**Table 1**). Venom of *C. d. ruruima* presents a great variability, with repercussions even in the color, what made the first researchers of this subspecies'venom to classify it in "white" and "yellow" venom (49), which was also observed in *C. d. terrificus* and *C. viridis helleri* venoms (50, 51). The yellow color of venoms is closely related to the presence of L-amino acid oxidases (LAAOs) (52), which will be discussed later. In this section, the main identified and/or isolated protein classes from *C. d. ruruima* venom are explored.

Crotoxin (CTX)

One of the most abundant protein family in *C. durissus* snake venoms is the CTX family (44, 45, 53). Crotoxin is a heterodimer protein composed of a complex of a basic subunit named

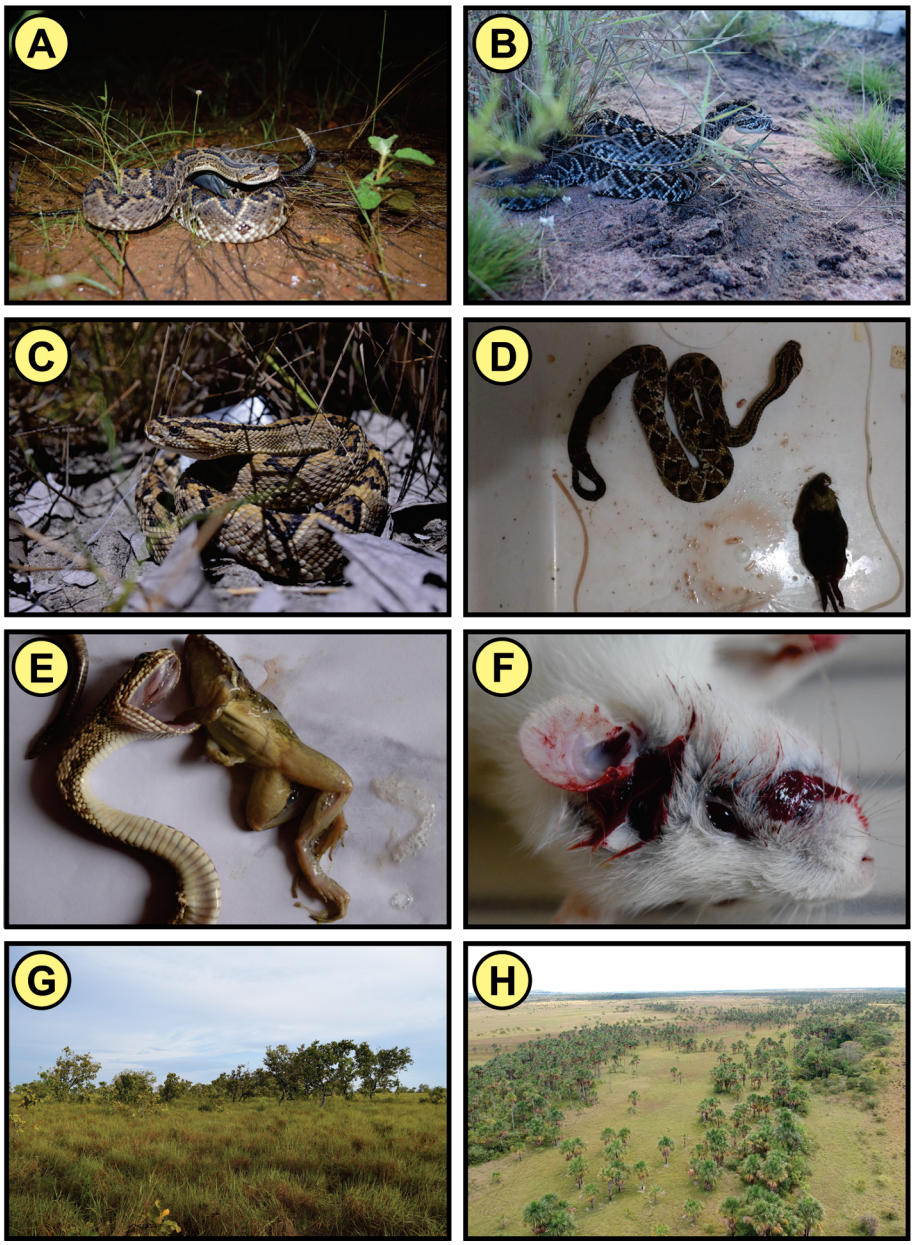

FIGURE 2 | **(A–C)** *Crotalus durissus ruruima*; **(D)** Specimen of *C. d. ruruima* with rodent found in its stomach contents; **(E)** Amphibian *Leptodactylus macrosternum* regurgitated by a juvenile specimen of *C. d. ruruima*; **(F)** Mouse recently bitten by a captive *C. d. ruruima* showing massive bleeding; **(G)** Region of savanna (*lavrado*) where *C. d. ruruima* occurs; **(H)** Aerial image of the savanna (*lavrado*) area where *C. d. ruruima* occurs. Pictures: Anderson Maciel Rocha.

phospholipase A_2 (PLA_2) and a non-toxic acidic subunit, crotapotin, which prevents the PLA_2 binding in to non-specific sites (54–56). Among subspecies CTXs' abundance varies from 48.5 to 82.7% of total venom composition, found more abundant in *C. d. ruruima* venom (**Table 1**) (5). Crotoxin-rich venoms from Neotropical *Crotalus* subspecies are classified into type II phenotype, characterized by a high lethality (57). CTX is responsible for neurotoxicity and myotoxicity, both important events on pathophysiology (5, 58–60). Studies have also brought novel perspectives for CTX as a possible pharmacological strategy due to its anti-inflammatory/immunosuppressive, anti-tumoral, and microbicidal effects (61–63).

Phospholipases A_2 (PLA_2s)

Snake venom PLA_2s constitute a puzzling group of molecules since, despite having a similar three-dimensional structure and highly conserved molecular regions, display a plethora of pharmacological activities such as myotoxic, neurotoxic, anticoagulant, hypotensive, hemolytic, platelet aggregation inhibition, bactericidal, and pro-inflammatory activities (64).

TABLE 1 | Venomic comparison of Brazilian *Crotalus durissus* subspecies.

C. durissus	Crotoxin	SVSP	CTL	SVMP				Crotamine	LAAO	BIP	Others	Ref.
				I	II	III	IV					
ruruima	82.7	8.1	4.3	–	–	2.9	–	1.5	<0.5	<0.1	–	(5)
cascavella	72.5	1.2	<0.1	–	–	<0.1	–	–	<0.1	–	20.3	(44)
collilineatus	67.4	1.9	<0.1	–	–	0.4	–	20.8	0.5	–	13.8	(44)
durissus							NR					
marajoensis							NR					
terrificus	48.5-82.7	0.7-25.3	<0.1-2.7			0.09-5.5		1-19	0.6-4.5	1.8	0.5-22.3	(5, 45–48)

BIP, bradykinin-inhibitory peptide; CTL, C-type lectin-like; LAAO, L-amino acid oxidase; NR, not reported; SVMP, snake venom metalloprotease; SVSP, snake venom serine protease.

Secretory PLA_2s occur in a large variety of venoms (*e.g.* snakes, arthropods, and mollusks), being able to cleave the *sn*-2 acyl bond of glycerophospholipids, releasing free fatty acids and lyso-phospholipids (65). Several studies have shown that *C. d. ruruima* venoms contains various isoforms of PLA_2 with distinct functions (6, 66–68). Cdr-12 and Cdr-13 are two isoforms that were isolated from *C. d. ruruima* snake venom. They present high content of Lys, Tyr, Gly, Arg, and 14 half-Cys residues, typical of a basic PLA_2 and show high homology to other D49 PLA_2s isolated from venoms of crotalic snakes (66).

Also, the crotoxin-like toxins were capable to induce myonecrosis and edema in mice, and a potent blockade of neuromuscular transmission in chicken biventer cervicis preparation (66). Cavalcante and colleagues (2015) also observed that a crotoxin isolated from *C. d. ruruima* "white" venom was capable to induce a neuromuscular blockade of indirectly evoked twitches of mice phrenic-diaphragm preparations (68).

Another PLA_2 isolated from *C. d. ruruima* venom is PLA_2A, a calcium dependent enzyme which shows antibacterial activity (67). The most recent PLA_2 isolated from *C. d. ruruima* is CBr (basic crotoxin), which presents its 20 amino-terminal residues identical to CB1 from *C. d. terrificus* venom. The CBr along with the whole venom were able to activate macrophages with focus on the formation of lipids droplets and synthesis of lipid mediators, suggesting its role on the production of inflammatory mediators during envenomings (6). Moreover, these PLA_2s could be responsible to potentiate toxicity of venoms, according to the synergism phenomenon (69).

Snake Venom Serine Proteases (SVSPs)

SVSPs comprise a group of extensively studied enzymes, widely found in the venom of terrestrial snakes from Viperidae, Elapidae, and Crotalidae families (70). SVSPs are catalytically active proteins able to degrade fibrinogen (71), resulting in several biological effects such as hemorrhagic, procoagulant, anticoagulant, platelet activation, and bradykinin-release (72).

Based on their functions, the procoagulant snake venom proteases are classified as factors I (snake venom thrombin-like enzymes, SVTLEs), V, VII and X, and groups C and D prothrombin activators (73). SVTLEs are the prevalent class of SVSPs from Viperidae venoms and present similar activities to human thrombin (74, 75). The SVTLEs are considered multifunctional toxins due to their broad substrate specificity and can act on different biological systems of the preys or the victims. Therefore, the investigation of the intrinsic pathways involved in the variety of biological activities of these molecules may contribute to expanding their potential applications (76).

Although *C. d. ruruima* venom presents SVSPs (8.1%) (5), these enzyme classes have never been isolated from this venom, unlike from *C. d. terrificus* (0.7-25.3%; **Table 1**) (5, 45–48), from which two SVSPs have been isolated, gyroxin and CdtSP2, presenting important roles in coagulation disturbances, neurotoxicity, and inflammation (77–79). Studies have also shown potential therapeutic use of SVSPs from *C. durissus* subspecies, such as the use for fibrin sealant in the treatment of dermatological ulcers (80). Also, an SVSP from *C. d. collilineatus* venom (1,9%) (44, 81) was recently shown to modulate ion channels (hEAG1, Kv10.1, KCNH1), helping to understand and reveal the complex and pluripotent pharmacology of SVTLEs, and also to open perspectives in terms of applicability particularly in the field of oncology due to its action on the oncogenic *ether-a-go-go* 1 voltage-gated potassium channel (70).

C-Type Lectin-Like Proteins (CTLs)

C. d. ruruima is the Brazilian subspecies that presents the highest abundance of CTLs (4.3%) (5), while the others subspecies present lower proportions in their venoms (<2.7%) (5, 45–48). Snake venoms CTLs are heterodimeric proteins, composed of two homologous subunits α and β (with 14-15 kDa and 13-14 kDa, respectively), which can be arranged in oligomeric complexes (82, 83). They show low primary sequence similarities to venom sugar-binding lectins, with no ability to interact with glycans. However, CTLs show an indiscriminate ligand spectrum, targeting clotting factors and several receptors on platelets, endothelial cells, and immune cells. This results in a wide range of pharmacological activities, acting in hemostasis and inflammation (84). There are no studies that disclose the isolation and characterization of this protein class from *C. d. ruruima*. Nonetheless, others CTLs have been described in *C. durissus* venoms. Convulxin, isolated from *C. d. terrificus* consists of the most studied CTLs, acting as a platelet-aggregating agonist that acts on the p62/GPVI collagen receptor in platelet surface (85). Other CTL is crotacetin, a convulxin-homologue toxin isolated from *C. d. terrificus*, *C. d. cascavella*, and *C. d. collilineatus* venoms with platelet aggregating and antibacterial activity (86).

Snake Venom Metalloproteases (SVMPs)

SVMPs comprise a group of metal-dependent proteases, playing a critical role in the proteolytic and biological activities of the venom (87). Zinc-dependent SVMPs belong to the metzincin family, which has a zinc-binding domain in common and structures very similar to each other. The zinc-binding site of this family has the amino acid sequence that is common to all subfamilies (88).

Metalloproteases are classified into three classes (PI, PII, and PIII) according to the organization of their multi-domains, considering the presence or absence of non-proteolytic domains observed in mRNA transcripts and in isolated proteins from snake venom (89). The PI-SVMPs are composed only of a metalloprotease domain. The PII-SVMPs have a metalloprotease domain followed by a disintegrin domain, which are often separated by a post-translational proteolytic cleavage. Both proteolytic products are stable (89). Finally, the PIII-SVMPs have a cysteine-rich domain (Cys-rich), in addition to metalloprotease and disintegrin-like (dis-like) domains. The PIII is subdivided into subclasses (PIIIa, PIIIb, PIIIc, and PIIId), reflecting the potential proteolytic processing and formation of dimeric structures, for which PIII has an additional lectin-like domain (89, 90).

According to the venomic approach, *C. d. ruruima* presents only PIII-SVMP in its venom composition (2.9%) (5), as well as *C. d. cascavella* (<0.1%) and *C. d. collilineatus* (0.4%) (44), although the latter also presents disintegrins (44, 53), while *C. d. terrificus* presents varied proportions of the SVMPs group (0.09-5.5%; **Table 1**) (5, 45–48). Although SVMPs from *C. durissus* subspecies are poorly studied, this group of toxins from *Crotalus* spp. from North and Central Americas are more abundant and have been widely investigated. These studies have shown hemostatic effects such as fibrino(geno)lytic and inhibition of platelet aggregation activities as well as local hemorrhage caused by degradation of the capillary basement membrane and muscle damage with lower regeneration (91–93). As *C. d. ruruima* venom composition differs from other more prevalent *C. durissus* subspecies envenomings, an antivenom based on oligoclonal mixtures of antibodies could be a strategy to improve treatment.

Crotamine

Crotamine, which may or may not be present, is one of the main components of rattlesnake venoms. This particularity indicates a Mendelian character of the toxin, since its frequency increases according to the east-west and north-south axes in Brazilian territory (44, 94). Although *C. d. ruruima* venom can present crotamine in their venom cocktail, its abundance is very low (1.5%) as shown in preliminary results (5) when compared to *C. d. collilineatus* venom (20.8%) (44). Crotamine has an evident myotoxic action during envenomings, which was demonstrated through increased levels of creatine phosphokinase (CK) *in vivo* (53, 58, 95), as well as neuromuscular blocking effects (96) and hemostasis modulation (79, 97).

Bradykinin-Inhibitory Peptide (BIP)

C. d. ruruima and *C. d. terrificus* are the only Brazilian rattlesnakes presenting BIP, even in a low proportion (<1.8%) (5). This molecule was found in other Crotalinae venoms (e.g. *C. viridis viridis*, *Lachesis muta*, and *Agkistrodon bilineatus* venoms), which present a fully conserved primary structure, suggesting a conserved biological role for this toxin (98). BIPs present antagonistic effects on the vasodilatation induced by bradykinin at bradykinin receptor type 2 (B2 receptors), which are expressed in vascular endothelial and smooth muscle cells (98, 99), resulting in vasoconstriction effects by disrupting the functioning of the cardiovascular system, thus also contributing to clinical effects of envenomings (98).

