

P. t. verus and *P. t. vellerosus* apes, the presence of SIVcpz infection in *P. t. troglodytes* and *P. t. schweinfurthii* apes, and the phylogenetic separation of SIVcpzPtt from SIVcpzPts viruses (4, 7, 9, 15). HIV-1 groups M, N, and O each resulted from independent cross-species transmissions of SIVcpzPtt from *P. t. troglodytes* to humans early in the 20th century (17–19). We show here that the SIVcpzPtt strain that gave rise to HIV-1 group M belonged to a viral lineage that persists today in *P. t. troglodytes* apes in southeastern Cameroon. That virus was probably transmitted locally. From there it appears to have made its way via the Sangha River (or other tributaries) south to the Congo River and on to Kinshasa where the group M pandemic was probably spawned (20). HIV-1 group N, which has been identified in only a small number of AIDS patients from Cameroon (21, 22), derived from a second SIVcpzPtt lineage in south central Cameroon and remained geographically more restricted. The source of HIV-1 group O remains unknown but will probably yield to further study of wild ape populations not yet sampled. Given the extensive genetic diversity and phylogeographic clustering of SIVcpz now recognized, and the vast areas of west central Africa not yet sampled (Fig. 1), it is quite possible that still other SIVcpz lineages exist that could pose risks of human infection and prove

problematic for HIV diagnostics and vaccines. The present report describes molecular tools and noninvasive strategies that can be used to explore these possibilities as well as the molecular ecology of pathogens in endangered species more generally.

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14. In contrast to plasma samples from uninfected humans, which exhibit false positive Western blot reactivity to HIV-1 p24 in as many as 10 to 15% of individuals (www.fda.gov/cber/products/hiv1cam052898.htm), we have found no such nonspecific cross-reactivity of chimpanzee immunoglobulin extracted by the RNeasy method from over 2000 fecal specimens.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/1126531/DC1

Materials and Methods

Fig. S1

Tables S1 to S4

References

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Mast Cells Can Enhance Resistance to Snake and Honeybee Venoms

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Snake or honeybee envenomation can cause substantial morbidity and mortality, and it has been proposed that the activation of mast cells by snake or insect venoms can contribute to these effects. We show, in contrast, that mast cells can significantly reduce snake-venom-induced pathology in mice, at least in part by releasing carboxypeptidase A and possibly other proteases, which can degrade venom components. Mast cells also significantly reduced the morbidity and mortality induced by honeybee venom. These findings identify a new biological function for mast cells in enhancing resistance to the morbidity and mortality induced by animal venoms.

Venomous reptiles and their prey have coexisted for ~200 million years (1), and snake envenomation still accounts for considerable human morbidity and mortality worldwide (2, 3) (SOM Text 1). The mechanisms by which snake envenomation can produce tissue injury and death have been studied extensively (3–5), and it is known that many components of snake venoms can induce

mammalian mast cells (MCs) to release potent biologically active mediators (6, 7). These MC products in turn can promote an increase in vascular permeability, local inflammation, abnormalities of the clotting and fibrinolysis systems, and shock (8, 9).

Accordingly, it has been considered that the activation of tissue MCs can contribute importantly to the local tissue injury, systemic distribution of venom components, and death associated with snake envenomation (6, 7). This hypothesis is consistent with the well-understood role of MCs in the pathology of allergic disorders such as anaphylaxis and asthma (8–11). However, MCs can enhance survival in certain models of innate immunity to bacterial infection

(12–15). In one such model, MCs can reduce morbidity and mortality in part by promoting the degradation of the potent endogenous vasoconstrictor peptide endothelin-1 (ET-1) (16). The most toxic components of the venom of *Atractaspis engaddensis* (the burrowing asp or Israeli mole viper) are the sarafotoxins, which exhibit a very high homology (~70% at the amino acid level) to ET-1 (17).

When various amounts of *A. engaddensis* venom (*A.e.v.*) were administered intraperitoneally, wild-type mice developed significant reductions in body temperature at a dose of 5 µg, and death occurred at 50 µg (fig. S1). By contrast, as little as 5 µg of *A.e.v.* induced death in *Kit^{W-sh}/Kit^{W-sh}* mice, which are genetically deficient in MCs (18). Levels of sarafotoxins in the peritoneal cavity of wild-type mice were significantly lower than those in the corresponding *Kit^{W-sh}/Kit^{W-sh}* mice at all amounts of *A.e.v.* tested that were ≥5 µg (fig. S1). Although intraperitoneal injection has been recommended for analyses of the systemic toxicity of snake venoms (4), many snake bites are to the skin and subcutaneous tissue. MC-deficient mice were also much more susceptible than wild-type mice to the development of hypothermia and death when *A.e.v.* (10 µg) was injected subcutaneously (fig. S2).

A.e.v. contains several toxic compounds, including sarafotoxins 6a, 6b, 6c, and 6d, and hemorrhagins, but the most toxic of these is

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sarafotoxin 6b (S6b) (19). When injected intraperitoneally with 1 nmol of S6b, each MC-deficient *Kit^W/Kit^{W-v}* mouse developed severe hypothermia and died within 1 hour, whereas the wild-type *Kit^{+/+}* mice developed only a slight drop in temperature and recovered completely, even when injected with 10 times the amount of S6b (Fig. 1A). Injection of unfractionated venom produced similar results (Fig. 1B). Similar results also were obtained when C57BL/6 and MC-deficient *Kit^{W-sh}/Kit^{W-sh}* were injected intraperitoneally with S6b or *A.e.v.* (fig. S3). Intraperitoneal injection of S6b or *A.e.v.* also induced extensive degranulation of peritoneal MCs (PMCs) (fig. S4, A and B), a finding readily detected at 10 min after injection of *A.e.v.* (fig. S4), and levels of sarafotoxins were almost undetectable in the peritoneal cavity of

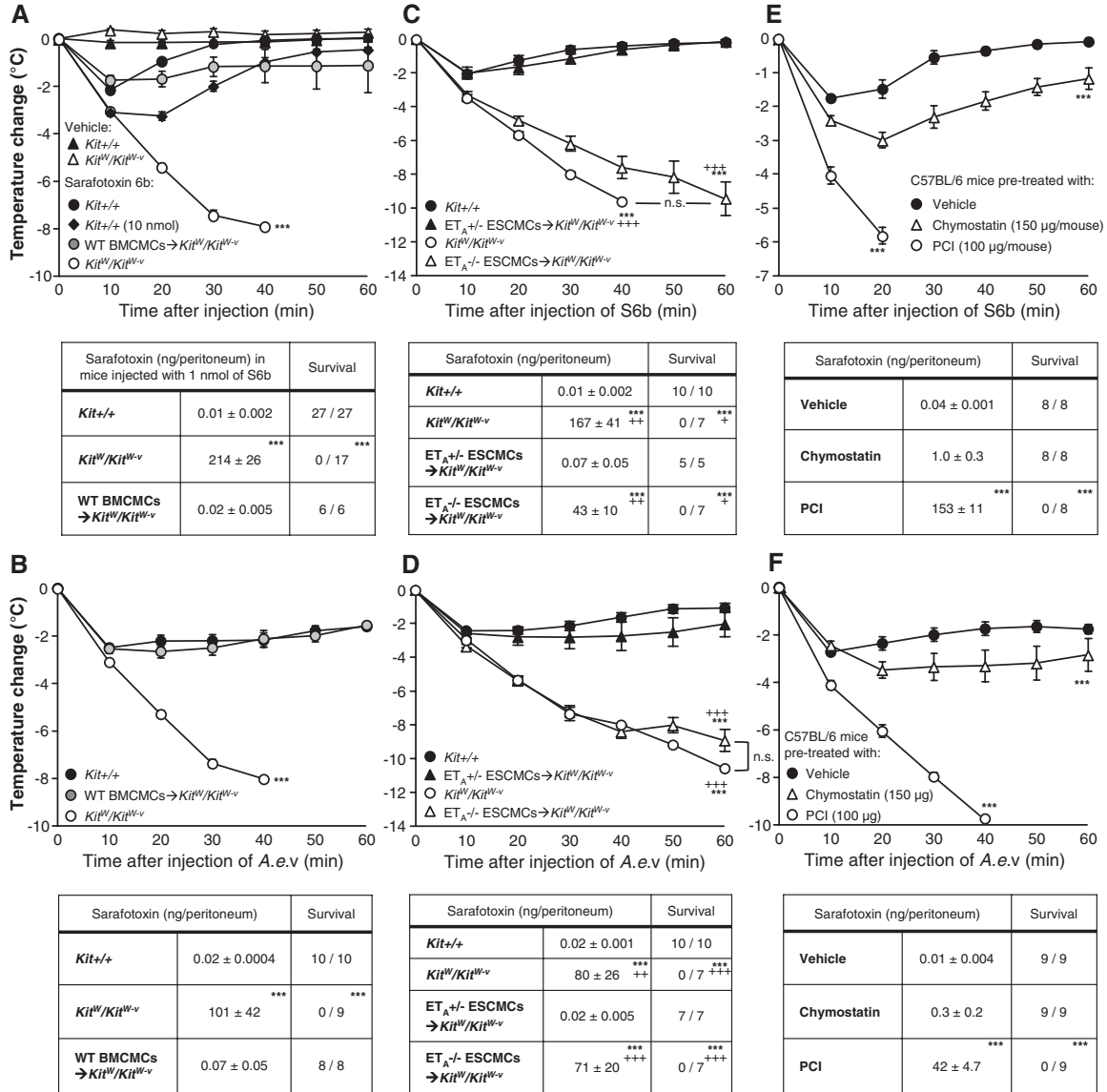
Kit^{+/+} mice 60 min after S6b or *A.e.v.* injection, whereas the peritoneal cavity of *Kit^W/Kit^{W-v}* mice contained high levels of sarafotoxins (Fig. 1, A and B).

To assess whether the differences in the responses of *Kit^{+/+}* versus *Kit^W/Kit^{W-v}* mice specifically reflected the lack of MCs in the *Kit^W/Kit^{W-v}* mice, we examined *Kit^W/Kit^{W-v}* mice that had been engrafted with bone marrow-derived cultured MCs (BMCMCs) derived from the *Kit^{+/+}* mice (wild-type BMCMCs → *Kit^W/Kit^{W-v}* mice) (15, 20). Upon injection of S6b or *A.e.v.*, MC-engrafted *Kit^W/Kit^{W-v}* mice exhibited very low sarafotoxin levels in the peritoneal cavity and were protected against hypothermia and death (Fig. 1, A and B); similar results were obtained with wild-type MC-engrafted *Kit^{W-sh}/Kit^{W-sh}* mice (fig. S3).

