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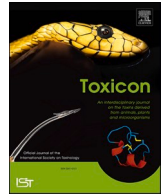
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Short communication

Discovery of a human monoclonal antibody that cross-neutralizes venom phospholipase A₂s from three different snake genera

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ABSTRACT

Despite the considerable global impact of snakebite envenoming, available treatments remain suboptimal. Here, we report the discovery of a broadly-neutralizing human monoclonal antibody, using a phage display-based cross-panning strategy, capable of reducing the cytotoxic effects of venom phospholipase A₂s from three different snake genera from different continents. This highlights the potential of utilizing monoclonal antibodies to develop more effective, safer, and globally accessible polyvalent antivenoms that can be widely used to treat snakebite envenoming.

Snakebite envenomings remain a critical global health issue. According to the World Health Organization (WHO), an estimated 1.8 to 2.7 million people globally suffer from snakebite envenomings each year. Tragically, between 81,000 and 138,000 of these cases result in fatalities, and many survivors experience permanent sequelae, including loss of limbs and disfigurements (Gutiérrez et al., 2017; Kasturiratne et al., 2008; Roberts et al., 2022). Although the burden is global, the impact of snakebite envenomings is felt most acutely in Africa, South Asia, and Central and South America (Harrison et al., 2009).

Snake venoms are intricate mixtures of proteins and peptides, unique to each species, although they often exhibit intra-species variation due to factors like geographical location and age of the snakes (Calvete et al., 2010, 2011; Casewell et al., 2020; Madrigal et al., 2012). Despite this complexity, venom toxins can be classified into several major families, with the phospholipase A₂s (PLA₂s), three-finger toxins (3FTxs), snake venom metalloproteases (SVMPs), and snake venom serine proteases (SVSPs) being the medically most important ones across most snake species (Bermúdez-Méndez et al., 2018). Notably, PLA₂s, known to inflict diverse toxic effects such as myotoxicity, cytotoxicity, neurotoxicity, and hemotoxicity, are universally present in the venoms of all known venomous snake species (Borges et al., 2017). Traditional

antivenoms are currently the only specific treatment option for snakebite envenoming. However, these are often limited by their high cost of goods sold (COGS) and the potential adverse immune reactions they may cause (Gutiérrez et al., 2017; Kasturiratne et al., 2008; Roberts et al., 2022), which emphasizes the urgent need for improved and more accessible snakebite therapies.

Efforts are currently underway to improve existing antivenom therapies and to develop alternatives. Among these, monoclonal antibodies (mAbs) and small molecule inhibitors have been demonstrated to hold promise (Albulescu et al., 2020; Glanville et al., 2022; Jones et al., 2022; Laustsen et al., 2018; Ledsgaard et al., 2022a, 2023; Xie et al., 2020). In this relation, recombinant antivenoms, composed of such monoclonal antibodies have therefore been hypothesized as a promising therapy for snakebite envenoming therapy (Laustsen, 2016; Laustsen, 2016, 2017, 2018). For such recombinant antivenoms, the discovery of broadly-neutralizing monoclonal antibodies (bnAbs) against multiple toxins could simplify the antivenom composition by limiting the number of unique antibodies needed for broad neutralization, thereby making their manufacturing more feasible (Ledsgaard et al., 2023; Zhang et al., 2003).

Building upon our prior work using a phage display-based cross-

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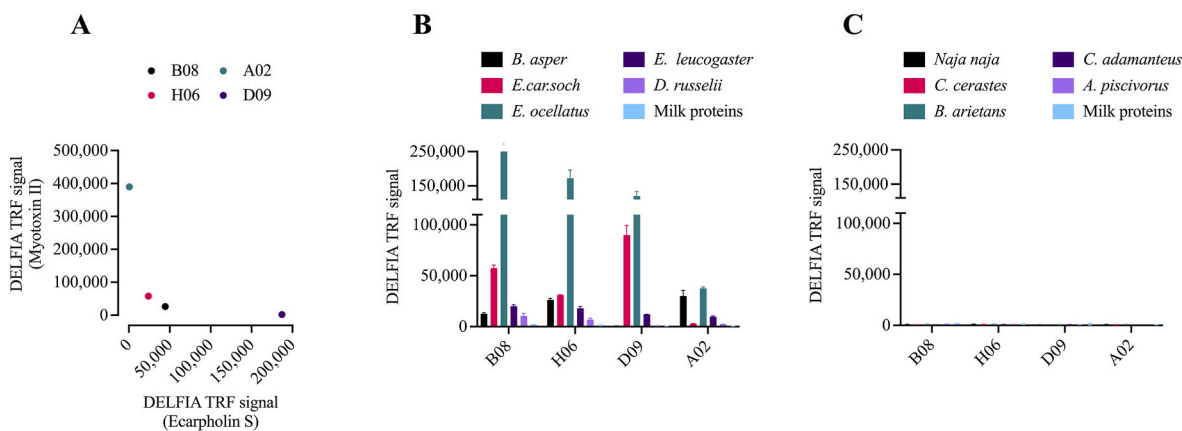