L-Amino Acid Oxidases From Snake Venoms (SV-LAAOs)

SV-LAAOs are widely distributed in venomous snake families of Viperidae, Crotalidae, and Elapidae (52). LAAOs are flavoenzymes that catalyze the stereospecific oxidative deamination of an L-amino acid substrate, producing α-keto acid, ammonia, and hydrogen peroxide (H_2O_2) (100–102). With a molecular mass of around 110-150 kDa under non-denaturating conditions, SV-LAAOs are homodimeric glycoproteins linked to flavin adenine dinucleotide (FAD) (103, 104). As a prosthetic group of LAAOs, FAD has riboflavin, which characterizes the yellow color of venoms (52).

An interesting aspect of *C. d. ruruima* venom concerns its color, presenting a "white" and "yellow" variation with particular biological activities (49). The proteome analysis performed by Calvete and colleagues from *C. d. ruruima* was performed with a pool of "white" venom, and showed a low abundance of LAAO (<1%) (5). In other, *C. durissus* subspecies venoms, LAAOs are also present in low abundance (44), with a highest concentration of 4.5% found in a *C. d. terrificus* sample (**Table 1**) (5, 45–48). LAAO's physiological role is still unknown; it is speculated that they may be related to venom conservation and stabilization of the venom glands, due to their antibacterial properties (102). LAAOs have been isolated from *C. durissus* venoms, showing hemolytic activity, induced plasma clot and platelet aggregation (105–107). Many of these could be related, at least in part, with H_2O_2 produced during the chemical reaction catalyzed by LAAOs, contributing to envenoming toxicity, due to oxidative stress (108, 109).

C. d. Ruruima Specific Features

Although the main rattlesnake toxins were described in *C. d. ruruima* venom using proteomics (5), many toxins encountered in other Brazilian *C. durissus* subspecies (e.g. disintegrins, hyaluronidases, growth factors, nucleases, and nucleotidases), which are also determinants for the toxicity of venoms, have not been described for this subspecies (5, 43–46, 53, 110, 111). However, we do not exclude the possibility of *C. d. ruruima* presenting other compounds. Dos-Santos and colleagues evidenced individual venom variations between six *C. d. ruruima* snakes: 1) the toxicity of white venoms was higher than yellow venoms; 2) PLA_2 activity also varied, being higher in yellow venom; 3) only one white venom showed hemorrhagic activity; 4) myotoxicity degree and edematogenic activity varied between venoms; and 5) coagulant activity in human plasma or

bovine fibrinogen also varied between tested venoms (112). Another study with white and yellow venoms from *C. d. ruruima* demonstrated that they have similar lethality and coagulant activity, while presenting small differences regarding proteolytic, hemorrhagic, and necrotic activities (49). Cavalcante and colleagues (2015) also observed that *C. d. ruruima* "white" venom was capable of inducing a neuromuscular blockade of indirectly evoked twitches of mice phrenic-diaphragm preparations (68).

C. d. ruruima venom is the one with the highest abundance of crotoxin. Based on the high proportion of crotoxin, *C. d. ruruima* venom could be considered the most toxic Brazilian rattlesnake venom, since its "lethal neurotoxicity coefficient" (LNC) is higher than the other Brazilian rattlesnakes already studied (5, 44). This coefficient is determined through the ratio of average venom LD_{50} and the neurotoxins concentration (crotoxin and crotamine) (5). This fact could justify the severe cases of envenoming and lethality caused by *C. d. ruruima* snakes (26, 59).

HUMAN ENVENOMATIONS CAUSED BY *C. D. RURUIMA*

Epidemiological Aspects

In Brazil, snakebites are compulsorily recorded by the *Sistema de Informação de Agravos de Notificação* (SINAN, Brazilian Notifiable Diseases Surveillance System), based on data report forms used in the investigation and follow-up of cases of envenomings caused by animals. In the state of Roraima, in the extreme north of Brazil, *C. d. ruruima* is known to be the unique subspecies of *C. durissus* responsible for the local envenomings. Thus, the description of crotalid envenomings registered in SINAN in this state can provide important information about the epidemiological and clinical features of envenomings by *C. d. ruruima*. Of a total of 3,616 snakebites (71.3 cases/100,000 persons/year) reported to SINAN from January 1, 2010, to December 31, 2019, in Roraima, 396 (10.9%) cases were caused by rattlesnakes, resulting in an incidence rate of 7.8 cases/100,000 people/year.

Spatial distribution of rattlesnake bites across the Brazilian Amazon showed higher incidence in areas of savanna in high altitude areas (113). In Roraima, the geographic distribution of *Crotalus* envenoming shows the highest incidence rate in the northernmost municipalities, in the border with the Guyana and Venezuela, namely in the municipalities of Uiramutã and Normandia (**Figure 3**), coinciding with savannas that occur in mountainous areas in the northern areas of the state. There was a slight variation in the annual incidence rates during the study period. Incidence was higher in 2018 (94 cases; 16.3 per 100,000 inhabitants) and 2017 (54 cases; 10.3 per 100,000) and lower in 2010 (11 cases; 2.4 per 100,000). The seasonality of the cases was modest, with a slight increase in cases from the beginning of the rainy season.

Most of the snakebites occurred in males (77.3%). The most affected age group was between 16 and 45 years old (51.0%). Strikingly, Amerindians represent 11% of the Roraima's total population but suffered 64.9% of reported *Crotalus* envenomings; this incidence is ~12 times higher than in the non-Amerindian population. In the Amazon, the distribution of the snakebite burden is disproportional among indigenous and non-Amerindian populations. In the state of Amazonas, the prevalence is 7.5 times higher among Amerindians (114). Indigenous people's way of life, and their daily subsistence activities, involve daily interaction with snakes, without personal protective equipment, thus increasing the risk of snakebites (114). Regarding the area of occurrence, 93.0% were reported in rural areas; 24.3% of the snakebites were related to work activities. Regarding time elapsed from the bite until medical assistance, 57.0% of the cases received treatment within the first six hours after the snakebite, 22.7% within

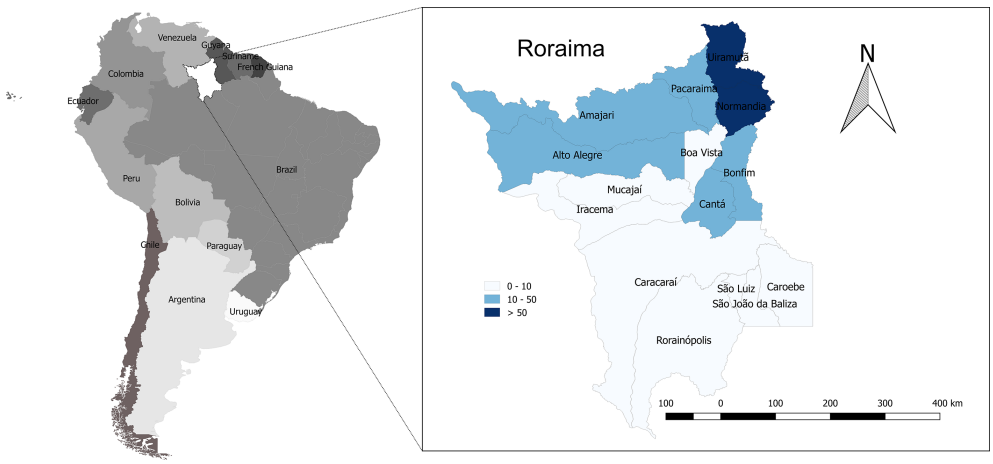

FIGURE 3 | Geographic and temporal distribution of *Crotalus durissus ruruima* snakebites in the state of Roraima, Brazilian Amazon. Incidence rate per municipality (cases/100,000 inhabitants/year, showing a higher incidence in the north of the state).

6–24 hours, and 20.3% with more than 24 hours after the bite. Among the victims, 14.6% were illiterate, and 36.1% had ≤4 years of study. Most of the snakebites occurred in the lower (85.6%) and upper limbs (13.1%) (**Table 2**). This epidemiological profile confirms small case series previously published in the state of Roraima (25, 26, 115). Asato and colleagues showed that the *C. ruruima* envenomings attended to in Boa Vista, the capital of the state of Roraima, predominantly involved farmers during morning or afternoon work in their fields (26).

TABLE 2 | Epidemiological characteristics of *C. d. ruruima* envenomings reported in the state of Roraima, northern Brazilian Amazon, from 2010 to 2019.

Variable (Completeness)	Number	%
Sex (n=396; 100%)		
Male	306	77.3
Age range, in years (n=396; 100%)		
0-15	113	28.5
16-45	202	51.0
46-60	51	12.9
≥61	30	7.6
Ethnicity (n=379; 95.7%)		
White	10	2.6
Black	13	3.4
Asian	2	0.5
Mixed	108	28.4
Amerindian	246	64.9
Area of occurrence (n=384; 97%)		
Rural	357	93.0
Urban	27	7.0
Work-related (n=296; 74.7%)		
Yes	72	24.3
Education level, years (n=219; 55.3%)		
Illiterate	32	14.6
≤4	79	36.1
5-8	49	22.4
>8	59	26.9
Site of the bite (n=388; 98%)		
Head	4	1.0
Trunk	1	0.3
Lower limbs	332	85.6
Upper limbs	51	13.1
Time from bite to medical assistance, hours (n=375; 94.7%)		
≤6	214	57.0
>6-24	85	22.7
>24	76	20.3

Clinical Aspects and Physiopathology

There is very little information in the literature on the clinical manifestations of envenomings caused by *C. d. ruruima*. However, the few reports suggest some differences in relation to the clinical pictures resulting from different subspecies of *C. durissus* found in South America. The main difference pointed out is the high frequency of inflammatory manifestations at the bite site and coagulopathy (26, 115). However, the small number of published cases prevents a more accurate comparison with data from other regions. To explore this review's mentioned hypothesis and to present a more accurate comparison of envenomings caused by different subspecies of *C. durissus*, we analyzed the data gathered by SINAN, from 2010 to 2019, comparing envenomings cases of *Crotalus* occurring in six regions of the country in which the snake is present (**Figure 4**). Only country units (states) without subspecies overlap in geographical distribution of six *C. durissus* subspecies, according to the current knowledge (1, 2), were selected for comparison, using the group of patients envenomed by *C. d. ruruima* (cases from the state of Roraima) as the reference. As the epidemiological characteristics of the cases are different in relation to demographics and access to the health system, the results were statistically adjusted by sex, age, and time until the assistance. Results must be interpreted with caution, since a limitation of this approach is related to the lack of standardization in the clinical management of cases and data collection in different country regions, and the number of cases described for each subspecies differs widely. However, we believe that the broad population coverage allowed the acquisition of valuable information to guide future studies.

Bite Site Manifestations

In general, the authors report that local manifestations are usually discreet during envenomings caused by *C. durissus*, as a result of the low inflammatory activity of the venom (116–118). The most frequent local manifestation among *Crotalus* envenomings reported to SINAN was pain, ranging from 92.1% in *C. d. terrificus* to 98% in *C. d. durissus* (**Figure 4**). In *C. d. ruruima* envenomings, frequency of pain (95.6%) was significantly higher only than *C. d. terrificus* (**Figure 4**). Some studies have shown that pain may be absent in a large proportion of patients in São Paulo, where *C. d. terrificus* is the snake mainly responsible for envenomings (117). Edema (81.8%) was also very common in *C. d. ruruima* cases, at a higher frequency than observed for *C. d. cascavella* (66.4%), *C. d. terrificus* (58%), and *C. d. collilineatus* (48.9%) (**Figure 4**). Unfortunately, paresthesia is not reported by SINAN but is also a very common manifestation in *Crotalus* bites. In *C. d. ruruima* envenomings, paresthesia frequency ranges from 12.5 to 16.2% (26, 115). In the state of São Paulo, paresthesia was reported in 47.6% of the children (117) and 26.1% (116) of the general cases of *Crotalus* bites.

Local complications such as secondary infections and compartment syndrome seem to appear at very low frequency in *Crotalus* bites (119, 120). Data from SINAN show that secondary infections frequency ranged from 0.7% in *C. d. cascavella* and *C. d. collilineatus*, to 5.6% in *C. d. durissus*. In *C. d. ruruima* envenomings, the frequency of secondary infections (3.2%) was significantly higher than *C. d. cascavella* and *C. d. collilineatus*, but lower than *C. d. durissus* (**Figure 4**). Necrosis, compartment syndrome, functional loss and amputation were rare, and their frequencies were similar between envenomings caused by different *C. durissus* subspecies.

Local manifestations are commonly associated with the inflammatory properties that venom can trigger once inoculated. Although toxins from *C. durissus* subspecies are known to present low immunogenicity, studies have shown the capacity of venoms and toxins to promote local and systemic inflammatory responses in animal models (78, 121, 122). As previously described, *C.d. ruruima* "yellow" and "white" venom variations are responsible for inducing mouse paw edema (112). Considering that the venom components crotoxin and serine

proteases are found to trigger an inflammatory response (78, 122), the higher frequency in local manifestations, such as edema, found in Roraima state could be associated with *C. d. ruruima* venom composition, in which the two toxins classes comprise over 90%. Moreover, although the low abundance of SVMP found in *C. d. ruruima* venom can be associated with rare cases of local sequelae, this group of toxins could be an additional factor for local injury as observed for North American *Crotalus* spp. venoms (93).