Thus, MCs can significantly reduce levels of sarafotoxins in the peritoneal cavity after intraperitoneal injection of S6b or *A.e.v.* in vivo and can markedly limit the systemic toxicity and death induced by either S6b or unfractionated *A.e.v.*

S6b, like ET-1, activates the ET_A receptor, which is expressed by MCs (16). We generated two groups of mice that contained MCs that either expressed or lacked ET_A. Because ET_A^{-/-} mice are not viable, we generated MCs from ET_A^{+/-} or ET_A^{-/-} embryonic stem (ES) cells (21) and then adoptively transferred these ES cell-derived cultured MCs (ESCMCs) into *Kit^W/Kit^{W-v}* mice (Fig. 1, C and D). Although ET_A^{+/-} and ET_A^{-/-} ESCMC-engrafted mice contain similar numbers of MCs in the peritoneum (16), those that received ET_A^{-/-} ESCMCs developed severe hypo-

Fig. 1. MCs can protect mice from *A.e.v.*- and S6b-induced morbidity and mortality. Shown are changes in rectal temperatures, levels of sarafotoxins in the peritoneal lavage fluid (at time of death or at 60 min, whichever was sooner), and 24-hour survival after intraperitoneal injection of S6b, *A.e.v.*, or saline. (A, C, and E) Results after injection of 1 nmol or [in (A) only] 10 nmol of S6b in 250 μ l saline (0.9% sterile NaCl) or 250 μ l saline alone. In (A), survival and sarafotoxin data in the table are only for mice injected with 1 nmol S6b; survival in all other groups was 100%. (B, D, and F) Results after injection of *A.e.v.* [10 μ g in 250 μ l phosphate-buffered saline (PBS)]. Subjects were (A and B) *Kit^{+/+}* mice, MC-deficient *Kit^W/Kit^{W-v}* mice, and *Kit^W/Kit^{W-v}* mice that had been engrafted intraperitoneally with MCs derived in vitro from the bone marrow of *Kit^{+/+}* mice (WT BMCMCs → *Kit^W/Kit^{W-v}*); (C and D) *Kit^{+/+}* mice, MC-deficient *Kit^W/Kit^{W-v}* mice, and *Kit^W/Kit^{W-v}* mice that had been engrafted intraperitoneally with ES cell-derived cultured mast cells (ESCMCs) that did (ET_A^{+/-}) or did not (ET_A^{-/-}) express ET_A; and (E and F) C57BL/6 mice that had received intraperitoneal injections of vehicle (saline, 300 μ l), chymostatin (150 μ g in 300 μ l of saline), or PCI (100 μ g in 300 μ l of saline) 10 min before injection of S6b or saline. (A and B) ****P* < 0.005 versus



corresponding values for *Kit^{+/+}* or WT BMCMCs → *Kit^W/Kit^{W-v}* mice. (C and D) ****P* < 0.005 versus *Kit^{+/+}* mice; +*P* < 0.05, ++*P* < 0.01, or +++*P* < 0.005 versus ET_A^{+/-} ESCMCs → *Kit^W/Kit^{W-v}* mice; n.s., not significant (*P* > 0.05). (E and F) ****P* < 0.005 versus vehicle.

thermia (like that in *Kit^W/Kit^{W-sh}* mice) and died rapidly after intraperitoneal injection of S6b or *A.e.v.* (Fig. 1, C and D). Consistent with their survival responses, ET_A^{+/-} ESCMCs→*Kit^W/Kit^{W-sh}* mice, like *Kit^{+/+}* mice, exhibited extensive peritoneal mast cell (PMC) degranulation (fig. S4, C and D) and strongly reduced sarafotoxin levels in the peritoneal cavity (Fig. 1, C and D). In contrast, ET_A^{-/-} ESCMCs→*Kit^W/Kit^{W-sh}* mice exhibited significantly lower levels of MC degranulation (fig. S4, C and D) and much higher intraperitoneal levels of sarafotoxins (Fig. 1, C and D).

To assess potential mechanisms by which MCs might reduce venom toxicity, we tested inhibitors of two candidate MC-derived mediators, chymase, an endopeptidase (16), and carboxypeptidase A (CPA), an exopeptidase (22) [i.e., chymostatin and potato carboxypeptidase inhibitor (PCI), respectively]. Wild-type C57BL/6 mice pretreated intraperitoneally with PCI developed severe hypothermia and died within 1 hour of S6b or *A.e.v.* injection (Fig. 1, E and F). The extent of PMC degranulation did not differ in wild-type mice pretreated with PCI, vehicle, or chymostatin (fig. S4, E and F). However, sarafotoxin levels in the peritoneum of PCI-pretreated wild-type mice were much higher than those in the vehicle- or chymostatin-pretreated wild-type mice (Fig. 1, E and F), although lower than those in S6b- or *A.e.v.*-injected MC-deficient mice (Fig. 1, A and B). By contrast, compared with the effect of vehicle alone, intraperitoneal pretreatment with chymostatin resulted in only a small reduction in body temperature and a small increase in intraperitoneal levels of sarafotoxins (Fig. 1, E and F). PCI also markedly inhibited the ability of mouse peritoneal cells to degrade S6b *ex vivo*, whereas chymostatin had little or no effect (fig. S5).

These results were unexpected, because our previous pharmacological experiments have suggested an important role for MC-associated chymase in the degradation of ET-1 (16). To examine this further, we undertook a genetic approach to distinguish the relative roles of chymase versus CPA in limiting *A.e.v.* toxicity. The only protease with chymotryptic activity in mouse PMCs is mouse mast cell protease-4 (mMCP-4) (23). In mMCP-4^{-/-} and the respective wild-type control mice, intraperitoneal injection of S6b or *A.e.v.* induced comparable levels of mild hypothermia, intraperitoneal sarafotoxins, and PMC degranulation; moreover, all mice survived (fig. S6). Similar results were obtained when wild-type and mMCP-4^{-/-} mice were injected intraperitoneally with ET-1 (fig. S7, A to C). These findings are in accord with the results of *in vitro* studies of rat (22) or mouse MCs (fig. S7, G and H) and our *in vivo* experiments in mice (fig. S7, D to F), all of which indicate that CPA is more effective than chymase in degrading ET-1.

We also used a lentivirus-based short hairpin-mediated RNA (shRNA) interference

approach to generate mice with MC populations with normal or significantly decreased (>75%) levels of CPA activity (Fig. 2A). *Kit^{W-sh}/Kit^{W-sh}* mice that had been engrafted with either control (i.e., empty vector)-transduced MCs or CPA shRNA-transduced MCs exhibited similarities in numbers of PMCs (Fig. 2B), levels of PMC degranulation in response to S6b or *A.e.v.* (Fig. 2C), and appearance and distribution of MCs in the mesentery (Fig. 2D). However, upon intraperitoneal injection of S6b or *A.e.v.*, CPA

shRNA MC-engrafted *Kit^{W-sh}/Kit^{W-sh}* mice developed severe hypothermia, retained high concentrations of sarafotoxins in the peritoneal cavity, and died within 1 hour (Fig. 2, E and F), findings very similar to those observed in either MC-deficient *Kit^{W-sh}/Kit^{W-sh}* (Fig. 2, E and F) or *Kit^W/Kit^{W-sh}* (Fig. 1, A and B) mice, or in C57BL/6 wild-type mice that had been pretreated with PCI (Fig. 1, E and F).

To assess whether MCs could reduce the toxicity of snake venoms that do not contain