Fig. 1. Assessment of scFv cross-reactivity. **A)** DELFIA-based assessment of the four selected scFvs plotted with their binding to ecarpholin S and myotoxin II on the x and y axis, respectively, as published in (Sørensen et al., 2023b). **B and C)** The four selected scFvs were tested for their binding to 10 different snake venoms plus milk proteins as a negative control antigen. Binding was measured as the TRF signal using a wavelength of 320 nm for excitation and 615 nm for emission on a Victor Nivo Multimode Microplate Reader.

panning strategy for the discovery of cross-reactive single-chain variable fragments (scFv) (Ahmadi et al., 2020; Laustsen et al., 2021; Ledsgaard et al., 2022b; Sørensen et al., 2023b), in this study, we aimed to assess the degree of cross-reactivity in a selected subset of scFvs and whether their cross-reactive binding properties translate into broad toxin neutralization. Using a combination of immunoassays and *in vitro* cytotoxicity assays using muscle stem cells (myoblasts), we identified a PLA₂-specific antibody that reduces the cytotoxic effects of viperid snake venoms from Central America, Africa, and Asia. More specifically, two types of experiments were carried out: Dissociation-Enhanced Lanthanide Fluorescent Immunoassays (DELFIAs) and myoblast-based neutralization assays. DELFIAs were performed by coating black MaxiSorp plates (Nunc) with whole snake venoms (20 µg/mL of *Bothrops asper*, *Echis carinatus sochureki*, *E. ocellatus*, *E. leucogaster*, *Daboia russelii*, *Naja naja*, *Cerastes cerastes*, *Bitis arietans*, *Crotalus adamanteus*, or *Agkistrodon piscivorus*, all obtained from Latoxan) overnight at 4 °C. After blocking using 3% (w/v) skimmed milk in phosphate-buffered saline (MPBS), individual bacterial expression supernatants containing monoclonal scFvs in 3% MPBS were added (25 µL supernatant in 25 µL 6% MPBS). Bound scFvs were detected by the addition of a europium-conjugated anti-FLAG IgG (Sigma, F3165) and DELFIA Enhancement solution (PerkinElmer, 4001-0010), followed by measuring the time-resolved fluorescence (TRF) signal with excitation at 320 nm and emission at 615 nm in a Victor Nivo Multimode Microplate reader. The intensity of the signal was directly correlated with the amount of scFv bound to the coated venom.

Myoblast-based neutralization assays were carried out using a murine (C2C12) myoblast cell line, which were cultured in Dulbecco's Modified Eagle Medium (DMEM) [with 1% (v/v) penicillin-streptomycin and 10% (v/v) foetal bovine serum (FBS)] at 37 °C with 5% CO₂. Cells were seeded at 10,000 cells per well in 100 µL of growth medium and allowed to adhere overnight. Next, cells were incubated with 100 µL of the PLA₂-like toxin myotoxin II (50 µg/mL and 100 µg/mL for optimization assays and 100 µg/mL for neutralization assays) purified from whole *B. asper* venom as described previously (Lomonte and Gutiérrez, 1989; Mora-Obando et al., 2014; Sørensen et al., 2023b) or venoms (15.6–1000 µg/mL of *B. asper*, *E. c. sochureki*, *E. ocellatus*, *E. leucogaster*, or *D. russelii* venom for optimization assays and 125 µg/mL for neutralization assays, except for *D. russelii* venom, where 250 µg/mL venom was used as this venom was less cytotoxic to C2C12 cells) in growth medium for 24 h. The viability of the cells was thereafter measured by employing an MTS tetrazolium-based assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega), where the MTS reagent was incubated for 3 h followed by measuring the absorbance at 490 nm in a microplate reader. For neutralization assays, the