Rhabdomyolysis and Acute Renal Failure

The myotoxic activity is a result of the venom-induced skeletal muscle breakdown, often associated with rhabdomyolysis (123). In the state of Roraima, dark urine (13.5%) and myalgia (~25%) were reported after *C. d. ruruima* envenomings (26, 115). In the state of São Paulo, myalgia and dark urine were detected in 38.6 and 36.1% of the patients, respectively (118). In Minas Gerais, myalgia was detected in 29% and dark urine in 40% of the patients (124). In children from the same region, frequencies of myalgia and dark urine were still higher, exceeding 70% of the patients (116, 117). In the analysis of the SINAN database, rhabdomyolysis was significantly higher in patients bitten by *C. d. ruruima* (16.1%) using univariable analysis, compared to all other subspecies. However, after adjusting by sex, age, and time to medical assistance, significant difference remains only for *C. d. collilineatus* (**Figure 4**). The non-standardized collection of these signs and symptoms may prevent accurate comparisons across studies. In *C. d. terrificus*, serum CK activity increases early, reaching a peak in the first 24 hours after the bite, remaining above reference levels for more than four days in some patients (116, 117). In envenomings caused by *C. d. terrificus* (117, 118, 124), *C. d. collilineatus* (124, 125), *C. d. marajoensis* (126), and *C. d. ruruima* (26), CK activity was above normal levels in ~90% of the patients (118, 124, 125). Serum activities of lactate dehydrogenase and liver transaminases are also elevated in most cases. Hypocalcemia may occur in patients with intense rhabdomyolysis after *C. d. terrificus* bites (116). Both crotoxin and crotamine are the major myotoxic components within *C. durissus* venoms (96, 127). Moreover, crotoxin exhibits a capacity to spread to distant muscles and induce rhabdomyolysis, due to its systemic distribution ability mediated by the heterodimeric complex formation of the toxin to bind to specific cell targets (128). *C. d. ruruima* crude venom showed myotoxic activity, as observed for increased plasma levels of CK and histopathological analysis of myonecrosis (49, 112). Isolated crotoxin from this venom also induced increased plasma levels of CK and LDH not only at intramuscular administration but also intraperitonealy, confirming the

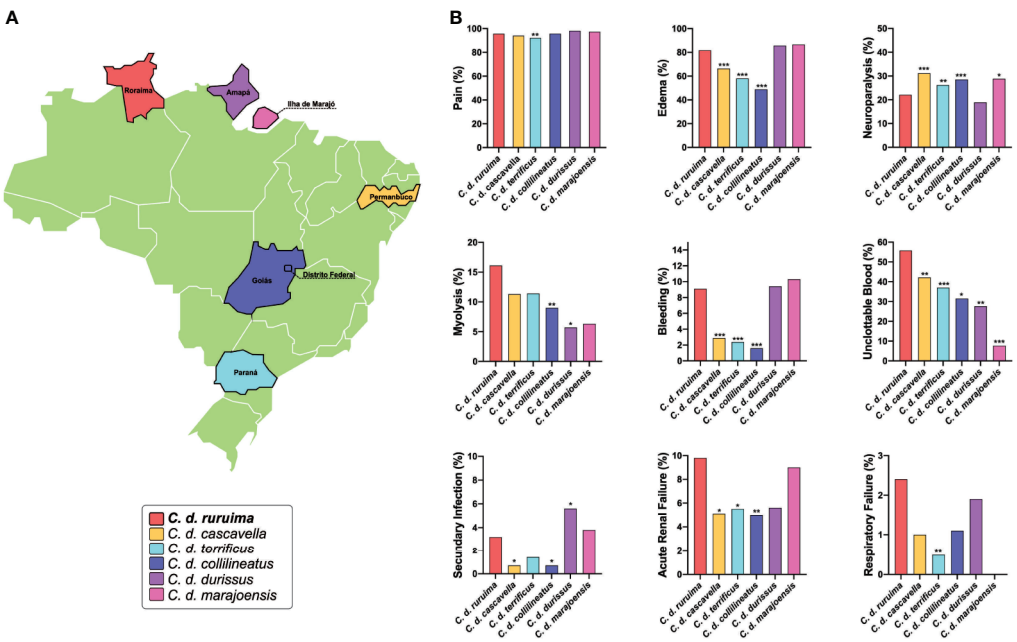

FIGURE 4 | Cross-country comparison of local and systemic manifestations in envenomings caused by different *Crotalus durissus* subspecies. Data was gathered from the SINAN, from 2010 to 2019. Information was stratified by state, considering the geographic distribution of six *C. durissus* subspecies. **(A)** Only states without subspecies overlap in geographic distribution were selected. For statistical comparison, X-square or Fisher's tests were performed in STATA software (StataCorp. 2013: Release 13. College Station, TX, USA), using the group of patients envenomed by *C. d. ruruima* as the reference. After the univariable analysis, any difference in the frequency of clinical manifestations between *C. d. ruruima* and another subspecies envenoming at a significance level of $p<0.20$ were included in a multivariable analysis, adjusting by age, sex, and time to medical assistance. Statistical significance was considered if $p<0.05$ in the Hosmer-Lemeshow goodness-of-fit test. **(B)** Only clinical manifestations with any significant difference are presented. Number of patients according *C. durissus* subspecies: *C. d. ruruima* (n=396), *C. d. cascavella* (n=990), *C. d. terrificus* (n=1,016), *C. d. collilineatus* (n=2,093), *C. d. durissus* (n=54), *C. d. marajoensis* (n=80); $^{*}p < 0.1$; $^{**}p < 0.05$; $^{***}p < 0.01$.

systemic effect of CTX (66). Considering the role of crotoxin in muscular damage events and its high abundance in *C. d. ruruima* venom, the high frequency of myonecrosis in patients from Roraima state could be associated with CTX activity.

As a consequence of rhabdomyolysis, a major complication following *Crotalus* snakebites is acute renal injury (123). The definitions of acute renal failure vary widely across studies, and this lack of standardization is also a bottleneck for comparisons. In *C. d. ruruima* envenomings, 13.5% of the patients presented acute renal failure (serum creatinine >1.5 mg/dL) (26). The frequency of acute renal failure was 18% in Minas Gerais (124) and 12.9% in São Paulo (118). In Central Brazil, 29% of the patients developed acute renal failure; of those, 24% required dialysis, and 10% died (125). In São Paulo, 20% of children envenoming developed acute tubular necrosis, and half of them needed hemodialysis (117). Delay for antivenom treatment (124, 125) and CK at admission >2000 U/L (125) were risk factors for acute renal failure development. The effect of age is controversial (124, 125). In a SINAN-based cross-country comparison, the frequency of acute renal failure was significantly higher in *C. d. ruruima* envenomings than in *C. d. cascavella*, *C. d. terrificus*, and *C. d. collilineatus*, even after adjustment by age, sex, and time to medical care (**Figure 4**).

Experimental models using *C. durissus* subspecies' venoms and isolated toxins showed important renal alterations, for which several mechanisms are proposed (129–132). Possibly the most critical concern is the damage to skeletal muscle by the myotoxins crotoxin and crotamine, as discussed above. As the major crotoxin-induced product into the circulation, myoglobin is responsible for inducing renal vasoconstriction, formation of intratubular casts, and the direct toxicity to kidney tubular cells, which can be followed by acute kidney injury (AKI) (133). *C. durissus*-induced inflammation is another possible mechanism associated with kidney function; previous studies have shown that inflammatory mediators released from venom-treated macrophages were responsible for renal disturbances (134, 135). Although no studies have been conducted to associate *Crotalus*-induced renal abnormalities with coagulation disorders, this is an important issue to be considered. As a consequence of intravascular coagulation and hemostatic components consumption observed in *C. durissus* patients (115, 136), vascular ischemia due to microthrombi deposition (118) could be responsible for impaired renal functions as observed in similar cases of *Bothrops* accidents (137).

Among venom components, crotoxin was responsible for significant changes in renal function, followed by serine protease inducing mild alterations and CTL with no effects (129). Therefore, the high frequency of AKI in *C. d. ruruima* patients could be a result of myolisis, coagulation disturbances, and inflammation, also with elevated frequency in patients from Roraima. In contrast, crotoxin as the major venom component can play an important role in these events' physiopathology.

Coagulation Disorders

Coagulopathy in *Crotalus* envenomings is attributed to the presence of hemostatically active components in the venom, which can lead to hypofibrinogenemia and unclottable blood (123). Unclottable blood is observed in most of the *Crotalus* envenomings in Brazil, but systemic bleeding seems to be less common (116, 118, 124, 125, 138). In their case series, Cupo and colleagues did not find hemorrhage or thrombosis in the necroscopic analysis (117). Bucaretchi and colleagues (2013) report a case of juvenile *C. d. terrificus* envenoming that evolved with coagulopathy as the main systemic manifestation, without systemic bleeding (136). Also, local bleeding persisting on admission is not a common feature in *Crotalus* envenomings. In Roraima, however, the proportion of the patients presenting to the hospital with bleeding at the bite site ranged from 12.5% (115) to 24.3% (26). In *C. d. ruruima* envenomings, unclottable blood was reported in 62.5% and hypofibrinogenemia in 50% of the patients (115). One fatal case of rattlesnake envenoming attended in Roraima evolved with increased prothrombin time, severe thrombocytopenia, and macroscopic hematuria (59). In the patients reported to SINAN-Roraima, clotting time was prolonged in 55.8%, and systemic bleeding was reported in 9.1% of the patients (**Table 3**). In the cross-country comparison using the SINAN database, blood unclottability was significantly more prevalent in *C. d. ruruima* compared to other *C. durissus* subspecies. Furthermore, systemic bleeding was more frequent in *C. d. ruruima* compared to *C. d. cascavella*, *C. d.*

TABLE 3 | Clinical characteristics of *C. d. ruruima* envenomings reported in the state of Roraima, northern Brazilian Amazon, from 2010 to 2019.

Variable	Number	%
Local manifestations		
Pain (n=364)	348	95.6
Edema (n=363)	297	81.8
Ecchymosis (n=358)	21	5.9
Systemic manifestations		
Neuroparalysis (n=376)	83	22.1
Myolysis (n=378)	61	16.1
Bleeding (n=375)	34	9.1
Vomiting/diarrhea (n=374)	31	8.3
Clotting time (n=113)		
Normal	50	44.2
Prolonged	63	55.8
Severity grade on admission* (n=381; 96.2%)		
Mild	151	39.6
Moderate	170	44.6
Severe	60	15.7
Antivenom administration (n=381; 96.2%)		
Underdosage	155	40.7
As recommended	226	59.3
Local complications		
Secondary infection (n=345)	11	3.2
Necrosis (n=343)	4	1.2
Compartment syndrome (n=344)	2	0.6
Functional loss (n=343)	3	0.9
Amputation (n=343)	1	0.3
Systemic complications		
Acute renal failure (n=336)	33	9.8
Respiratory failure (n=335)	8	2.4
Sepsis (n=335)	2	0.6
Shock (n=334)	5	1.5
Death (n=350)		
Yes	4	1.1

**According to Brazilian Ministry of Health guideline (139).*

terrificus, and *C. d. collilineatus* subspecies (**Figure 4**). Statistical analysis revealed that systemic bleeding was associated with unclottable blood in this group of patients (OR=4.77, CI95% 1.28-17.70; P=0.021).

Clotting time, usually performed as the Lee-White clotting time (LWCT) or the 20-minute whole-blood clotting test (20WBCT) usually employed to determine the clotting time, are used worldwide as reliable, low- cost techniques to assess snakebite coagulopathy. Prolonged clotting time assesses the consumption/deficiency in coagulations factors, as well as alterations in platelet count and function (140). The venom is a cocktail of toxins capable of interefering with hemostatic components, leading to coagulopathy. Prolonged clotting times are associated with antagonistic behavior of toxins, which can induce anticoagulant activity, as described for crotoxin and crotamine (79, 141), and procoagulant toxins, reported by serine proteases with thrombin-like activity, which induces the consumption of coagulation factor and hypofibrinogenemia (78, 79). The overall activity induces blood incoagulability, an important factor in bleeding genesis. Another relevant element in hemorrhagic events consists of platelet depletion and the impaired function, majorly promoted by the platelet aggregation agonist convulxin (85), with the backup of others toxins such as crotoxin and crotamine (97, 142) working as agonist and antagonist of platelet aggregation, respectively.

Another interesting aspect concerns the possible role of SVMP from *C. durissus* subspecies venom acting as hemorrhagins. Although their biological activities have not been investigated, SVMP found in North-America in *Crotalus* venoms have been described as inducing local hemorrhage through vessel basement membrane degradation (93), as also observed in *Bothrops* species (143). Considering its abundance in *C. d. ruruima* compared to other venoms, this group of toxins could be associated with the high frequency of bleeding events in patients from Roraima state.

Neurological Disorders

Neurological impairment observed in *C. durissus* envenomings is associated with the action of neurotoxins, which are found to act in both peripheral and central nervous systems (96, 144, 145). In the clinical description from the Extra-Amazonian region, neuroparalytic signs, especially the neurotoxic facies/palpebral ptosis, are the most frequent signs of *Crotalus* envenomings. In general, palpebral ptosis is observed in ~70% of the patients (118, 124) and in ~90% of the children (116, 117). Ophthalmoplegia and diplopia are also very frequent, appearing in 95% of general patients and 81% of the children, respectively (117). In the cases reported in the state of Pará, these neurological manifestations are present in almost all patients (126, 146, 147). Bucaretchi and colleagues report an envenoming case caused by a juvenile *C. d. terrificus* that evolved with coagulopathy as the main systemic manifestation, without neuromyotoxic features normally associated with bites by adult specimens (136). In *C. d. ruruima* envenomings, the frequency of palpebral ptosis ranged from 24.3 (26) to 50% (115), and diplopia was seen in 21.6% of the patients (26). Dizziness, difficulty in walking, and muscle weakness are manifestations less frequent in *Crotalus* snakebites, including those caused by *C. d. ruruima* (26, 59, 115). In the patients reported to SINAN-Roraima, neuroparalytic signs were detected in 22.1% of the patients (**Table 3**). Compared to other *Crotalus* subspecies, neuroparalytic signs were significantly less prevalent in *C. d. ruruima* compared to other *C. durissus* subspecies, except when comparing to *C. d. durissus* (**Figure 4**).

Respiratory failure is also a rare complication from *C. durissus* snakebites, being associated with severe cases of envenomings resulting from paralysis of the rib cage and diaphragm muscles (148, 149). A previous report on two cases of *C. d. terrificus* snakebite showed that respiratory manifestations emerged within the first 48 hours and were characterized by dyspnea, tachypnea, use of accessory muscles of respiration, and decreased blood pH and imbalanced pO_2 pCO_2 levels (148). SINAN database analysis revealed that the frequency of this complication ranged from 0% in *C. d. marajoensis* to 2.4% in *C. d. ruruima* envenomings. The frequency of respiratory failure was significantly higher in *C. d. ruruima* than in *C. d. terrificus* envenomings (0.5%) (**Figure 4**).

As neurological manifestations and respiratory failure are both associated with the venom neuroxicity, in which crotoxin plays a major role (127), it is possible that the difference in neurotoxic activity could be associated with the singular potency of crotoxin found in each venom. Cavalcante and colleagues showed that crotoxin isolated from *C. d. cumanensis* (inhabit Colombia, Venezuela, and Caribbean coast) presents a more potent neuromuscular blockade effect on mice phrenic-diaphragm preparations when compared to that of *C. d. ruruima* (68).

Other Manifestations

Gastrointestinal manifestations, such as nausea (21.6%), vomiting (8.1 to 12.5%), and abdominal pain (8.1%), are reported in *C. d. ruruima* envenomings (26, 115). Necrosis, compartment syndrome, functional loss, and amputation were rare, and their frequencies were similar between envenomings caused by different *C. durissus* subspecies. Headache prevalence ranged from 12.5 (115) to 24.3% (26); dizziness in 12.5% (115); and fever in 8.1% of the patients (26).

Risk Factors for Severity

In Brazil, severity classification of *Crotalus* envenomings takes into account the neurological and myolytical signs and symptoms, added to the presence of acute renal failure (139) (**Table 4**). However, bite site manifestations and coagulopathy are not listed as predictors of severity in the Brazilian guideline.

Table 5 summarizes the results of the univariable and multivariable logistic regression models evaluating factors associated with severity in *C. d. ruruima* envenomings reported to SINAN-Roraima from 2010 to 2019. Antivenom underdosage [RR=1.91 (95%CI=1.18–3.09); (p=0.008)], time to medical care >6 hours [RR=1.85 (95%CI=1.14–2.99); (p=0.013)], and Amerindian ethnicity [RR=1.84 (95%CI=1.04–3.26); (p=0.036)] were independently associated with the risk of severity. Bites in lower limbs [RR=0.44 (95%CI=0.26–0.76); (p=0.003)] were associated with protection from severity.

Antivenom underdosage was previously reported as a risk factor for death in the Brazilian Amazon (150), being more

TABLE 4 | Severity classification of *Crotalus* bites according the Brazilian Ministry of Health.

Signs/symptoms	Case severity		
	Mild	**Moderate**	**Severe**
Neuroparalytic manifestations (myasthenic facies and others)	Mild	Evident	Evident
Myalgia	Absent	Mild	Evident
Dark urine	Absent	Mild	Evident
Oliguria/anuria	Absent	Absent	Evident

Source: Brazilian Ministry of Health (139).

TABLE 5 | Factors associated with severity in *C. d. ruruima* envenomings reported in the state of Roraima, northern Brazilian Amazon, from 2010 to 2019.

