Evidence that neutrophils do not promote *Echis carinatus* venom-induced tissue destruction

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Katkar et al.\(^1\) propose that neutrophils can mediate tissue damage induced by venom of the saw-scaled viper (*Echis carinatus*) by generating neutrophil extracellular traps (NETs), which can entrap venom toxins at the injection site, thereby accelerating local tissue destruction but protecting from systemic damage. Each year, snakebites are responsible for more than 400,000 amputations and 125,000 deaths worldwide. Together, the Indian cobra (*Naja naja*), the krait (*Bungarus caeruleus*), the Russell’s viper (*Daboia russelii*), and *E. carinatus* compose the group of snakes referred to as the “Big Four”, which account for the majority of snakebite-related deaths in India. *E. carinatus* venom (ECV) induces particularly strong tissue destruction at the bite site, but the cellular and molecular mechanisms regulating this tissue damage are unclear.

As part of this study, Katkar et al. report that treatment of mice with cyclophosphamide reduces tissue damage induced by *E. carinatus* carinatus (South Indian saw-scaled viper) venom, an effect they attribute to deficiency of neutrophils in cyclophosphamide-treated mice. However, when we treat mice with cyclophosphamide before injecting venom from *E. carinatus sochureki* in the tail, we find that cyclophosphamide has very little effect on mortality and venom-induced tissue destruction, as assessed by the same tail injury scoring method used by Katkar et al.\(^1\) (Fig. 1a, e). This distinction might reflect differences in the types and amounts of venom used, so we confirm our results using venom from two other *E. carinatus* subspecies: *E. carinatus multisquamatus* and *E. carinatus pyramidium*. Again, treatment with cyclophosphamide does not reduce tail injury and has no effect on mortality after injection of venoms from these two subspecies of *E. carinatus* (Supplementary Fig. 1). As expected, treatment with cyclophosphamide induces strong neutropenia (Supplementary Fig. 2a), but this broad immunosuppressive drug also reduces the number of circulating monocytes, T cells, and B cells (Supplementary Fig. 2b–d), and the spleen weight by 40% (Supplementary Fig. 2e, f). Therefore, we do not think the effect of this pleiotropic drug in the article by Katkar et al.\(^1\) is attributable to neutrophils.

Because cyclophosphamide suppresses many cell populations, and different batches of the drug could differentially affect degree of neutropenia (which might also contribute to the differences between our results and those of Katkar et al.\(^1\)), we reevaluate the role of neutrophils in response to ECV using antibody or genetic approaches to deplete neutrophils in mice. Similar to our results with cyclophosphamide, treatment of mice with neutrophil-depleting anti-Ly6G antibodies has no effect on *E. carinatus sochureki* venom-induced tail injury (Fig. 1b) or mortality (Fig. 1f). We also use PMN\(^{DTR}\) mice, which express the diphtheria toxin (DT) receptor specifically on neutrophils, and in which >95% of circulating and tissue neutrophils can be depleted upon injection of DT\(^4\). ECV-induced tail injury and mortality in DT-treated neutrophil-deficient PMN\(^{DTR}\) mice compared to DT-treated neutrophil-sufficient PMN\(^{WT}\) littermates is not affected (Fig. 1c, g). The lack of effect after antibody-mediated or DT-mediated neutropenia induction is not due to persistence of circulating neutrophils in blood (Supplementary Figs. 3a, b and 4a, b) or at the venom injection site (Supplementary Figs. 3c and 4c) 5 h after venom injection, a time point at which important tail injury already occurs. We further confirm our results using mice deficient for growth factor independence-1 (Gfi1), a transcription repressor that enables neutrophil differentiation\(^2\). Indeed, similar tail injury and mortality occurred after ECV injection in neutrophil-deficient Gfi1\(^{−/−}\) mice\(^6\) compared to neutrophil-sufficient Gfi1\(^{+/+}\) littermates (Fig. 1d, h). At a lower dose of venom, both tail injury 8 h after venom injection and mortality are increased in neutrophil-deficient Gfi1\(^{−/−}\) mice as compared to Gfi1\(^{+/−}\) mice (Supplementary Fig. 5a, b). These responses might be a consequence of neutropenia in Gfi1\(^{−/−}\) mice (Supplementary Fig. 5c) or of other phenotypic abnormalities associated with Gfi1 deficiency\(^5\).

Interestingly, Katkar et al.\(^1\) report that treatment of mice with DNAse I, which can degrade extracellular DNA, reduces tissue damage at the site of venom injection. The authors suggest that these effects are due to the clearance of NETs by DNAse I. We confirm that treatment with DNAse I can reduce ECV-induced tail injury (Fig. 1d). However, DNAse I has similar effects in both neutrophil-sufficient Gfi1\(^{+/−}\) and neutrophil-deficient Gfi1\(^{−/−}\) mice (Fig. 1d). Katkar et al.\(^1\) also report increased ECV-induced...
mortality of mice treated with DNase \(^1\). We too observe an increase in mortality after ECV injection of DNase I-treated mice, but only with neutropenic Gfi1\(^{KI/KI}\) mice and not neutrophil-sufficient Gfi1\(^{KI/+}\) littermates (Fig. 1h).

Altogether, our data indicate that neutrophils do not promote ECV-induced tissue damage at the site of venom injection. Our data using a low dose of venom in Gfi1\(^{KI/KI}\) mice even suggest that neutrophils might help to reduce tail injury. However, using different approaches, we confirm the finding by Katkar et al. that neutrophenic mice are more susceptible to mortality after ECV injection. While treatment with DNase I might be of great interest to reduce local tissue damage induced by the venom, the effects of the drug in our study are probably not a result of the degradation of NETs. Some toxins in ECV have strong cytotoxic effects of the drug in our study are probably not a result of the degradation of NETs. Some toxins in ECV have strong cytotoxic effects. Therefore, it is possible that neutrophils contribute to the degradation of NETs.