antibody was preincubated for 30 min at 37 °C with myotoxin II (1:1, 1:2, and 1:2.7 toxin:antibody molar ratio) or whole venoms (3 mg/mL antibody for all venoms, except *D. russelii* venom, for which 6 mg/mL antibody was used) before addition to the cells. The signal of control cells cultivated with only medium was considered as 100% viability to calculate the effect of venom/toxin with and without antibody addition.

The discovery and initial screening of scFvs and antibodies tested in this study is described in our previous study (Sørensen et al., 2023b). In short, a naïve scFv antibody phage display library was used for phage display selection campaigns on venom fractions containing the PLA₂-like snake toxins ecarpholin S (P48650) from *E. c. sochureki* and myotoxin II from *B. asper* (P24605) (Sørensen et al., 2023b). Based on the initial scFv characterization published in Sørensen et al. 2023, four scFvs were selected for further cross-reactivity testing – two scFvs (126_01_B08 (B08) and 127_02_H06 (H06)) which showed cross-reactive binding against ecarpholin S and myotoxin II and two scFvs (125_01_D09 (D09) and 127_02_A02 (A02)) that showed mono-specificity against either ecarpholin S or myotoxin II, respectively (Fig. 1A).

While examining the breadth of the binding of the anti-PLA₂ scFvs to ten different snake venoms, it was discovered that the two scFvs, which had already shown cross-reactive potential (Fig. 1A), could bind to the venoms from *B. asper*, *E. c. sochureki*, *E. ocellatus*, *E. leucogaster*, and *D. russelii* (Fig. 1B). Further, the anti-ecarpholin S scFv, 125_01_D09, was observed to bind to the venoms from *E. c. sochureki*, *E. ocellatus*, and *E. leucogaster*, which is likely because of intra-genus venom/toxin similarities. Surprisingly, the myotoxin II binding scFv (127_02_A02) showed binding to venoms from *B. asper*, *E. ocellatus*, and *E. leucogaster*, but showed no binding to the venom from *E. c. sochureki*, hereby highlighting intra-genus venom/toxin similarities and inter-genus venom/toxin differences. None of the four selected scFvs showed any binding to the venoms of *N. naja*, *C. cerastes*, *B. arietans*, *C. adamanteus*, and *A. piscivorus*. The two scFvs that showed broad cross-binding properties, with binding to at least five different snake venoms, originate from the use of cross-panning strategies with venom fractions containing mainly ecarpholin S (P48650) from *E. c. sochureki* venom and myotoxin II (P24605) from *B. asper*, respectively (Sørensen et al., 2023b). While the observed binding of anti-PLA₂ scFvs to snake venoms within the *Echis* or *Bothrops* genera could be expected due to intra-genus venom/toxin similarities to the toxins used during discovery, the ability of these scFvs to also bind to *D. russelii* venom components was less expected, as no *Daboia* toxins were involved in the discovery process. Additionally, the significantly higher binding signals exhibited by the scFvs towards *E. ocellatus* venom compared to *E. c. sochureki* are noteworthy as *E. c. sochureki* has been reported to have a higher amount of PLA₂s than the