Severity#	RR	95% CI	*P*	aRR	95%CI	*P*
Underdosage	1.69	1.06-2.69	**0.026**	1.91	1.18-3.09	**0.008**
Time to care >6 hours	1.79	1.11-2.91	**0.018**	1.85	1.14-2.99	**0.013**
Amerindian ethnicity	1.45	0.85-2.48	**0.166**	1.84	1.04-3.26	**0.036**
Site of the bite (lower limbs)	0.51	0.30-0.87	**0.014**	0.44	0.26-0.76	**0.003**
Schooling (years)						
Illiterate	1					
≤4	0.81	0.30-2.19	0.687			
5-8	1.03	0.37-2.87	0.950			
>8	0.94	0.34-2.57	0.913			
Gender (Women)	0.67	0.35-1.27	0.227			
Work-related	1.35	0.69-2.66	0.371			
Rural zone	1.37	0.46-4.07	0.568			
Age (years)						
≤15	1					
16-45	0.97	0.55-1.67	0.906			
46-60	1.08	0.50-2.33	0.842			
>60	1.09	0.44-2.72	0.844			

Proportions of severe cases and deaths were compared by Chi-square test (corrected by Fisher's test if necessary); differences were considered statistically significant for p<0.05. The crude Relative Risk (RR) with its respective 95% Confidence Interval (95% CI) was determined considering severity and death as the dependent variables. Logistic regression was used for the multivariable analyses, and the adjusted RR with 95% CI were also calculated. All variables associated with the outcomes at a significance level of p<0.20 in the univariable analysis were included in the multivariable analysis. Statistical significance was considered if p<0.05 in the Hosmer-Lemeshow goodness-of-fit test. aRR, Adjusted Relative Risk.
Bolded values represent variables significantly associated to severity.

frequent in Amerindian patients (114). Incomplete treatments may be associated to with the lack of trained health professionals assisting snakebites and antivenom shortage, forcing professionals to rationalize the limited stock among patients. As also discussed in previous reports (150, 151), late medical care was found to be a risk factor of severity. In these cases, venom toxins acting longer in the body increase the possibility of systemic complications due to more intense myolysis (59, 117). Amerindian ethnicity was also independently associated with the risk of severity, it is necessary to consider the main challenges with cultural, social, and economic disparities between Amerindians and non-Amerindians to understand this association. Firstly, Amerindian villages have their own and very verticalized health system, presenting a low performance in treating snakebites resulting in a higher frequency of long-term disabilities and deaths in these groups (114). Moreover, traditional therapeutic practices within the community involving healers such as shamans, those with knowledge of medicinal plants, and animal-based medicines are often provided in the villages (152). This has a consequence in the time to medical assistance, and the use of deleterious procedures is not discarded (114). Bites in lower limbs were independently associated with milder envenomings. We do not understand the mechanism of this finding, but it may be related to a different bioavailability profile of the venom in the patient's body according to the bite site. In other studies, older age was associated with severity in rattlesnake envenomings (113, 153). This association was not found in *C. d. ruruima* envenomings reported to SINAN-Roraima, possibly because the number of cases in patients ≥61 years was very small. Indeed, statistical analysis revealed that cases in the state of Roraima occurred mostly in the younger population, as compared to other regions of Brazil (P<0.0001).

THERAPEUTICS AGAINST *C. D. RURUIMA* ENVENOMINGS

Specific Treatment

Specific antivenom is crucial for the efficiency of the treatment of rattlesnake envenomings (154). For the treatment of *Crotalus* envenomings in areas where *C. d. ruruima* occurs, there are three types of antivenoms, two produced in Brazil and one in Venezuela. Characteristics of these products are described in **Table 6**. Although the Brazilian antivenoms are produced using only one or two rattlesnake subspecies venoms (*C. d. terrificus* 100% or *C. d. terrificus* 50% plus *C. d. collilineatus* 50%), both

have been used to treat envenomings caused by all the six *Crotalus* subspecies in Brazil (*C. d. durissus*, *C. d. terrificus*, *C. d. cascavella*, *C. d. ruruima*, *C. d. marajoensis*, and *C. d. collilineatus*) (26, 149, 155). The three producers of crotalid antivenoms in the country follow the guidelines of the *Agência Nacional de Vigilância Sanitária* (Brazilian National Health Surveillance Agency, ANVISA). While each producer uses its own crotalid venoms, all of them use crotamine-abundant venoms in their antigen mixtures (156) (for details, see Section 2). The antivenoms are distributed by the government free of charge. The dosage can vary according to the envenoming severity, though there are standardized recommendations of dosage according to the severity of the case (157). Likewise, Venezuelan antivenom is produced using *C. d. cumanensis* venom, and it is used to treat *Crotalus* envenomings caused by different subspecies occurring in Venezuela (158). Although with adequate, specific and general treatment, most victims envenomed by rattlesnakes can recover and survive, there is some evidence suggesting that the *Crotalus* antivenom cannot neutralize all the deleterious actions of the venom (49). Although crotalid antivenom was effective in neutralizing the lethal, myotoxic, and *in vitro* coagulant activities of the venoms of *C. d. terrificus*, *C. simus*, and *C. d. cumanensis* (159), as well as the lethal effect of *C. d. ruruima* (49) and *C. basiliscus* venoms (160), this antivenom was ineffective in the neutralization of hemorrhagic activity of *C. d. ruruima* venom (49, 159). Hemorrhage was inhibited in the experimental *C. d. ruruima* envenoming only by using the *Bothrops-Crotalus* antivenom, possibly due to the sharing of hemorrhagic toxins with *Bothrops* species (49). Follow-up of four patients envenomed by *C. d. ruruima* has shown that neurotoxic signs ceased, and hemostasis parameters and CK values returned to normal 24 hours after treatment with the *Bothrops-Crotalus* antivenom (115). Otherwise, preliminary reports from the Snakebite Roraima group (Boa Vista, RR, Brazil— www.snakebiteroraima.com) have documented that, in some *C. d. ruruima* envenoming cases, the antivenom does not seem to reverse the venom-induced signs (59). Indeed, antivenomic studies or detailed *in vivo* neutralizing assays with this subspecies venom to elucidate the antivenom efficacy are lacking. Venomics results (see **Table 1**) suggest that different subspecies venoms induce a different proportion of specific antibodies when inoculated in horses; thus, the antivenom manufactured for *C. d. terrificus* and *C. d. colillineatus* venoms may not contain neutralizing antibodies for all *C. d. ruruima* toxins.

The need for a cold chain and physicians to prescribe antivenoms usually restricts access to effective snakebite treatment (161). However, as presented above, *C. d. ruruima* envenomings are reported mostly in Amerindian populations, and it is necessary to consider challenges related to cultural, social, and economic disparities between Western and indigenous cultures, contributing to late medical care among the latter (114). In Roraima, some Yanomami sanitary districts have antivenom available for mild to moderate snakebite cases (162), but in the other indigenous groups, referral to urban areas is needed. The transfer of the indigenous to urban areas is a critical event in these individuals lives, due to the breakdown of relations with the village and eating habits, causing treatment refusals (114). From an economic point of view, the public health system spends thousands of dollars per year to transport indigenous patients by plane or helicopter (~US$600 and ~US $900/hour of flight, respectively), in addition to hospital costs and loss of productivity, which are also very high (US$8 million in 2015, in the Amazon region) (163). Besides the specific treatment, supportive assistance during severe rattlesnake treatment is crucial but possible only in the capital, Boa Vista. Strategies that guarantee this affected population's cases timely access to treatment and proper training of health professionals who deal with indigenous populations are key to providing the best care to the indigenous communities. In a previous study, we discuss a plan for the decentralization of antivenom treatment to local healthcare facilities as an intervention to increase the indigenous population's access to proper healthcare (114).

An important fact to be mentioned is the absence of antivenoms in the health facilities of neighboring countries where *C. d. ruruima* envenomings are also common. In

TABLE 6 | *Crotalus* antivenoms available in areas where *Crotalus durissus ruruima* area reported, in Brazil and Venezuela.

Laboratory (Country)	Antivenom	Description of the antivenom	Venoms used for production	Dosage
Butantan Institute (IBU), Vital Brazil Institute (IVB), and Ezequiel Dias Foundation (FUNED) (Brazil)	*Crotalus* AV	Each vial contains heterologous horse F(ab')2, neutralizing at 15 mg of the reference venom of *C. d. terrificus*, in mice, phenol (35 mg maximum) and physiological solution 0.85% q.s. 10 mL	*C. d. terrificus* 50.0% plus *C. d. collilineatus* 50.0% (IBU) or *C. d. terrificus* 100% (IVB and FUNED)	5 vials to mild cases, 10 vials to moderate cases, 20 vials to severe cases
Butantan Institute (IBU), Vital Brazil Institute (IVB), and Ezequiel Dias Foundation (FUNED) (Brazil)	*Bothrops-Crotalus* AV	Each vial contains heterologous horse F(ab')2, neutralizing at least 50 mg and 15 mg of the reference venoms of *Bothrops jararaca* and *C. d. terrificus*, respectively, in mice, phenol (35 mg maximum) and physiological solution 0.85% q.s. 10 mL	*Bothrops* genus (*B. jararaca* 50.0%, *B. alternatus* 12.5%, *B. jararacuçu* 12.5%, *B. moojeni* 12.5% and *B. neuweidi* 12.5%), *Crotalus* genus (*C. d. terrificus* 50.0% plus *C. d. collilineatus* 50.0% - IBU, or *C. d. terrificus* 100% - IVB and FUNED)	5 vials to mild cases, 10 vials to moderate cases, 20 vials to severe cases
BIOTECFAR - Biotechnology Center, Venezuela Faculty of Pharmacy of the Venezuela University Center (Venezuela)	*Bothrops-Crotalus* AV	Each vial contains heterologous horse F(ab')2, neutralizing at least 20 mg of the reference venom of *Bothrops colombiensis* and 15 mg of the reference venom of *C. cumanensis*, in mice, phenol (30 mg maximum) and physiological solution 0.85% q.s. 10 mL	*Bothrops colombiensis* and *C. d. cumanensis*	10 vials to moderate cases and 15 vials to severe cases

Guyana, for example, health authorities have only recently announced the acquisition of antivenoms (the polyvalent antivenom PoliVal-ICP, Instituto Clodomiro Picado, Costa Rica), which are available only in Georgetown, the country's capital (164). The country has one of the highest case fatality rates (~ 8%) on the continent, which is explained by the fact that few patients visit the health facilities offering proper treatment (165). Envenomings by *C. d. ruruima* are reported in the Guianan savanna, especially in the Lethem region, on the border with Brazil (59). In the absence of antivenoms in these localities, and because of the nearly nonexistent evacuation possibilities to the capital, these patients seek treatment in Brazil. In Venezuela's case, the Laboratorios BIOTECFAR, a public producer maintained by Universidad Central de Venezuela, responsible for the production of antivenoms in the country, has experienced difficulty maintaining continuous production in the last years (158). The shortage of antivenom in Venezuelan is suggested as the cause of patients seeking treatment in Brazil after severe snakebites (166).

Heterologous Antivenom Disadvantages and Next-Generation Crotalid Antivenoms

Although promising results suggest that the PLA_2 inhibitors varespladib and methyl-varespladib are effective in preventing neurotoxic manifestations induced by *C. d. terrificus* (167), crotalid heterologous antivenoms are the only available effective treatment for rattlesnake envenomings in endemic areas. As other heterologous antivenoms, they may present undesirable problems, such as anaphylactic reactions, and serum sickness. Moreover, many of the antibodies from antivenoms (~70%) are composed of non-neutralizing antibodies [for review, see (168)]. Thus, it is evident that the century-old crotalid therapy introduced by Vital Brazil in 1901 (169) needs to be improved. The information on the safety of *Crotalus* antivenoms is scarce, but it was estimated that 80% of patients developed developed an early, adverse event of early adverse event after using the available Brazilian *Crotalus* antivenom, with no significant difference in the frequency of patients with early reactions between the groups that were and were not pretreated with antihistamines and corticosteroids (116). Even though the antivenom purification process has, over time, contributed to reducing this frequency to around 20% today (115), the possibility of early reactions generates fear among health professionals and is a major obstacle for antivenom decentralization to remote areas (114).

The first approach to decreasing the size of the immunocomplexes generated by heterologous antivenoms and preventing side effects (e.g., serum sickness) treated all immunoglobulins (IgGs) with specific enzymes. This methodology results in antigen-binding fragments $F(ab')_2$s or Fabs (170), which are much smaller, corresponding to 115 and 50 kDa, respectively (a whole IgG presents ~150 kDa) (171). At the moment, most of the animal-derived crotalid antivenoms are composed of antibody fragments (e.g., CroFab (172), BIOTECFAR from Venezuela, and both crotalid antivenoms from Brazilian producers). Although the antibody size reduction was important for improving antivenom therapy (173), the molecules continue to cause side effects in keeping their heterologous nature. Indeed, the Instituto Clodomiro Picado from Costa Rica still produces crotalid antivenoms composed of whole IgGs (174).

Animal plasma-derived antivenoms will continue to be the cornerstone of envenoming therapy for many years to come, and it may indeed be warranted to further improve these life-saving medicines e.g. *via* improved downstream processing or improved immunization approaches (175). However, further into the future, it is not unlikely that recombinant DNA technology and the application of human monoclonal antibodies and/or nanobodies may find their way into the field (176). Indeed, the development of monoclonal antibodies to treat envenomings caused by venomous animals is now being extensively investigated (177–181), although none have so far been tested in human patients. Here, we will explore only the discoveries regarding experimental crotalid antivenoms.

Antibody phage display (182) has been used as the main successful technology to generate monoclonal antibodies against toxins. In 1995, the technique was first used for the discovery of monoclonal antibody fragments against animal toxins (176). Interestingly, the target toxin was a crotoxin obtained from the snake *C. d. terrificus* (183). Although the mouse-derived library construction and the antibody panning were performed appropriately, the selected antibody failed to inhibit the PLA_2 activity induced by crotoxin. In 1997, the first human monoclonal antibody generated by phage display against crotoxin was obtained. The research group discovered scFvs targeting crotoxin from a human semi-synthetic antibody phage display library, and the antibodies demonstrated to bind the toxin in ELISA assays (184).

In 2009, a non-immune human single-chain fragment variable library (Griffin.1) was used to select antibodies against *C. d. terrificus* PLA_2s. Two clones demonstrated the ability to partially inhibit the PLA_2 activity *in vitro*, to reduce the myotoxic and edema activities of basic crotoxin *in vivo*, and to inhibit the lethality of *C. d. terrificus* venom in an experimental envenoming (185). Later (2018), using previous phage display selections, the same research group discovered scFv clones able to cross-inhibit *in vitro* indirect hemolysis and plasma-clotting effects of *C. d. terrificus* and *Bothrops jararacussu* venoms (186). Using a phage display peptide library, Titus and colleagues (2017) also developed peptides against a PLA_2 consensus peptide from North American rattlesnakes, *C. adamanteus*, *C. atrox*, *C. scutulatus*, *Agkistrodon piscivorus*, and *A. contortrix laticinctus*. PLA_2s from these venoms had their *in vitro* enzyme activity slightly reduced by the selected peptides by 40% or less (187).