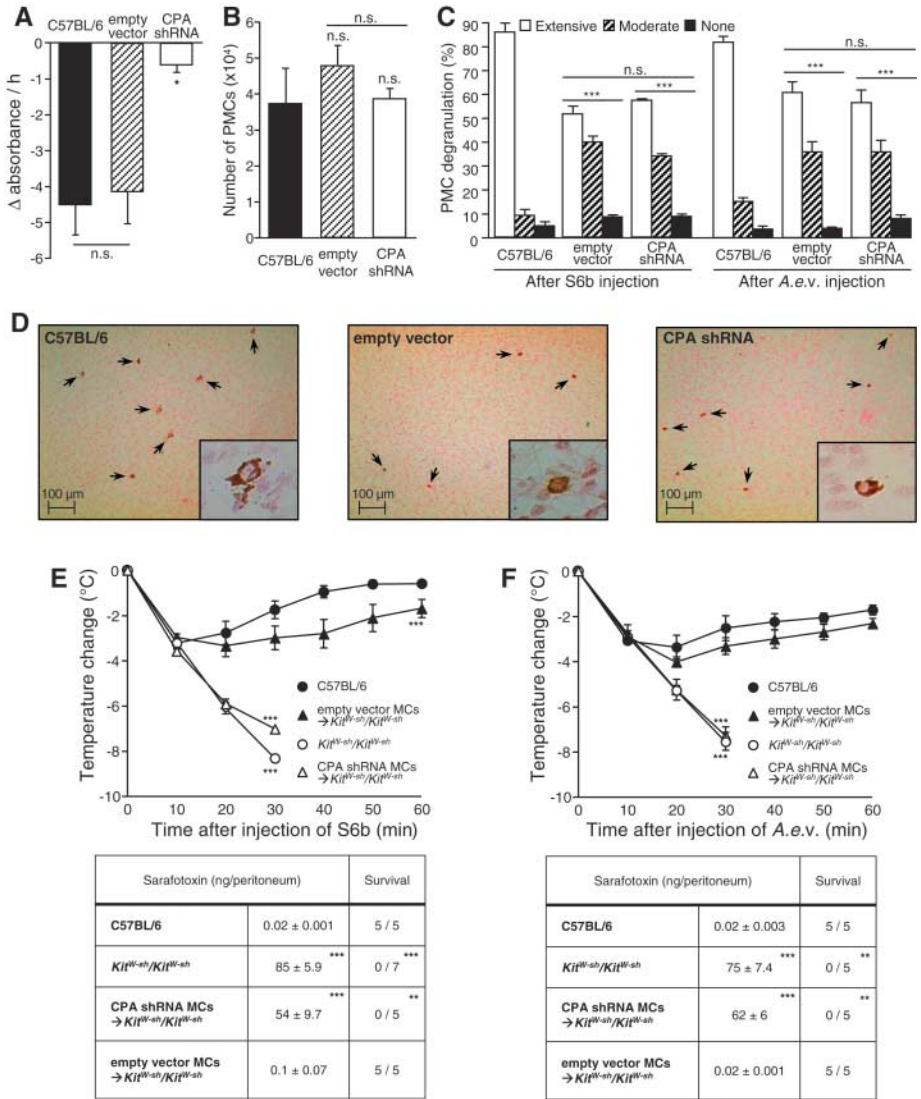


Fig. 2. Reduction of CPA using shRNA in MCs renders mice susceptible to *A.e.v.*- and S6b-induced morbidity and mortality. (A) CPA activity in peritoneal cells from C57BL/6 or *Kit^{W-sh}/Kit^{W-sh}* mice engrafted with BMCMCs that were transduced with CPA shRNA or empty vector (control). **P* < 0.05 versus C57BL/6 or empty vector. (B to D) *Kit^{W-sh}/Kit^{W-sh}* mice engrafted with BMCMCs transduced with CPA shRNA or empty vector exhibit (B) normalization of PMC numbers, (C) similar extent of PMC degranulation in response to S6b and *A.e.v.*, and (D) the appearance of MCs in the mesentery (arrows, MCs; scale bars, 100 μm). In (C), ****P* < 0.005 versus C57BL/6; in (B) and (C), n.s., not significant (*P* > 0.05) versus values for C57BL/6 mice or for the comparisons indicated by brackets. (E and F) Changes in rectal temperature, levels of sarafotoxins in the peritoneal lavage fluid (at time of death or at 60 min, whichever was sooner), and 24-hour survival in C57BL/6 mice or *Kit^{W-sh}/Kit^{W-sh}* mice that were engrafted intraperitoneally with empty vector (empty vector MCs→*Kit^{W-sh}/Kit^{W-sh}*) or CPA shRNA (CPA shRNA MCs→*Kit^{W-sh}/Kit^{W-sh}*) expressing BMCMCs, and then were injected 4 weeks later with (E) 1 nmol S6b or (F) 10 μg *A.e.v.* ***P* < 0.01 or ****P* < 0.005 versus C57BL/6 or empty vector MCs→*Kit^{W-sh}/Kit^{W-sh}* mice.

S6b or other ET-like peptides, we tested venoms from the western diamondback rattlesnake (*Crotalus atrox*, 150 μ g) or the southern copperhead (*Agkistrodon contortrix contortrix*, 75 μ g), species representative of pit vipers (Crotalidae), which account for the great majority of snake envenomations in North America (24). MC-deficient *Kit^W/Kit^{W-v}* mice injected intraperitoneally with *Crotalus atrox* venom (*C.a.v.*) or *Agkistrodon contortrix contortrix* venom (*A.c.c.v.*) exhibited significantly lower body temperatures than wild-type mice, and all of them died within 24 hours. By contrast, most *Kit^{+/+}* mice survived (87% in *C.a.v.*- and 100% in *A.c.c.v.*-injected mice) (Fig. 3, A and B), and these mice appeared to recover fully. Moreover, engraftment of *Kit^W/Kit^{W-v}* mice with wild-type BMCs resulted in levels of protection against hypothermia and death that were statistically indistinguish-

able from those in wild-type mice (Fig. 3, A and B). Very similar results were obtained when these experiments were repeated with C57BL/6, *Kit^{W-sh}/Kit^{W-sh}*, and wild-type MC-engrafted *Kit^{W-sh}/Kit^{W-sh}* mice (fig. S8).

We could not assess PMC degranulation in these experiments because of the excessive bleeding that had occurred within 60 min of intraperitoneal venom injection (or by the time of death) in all venom-injected mice. However, PMCs exhibited extensive degranulation when exposed to either type of venom ex vivo (fig. S9A) or, in C57BL/6 wild-type mice, at 10 min after intraperitoneal injection of venom in vivo (fig. S9, B and C).

MCs also diminished the extent of the hemorrhagic lesions that developed upon the injection of *C.a.v.* into the skin (fig. S10). Thus, MCs can diminish the local pathology induced

by this venom, as well as reduce its systemic toxicity after intraperitoneal injection.

In wild-type (C57BL/6) mice injected intraperitoneally with venom, PCI pretreatment significantly worsened *C.a.v.*- or *A.c.c.v.*-induced hypothermia and mortality (Fig. 3, C and D) but did not result in levels of hypothermia or death rates (Fig. 3, C and D) that were quite as high as those observed in MC-deficient mice, especially in mice injected with *C.a.v.* One possible explanation for these results is that MC-derived proteases other than CPA (and/or additional MC-derived mediators or functions) may also have some protective effects against such venoms, and/or in counteracting the pathology they induce.

Like venomous reptiles, honeybees (*Apis mellifera*) and other stinging insects can account for substantial venom-associated morbidity and mortality (SOM Text 2). Although it is well-known that honeybee venom contains compounds that can induce MC degranulation (25), it had been thought that this contributed to the pathology associated with such stings (26). However, we found that MCs can confer significant protection against the hypothermia and death induced by the subcutaneous injection of *A. mellifera* venom (Fig. 4). Although all mice

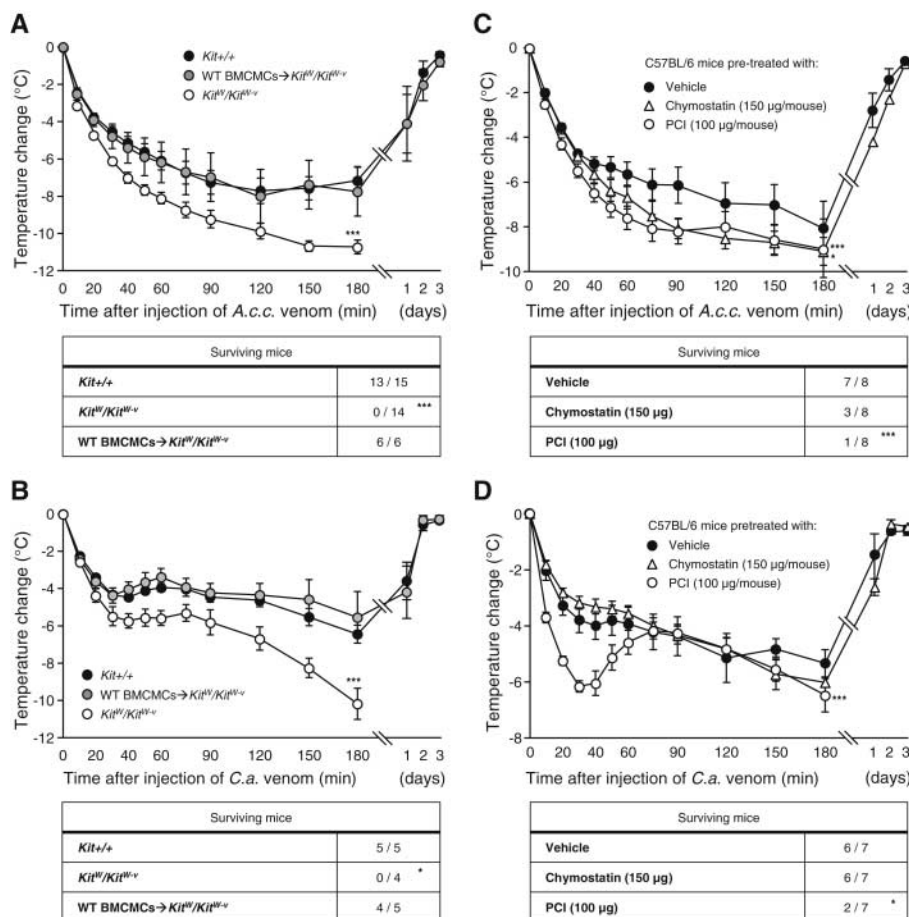


Fig. 3. MCs can limit the toxicity of western diamondback rattlesnake and southern copperhead venoms. Changes in rectal temperature and 24-hour survival after intraperitoneal injection of (A and C) *A.c.c.* venom (150 μ g in 250 μ l PBS) or (B and D) *C.a.* venom (75 μ g in 250 μ l) into (A and B) *Kit^{+/+}* mice, MC-deficient *Kit^W/Kit^{W-v}* mice, and *Kit^W/Kit^{W-v}* mice that had been engrafted intraperitoneally with BMCs from *Kit^{+/+}* mice (WT BMCs → *Kit^W/Kit^{W-v}*) or into (C and D) C57BL/6 mice that received intraperitoneal injections of vehicle (saline, 300 μ l), chymostatin (150 μ g in 300 μ l of saline), or PCI (100 μ g in 300 μ l of saline) 10 min before intraperitoneal injection of snake venom. Mice that survived for 3 hours all appeared healthy and returned to baseline body temperature within 1 to 3 days. Body temperatures at 1 to 3 days were measured in surviving mice in one of the three experiments whose data were pooled to give the depicted 24-hour survival and up to 3 days of body temperature data. (A and B) * P < 0.05 or *** P < 0.005 versus *Kit^{+/+}* or WT BMCs → *Kit^W/Kit^{W-v}* mice; (C and D) * P < 0.05 or *** P < 0.005 versus vehicle.