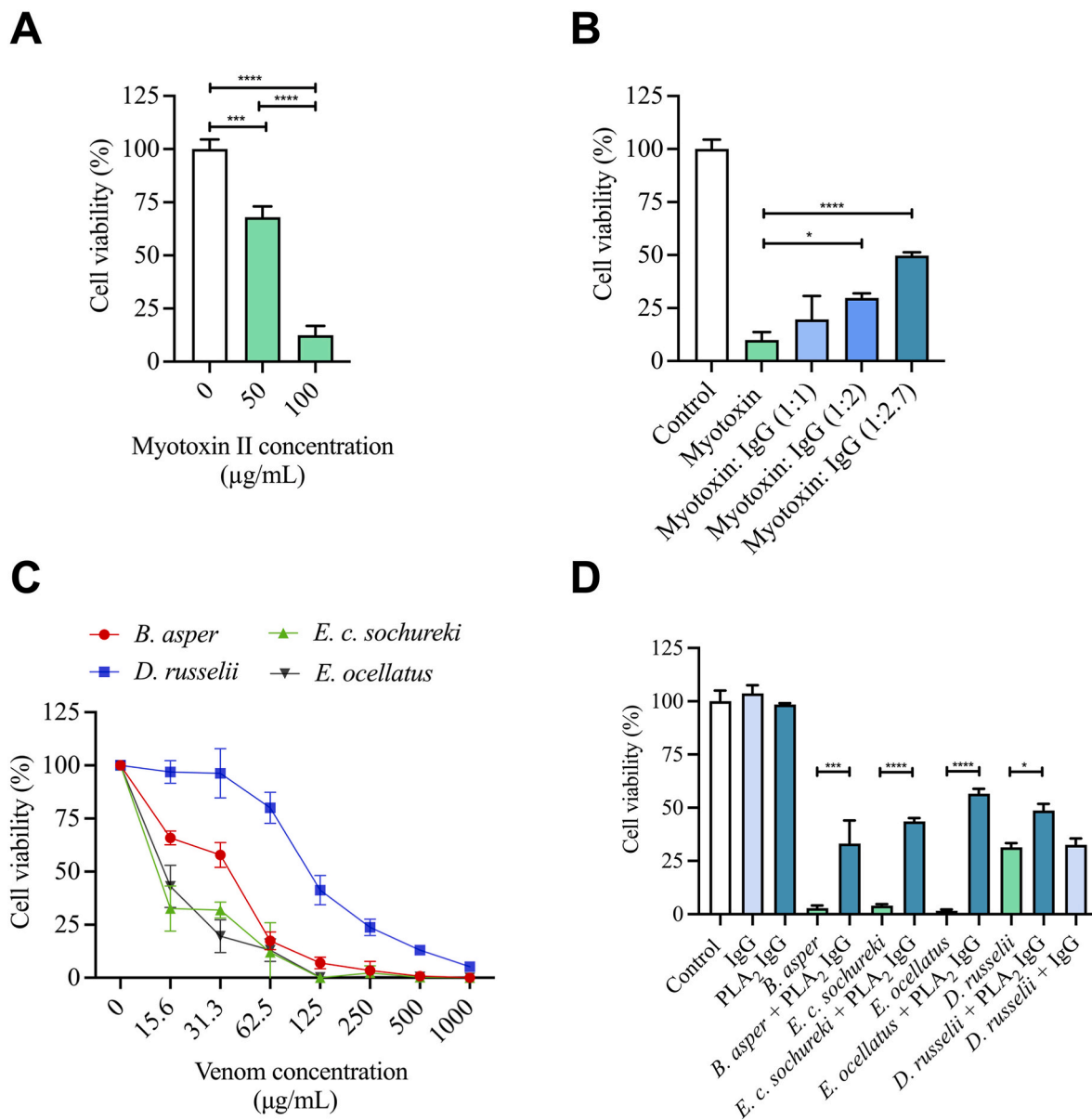


Fig. 2. Assessment of the neutralizing capacity of the B08 IgG in a murine C2C12 myoblast-based cytotoxicity assay. **A)** Establishment of the optimal myotoxin II quantity for neutralization assessment through dose optimization experiments. **B)** Different molar ratios of the B08 IgG were preincubated with 100 µg/mL of myotoxin II followed by cell viability measurements to test the ability of the IgG to neutralize myotoxin II. **C)** Establishment of the optimal venom quantity for neutralization assessment through dose optimization experiments. **D)** Neutralization of venom-induced toxicity using 125 µg/mL venom and 3 mg/mL IgG (PLA₂ IgG), except for *D. russelii*, where 250 µg/mL of venom and 6 mg/mL of the IgG was used instead due to the lower toxicity of the venom to the cells. A non-myotoxin-targeting IgG was included as an isotype control (IgG). Asterisks (**** = p-value <0.0001; *** = p-value <0.001; * = p-value <0.05) note significant statistical differences, and error bars represent the mean ± S.D. (n = 3). Statistics were carried out using a one-way ANOVA with Tukey's multiple comparison test.

venom from *E. ocellatus* (Casewell et al., 2009; Dam et al., 2018; Wagstaff et al., 2009). Thus, the higher binding signals could either be caused by a higher affinity to *E. ocellatus* PLA₂s, or they could be caused by *E. ocellatus* venom containing more PLA₂s that resemble ecarpholin S or myotoxin II, which were used in the discovery process.