Thus far, the discovery of monoclonal antibodies and peptides have only yielded inhibitors of crotoxin and PLA_2s from *Crotalus* spp, with no reports on monoclonal antibodies against crotamine or other rattlesnake venom compound. Part of the explanation for this may be that PLA_2s seem to dominate as antigens compared to other venom components. Campos et al. demonstrated that when snake whole venoms were used as targets in phage display panning rounds, antibodies were

primarily selected against PLA_2 proteins (180). To overcome this difficulty, key toxins that are important to neutralize, but which are not dominant in neither phage display campaigns or immunization schemes, can be carefully isolated or recombinantly expressed to provide more control over the antibody selection or immunization process (188–190). In the future, such efforts could hypothetically both be used to identify antibodies that could be used to fortify existing crotalid antivenoms (191), and thereby extend their species coverage, or improve their efficacy against non-immunogenic components (190). Finally, it is speculated that efforts focusing on the discovery and development of monoclonal antibodies against *Crotalus* toxins could possibly enable the design of fully recombinant antivenoms based on oligoclonal mixtures of antibodies targeting all medically important venom toxins (192). If such efforts became fruitful, they could lead to better envenoming therapies with improved safety and efficacy, allowing for the deployment of such products in remote areas due to a decreased risk of anaphylactic shock and other serious immunological reactions (193). Such potential improvements could have a profound impact on rural communities in regions such as the Amazon, however, it is important to stress that all research efforts reported so far are in very early development. If monoclonal antibodies will one day find their way into clinical development, this will likely be several years into the future. Meanwhile, plasma-derived antivenoms will remain the mainstay of envenoming therapy against *Crotalus* spp.

CONCLUDING REMARKS

C. d. ruruima bites represent an important public health issue in the northernmost state of Brazil, Roraima, given the predominance of this snake subspecies in the region and the severity of its bite. Envenoming pathophysiology includes pain, edema, myolysis, bleeding, uncoagulable blood, renal dysfunction, and respiratory impairment, which have been observed more frequently in patients envenomed by *C. d. ruruima* compared to other *C. durissus* subspecies. We identified that antivenom underdosage, time to medical care, and being part of an indigenous population are risk factors of rattlesnake envenoming in Roraima state. Therefore, *C. d. ruruima* snakebites deserve special attention in the clinical and therapeutic management by health professionals. Also, better strategies for accessing specific antivenom through public distribution policies are needed. It is evident that *C. d. ruruima* venom is still among the least explored venoms from *Crotalus* species, with little existing data on its toxins and induced effects.

AUTHOR CONTRIBUTIONS

MP, PB, and WM conceived the main idea of this work. AR, PV, RF, IO, IF, and ES conducted the bibliography search. MP, PB, IO, MS, and WM designed and wrote most of this review's topics. VS performed the statistical analysis. FC designed the figures of this review article. JS, FC, AL, and FW corrected the manuscript and provided important contributions during the development of this work. All authors contributed to the article and approved the submitted version.

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Venom-Induced Blood Disturbances by Palearctic Viperid Snakes, and Their Relative Neutralization by Antivenoms and Enzyme-Inhibitors

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Palearctic vipers are medically significant snakes in the genera *Daboia, Macrovipera, Montivipera*, and *Vipera* which occur throughout Europe, Central Asia, Near and Middle East. While the ancestral condition is that of a small-bodied, lowland species, extensive diversification has occurred in body size, and niche specialization. Using 27 venom samples and a panel of *in vitro* coagulation assays, we evaluated the relative coagulotoxic potency of Palearctic viper venoms and compared their neutralization by three antivenoms (Insoserp Europe, VIPERFAV and ViperaTAb) and two metalloprotease inhibitors (prinomastat and DMPS). We show that variation in morphology parallels variation in the Factor X activating procoagulant toxicity, with the three convergent evolutions of larger body sizes (*Daboia* genus, *Macrovipera* genus, and *Vipera ammodytes* uniquely within the *Vipera* genus) were each accompanied by a significant increase in procoagulant potency. In contrast, the two convergent evolutions of high altitude specialization (the *Montivipera* genus and *Vipera latastei* uniquely within the *Vipera* genus) were each accompanied by a shift away from procoagulant action, with the *Montivipera* species being particularly potently anticoagulant. Inoserp Europe and VIPERFAV antivenoms were both effective against a broad range of *Vipera* species, with Inoserp able to neutralize additional species relative to VIPERFAV, reflective of its more complex antivenom immunization mixture. In contrast, ViperaTAb was extremely potent in neutralizing *V. berus* but, reflective of this being a monovalent antivenom, it was not effective against other *Vipera* species. The enzyme inhibitor prinomastat efficiently neutralized the metalloprotease-driven Factor X activation of the procoagulant venoms. In contrast, DMPS (2,3-dimercapto-1-propanesulfonic acid), which as been suggested as another potential treatment option in the absence of antivenom, DMPS failed against all

venoms tested. Overall, our results highlight the evolutionary variations within Palearctic vipers and help to inform clinical management of viper envenomation.

Keywords: venom, antivenom, enzyme inhibition, coagulopathy, snakebite

INTRODUCTION

Snakebite affects millions of people annually, killing over 100,000 and leaving many more with severe permanent injuries (1–3). Snake venom affects all physiological pathways reachable by the bloodstream, with blood coagulation itself a particular target (4). Despite their clinical importance, research into coagulotoxins (toxins that disrupt blood coagulation) has lagged behind other toxin types due to inherent difficulties of working with two enzyme systems (blood and venom) concurrently.

While snakebite in Europe is much less common than in African or Asiatic counties, it is still a potentially deadly medical emergency (5, 6). Common symptoms of envenomations from these vipers include local effects at the bite site such as swelling (edema), necrosis, and compartment syndrome (sometimes requiring fasciotomy), and/or systemic effects such as blood disturbances (coagulotoxicity), neuromuscular paralysis, myotoxicity, and hypotension (7–13). Severe envenomations can lead to amputation and/or death (7–13).

The Palearctic region is dominated by a clade of viperid snakes that emerged 20 million years ago, consisting of the genera *Daboia, Macrovipera, Montivipera*, and *Vipera* (**Figure 1**), with (*Daboia* + *Vipera*) being sister to (*Macrovipera* + *Montivipera*) (14). The basal morphology of this clade is small and species occupy lowland areas in arid regions. On two convergent occasions, giganticism evolved, once in *Daboia* and again independently in *Macrovipera*. Within *Vipera, V. ammodytes* is notably larger than other *Vipera* species.

Daboia and *Macrovipera* are well-characterised as causing potent procoagulant toxicity by converting the zymogen Factor X into the activated enzyme form FXa, which in turn converts prothrombin into thrombin, with the endogenous thrombin converting fibrinogen into fibrin, ultimately resulting in the development of well-ordered fibrin clots (15). The toxin class responsible for the coagulopathy produced by these snakes is a derived type of snake venom metalloprotease (P-IIId SVMP) that is characterised by two lectin toxins covalently linked to each other to form a dimer, with this dimer in turn covalently linked to the metalloprotease enzyme (16–18). In prey animals, this results in subjugation by thromboembolic stroke-induction and cardiovascular collapse from pulmonary embolism, but in human patients, the dilution of venom into a much larger blood volume results in formation of microemboli. While individual microemboli are of no clinical consequence, a net incoagulable state with morbidity and mortality results from the

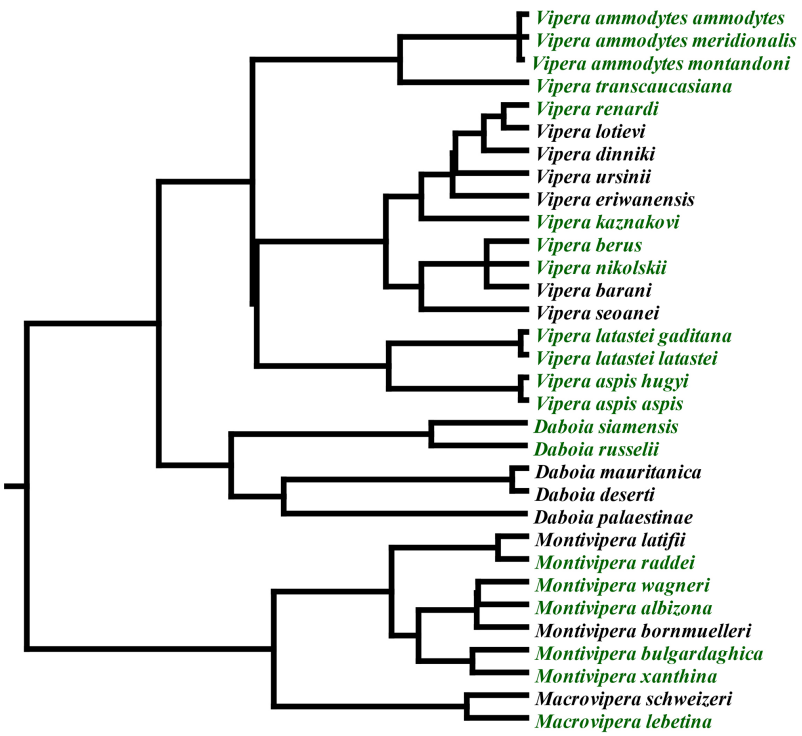

FIGURE 1 | Phylogenetic relationships of Palearctic vipers (14). Green indicates species included in this study.

consumption of clotting factors in the process of venom-induced consumption coagulopathy (19).

The genus *Vipera* is notable for being extremely widespread, radiating across Europe and Central Asia since its emergence 13 million years ago and having complex geographical histories (14, 20–22). Of particular clinical importance for human envenomings is coagulopathy (7–9, 12, 13). Documentation of Factor X activating P-IIId SVMP in at least one species (*V. ammodytes*) is consistent with its ability to produce severe coagulopathy (23). Sequence alignment shows that *Vipera* shares the characteristic cysteine used for the covalent linkage to the lectin dimer (**Figure 2**). The presence of FX activating P-IIId SVMP in *V. ammodytes* venom, like *Daboia* and *Macrovipera* venoms, combined with the observation of FX activation activity in *V. aspis* and *V. berus* venoms (24–26) and the documentation of congruent coagulopathy from *V. berus* envenomations (27) suggests that the presence of FX activating P-IIId SVMP is the basal state for the *Daboia*/ *Macrovipera*/ *Montivipera*/ *Vipera* clade.

The genus *Montivipera*, endemic to the Near and Middle East, is like *Vipera* in retaining the plesiomorphic small size but is notable for having uniquely specialized for montane habitats (28). The exception is *M. xanthina* which is derived relative to other species within this genus in being larger than the other species and radiating to occupy a lowland niche. Previous work has suggested that unlike the FX activating procoagulant basal state for the clade, this genus also has distinct anticoagulant venoms relative to the rest of the clade (29, 30) and induces systemic hemorrhage in victims rather than venom-induced consumptive coagulopathy (VICC) (31–34). The mechanism of action for this effect remains to be elucidated.

The divergence of venom documented for the *Daboia*/ *Macrovipera*/*Montivipera*/*Vipera* clade indicates these snakes are an excellent model system to examine the influence of morphology and ecology on pathophysiological venom effects and how this influences clinical treatment options. Furthermore, the development of *in vitro* assays to replace *in vivo* assays to assess antivenom efficacy has been stated as one of the main issues to be addressed for the development and improvement of antivenoms (35). In this study, we tested a wide array of species (**Figure 1**), ascertained the relative effects upon blood clotting, whether this was mediated by Factor X activation, and the efficacy of three antivenoms and two repurposed enzyme inhibiting drugs. These results not only elucidated the evolutionary processes shaping venom diversification but also how this variation might influence the ability of therapeutics to neutralise pathophysiological effects.

MATERIALS AND METHODS

Stocks Preparation

Venoms

All venom work was undertaken under the auspices of UQ IBSC approval #IBC134BSBS2015 and UQ NEWMA approval # 2021/

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K9JAW0 Daboia russelii      IILESGNVNDYEVVYPQKVTAMPKGAVKQPEQKYEDTMQYEFEVNGEPVVLHLEKNKILF
Q7T046 Macrovipera lebetina IILESGNVNDYEVVYPQKITALPEEAVQQPEQKYEDTMQYEFEVNGEPVVLHLEKNKDLF
MG958498 Vipera ammodytes   IILESGNVNDYEVVYPQKITALPKGAIQQPEQKYEDTIQYEFEVNGEPVVLHLEKNKGLF

K9JAW0 Daboia russelii      SEDYSETHYYPDGREITTNPPVEDHCYYHGHIQNDGHSSASISACNGLKGHFKLRGEMYF
Q7T046 Macrovipera lebetina SEDYSETRYSPDGRETTTKPPVQDHCYYHGRIQNDAYSSASISACNGLKGHFKLQGETYL
MG958498 Vipera ammodytes   SEDYSETHYSPDGREITTNPPVEDHCYYHGRIQNDAHSSASISACNGLKGHFQLQGETYL

K9JAW0 Daboia russelii      IEPLKLSNNEAHAVYKYENIEKEDETPKMCGVTQTNWESDKPIKKASQLVSTSAQ--FNK
Q7T046 Macrovipera lebetina IEPLKIPDSEAHAVYKYENIEKEDEAPKMCGVTQTNWESDEPIKKASQLVATSAKRKFHK
MG958498 Vipera ammodytes   IEPLKIPDSEAHAVYKYENVEKEDETPKMCGVTETNWESDEPIKKASQLVATSEQQRFPQ

K9JAW0 Daboia russelii      AFIELIIIVDHSMAKKC--NSTATNTKIYEIVNSANEIFNPLNIHVTLIGVEFWCDRDLI
Q7T046 Macrovipera lebetina TFIELVIVVDHRVVKKY--DSAATNTKIYEIVNTVNEIFIPLNIRLTLIGVEFWCNRDLI
MG958498 Vipera ammodytes   RFVELLIVVDLRMVKKYNNDSTAIRTRIYEMVNTVNEIYIPLNVRVRLIGVEFWCDRDLI

K9JAW0 Daboia russelii      NVTSSADETLDSFGEWRASDLMTRKSHDNALLFTDMRFDLNTLGITFLAGMCQAYRSVGI
Q7T046 Macrovipera lebetina NVTSSADDTLDSFGEWRGSDLLNRKRHDNAQLFTDMKFDLSTLGITFLDGMCQAYRSVGI
MG958498 Vipera ammodytes   NVTSSADDTLDSFAEWRGSDLLNRKRHDNAQLFTDMAFDLNTFGITFLAGMCQAYRSVGV

K9JAW0 Daboia russelii      VQVQGNRNFKTAVIMAHELSHNLGMYHDGKNCICNDSSCVMSPVLSDQPSKLFSNCSIHD
Q7T046 Macrovipera lebetina VQEHGNKNFKTAVIMAHELGHNLGMYHDRKNCICNDSSCIMSAVLSSQPSKLFSNCSNHD
MG958498 Vipera ammodytes   VKEH-NINFKTAVIMAHELGHNLGMSHDRKNCICGRSSCIMAPVLSDQPSKLFSNCSRDY

K9JAW0 Daboia russelii      YQRYLTRYKPKCILYPPLRKDIVSPPVCGNEIWEEGEECDCGSPADCQNPCCDAATCKLK
Q7T046 Macrovipera lebetina YRRYLTTYKPKCILNPPLRKDIASPPICGNEIWEEGEECDCGSPKDCQNPCCDAATCKLT
MG958498 Vipera ammodytes   YQRYLTRYKPKCILNEPLRKDIASRAVCGNEIWEEGEECDCGSPKDCQNPCCDAATCKLT

K9JAW0 Daboia russelii      PGAECGNGLCCYQCKIKTAGTVCRRARNECDVPEHCTGQSAECPRDQLQQNGQPCQNNRG
Q7T046 Macrovipera lebetina PGAECGNGLCCEKCKIKTAGTVCRRARDECDVPEHCTGQSAECPADGFHANGQPCQNNNG
MG958498 Vipera ammodytes   PGAECGNGLCCENCKIRTAGTECRPARDECDVPEHCTGQSAECPRDQFQQNGQPCLNNLG

K9JAW0 Daboia russelii      YCYNGDCPIMRNQCISLFGSRATVAKDSCFQENLKGSYYGYCRKENGRKIPCAPQDVKCG
Q7T046 Macrovipera lebetina YCYNGDCPIMTKQCISLFGSRATVAEDSCFQENQKGSYYGYCRKENGRKIPCAPQDIKCG
MG958498 Vipera ammodytes   YCYNGDCPIMTNQCISLFGSRATVAEDSCFEENLKGSYYGYCRKENGRKIPCAPQDIKCG

K9JAW0 Daboia russelii      RLFCLNNSPRNKNPCNMHYSCMDQHKGMVDPGTKCEDGKVCNNKRQCVDVNTAYQSTTGF
Q7T046 Macrovipera lebetina RLYCLDNSPGNKNPCKMHYRCRDQHKGMVEPGTKCEDGKVCNNKRQCVDVNTAY------
MG958498 Vipera ammodytes   RLYCLNNSPGNKNPCNMHYRCWDQHKGMVEPGTKCEDGKVCNNKRQCVDVNTAY------
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FIGURE 2 | Sequence alignment of representative Factor X activating P-IIId SVMP characteristic of the Palearctic viper clade. Highlighted is the diagnostic cysteine that forms the interchain disulfide bond to a covalently linked lectin dimer. Uniprot (*Daboia* and *Macrovipera*) and Genbank (*Vipera*) accession codes are given for each sequence.