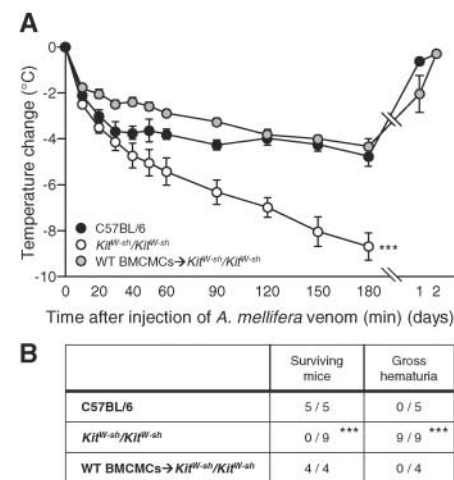


Fig. 4. MCs can limit the toxicity of honeybee venom. (A) Changes in rectal temperature and (B) 24-hour survival and occurrence of gross hematuria after subcutaneous injection of *A. mellifera* venom (*A.m.v.*) at five different sites (three injections distributed over the length of the back skin and two into the belly skin, each containing 100 μ g *A.m.v.* in 50 μ l PBS) into WT mice, mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice, or *Kit^{W-sh}/Kit^{W-sh}* mice that had been engrafted intradermally, 6 weeks earlier, with 1.5×10^6 BMCs into each of the five injection areas (WT BMCs → *Kit^{W-sh}/Kit^{W-sh}*). The amount of venom per injection (100 μ g) roughly reflects the amount that can be delivered by one bee sting (31). All of the WT or WT BMCs → *Kit^{W-sh}/Kit^{W-sh}* mice appeared healthy, and their body temperatures returned to baseline within 2 days. *** P < 0.005 versus either C57BL/6 or WT BMCs → *Kit^{W-sh}/Kit^{W-sh}* mice.

exhibited the same initial response to such injections, namely, intense scratching of the injection sites, all of the MC-deficient *Kit^{W-sh}/Kit^{W-sh}* mice, but none of the wild-type mice or the wild-type BMCMC-engrafted *Kit^{W-sh}/Kit^{W-sh}* mice, developed gross hematuria (Fig. 4).

Although the extent to which MCs might be able to enhance resistance to other animal venoms remains to be determined, components of the venoms of many different animals can activate MCs (7) (SOM Text 3). Moreover, it is quite possible that MC mediators, in addition to CPA and other proteases, also may contribute to the ability of MCs to reduce the morbidity and mortality associated with certain venoms. In 1965, Higginbotham hypothesized that MCs can reduce the toxicity of Russell's viper venom by degranulating and releasing heparin, which then binds highly cationic components of the venom and thereby reduces its toxicity (27). However, this interesting hypothesis has not yet been tested using MC-deficient and MC-engrafted mice (SOM Text 4). Finally, it is of course possible that MCs might contribute to (or have no effect on) the toxicity observed with some venoms.

We have identified a heretofore unproven role for MCs: enhancing innate host resistance to the toxicity of certain animal venoms. Our observations also provide a new perspective on the presence, within MCs, of prominent cytoplasmic granules that contain a large amount and, in some species, a large diversity, of proteases (28, 29). It is likely that mast cell proteases can have beneficial roles in many settings, not only in host defense (23, 28, 30). However,

we speculate that the storage in MC cytoplasmic granules of large amounts of proteases, which can be released to the exterior very rapidly upon suitable MC activation, reflects, at least in part, the selective pressure of the exposure of animals to diverse exogenous toxins (such as those in vertebrate and invertebrate venoms and perhaps those produced by certain microorganisms), as well as the advantage of being able to degrade and thereby control the toxicity of potent endogenous molecules such as ET-1 (16) (SOM Text 5).

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Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S10

References

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Multiple Phosphorylation Sites Confer Reproducibility of the Rod's Single-Photon Responses

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Although signals controlled by single molecules are expected to be inherently variable, rod photoreceptors generate reproducible responses to single absorbed photons. We show that this unexpected reproducibility—the consistency of amplitude and duration of rhodopsin activity—varies in a graded and systematic manner with the number but not the identity of phosphorylation sites on rhodopsin's C terminus. These results indicate that each phosphorylation site provides an independent step in rhodopsin deactivation and that collectively these steps tightly control rhodopsin's active lifetime. Other G protein cascades may exploit a similar mechanism to encode accurately the timing and number of receptor activation.

Rhodopsin may be biology's most precise single-molecule timekeeper. In retinal rod photoreceptors, the effective absorption of a single photon activates a single rhodopsin molecule, which triggers a highly amplified signal transduction cascade to produce a macroscopic change in the current flowing into the outer segment of the receptor. The electrical response evoked by a single photon shows much less trial-

to-trial variability than other familiar signals generated by single molecules, such as the time to decay of a radioactive particle or the charge flowing through an ion channel during a single opening (1–5). More generally, events controlled by a first-order process (Fig. 1, A and B) are inherently more variable than the responses to single photons. Previous studies indicate that the low variability in the rod's current responses arises

from low variability in the lifetime of light-activated rhodopsin (2–6). This poses a challenging molecular design problem: How is the active lifetime of a single molecule regulated so tightly?

Past work argues, largely by excluding other explanations, that reproducibility is produced by the deactivation, or shutoff, of a single rhodopsin molecule through a series of steps or transitions (2–6). The essence of this multistep shutoff model is simple averaging: The integrated rhodopsin activity averaged over multiple stochastic steps varies less than the activity controlled by a single step. Rhodopsin activity could be timed by the occurrence of each step before terminating with completion of the final step (Fig. 1, C and D), or rhodopsin activity could decline after each step (Fig. 1, E and F). In either case, the coefficient of variation ($CV = \text{standard deviation}/\text{mean}$) for rhodopsin's integrated activity is $1/\sqrt{N}$ for N independent steps that each control an equal frac-

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Supporting Online Material for

Mast Cells Can Enhance Resistance to Snake and Honeybee Venoms

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This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S10
References

Supporting Online Material

Materials and Methods

Animals.

C-*kit* mutant genetically mast cell-deficient (WB/ReJ-W⁺ x C57BL/6J-W^v/⁺)F₁-*Kit*^W/*Kit*^{W-v} (WBB6F1-*Kit*^W/*Kit*^{W-v}) (*Kit*^W/*Kit*^{W-v}) mice (S1) and the congenic normal WBB6F1-+/+ (*Kit*+/+) mice, and C57BL/6J mice, were purchased from Jackson Laboratories, Bar Harbor, Maine. Mast cell-deficient C57BL/6-*Kit*^{W-sh}/*Kit*^{W-sh} (*Kit*^{W-sh}/*Kit*^{W-sh}) mice and the congenic normal C57BL/6 mice, which were originally provided by Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, NY) (S2) and mast cell protease 4-deficient (mMCP4-/-) mice on the C57BL/6 background (S3), were bred and maintained at the Stanford University Research Animal Facility. Both adult *Kit*^W/*Kit*^{W-v} mice and adult *Kit*^{W-sh}/*Kit*^{W-sh} mice have a profound deficiency of mast cells, including virtually no mast cells in the peritoneal cavity or within the mesentery, and <1% wild type levels of mast cells in the skin (S4, 5, 6). All animal care and experimentation was conducted in accord with current National Institutes of Health and Stanford University Institutional Animal Care and Use Committee guidelines.

Mast-cell engraftment of *Kit*^W/*Kit*^{W-v} and *Kit*^{W-sh}/*Kit*^{W-sh} mice.

Some *Kit*^W/*Kit*^{W-v} and some *Kit*^{W-sh}/*Kit*^{W-sh} mice (female, 4-6-week-old) were repaired of their mast cell deficiency selectively and locally by the injection of growth factor-dependent BMCMCs, of WBB6F1-*Kit*+/+ (*Kit*+/+) or C57BL/6 mouse origin, respectively, into the peritoneal cavity (S7, 8). Briefly, femoral bone marrow cells from *Kit*+/+ or C57BL/6 mice were maintained *in vitro* for ~4 weeks in IL-3-containing medium until mast cells represented >95% of the total cells according to staining with May–Grünwald–Giemsa. 1.0 x 10⁶ mast cells in 200 µl of PBS, were injected i.p. (via a 26 gauge needle) into each mouse and the

mice were used for experiments, together with gender- and age-matched mast cell-deficient *Kit^W/Kit^{W-v}* mice and *Kit^{+/+}* WT mice, or mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice and C57BL/6 WT mice, 4–6 weeks after adoptive transfer of BMCMCs. Other *Kit^W/Kit^{W-v}* mice received, 4–6 weeks before injection of sarafotoxin 6b or *Atractaspis engaddensis* venom, i.p. injections of 1.0×10^6 ESCMCs that did (*ET_A^{+/-}*) or did not (*ET_A^{-/-}*) express *ET_A*; ESCMCs were generated *in vitro* from *ET_A^{+/-}* or *ET_A^{-/-}* Strain 129 ES cells (S9) as previously described (S10, 11). Other *Kit^{W-sh}/Kit^{W-sh}* mice received, four weeks before injection of sarafotoxin 6b, i.p. injections of 1.0×10^6 BMCMCs expressing an MC-CPA-targeting shRNA or empty vector (see below). Other *Kit^{W-sh}/Kit^{W-sh}* mice received, 6 weeks before s.c. injection of venom either one i.d. injection (via a 30 gauge needle) of 2.0×10^6 BMCMCs in 50 μ l of PBS (*Crotalus atrox* venom-injected mice) or 5 i.d. injections distributed over the length of the back skin (three injections) and the belly skin (two injections), with each injection containing 1.5×10^6 BMCMCs in 50 μ l of PBS (*Apis mellifera* venom-injected mice).

Lentiviral vector production.

pLentiLox 3.7 (pLL3.7) (kindly provided by Dr. L. Van Parijs (S12)), a vector engineered to co-express enhanced green fluorescent protein (GFP) as a reporter gene, permitting infected cells to be tracked by flow cytometry, was digested with XhoI and HpaI and the annealed oligos 5'-t-GAAACAGTTTGATGTGAAA-ttcaagaga-TTTCACATCAAACACTGTTTC-tttttc-3' and 5'-tcgagaaaaaa-GAAACAGTTTGATGTGAAA-tctcttgaa-TTTCACATCAAACACTGTTTC-a-3' were ligated into pLL3.7 to yield a CPA-directed shRNA-producing vector. The 19 nt CPA target sequences are indicated in capitals in the oligonucleotide sequence. Active viral stocks were created and concentrated as previously described (S13). Briefly, 293T cells were transfected with the transfer vector plasmid pLL3.7-

MC-CPA or pLL3.7 (empty vector), the VSV-G envelope-encoding plasmid pMD.G, and the packaging plasmid CMVΔR8.74 (S13) using the calcium phosphate method. The supernatants were harvested 48 and 72 h post-transfection, pooled, passed through a 0.45 μm filter, ultracentrifuged for 2 h 20 min at 19,200 rpm in an SW28 rotor, re-suspended in 100 μl of 0.1% BSA in PBS and stored at -80°C.

Lentiviral infection and characterization of CPA shRNA-containing mast cells.