To reformat the antibody to a therapeutically relevant format, the B08 scFv was converted to a human immunoglobulin G (IgG) by extracting the DNA sequences for the variable chains from the pSANG10-3 F vector and cloning these into a single expression vector containing the constant domain sequences of the respective human IgG heavy chain and human kappa light chain, as described previously (Sørensen et al., 2023a). The B08 IgG was then assessed for its capacity to neutralize venom-induced toxicities in myoblast-based cytotoxicity assays. First, two different toxin concentrations (50 µg/mL and 100

µg/mL) were evaluated to establish the optimal toxin quantity for neutralization assessment, which was found to be 100 µg/mL myotoxin II (Fig. 2A). Thereafter, different molar ratios of IgG were preincubated with 100 µg/mL of myotoxin II and applied to myoblasts for 24 h. The outcomes demonstrated significant reduction of cytotoxicity at toxin: IgG molar ratios of 1:2 and 1:2.7 (Fig. 2B). Finally, a preliminary venom dose optimization assay was carried out (Fig. 2C), followed by an evaluation of the neutralization of cytotoxicity by the B08 IgG against the venoms of *B. asper*, *E. c. sochureki*, *E. ocellatus*, and *D. russelii* (Fig. 2D). This experiment demonstrated that the IgG significantly reduced the cytotoxicity of all four tested venoms on mouse myoblasts. While the B08 IgG reduced the cytotoxicity of the venoms, some degree of cytotoxicity still occurred in the experiments, which could be due to the presence of PLA₂s not neutralized by the IgG or that other types of

toxins could have caused the observed cytotoxicity. Alternatively, the remaining cytotoxicity could be due to the IgG only being able to partially neutralize the PLA₂s. Nevertheless, as viperid snake venoms contain many cytotoxic proteins, especially from the PLA₂ and SVMP families, full neutralization of cytotoxicity by a single monoclonal antibody targeting PLA₂s would have been unexpected.

The findings in this study build upon and extend prior investigations into the use of phage display-based cross-panning strategies for the discovery of cross-reactive antibodies (Ahmadi et al., 2020; Laustsen et al., 2021; Ledsgaard et al., 2022b; Sørensen et al., 2023b). Here, we identified an antibody capable of neutralizing the cytotoxic effects on mouse myoblasts (which may directly reflect venom-induced myotoxicity) of viperid snake venoms from three different genera, originating from three different continents. Further, our study reveals specific binding of our IgG and other scFvs to *Echis*, *Bothrops*, and *Daboia* venoms, while no binding is observed to other tested venoms. This selectivity raises intriguing questions about how the underlying biochemical or structural characteristics of the toxins in each venom contribute to this specificity, which warrants further investigation.

In conclusion, our findings underscore the potential of using antibody phage display-based cross-panning strategies to discover broadly-neutralizing antibodies. Continuous development and optimization of the broadly-neutralizing antibodies discovered in this study and elsewhere could have the potential to improve the design of next-generation antivenoms. We therefore propose that this approach is used to facilitate the development of recombinant antivenoms with exceptionally broad neutralization profiles for world-wide use to treat snakebite envenoming.

Credit author statement

Conceptualization: CVS, AL, SV, AHL. Methodology: CVS, JRA, AHL. Investigation: CVS, JRA, ERT. Visualization: CVS, JRA, MFB. Funding acquisition: AHL, BV, SV. Project administration: CVS, AHL. Resources: CVS, JRA, SS, SV, AHL. Supervision: AL, SV, AHL. Writing – original draft: CVS, MFB. Writing – review & editing: CVS, JRA, MFB, ERT, SS, AL, SV, AHL.

Ethical statement

No experimentation on human or animal subjects was involved in this study.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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