AE000075. Pooled venoms (N = 3 captive adults unknown sex) which were immediately snap frozen in liquid nitrogen and kept at -80 until lypholisation and founder locality (if known) tested were: *Daboia russelii* (Pakistan), *D. siamensis* (Taiwan), *Macrovipera lebetina turanica* (Turkmenistan), *M. schweizer*i (Greece), *Montivipera albizona, M. bulgardaghica, M. raddei, M. wagneri, and M. xanthina (Turkey), Vipera ammodytes* (Krk Island, Croatia), *V. ammodytes* (Maribor, Slovenia), *V. ammodytes* (Ada Island, Montenegro), *V. ammodytes* (Lake Skadar, Montenegro), *V. ammodytes* (Slunj, Croatia), *V. a. meridionalis* (Greece), *V. a. montandoni* (Bulgaria), *V. aspis aspis* (France), *V. aspis hugyi* (Italy), *V. berus* (Norway), *V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia), *V. kaznakovi* (Turkey), *V. latastei latastei* (Burgos, Spain), *V. latastei gaditana* (Spain), *V. nikolskii* (Russia), *V. renardi* (Russia), and *V. transcaucasiana* (Turkey). These lyophilized venoms were reconstituted to 1 mg/ml concentrated venom stock (concentration checked using 280 nm wavelength on a Thermo Fisher Scientific™ NanoDrop 2000 UV–Vis Spectrophotometer (Thermofisher, Sydney, Australia) following the manufacturer's instructions 1 Abs = 1 mg/ml which is recommended when analyzing heterogenous solutions) by adding 50% glycerol and deionized water and stored at -20°C for further use.

Plasma

All plasma work was undertaken under the UQ IBSC approval #IBC134BSBS2015. Two bags of pooled 3.2 % citrated plasma (Label #A540020754341 & #A540020764777) were obtained from the Australian Red Cross (Research agreement #18-03QLD-09 and University of Queensland Human Ethics Committee Approval #2016000256). The two lots of plasma were pooled, aliquoted to 1 ml quantities, flash-frozen in liquid nitrogen, and stored at -80°C until required for testing. Aliquots were defrosted in at 37°C in a Thermo Haake ARCTIC water bath with a SC150-A40 circulator. Plasma aliquots were only used for up to an hour post-defrosting, after-which fresh aliquots were defrosted.

Fibrinogen

To ascertain the effect of venom on human fibrinogen clotting time, 100 mg of fibrinogen (Lot# SLBZ2294 Sigma Aldrich, St. Louis, Missouri, United States) was mixed with Owen Koller (OK) buffer (Stago catalogue #00360) to achieve a concentration of 4 mg/ml, then aliquoted to 1 ml quantities, flash-frozen, and stored at -80°C until further use. Defrosting steps and use were as per 2.1.2.

Antivenom (AV)

Antivenoms tested (and immunizing species for each antivenom) were as follows. Inoserp Europe (lot # 9IT03006), a 22.5 mg/ml $F(ab')_2$ antivenom made using an immunizing mixture consisting of *Macrovipera lebetina cernovi, M. l. obtusa, M. l. turanica, M. schweizeri, Montivipera xanthina, Vipera ammodytes, V. aspis, V. berus, and V. latastei*). MicroPharm VIPERFAV (lot #P4A281V), a 100 mg/ml $F(ab')_2$ antivenom made using an immunizing mixture consisting of *Vipera ammodytes, V. aspis, V. berus*). MicroPharm ViperaTAb (lot #VPT 002000), a 24.6 mg/ml Fab antivenom made using *V. berus* as the sole venom in the immunizing mixture. Both VIPERFAV and ViperaTAb came in a concentrate (1 X 4ml and 2 X 4ml vials respectively) solution, while Inoserp Europe was supplied in a lyophilized form, which was reconstituted with 10 ml of deionized water, according to company instructions. All antivenoms were centrifuged (RCF 14000) at 4°C for 10 min, followed by filtration of the supernatant (to remove insoluble material) using 0.45 µm Econofltr PES (Agilent Technologies, Beijing, China), aliquoted, and then stored at 4°C for future use. For tests (see 2.2.1.2), 5% AV solution was prepared by diluting with Owren Koller (OK) buffer (Stago catalogue #00360) for Inoserp, 4% for ViperaTAb, and 2% VIPERFAV. The percentages were calculated relative to the different antivenom treatment volume (10 ml, 8 ml, and 4 ml respectively) and thus the same proportion of each vial was used for the tests, thus allowing for a vial-to-vial comparison of efficacy. Therefore, calculating the ratios, the above-mentioned percentages were determined.

Enzyme Inhibitors

We set out to determine the efficacy of two small molecule inhibitors prinomastat hydrochloride ((S)-2,2-Dimethyl-4- ((p-(4-pyridyloxy) phenyl) sulfonyl) -3- thio- (catalogue# PZ0198) and DMPS 2,3-Dimercaptopropanesulfonic acid sodium salt monohydrate (catalogue # D8016) from Sigma-Aldrich. The powder was first dissolved in 10 % dimethyl sulfoxide (DMSO) and further diluted using deionized water to form 10 mM and 20mM stock solutions, respectively and stored at -80°C.

Assay Conditions

Effects Upon Clotting Times of Plasma and Fibrinogen

Coagulotoxicity Effects on Plasma and Fibrinogen

Determination of venom effects upon coagulation was done using the STA-R Max® (Stago, Asnières sur Seine, France) coagulation analyser and adapted from validated coagulation assay protocols (36–39). 1mg/ml venom stocks (50% glycerol/ 50% deionized water) were diluted to 100 µg/ml with OK Buffer (Stago catalogue #00360) to prepare the working stock, which was later loaded into the analyser for running 8-point concentration curves with serial dilutions of 1, 1/2, 1/5, 1/12.5, 1/30, 1/80, 1/160, and 1/400 (final reaction concentrations of venom). In an automated process, 50 µl venom stock (100 µg/ml starting concentration and serially diluted to form final reaction concentrations as noted above) were added to a cuvette, followed by the addition of 25 µl of OK buffer, 50 µL of 0.025 M calcium chloride (Stago catalogue # 00367), and 50 µl of phospholipid (Stago catalogue #00597), and then the mixture incubated for 2 minutes at 37°C. Subsequently 75µl of plasma or 75µl of 4 mg/ml fibrinogen was added after incubation, and clotting time was recorded immediately. To avoid abnormal results due to venom degradation, venom was changed after each set. As a positive control, coagulation activator kaolin (Stago C·K Prest standard kit, Stago catalogue #00597) was used to check for consistent plasma responses, and 25 µL of

the thrombin (Stago catalogue #115081) as a positive control to check for consistent plasma responses. The negative control for both plasma and fibrinogen studies was 50% glycerol/deionized water that was diluted to the same amount as the venom 50% glycerol venom and positive control stocks (1% final concentration for venom, positive controls, and negative controls).

Vial to Vial Antivenom Efficacy and Enzyme-Inhibitor Efficacy

Efficacy of antivenoms or enzyme inhibitors in neutralizing toxic effects of venom upon blood clotting was tested by repeating the above mentioned 8-point concentration curves, but the 25 µl of OK buffer (added to the cuvette before incubation) was replaced with 25 µl of antivenom (final reaction concentration of Inoserp Europe 0.5%, ViperaTAb 0.4%, and VIPERFAV 0.2%. The differences in concentration were reflective of the different antivenom vial size (10 ml, 8 ml, and 4 ml respectively) and thus the same proportion of each vial was used for the tests, thus allowing for a vial-to-vial comparison of efficacy. Similarly, enzyme-inhibitors at 2 mM working stock for prinomastat and DMPS, (final reaction concentration of 0.2 mM) was tested against *Daboia russelii* (Pakistan), *D. siamensis* (Taiwan), *Macrovipera lebetina turanica* (Turkmenistan), *M. schweizer*i (Greece), *V. ammodytes* (Ada Island, Montenegro), *V. aspis hugyi* (Italy).

Thromboelastography

To evaluate the strength of the clot and total thrombus generated by the venoms in plasma, further investigation was carried out by using TEG5000 haemostasis analyzers (Haemonetics®, Haemonetics.com, catalogue # 07- 033). The assay included consecutive addition of 72 µl of 0.025M $CaCl_2$, 72 µl phospholipid, 20 µl of the OK buffer, 7 µl of 1 mg/ml of venom, and 189 µl plasma to the reaction cup, followed by automated measurement. For spontaneous clotting of plasma (negative control), 7 µl 50% deionized water/glycerol was replaced with venom. Similarly, 7 µl of thrombin (Stago catalogue #115081 Liquid Fib) or 7 µl Factor Xa (Stago catalogue #253047 Liquid Anti-Xa) was replaced with venom, for two positive controls. Each reaction ran for 30 minutes.

Clotting Factor Activation Assays

Fluoroskan Ascent™ (Thermo Scientific, Vantaa, Finland) was employed to detect Factor X and prothrombin activation based on the results of the above methods (38, 39). **Table 1** reagents were manually plated in 384-well plates (black, lot#1171125, Nunc™ Thermo Scientific, Rochester, NY, USA), followed by automated pipetting of 70 µl of buffer containing 5 mM CaCl2,150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) and Fluorogenic Peptide Substrate, (ES011Boc-Val-Pro-Arg-AMC. Boc: t-Butyloxycarbonyl; 7-Amino-4-methylcoumarin; R & D systems, Cat# ES011, Minneapolis, Minnesota) in 500:1 ratio to start the reaction; with the plate warmed up at 37°C and shaken for 3 s before each measurement. The reaction was carried out 300 times at 390/460 nm (excitation/emission) and every 10 s the fluorescence generated by the cleavage of the substrate was measured by Ascent® Software v2.6 (Thermo Scientific, Vantaa, Finland). To obtain results, blank (background) values were subtracted from all other reactions, followed by subtraction of "venom without zymogen" values from "venom with zymogen" values (to nullify artificial increment of the fluorescence values caused some venoms which work directly on the substrate). Finally, the resultant values from the subtractions were normalized as a percentage relative to FXa or thrombin by organizing in Excel and then analysing in GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA).

TABLE 1 | Fluorescent substrate activation assay.

Blank	20 µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10ul PPL)
Control with Activated Enzyme	10µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10ul PPL + 10µl (10 µg/ml FXa (Haematologic Technologies catalog # GG0621) or 1 µg/ml Thrombin (Haematologic Technologies catalog # JJ0701))
Control with Zymogen	10µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10ul PPL + 10µl (10 µg/ml FX (Haematologic Technologies catalog # HH0821) or 1 µg/ml prothrombin (Haematologic Technologies catalog # HH1010))
Venom without Zymogen	10µl of enzyme buffer without calcium (150 mM NaCl, and 50 mM Tris-HCl (pH 7.3) + 10ul PPL + 10µl venom
Venom with Zymogen	10µl zymogen (10 µg/ml FX or 1 µg/ml prothrombin) + 10ul PPL + 10µl venom (1 µg/ml FX (Haematologic Technologies catalog # HH0821) or 0.1 µg/ml prothrombin (Haematologic Technologies catalog # HH1010))

Clotting Factor Inhibition Assays

Samples were diluted at 1:10 with OK buffer. 50 µl of venom + 50 µl of $CaCl_2$ (0.025M, Stago Cat#11851) + 50 µl of phospholipid solution (STA C.K. Prest standard kit, Stago Cat#12207, solubilized in 5 ml OK buffer) + 25 µl of clotting factor (thrombin, FIXa, FXa, FXIa, and FXIIa) were incubated for 120s at 37°C. Subsequently 75 µl of plasma was added, and clotting time was measured. OK buffer control was used as a negative control.

Statistical Analyses

All tests were run in triplicate. All data plotting and statistical analysis were done by using GraphPad PRISM 8.1.1 (GraphPad Prism Inc., La Jolla, CA, USA). Determination of the AV efficacy against venom, the area under the curve (AUC) for both venom and venom + antivenom was calculated using the software, followed by generation of X-fold magnitude of shift in Excel (formulae [(AUC of venom incubated with antivenom/AUC of venom) - 1]). The resulting values for X-fold magnitude of shift; if 0, indicated no neutralization (no change of clotting time curve), and if over 0, demonstrated venom neutralization (change in clotting time curve).