2-5 week old BMCMCs were infected with virus carrying the CPA-targeting shRNA or the empty vector. Since pLL3.7 carries a CMV-GFP cassette, BMCMCs were sorted for GFP expression at 72-96 h after infection using FACS Aria (Becton Dickinson) and then were cultured in IMDM + 10 ng/ml IL-3 (Peprotech) + 10 ng/mL SCF (a gift from Amgen Inc.). 1.0×10^6 infected BMCMCs were injected i.p. into *Kit^{W-sh}/Kit^{W-sh}* mice and experiments were performed 4 weeks later. Since, to our knowledge, this is the first time shRNA-targeted mast cells have been used *in vivo*, we assessed the CPA enzymatic activity in the peritoneal mast cells (PMCs) which arose from the adoptively-transferred CPA shRNA- or empty vector-containing mast cells by measuring reduction of absorbance of a chromogenic substrate (N-[4-Methoxyphenylazoformyl]-Phe-OH) specific for CPA. We also counted the numbers of mast cells in the peritoneum (as assessed by counting toluidine blue-positive PMCs in an improved Neubauer chamber), and evaluated the appearance of mast cells in the mesentery. The latter point is important, since the migration of adoptively-transferred mast cells into the mesentery appears to be required for such mast cells to be able to provide protective function in a model of innate immunity to bacterial infection (S14). Mesenteric windows were prepared and stained with Csaba stain for mast cell detection (S6). We also evaluated levels of mMCP-5 in Western blots of CPA shRNA PMCs, which revealed that levels of mMCP-5 protein are

greatly reduced in such cells, a finding which has also been reported in mast cells derived from CPA-deficient mice (S15).

Sarafotoxin and ET-1 measurements.

Sarafotoxin concentrations were measured using an endothelin EIA with 100% cross-reactivity for sarafotoxins (Cayman Chemical, Ann Arbor, MI), and ET-1 levels were measured by ELISA (Biomedica, Vienna). All measurements of amounts of sarafotoxins or ET-1 in peritoneal fluid were made at 60 min after injection or at the time of death, if that occurred sooner.

Venoms.

Venom from *Atractaspis engaddensis*, obtained as described (S16) and stored at -20°C, was kindly provided by Elazar Kochva and Avner Bdolah (Department of Zoology, Tel Aviv University, Israel). *Crotalus atrox* and *Agkistrodon contortrix contortrix* venoms were purchased from Natural Toxins Research Center (Kingsville, TX); *Apis mellifera* venom was purchased from Vespa Laboratories (Spring Mills, PA).

Assessment of peritoneal mast cell degranulation.

Cytospins were prepared from cells collected by peritoneal lavage, stained with May-Grünwald-Giemsa stain and examined under a light microscope at x 1,000 magnification to quantify the extent of peritoneal MC (PMC) degranulation. The slides were coded so that the observers assessing the extent of PMC degranulation were not aware of the identity of the individual slides. MC degranulation is expressed as the % of the PMCs examined (at least 60 PMCs per cytospin) in which >50% (“Extensive” degranulation of that cell), 10-50% (“Moderate” degranulation of that cell) or <10% (“None”, indicative of no significant

evidence of degranulation of that cell) of the cytoplasmic granules of that PMC exhibited morphological evidence of degranulation, namely, alterations in the staining characteristics, size or distribution of the granules (S11, 17, 18).

Statistical analysis.

The differences in percentages of mice exhibiting death by 24 h after injection with S6b or various venoms was compared by Fisher's exact test, whereas analysis of variance (ANOVA) for repeated measures was used to assess differences in body temperature responses. Unless otherwise specified, all other data were tested for statistical significance using the unpaired two-tailed Student's *t*-test or, to compare values for the extent of mast cell degranulation, the Chi-square test. $P < 0.05$ is considered statistically significant. Unless otherwise specified, all data are presented as mean $\pm$ s.e.m. or mean + s.e.m.

Text Notes

1. Currently, ~ 2.5 million people are bitten by poisonous snakes annually worldwide, and it has been estimated that ~ 50,000 to 125,000 individuals die each year as a result of snake bites; most of these deaths occur in the developing world (S19, 20). However, not all snake bites result in the injection of the maximum potential amount of that snake's venom and some bites are actually "dry", in that the fangs penetrate the skin but, based on clinical observations and other types of assessment, little or no venom is injected (S21, 22). Depending on the species of venomous snake, <10% to as many as 80% of the bites can be dry, with the average of all poisonous snake bites that are dry estimated at ~ 50% (S20). The amount of venom injected by a snake of a particular species can also vary according to the age and size of the snake; compared to adults, juvenile snakes inject smaller amounts of venom (albeit sometimes of relatively high potency) (S23).

2. Death can be induced in some mouse strains by a single sting of worker bees of either the European (*Apis mellifera mellifera*) or “Africanized” (*Apis m. scutellata*) honeybee (S24). It has been estimated that ~ 150-1200 stings can deliver the LD₅₀ of honeybee venom in humans (S25), and the introduction of the “Africanized” honeybee into North America has resulted in an increased incidence of human deaths due to multiple honeybee stings (S25, 26).

3. Evidence was recently reported that the venom system in snakes and lizards derives from a single early origin in squamate evolution (~200 million years ago) and that such venoms share some similar or identical toxins (S27), including nerve growth factor, crotoamine and B-type natriuretic peptide; in each case the respective reptile toxin (S28) or a highly homologous mammalian peptide or protein (S29, 30) has been shown to induce mast cell degranulation .

Moreover, a variety of venoms derived from several animal species other than reptiles and honeybees also have been shown to activate mast cells, including those from the platypus (*Ornithorhynchus anatinus*) (S31), scorpions (Scorpionida) (S31), ants (Formicidae) (S32) and the Portuguese man-of-war (*Physalia* spp.) (S33). It will be of interest to assess whether mast cells might enhance resistance to the venoms of these and other species, as well as to those of the honeybee and the three snake species tested in the present study.

4. Higginbotham’s hypothesis was based on morphological observations of mast cell degranulation at sites of s.c. injection of Russell’s viper (*Daboia russellii*) venom and other lines of evidence. For example, the mast cell-associated proteoglycan, heparin, which is highly anionic, can bind to certain cationic components of Russell’s viper venom and can reduce the toxicity of the venom when admixed with it prior to its injection into mice (S34).

However, the MC deficiency of *Kit^W/Kit^{W-v}* mice had not yet been reported (S1) at the time of Higginbotham's study (which was published in 1965), so his hypothesis that mast cells could confer protection against snake venom-induced toxicity could not be tested directly. Moreover, many toxic components of snake venoms are not highly cationic (S35, 36) and therefore would not be expected to bind strongly to heparin.

In addition to heparin, other secreted products of mast cells have the potential to induce effects which might contribute to the reduction of the toxicity of animal venoms. For example, mast cell-dependent enhancement of local vascular permeability (through the effects of several of its secreted mediators) would be expected to enhance the extravasation of plasma inhibitors of venom metalloproteases and other toxins (S37). Indeed, it is possible that mast cells and their secreted products can enhance host resistance to venoms by a variety of direct or indirect mechanisms. Such potentially protective mechanism include not only the induction of local effects on the vasculature, or on other cellular, interstitial and/or humoral elements, at the site of envenomation, but also certain systemic effects of mast cell mediators released into the circulation.

5. Mast cells have been defined largely based on studies of these cells in vertebrates, especially mammals. However, cells which share several features of vertebrate mast cells have been reported in representatives of the subphyla of invertebrate chordates, *Uruchordata* (commonly known as sea squirts), which are phylogenetically close to vertebrate chordates (S38, 39). For example, in the ascidian *Styela plicata*, test cells, which are located in large numbers at the periphery of *S. plicata* oocytes, remain in the perivitelline space until hatching and then continue to surround the developing larva; such test cells resemble mast cells in possessing prominent cytoplasmic granules that contain histamine and heparin, and treatment of *S. plicata* oocytes with compound 48/80 *in vitro* can induce test cells to undergo

degranulation and to release heparin (S38). Moreover, it has been reported (although based on “data not shown”) that tryptases were detectable in the supernatants of test cell-enriched populations of *S. plicata* cells which had been incubated *in vitro* with the mast cell secretagogue, compound 48/80 (S38).

Based on such findings, Cavalcante *et al.* hypothesized that “ascidian test cells may be the invertebrate counterpart of mammalian mast cells” (S39). This group also stated that the “biochemical and functional properties of ascidian test cells, as well as their localization (they are located immediately beneath a layer of follicle cells around the oocyte, which is in contact with the external environment), strongly suggest that these cells may be involved in defense mechanisms, to protect the oocyte against microorganisms invasion” (S39). This interesting hypothesis apparently remains to be tested. However, based on our findings with mouse mast cells, it is intriguing to speculate that ascidian test cells might be able to contribute to host defense at least in part by enhancing resistance to exogenous toxins present in the aquatic environment, whether these toxins are derived from microorganisms or other sources.