In conclusion, our study confirms that treatment with DNase I can reduce tissue damage induced by ECV, which may have important clinical implications, but we highlight the fact that more research is needed before definite conclusions can be made on the role of neutrophils and NETs in this process.

**Methods**

Mice. B6Grlw/SwiSS mice were purchased from Janvier Labs. PMN\(^{DPTR}\) mice (NRPr8-Cre; DT\(^{DPTR}\)) on a C57BL/6 background were described previously. Neutrophil-deficient Gfi1\(^{KI/KI}\) mice on a C57BL/6 background were provided by T. Moro (Montreal University, Montreal, QC, Canada), and we used neutrophil-sufficient Gfi1\(^{KI/+}\) as littermate controls. Mice were bred and maintained at the Institut Pasteur animal facility, or housed in our animal facility for at least 7 days before starting experiments. We used age- and sex-matched mice for all experiments. All animal experiments were conducted with the specific approval of the CEEA ethics committee number 89 (Institut Pasteur, Paris, France) under #dap160072.

**Venom-induced mouse tail tissue destruction and lethality.** Lyophilized *E. carinatus sochureki* venom was purchased from Kentucky Reptile Zoo (USA), and used for most experiments (Fig. 1 and Supplementary Figs. 2–5). In Supplementary Fig. 1, we used lyophilized *E. carinatus multisquamatus* and *E. carinatus pyramidalum* venoms purchased from Latoxan (France). All venoms were resuspended in sterile phosphate-buffered saline (PBS) at a concentration of 100 mg/mL and stored at \(-20^\circ\text{C}\). *E. carinatus* venom was administered at the indicated dose subcutaneously 3 cm distal to the base of the tail in a volume of 25 μL. Local damages were assessed at different time points according to a 10-point scale described by Katkar et al. \(^1\) 0: no visible injury; 1: edema; 2: edema with minor hemorrhage; 4: edema with hemorrhage causing <25% tail discoloration; 6: edema and major hemorrhage or wound causing 25–50% tail discoloration; 8: edema and major hemorrhage or wound causing >50–75% tail discoloration; and 10: edema and major hemorrhage or wound causing >75% tail discolouration. Mice were observed for mortality at least four times daily. Mice that were clearly moribund were euthanized by CO\(_2\) inhalation.
Treatment with cyclophosphamide. RjOr1:SWISS mice were treated with cyclophosphamide as described by Katkar et al. Mice were injected intraperitoneally (i.p.) with cyclophosphamide (Sigma-Aldrich) twice (150 mg/kg on day one and 100 mg/kg on day four, in 500 µL PBS) and E. carinatus venom was injected 24 h after the last injection of cyclophosphamide. In the experiments presented in Supplementary Fig. 2, groups of mice were sacrificed 24 h after the last injection of cyclophosphamide to assess effects of the drug on neutrophils and other cell populations in the blood by flow cytometry.

Treatment with DNase I. Recombinant DNase I was purchased from Roche Diagnostics. DNase I was co-incubated with E. carinatus venom for 30 min and co-injected in mice at a final dose of 500 U in 25 µL PBS.

Antibody-mediated neutrophil depletion. Neutrophil-depleting anti-Ly6G antibodies were purified in our laboratory (using protein G columns) from culture supernatants of the NIMP-R14 hybridoma provided by Dr. Claude Leclerc (Institut Pasteur, France). Mice were injected i.p. with anti-Ly6G antibodies (NIMP-R14), or rat IgG2b isotype control antibodies (clone 2F11; BioXcell, each at 300 µg in 200 µL PBS) 24 h before, 2 h before, and 24 h after injection of venom. The efficiency of neutrophil depletion was verified by immunocytochemistry at the site of venom injection and by flow cytometry in the blood and spleen 5 h post injection. These data are presented in a Supplementary Fig. 3.

DT-mediated neutrophil depletion. PMN^DTR mice were injected i.p. with DT (500 ng in 200 µL PBS; Sigma-Aldrich) to selectively deplete neutrophils. DT injections were performed 24 h before, 2 h before, and 24 h after injection of venom. We used DT-treated PMN^WT littermates as neutrophil-sufficient control mice. The efficiency of neutrophil depletion was verified by immunocytochemistry at the site of venom injection and by flow cytometry in the blood and spleen 5 h post injection. These data are presented in a Supplementary Fig. 4.

Flow cytometry. We used flow cytometry to identify and enumerate immune cell populations in peripheral blood and spleen. Briefly, red blood cells were lysed by treatment with red blood cell lysis buffer (BD Biosciences). Cells were stained with a combination of antibodies on ice for 30 min. Immune cell populations were detected by staining with red blood cell lysis buffer (BD Biosciences). Cells were stained with secondary antibodies only, which revealed no unspecific staining. Samples were imaged by using a confocal microscope. This work was supported by the Institut Pasteur, the French “Institut National de la Santé et de la Recherche Médicale” (INSERM) and the European Research Council Seventh Framework Programme (ERC-2013-CoG 616050). J.S. acknowledges support from Ecole Normale Supérieure Paris-Saclay. B.B. is supported by a stipend from the Pasteur—Paris University (PPU) International Ph.D. Program. F.J. is an employee of the Centre National de La Recherche Scientifique (CNRS). L.L.R. acknowledges support from the European Commission Marie Skłodowska-Curie Individual Fellowship (H2020-MSCA-IF-2014 656066).

Author contributions
L.L.R. supervised the project, J.S., B.B., B.T., O.G., and B.I. did experiments. All authors contributed to discussing results and preparing the manuscript.

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