RESULTS

Effects Upon Clotting Times of Plasma and Fibrinogen

Coagulotoxicity Effects

The *Vipera* venoms all displayed a clotting effect upon plasma, but with a wide range of values. The maximum velocity clotting times

(in seconds) at the highest concentration (20 µg/ml) of (seconds +/- SD) (with smaller numbers indicating stronger effect) was: 15.4 +/- 0.9 *V. ammodytes* (Lake Skadar, Montenegro); 16.0 +/- 0.1 *V. ammodytes* (Ada Island, Montenegro); 18.4 +/- 1.9 *V. ammodytes* (Maribor, Slovenia); 20.4 +/- 0.5 *V. ammodytes* (Slunj, Croatia); 23.0+/- 0.6 *V. ammodytes* (Krk Island, Croatia); 23.9 +/- 0.1 *V. berus* (Slovenia); 23.9 +/- 0.1 *V. berus* (Snežnik Mountain, Slovenia); 26.7 +/- 0.3 *V. latastei gaditana* (Spain); 28.7 +/- 0.2 *V. aspis hugyi* (Italy); 30.3 +/- 0.4 *V. a. meridionalis* (Greece); 33.0 +/- 2.4 *V. berus* (Norway); 35.3 +/- 0.4 *V. kaznakovi* (Turkey); 44.1 +/- 0.4 *V. renardi* (Russia);66.2 +/- 3.4 *V. a. montandoni* (Bulgaria); 98.9 +/- 0.3 *V. aspis aspis* (France);107.8+/- 1.4 *V. transcaucasiana* (Turkey); 179.7 +/- 0.3 *V. nikolskii* (Russia); and 359.8 +/- 4.5 *V. latastei latastei* (Spain) (**Figure 3**). The plasma kaolin positive control was 51.0 +/- 0.34, while the negative control (spontaneous clotting) was 645.2 +/- 9.8

To check significant differences the values One Way ANOVA was carried out on the clotting times. There were no significant differences ($p > 0.05$ at 95.00 % confidence interval) between *V. ammodytes* (Krk Island, Croatia) versus (*vs.*), *V. ammodytes* (Slunj), Croatia) *V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia) and *V. latastei gaditana* (Spain); *V. ammodytes* (Maribor, Slovenia) *vs. V. ammodytes* (Ada Island, Montenegro), *V. ammodytes* (Lake Skadar, Montenegro) and *V. ammodytes* (Slunj, Croatia); *V. ammodytes* (Ada Island, Montenegro) *vs. V. ammodytes* (Lake Skadar, Montenegro) and *V. ammodytes* (Slunj, Croatia); *V. ammodytes* (Slunj, Croatia) *vs. V. berus* (Slovenia), *V. berus* (Snežnik Mountain, Slovenia); *V. a. meridionalis* (Greece) *vs. V. aspis hugyi* (Italy), *V. berus* (Norway) and *V. latastei gaditana* (Spain); *V. aspis hugyi* (Italy) *vs. V. berus* (Slovenia), *V. berus* (Norway) and *V. latastei gaditana* (Spain); *V. berus* (Norway) *vs. V. kaznakovi* (Turkey); *V. berus* (Slovenia) *vs. V. berus* (Snežnik Mountain, Slovenia) and *V. latastei gaditana* (Spain); *V. berus* (Snežnik Mountain, Slovenia) *vs. V. latastei gaditana* (Spain), while there were significant difference between clotting times between all other vipers.

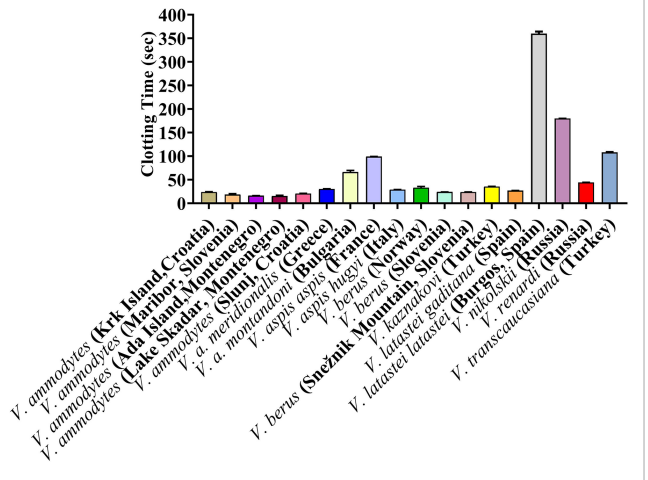

FIGURE 3 | Clotting times on human plasma produced by *Vipera* venoms (20 µg/ml).

However, like shown previously for *Daboia* and *Macrovipera* venoms (38) none of the *Vipera* venoms clotted fibrinogen with the assay measurements reaching the machine maximum of 999 seconds. The clotting time for the thrombin positive control was (seconds +/- SD) 3.6 +/- 0.1 seconds. This suggested that the clotting action shown for plasma was due to the activation of a clotting factor, which was explored further (see 3.3 below).

In contrast, all *Montivipera* venoms demonstrated potent anticoagulant actions on plasma, with the test sets all reaching the machine maximum reading time of 999 seconds. This included the derived *M. xanthina* which has secondarily colonized a lowland habitat relative to the other species in this clade (40) and also evolved a larger body size. Despite these derivations, it retains the potent anticoagulant venom characteristic of the *Montivipera* genus.

Antivenom and Enzyme Inhibitor Efficacy

Inoserp and VIPERFAV were comparable against all *V. ammodytes* populations and *V. a. meridionalis*, while VIPERFAV was moderately less effective for other species, except for *V. latastei gaditana* against which it performed comparatively poorly (**Figures 4** and **5**). Interestingly, both Inoserp and VIPERFAV, which had *V. ammodytes* as their immunizing species, showed lower level potency against *V. ammodytes* species hailing from Montenegro compared to other *V. ammodytes* populations. Consistent with ViperaTAb having only *V. berus* as an immunizing species, it performed extremely well against *V. berus* but compared to the other two antivenoms, it performed poorly against the other species except for moderate levels of neutralization of *V. renardi*.

While the antivenoms had variable differences, prinomastat highly neutralized not only *Vipera* representatives but also *Daboia* and *Macrovipera* (major metalloprotease dependent venoms) representatives at 0.2 mM concentration (**Figure 6**). In contrast, DMPS performed extremely poorly against all venoms (**Figure 6**).

Thromboelastography

Consistent with the activation of a clotting factor and the resulting generation of endogenous thrombin, all venoms produced strong, stable clots in the thromboelastography assays (**Figure 7**).

Clotting Factor Zymogen Activation

All venoms displayed the ability to activate Factor X but only negligible activation of prothrombin (**Figure 8**). The relative potencies were congruent with the action of respective venoms on plasma (**Figures 3** and **7**). *V. ammodytes* (Ada Island, Montenegro), which was the fastest on plasma, showed the highest activation of FX. Conversely, *V. renardi* was the slowest on plasma and also activated FX the least.

Clotting Factor Inhibition

As the *Montivipera* venoms were shown to be potently anticoagulant, tests were undertaken to ascertain if the

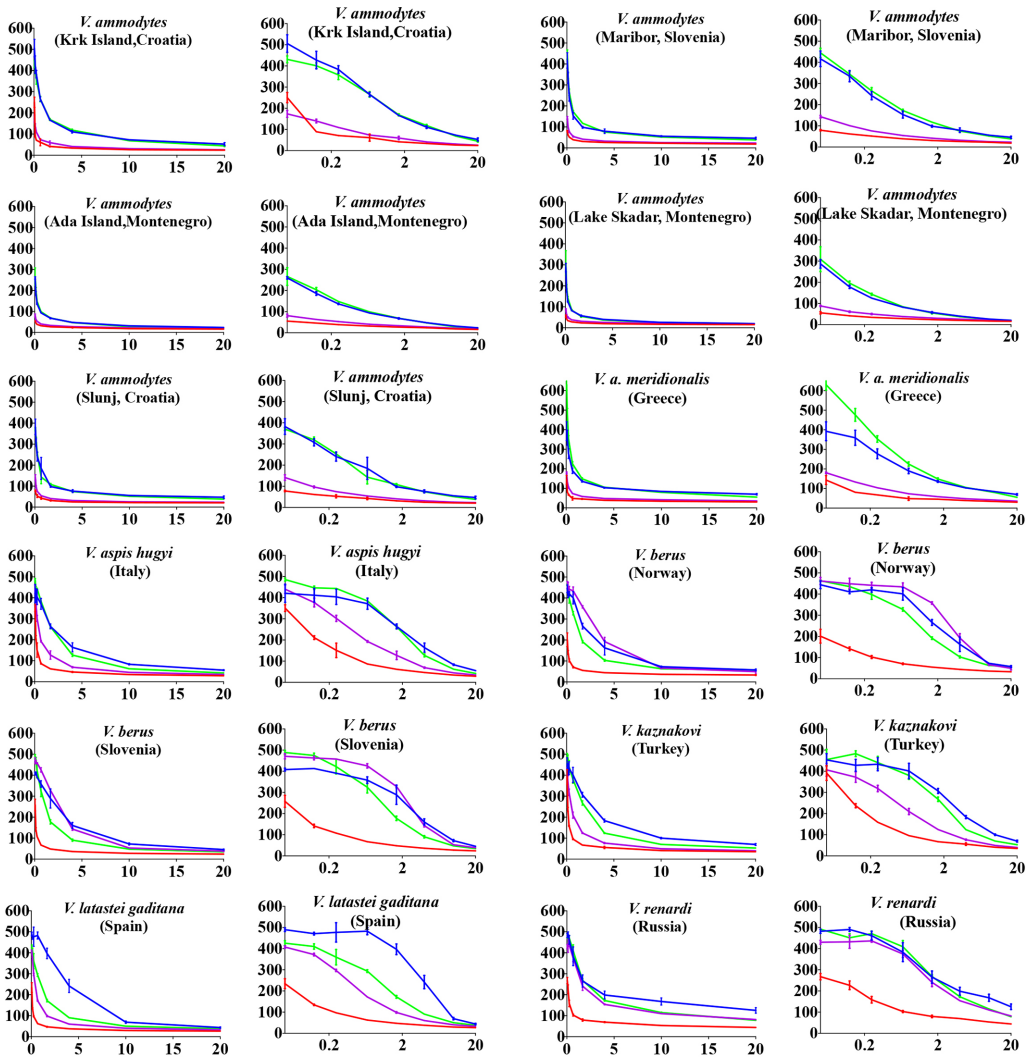

FIGURE 4 | 8-point concentration curves, x-axis showing concentrations of venom in µg/ml and y-axis showing clotting times in seconds of human plasma with venom and relative antivenom efficacy. For each species, linear graphs are presented on the left and logarithmic views on the right. Shown are: venom-induced clotting times (red curves); venom-induced clotting times after preincubation with Inoserp AV (final concentration 0.5%; spontaneous control- 420.2 +/27.7) (blue curves), ViperaTAb AV (final concentration 0.4%; spontaneous control- 478.4 +/33.3) (purple curves), or VIPERFAV AV (final concentration 0.2%; spontaneous control- 509.7 +/13.5) (green curves). Values are mean ± SD of N = 3 and shown as dots with error bars. Some error bars are too small to see.

inhibition was due to the inhibition of thrombin, FIXa, FXa, FXIa, or FXIIa. Only FXa was shown to be inhibited, with all the species having this action at comparable potency (**Figure 9**).

DISCUSSION

Our study set out to examine changes in venom biochemistry relative to two distinct types of derivation in this clade away from the diminutive, low-land niche occupying last common ancestor: that of the three convergent evolutions of giganticism (*Daboia* genus, *Macrovipera* genus, and *Vipera ammodytes* as the significantly largest member of the *Vipera* genus); and the occupation of the high-land niche by the *Montivipera* genus and *Vipera latastei latastei* as a unique high-land specialist within the *Vipera* genus.

Consistent with previous results demonstrating that *Daboia* and *Macrovipera* species have extremely potent procoagulant (Factor X activating) venoms (38), this study revealed that the largest *Vipera* species (*V. ammodytes*) was also the most potent *Vipera* in activating Factor X, with one population (Lake Skadar, Montenegro) even approaching that of *Daboia* and *Montivipera* speed of action. Consistent with the link between size and relative FX activation levels of the venoms, the more diminutive sister species *V. transcaucasiana* and all other smaller *Vipera* species were comparably less potent than *V. ammodytes*. This is in

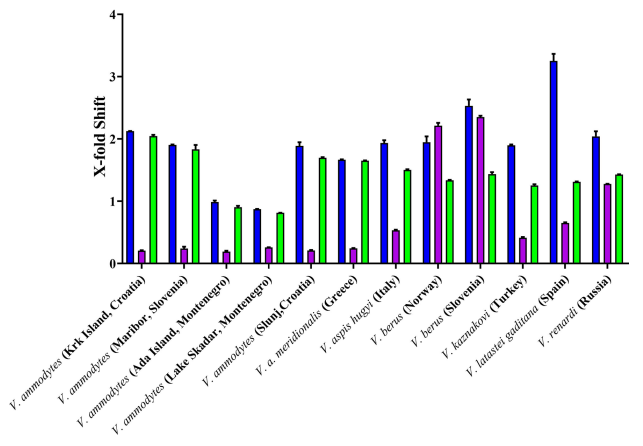

FIGURE 5 | X-fold magnitude of shift of plasma clotting time due to incubation of antivenoms indicated by: Inoserp in blue bars, ViperaTAb in purple bars; and VIPERFAV in green bars. X-fold magnitude of shift was calculated by the formula [(AUC of antivenom + venom/AUC of venom) -1]. A value of 0 indicates no shift (no neutralization by antivenom), while a value above 0 indicates neutralization by antivenom. Values are mean ± SD of N = 3.

contrast to other snakes, such as the *Bitis* genus in which gigantism evolved on two separate occasions (41), neither of which were linked to notable changes in coagulotoxicity (42).

The strongest divergence in venom action was for the *Montivipera* species. The diversification into a unique high-altitude niche relative to the low-altitude last common ancestor of the Palearctic viper clade was accompanied by a change from the procoagulant ancestral trait to potent anticoagulant toxicity. Specifically, instead of activating Factor X into Factor Xa like the last common ancestor of the Palearctic viper clade, the venom of these snakes was shown in this study to inhibit Factor Xa. This radical change in venom biochemistry was retained in *M. xanthina.* This is significant as this species is nested deep within the *Montivipera* genus (**Figure 1**), and has secondarily evolved to occupy a low-land niche from within this montane specialist genus (40). However, the relatively recent shift from high-land to low-land niche has not yet been accompanied by a change in venom biochemistry. The relationship between a secondary losses of the ancestral procoagulant trait accompanying the specialization for a high-land niche was reinforced by the convergent action within *V. latastei*, which has two subspecies: the low-land *V. latastei gaditana* and the high-altitude (2,900 to 3,600 feet) subspecies *V. latastei latastei. V. l. gaditana* is like other *Vipera* in having a FX activating procoagulant venom, while *V. latastei latastei* has lost the procoagulant trait (21).

Antivenom neutralization patterns broadly followed the immunizing mixtures. Reflective of having *V. berus* in the immunizing mixture, ViperaTAb was strongly effective only against the *V. berus* venoms. The multi-species immunizing mixture of VIPERFAV (*Vipera ammodytes, V. aspis, V. berus*) was reflected in its multi-species neutralisation potential. Consistent with Inoserp Europe having a very complex immunizing mixture (the *Vipera* species *V. ammodytes, V. aspis, V. berus*, and *V. latastei*, in addition to the *Macrovipera* species *M. lebetina cernovi, M. schweizeri, M. l. obtusa, M. l. turanica* and the *Montivipera* species *M. xanthina*), it displayed the greatest paraspecificity. Consistent with the variation between species and populations within a species observed in this study, previous work using other methods to ascertain variations in other characteristics have similarly reported such variations (43–47). The discrepancy of antivenom neutralizing property on the venom of same species from different locations or within genus is evident in other studies, which is a major issue in antivenom production (42, 48–58).

The comparative testing of the enzyme inhibitors prinomastat and DMPS revealed highly contrasting differences in their specific abilities to neutralize the P-IIId SVMP responsible for FX activation by the venoms in this study. This is congruent with recent data published for FX activation of neonate *Crotalus culminatus* venom, which prinomastat neutralised but DMPS at the same concentration did not (59) and another report of DMPS requiring long incubation times to exert a discernable effect (60).