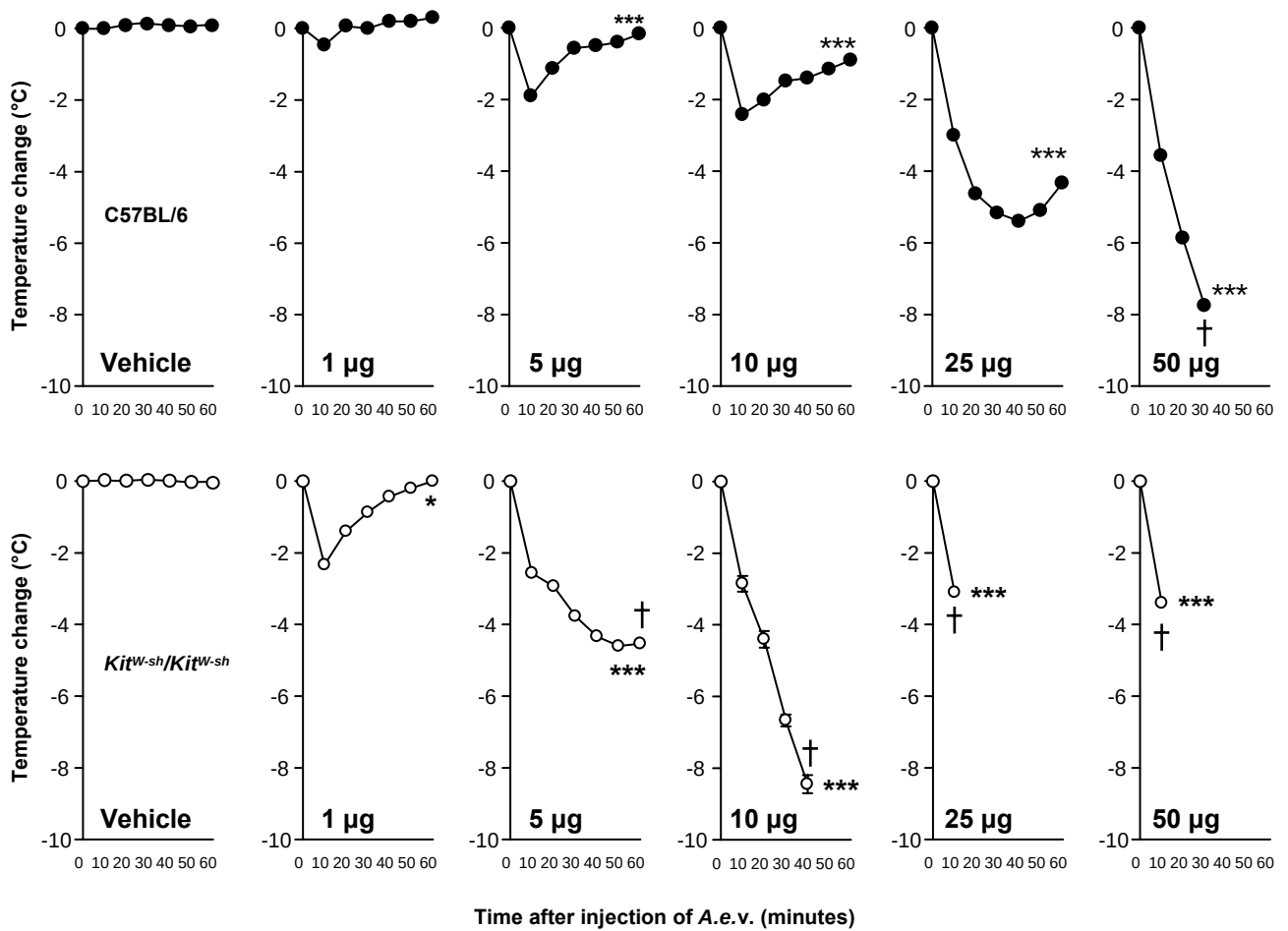
Our study focused on the role of mast cells in innate responses to venoms. However, it is well known that components of animal venoms can induce mammals to develop acquired immune responses to these substances, and that some individuals sensitized to venom components can develop anaphylaxis (S40, 41, 42, 43). In humans and other mammals, anaphylaxis occurs when certain individuals previously sensitized to a particular antigen are exposed to even very small amounts of that antigen; this rapidly triggers the extensive local and systemic antibody- and antigen-dependent activation of mast cells and certain other effector cells, which then secrete large amounts of biologically-active mediators that result in severe and sometimes fatal pathophysiological responses in the unfortunate victim (S44, 45, 46, 47). Mast cell activation is rapidly induced in this setting (within minutes of antigen exposure) because the mast cells in such sensitized subjects already have bound, to their cell

surface FcεRI receptors, IgE antibodies which recognize the provoking antigens (S44, 45, 47). Because anaphylaxis can be triggered by quite small amounts of what often are intrinsically innocuous substances (such as components of peanuts or other foods), anaphylaxis is rightly regarded as the most grotesque example of a maladaptive immune response.

However, in addition to certain antigens which are otherwise harmless, substances in the venoms of stinging insects, including the honeybee, represent common inducers of anaphylaxis in humans (S48). Components of the venoms of animals other than insects, including reptiles and terrestrial and marine invertebrates, also can induce mammals to develop anaphylactic reactivity (S41, 42, 43). Indeed, the first scientific description of anaphylaxis reported the occurrence of this phenomenon in dogs that had been immunized with and then were re-exposed to preparations of the venomous marine organism, *Physalia physalis* (the Portuguese man-of-war) (S49).

If we are correct in our hypothesis that enhancing innate resistance to the toxicity of certain venoms and other toxins represents a phylogenetically ancient mast cell function, that developed before the evolution of acquired immunity in vertebrates (S50, 51), then we might also speculate that anaphylaxis represents the most extreme and maladaptive end of a spectrum of acquired immunological responsiveness to venom components that includes responses that can enhance resistance to venom toxicity. Specifically, it will be of interest to assess whether, in some settings, the ability of mast cells to bind to their surface antibodies that recognize venom components, a process that primes these mast cells to undergo especially rapid and extensive activation upon subsequent exposure to that venom, actually can enhance host defense against the toxicity of such venoms compared to that observed in animals exposed to that venom for the first time. By contrast, in other circumstances, perhaps because of factors in individual subjects or species that can enhance the intensity of such acquired immune responses or diminish the effects of those elements which limit their extent,

antibody- and mast cell-dependent responses to venom components (or to other antigens that can elicit the same type of immune response) can become inordinately extensive, with potentially catastrophic systemic manifestations. In such individuals, exposure to the offending antigen can result in anaphylaxis.



Sarafotoxin levels in the peritoneum (ng/peritoneum) after 60 min or death

A.e.v. i.p.	1 µg	5 µg	10 µg	25 µg	50 µg
C57BL/6	not detectable	0.005 ± 0.005	0.02 ± 0	0.03 ± 0.07	0.1 ± 0.02
<i>Kit^{W-sh}/Kit^{W-sh}</i>	not detectable	0.8 ± 0.7 ⁺	59 ± 14 ⁺⁺	191 ± 27 ⁺	433 ± 4 ⁺

Fig. S1. Toxicity of intraperitoneal (i.p.) injection of *Atractaspis engaddensis* venom is markedly increased in mast cell-deficient mice. Changes in rectal temperatures and levels of sarafotoxins in the peritoneal lavage fluid (at time of death or at 60 min, whichever was sooner) after i.p. injection of vehicle (PBS) or *Atractaspis engaddensis* venom (A.e.v., in 250 µl PBS) into wild type (C57BL/6) or mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice (3 mice/group, except for 10 µg, for which 5 mice/group were used). * $p < 0.05$ or *** $p < 0.005$ versus values for mice injected with vehicle (ANOVA); + $p < 0.05$ or ++ $p < 0.01$ versus values for C57BL/6 mice (Mann-Whitney U test, 2-tailed); † = all mice dead (all of the mice in the other groups survived).

Fig. S1

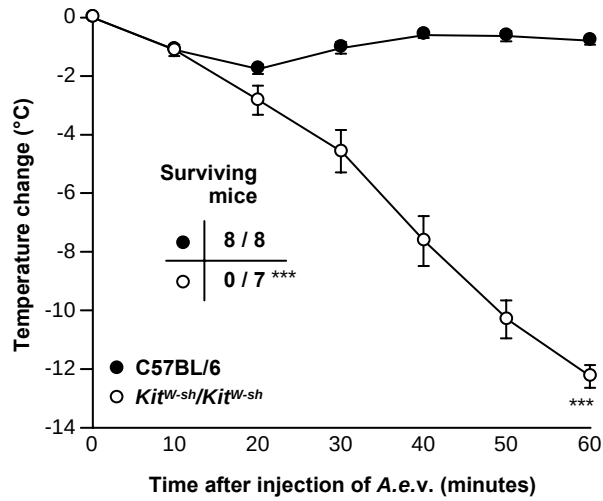
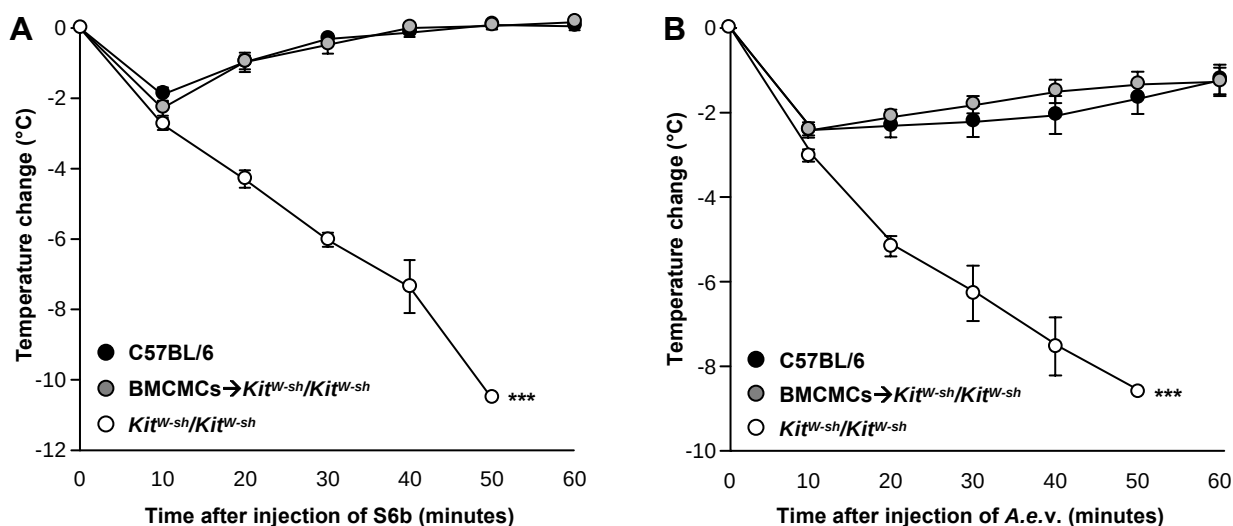


Fig. S2. Toxicity of subcutaneous (s.c.) injection of *Atractaspis engaddensis* venom is markedly increased in mast cell-deficient mice. Changes in rectal temperatures and survival after s.c. injection of *Atractaspis engaddensis* venom (A.e.v., 10 µg in 100 µl PBS) into wild type (C57BL/6) or MC-deficient *Kit^{W-sh}/Kit^{W-sh}* mice. *** $p < 0.005$ versus C57BL/6 mice (by ANOVA for temperature changes; by Fisher's exact test for survival).



	Sarafotoxin (ng/peritoneum)	Survival
C57BL/6	0.02 ± 0.003	11 / 11
<i>Kit^{W-sh}/Kit^{W-sh}</i>	191 ± 30 ***	0 / 13 ***
WT BMCMCs→<i>Kit^{W-sh}/Kit^{W-sh}</i>	0.04 ± 0.006	5 / 5

	Sarafotoxin (ng/peritoneum)	Survival
C57BL/6	0.02 ± 0.003	7 / 7
<i>Kit^{W-sh}/Kit^{W-sh}</i>	45.3 ± 8 ***	0 / 7 ***
WT BMCMCs→<i>Kit^{W-sh}/Kit^{W-sh}</i>	0.02 ± 0.004	6 / 6

Fig. S3. Mast cells (MCs) can protect mice from *A. engaddensis* venom (A.e.v.)- and sarafotoxin 6b (S6b)-induced morbidity and mortality. (A, B) Changes in rectal temperatures, levels of sarafotoxins in the peritoneal lavage fluid (at time of death or at 60 min, whichever was sooner) and 24 h survival after i.p. injection of S6b (A, 1 nmol in 250 µl saline [0.9% sterile NaCl]) or A.e.v. (B, 10 µg in 250 µl PBS) into C57BL/6 mice, MC-deficient *Kit^{W-sh}/Kit^{W-sh}* mice and *Kit^{W-sh}/Kit^{W-sh}* mice that had been engrafted i.p. with MCs derived *in vitro* from the bone marrow of C57BL/6 mice (WT BMCMCs→*Kit^{W-sh}/Kit^{W-sh}*). *** $p < 0.005$ versus corresponding values for C57BL/6 or WT BMCMCs→*Kit^{W-sh}/Kit^{W-sh}* mice (by ANOVA for temperature changes; by Fisher's exact test for survival).

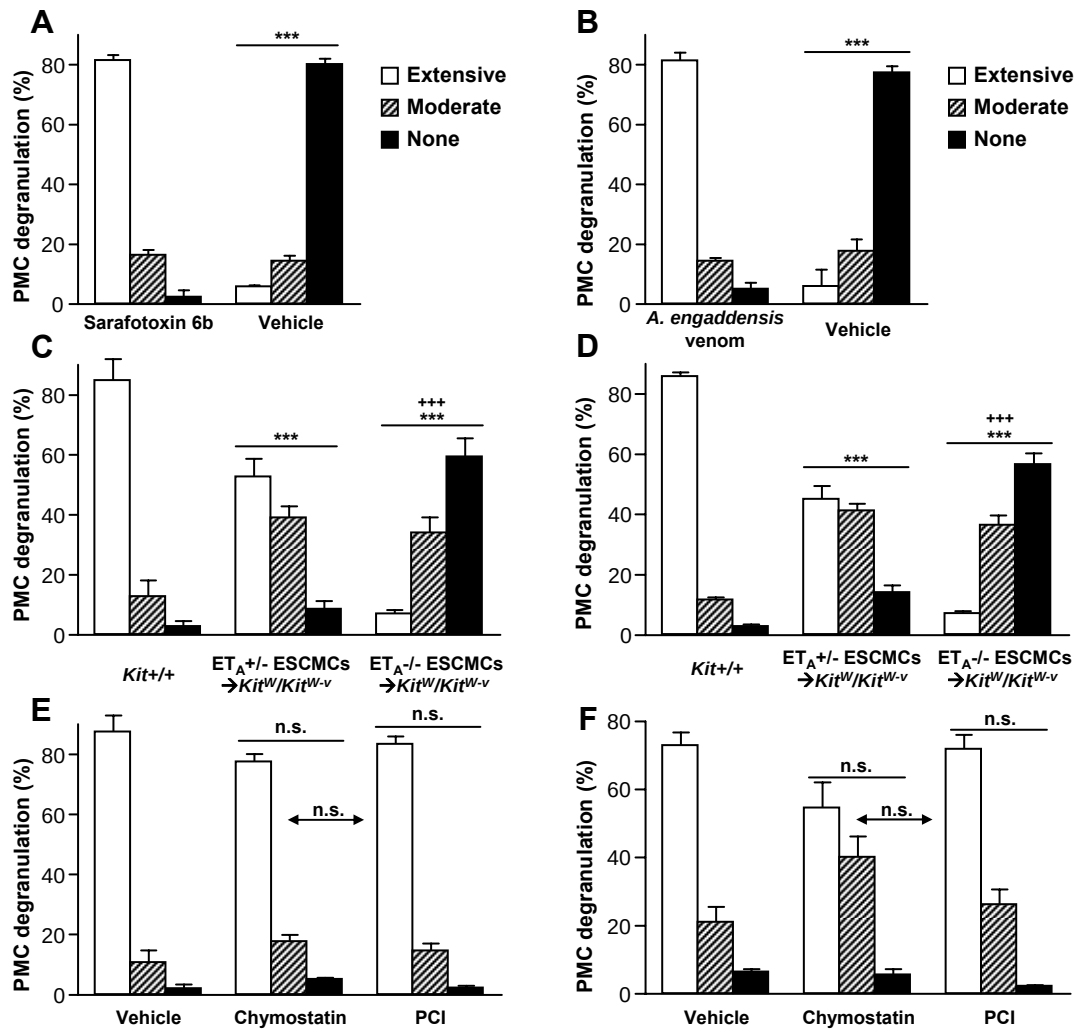


Fig. S4. Sarafotoxin 6b (S6b) and *Atractaspis engaddensis* venom (A.e.v.) induce degranulation of peritoneal mast cells (PMCs) via ET_A . (A to F) Degranulation of PMCs after i.p. injection of S6b (1 nmol in 250 μ l saline, **A**, **C** and **E**), A.e.v. (10 μ g in 100 μ l PBS, **B**, **D** and **F**) or saline or PBS alone (250 μ l [“vehicle” in **A** and **B**]) in *Kit*^{+/+} mice (**A** and **B**), or in *Kit*^{W/Kit}^{W-v} mice engrafted i.p. with *ET*_A^{+/-} (*ET*_A^{+/-} ESCMCs→*Kit*^{W/Kit}^{W-v}) or *ET*_A^{-/-} (*ET*_A^{-/-} ESCMCs→*Kit*^{W/Kit}^{W-v}) ESCMCs (**C** and **D**). (**E** and **F**) Degranulation of PMCs in C57BL/6 mice that received i.p. injections of vehicle (saline, 300 μ l), chymostatin (150 μ g in 300 μ l of saline) or potato carboxypeptidase inhibitor (PCI, 100 μ g in 300 μ l of saline) 10 min before S6b (**E**) or A.e.v. (**F**) injection. Mast cell degranulation is expressed as % of PMCs exhibiting >50% “Extensive”, 10-50% “Moderate” or <10% “None” degranulation 60 min after i.p. injection of S6b, A.e.v. or saline. *** *p* < 0.005 versus values for: (A) S6b, (B) A.e.v. (C and D) *Kit*^{+/+} or (E and F) vehicle groups; +++ *p* < 0.005 versus values for *ET*_A^{+/-} ESCMCs→*Kit*^{W/Kit}^{W-v} mice; n.s. = not significant (*p* > 0.05) versus values for Vehicle-treated mice or

for values indicated by double-headed arrows (E and F) (Chi-square test). n = 12 mice/group (A); n $\geq$ 10 mice/group (B); n $\geq$ 5 mice/group (C); n $\geq$ 7/mice group (D); n = 8 mice/group (E); n = 4 mice/group (F). At 10 min after i.p. injection of *A.e.v.* (10 μ g in 100 μ l PBS) or vehicle (PBS) into C57BL/6 mice as in A above, the % of PMCs that exhibited extensive, moderate or no degranulation were 68.8 ± 6.8 % (*A.e.v.*) versus 7.2 ± 0.3 % (PBS), 23.8 ± 6.0 % (*A.e.v.*) versus 19.1 ± 2.7 % (PBS), and 7.4 ± 1.0 % (*A.e.v.*) versus 73.7 ± 2.9 % (PBS); the difference in the extent of PMC degranulation 10 min after injection of *A.e.v.* versus PBS was highly significant ($p < 0.005$) (Chi-square test).

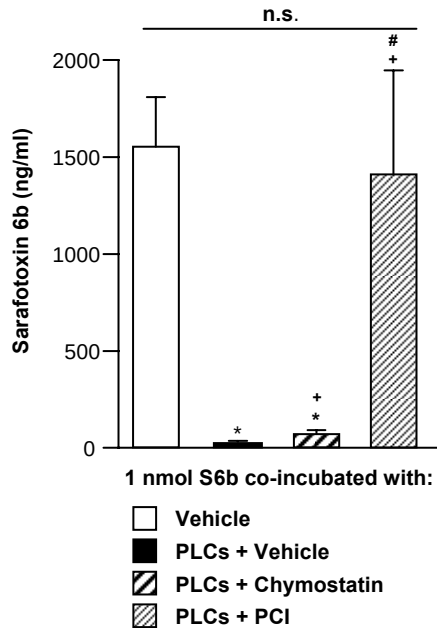


Fig. S5. Evidence that carboxypeptidase A is more important than chymase in mediating mast cell-cell dependent degradation of sarafotoxin 6b (S6b) *ex vivo*. S6b (1 nmol in 300 μ l) was incubated *ex vivo* at 37° C for 30 min with vehicle alone (DMEM), or with vehicle containing peritoneal lavage cells (PLCs) from C57BL/6 mice (1×10^6 total cells, containing 2×10^4 PMCs) which were co-incubated with vehicle alone, chymostatin (100 μ M) or PCI (400 μ g/ml). Remaining levels of S6b were then measured by ELISA; * $p < 0.05$ versus incubation with vehicle; + $p < 0.05$ versus incubation with PLCs + vehicle; # $p < 0.05$ versus incubation with PLC + chymostatin; n.s. = not significant ($p > 0.05$) for values indicated by brackets (Mann-Whitney U test, 2-tailed). Data are the mean + s.e.m. calculated from the average of duplicate values derived from the results of three separate experiments ($n = 3/\text{group}$).