Additional important caveats are that this study only examined the neutralization capacity of antivenoms and enzyme-inhibitors using *in vitro* systems. Neutralization assays conducted in this study were under preincubation conditions and as such, demonstrated failure under such idealized conditions are strongly suggestive of failure under dynamic physiological conditions. Thus the limited taxonomical range of ViperaTAb antivenom in this study is strongly suggestive of limited taxonomical range when tested *in vivo* and real-world use, as is the failure of DMPS suggestive of *in vivo* and real-world failure. However, success under such *in vitro* conditions does not automatically translate into success in *in vivo* models or real-world clinical cases. Therefore additional studies must be undertaken using *in vivo* assays and then clinical studies before clinical recommendations can be made. Thus while the Inoserp and VIPERFAV antivenoms, and the enzyme-inhibitor prinomastat, are predicted by this study to have broad utility, this must be corroborated by *in vivo* and subsequent clinical studies.

Another important caveat is that this study focused solely upon the coagulotoxic effects of these venoms. Each of these snake venoms has clinically relevant profile extending beyond coagulopathy, such as neurotoxicity, another major lethal trait (61). In addition to P-IIId SVMP, these venoms contain myriad other toxins, including snake venom serine protease (SVSP), phospholipase A_2 (PLA_2), and L-Amino acid oxidase (LAAO) (62). In a study on *V. berus* venom, the complex presence of basic phospholipases, SVSPs, LAAO, SVMP, were responsible for hemotoxicity, myotoxicity, cytotoxicity and neurotoxicity (63). *V. aspis* shared similar toxins with *V. berus* along with PLA_2 (ammodytoxin B-like PLA_2: neurotoxic effect), SVMP inhibitor, SVMP, SVSP, and disintegrins; however, a higher presence of disintegrins were seen in *V. aspis* compared to *V. berus* (64, 65). In separate studies on *V. kaznakovi* and *V. anatolica* venom, an abundance of SVMP was evident. However, *V. kaznakovi* had a

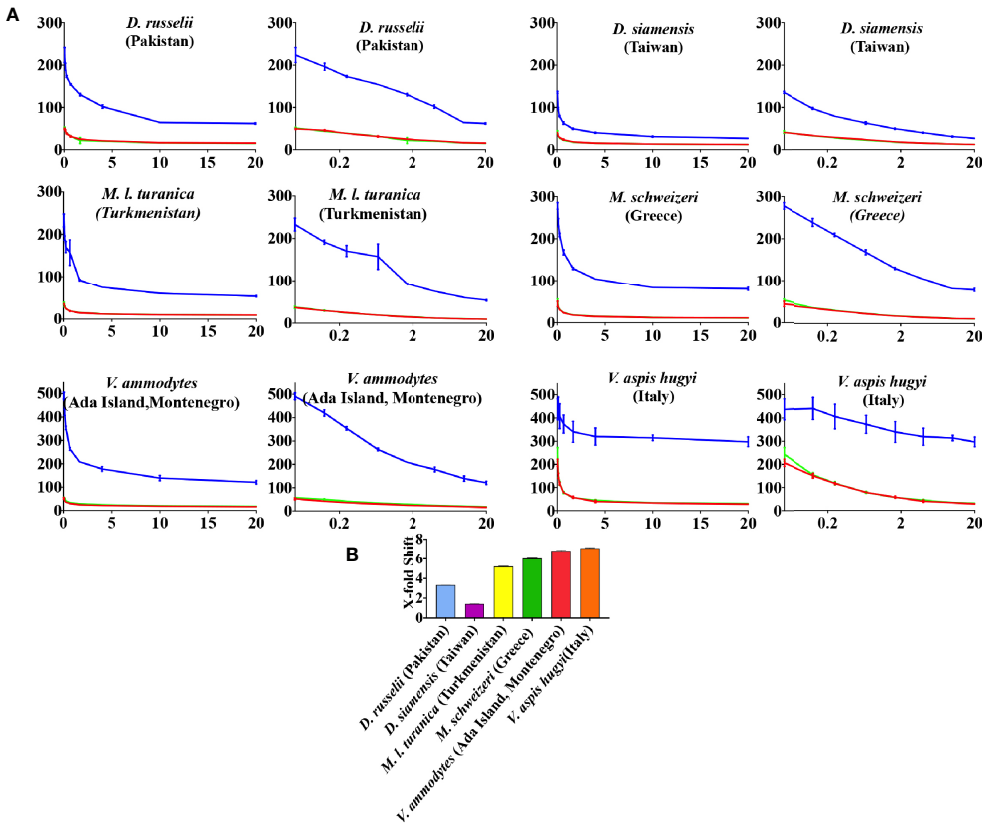

FIGURE 6 | **(A)** 8-point concentration curves, x-axis showing concentrations of venom in μg/ml and y-axis showing clotting times in seconds of human plasma with venom and relative inhibitor efficacy. For each species, linear graphs are presented on the left and logarithmic views on the right. Shown are venom-induced clotting times (red curves), effect of venoms after preincubation with prinomastat (final concentration 0.2 mM%; spontaneous control- 484.8 +/- 11.0) (blue curves), and effect of venoms after preincubation with DMPS (final concentration 0.2 mM %; spontaneous control- 425.8 +/3.3) (green curves). Values are mean ± SD of N = 3 and shown as dots with error bars. Some error bars are too small to see and the failure of DMPS to shift the curves results in an identical line to the red (venom only) curves. **(B)** Bar graphs of X-fold magnitude of shift of plasma clotting time due to induction of prinomastat. X-fold magnitude of shift was calculated by the formula [(AUC of inhibitor + venom/AUC of venom) -1]. A value of 0 is no shift (no neutralization by inhibitor), while a value above 0 indicates neutralization by inhibitor. Values are mean ± SD of N = 3.

higher percentage of PLA_2 and SVSP compared to *V. anatolica* (46, 66). *V. nikolskii*, *V. orlovi*, *V. renardii*, and *V. kaznakovi* were all reported to have greater PLA_2 percentage compared to other toxins followed by SVMPs (67–69). Great diversity may exist within each of these toxin classes. For example, a SVSP isolated from *V. ammodytes* venom (VaaSP-VX) has been shown to activate Factor X (FX) and Factor V (FV) simultaneously, a function congruent with the metalloproteases in this study (although this toxin is in much lower levels in the venom than the metalloprotease), while another *V. ammodytes* SVSP (VaF1 toxin) has α-fibrinogenolytic activity (70, 71). Another *V. ammodytes* toxin, a myotoxic secreted PLA_2 analogue ammodytin L (AtnL) was reported to cause irreversible atrioventricular (AV) blockade (72, 73). *V. ammodytes meridionalis* has been shown to share similar toxins as well as vipoxin (PLA_2, postsynaptic neurotoxin) (74). The presence of additional toxin actions beyond those examined in this study is reflected in complex envenomation clinical profiles (61). Thus, more comprehensive *in vitro* assays and also *in vivo* studies must be conducted before clinical recommendations can be finalised regarding treatment options for particular species or populations within a species.

This work provides data useful for predicting potential clinical effects and contributing to the evidence-based design of clinical management strategies. As with *Daboia* and *Macrovipera*, an increase in *Vipera* species sizes was correlated with an increase in the FX activation potency, with the largest species (*V. ammodytes*) possessing the most potently procoagulant venom. Consistent with their multi-species immunizing mixture, both Inoserp Europe and VIPERFAV showed broad paraspecificity. In contrast, and consistent with *V. berus* as the sole immunizing venom, ViperaTAb strongly specific for *V. berus*. While the small molecule inhibitor prinomastat nullified the effects being tested in representative venoms, DMPS failed to do so at the same molar

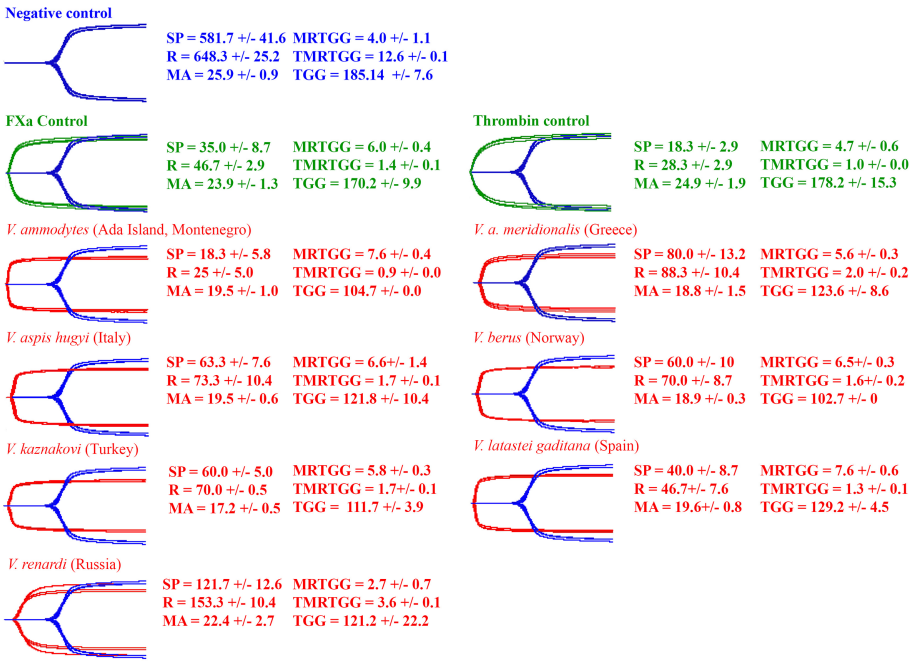

FIGURE 7 | Overlaid thromboelastography traces of spontaneous clotting negative control (blue), Factor X and thrombin positive controls (green) and venom-induced clotting experimental condition (red). Parameters: SP = the split point (time till clot formation begins) (sec); R = time until detectable clot (2 mm +) is formed (sec); MA = maximum amplitude of clot (mm); MRTG = maximum rate of thrombus generation (dynes/cm2/s); TMRTG = time to maximum rate of thrombus generation (min); and TGG = total thrombus generated (dynes/cm2). Values are mean ± SD of N = 3.

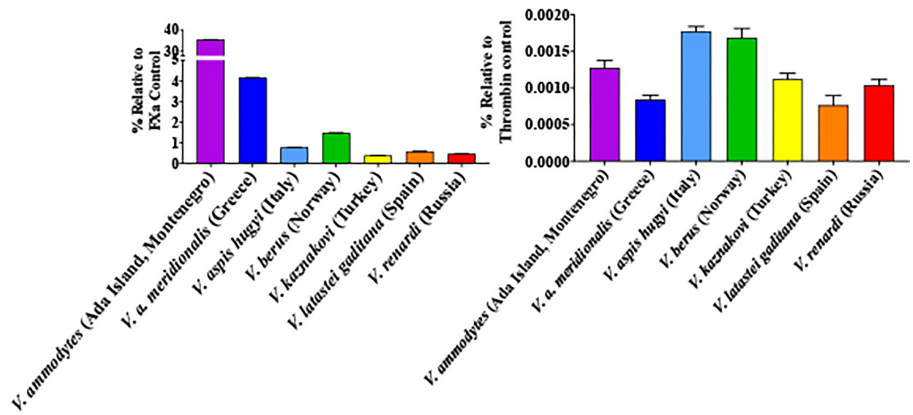

FIGURE 8 | Ability of venoms to activate Factor X (left) compared to prothrombin (right). Results are presented relative to the control of the same amount of the corresponding activated enzyme form (note difference in y-axis scales between the two graph sets). Data points are N = 3 mean ± SD.

concentrations and experimental conditions, including incubation times. In contrast to the retention of the FX activating procoagulant trait in *Vipera* species, *Montivipera* venoms were shown to be unique for the clade, exhibiting anticoagulant activity through the inhibition of FXa, paralleling its specialization for a unique montane habitat. This trend has also been noted for the lowland *V. l. gaditana*, which retained the FX activating procoagulant trait, while the derived high-altitude subspecies *V. l. latastei* had a secondary reduction in FX potency. This study therefore underscores the importance of studying evolution in parallel to venom biochemistry in order to provide data essential for understanding potential clinical effects of particular species or populations, and the relative therapeutic options.

Future work should examine for prey specific effects to reconstruct the evolutionary shaping pressures by testing several hypotheses emerging from this work regarding the

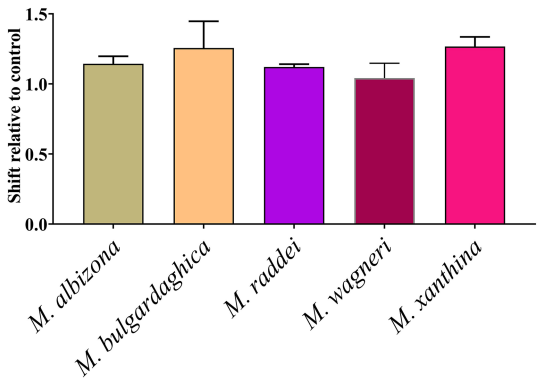

FIGURE 9 | Shift of clotting time relative to control due to inhibition of Factor Xa (no effect relative to control would be a value of 0). Data points are N = 3 mean ± SD.

selection pressures exerted by prey type, prey retaliation potential, and prey escape potential, all of which have been shown to be major drivers of venom evolution (75–77). First is the hypothesis that as mammals are particularly sensitive to procoagulant toxins due to the high circulatory rates making them rapidly subjugated by stroke as a consequence of the large blood clots formed by the venoms, the evolution larger body size is linked to an increased proportion of mammalian prey in the diet (78). This is in turn linked to a second testable hypothesis, that the greater proportion of mammals in the diet, leads to an increased chance of prey retaliation and thus a selection pressure for the increased levels of stroke-inducing procoagulant toxins in order to rapidly subjugate such dangerous prey, as has been seen for mammal specialists such as Australian elapids in the *Oxyuranus* and *Pseudonaja* genera (37). A third testable hypothesis is that the specialization into a high-altitude shift is linked to either a shift in prey type towards amphibian or reptilian prey, thus providing the selection pressure for the down-regulation of the procoagulant venom phenotype and, in the case of *Montivipera* the evolution of the anticoagulant phenotype. A linked fourth testable hypothesis is that the relative prey escape potential is another significant variable driving venom evolution, whereby montane habitats occupied by *Montivipera* and *V. l. latastei* with abundant rock cracks result in higher chance of prey escaping into inaccessible areas, versus snakes living in lowland arid habitats which are able to scent track prey over considerable distances. Such variation in relationship to altitude and prey type and prey escape potential has been noted for the rattlesnake species *Crotalus helleri* (79). In addition, as all venom samples used in this study from adult specimens, future work should examine ontogenetic shifts to ascertain if juvenile snakes have differentially procoagulant venoms, which has been noted for other species (59, 78, 80, 81).

This work thus has a broad impact, contributing to the understanding of the lethal coagulopathy produced by some species, while also providing a starting point for diverse evolutionary studies. We hope these findings stimulate further research into the evolution of venom in this group of fascinating snakes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UQ Human Ethics Committee Approval #2016000256 using pooled plasma from anonymous patients, supplied by the Australian Red Cross under Research Agreement #18-03QLD-0. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by UQ NEWMA approval # 2021/AE000075.

AUTHOR CONTRIBUTIONS

Study conception: BF. Study design AC, CZ, ML, RC, MA, and RS. Resources ML, RC, TJ, HH, MA, and RS. Conducting of experiments. AC, CZ, and BF. Data analysis. AC, CZ, and BF. Primary draft writing AC and BF. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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