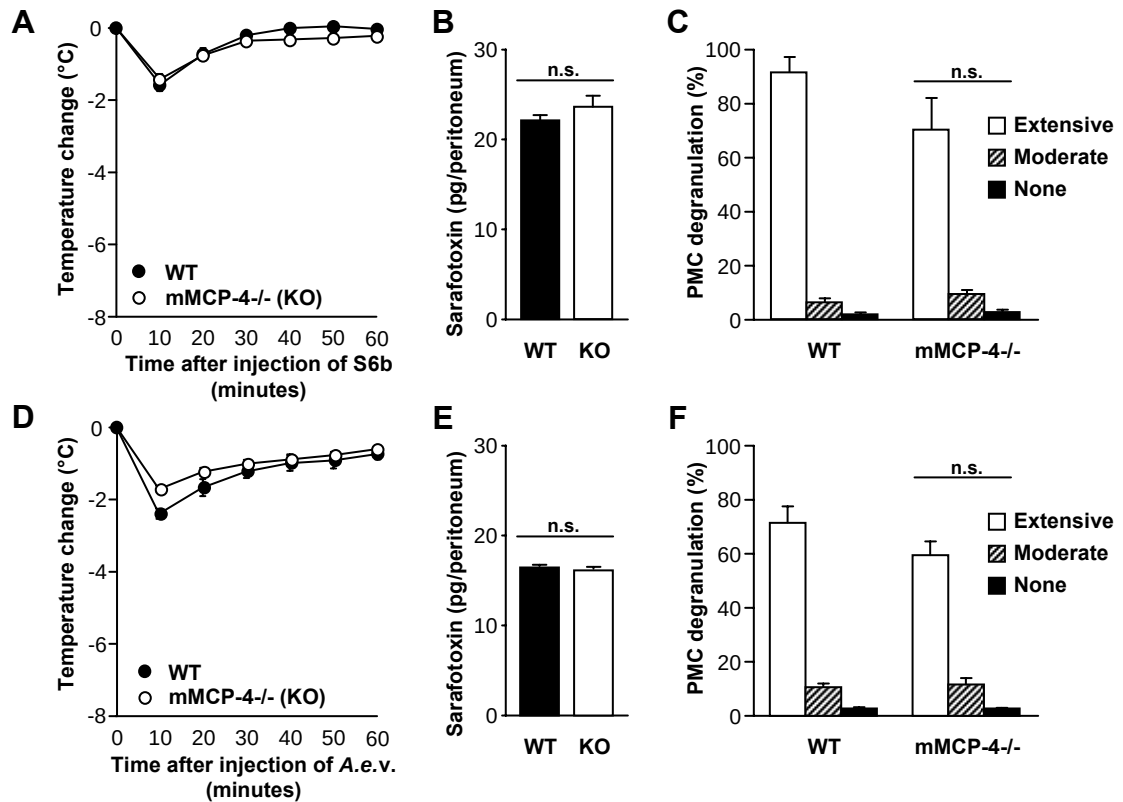


Fig. S6. Mouse MCP-4 (mMCP-4) does not contribute significantly to protection against *A. engaddensis* venom-induced morbidity and mortality. (A and D) Changes in rectal temperature, (B and E) levels of sarafotoxins in the peritoneal lavage fluid (at 60 min after injection) and (C and F) peritoneal mast cell (PMC) degranulation after i.p. injection of sarafotoxin 6b (S6b; 1 nmol in 250 μ l saline, in [C]) or *A. engaddensis* venom (A.e.v.; 10 μ g in 100 μ l PBS) into wild type (WT) C57BL/6 mice or mMCP-4-/- (KO) mice. (C and F) Percentage of PMCs exhibiting >50% “Extensive”, 10-50% “Moderate” or <10% “None” degranulation 60 min after injection of S6b or saline (Chi-square test). n.s. = not significant ($p>0.05$) versus values for WT mice (C) (Chi-square test) or for values indicated by brackets (B and E) (by Student’s *t* test, 2-tailed). $n = 4$ (C57BL/6) or 5 (mMCP-4-/-) mice/group; all of the mice survived the injection of S6b or A.e.v. and the differences in the temperature change responses in WT versus KO mice (A and D) were n.s. ($p>0.05$) (ANOVA).

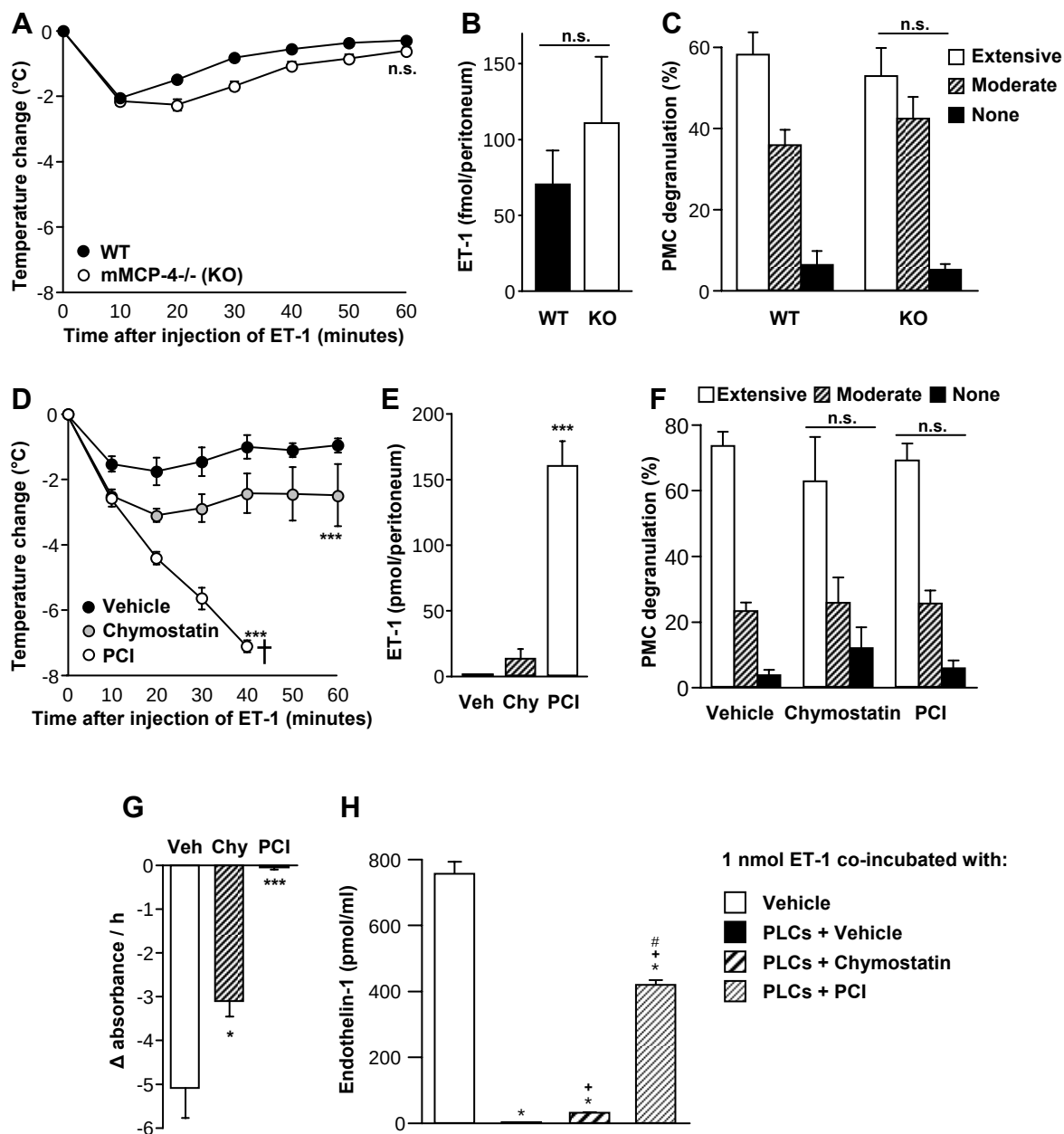
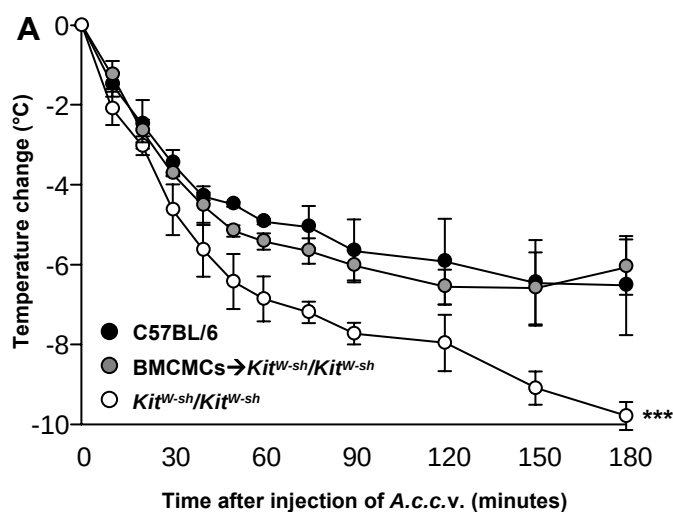
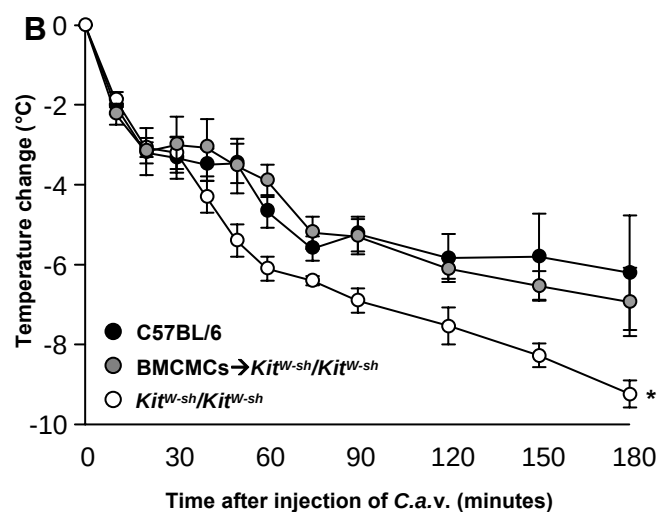


Fig. S7. Evidence that carboxypeptidase A is more important than mMCP-4 in limiting endothelin-1 (ET-1)-induced toxicity. Since S6b and ET-1 are highly homologous (S19), we also assessed whether CPA might be the dominant mast cell-associated enzyme involved in the degradation of ET-1 *in vivo*. I.p. injection of 1.2 nmol ET-1 (in 300 μ l saline) did not result in statistically significant differences of (A) body temperature, (B) intra-peritoneal ET-1 levels (measured at 60 min), or (C) levels of PMC degranulation between WT and mMCP-4^{-/-} (KO) mice, and all of the mice survived the injection of ET-1. Pre-treatment of ET-1-injected WT mice with potato carboxypeptidase inhibitor (PCI, 100 μ g in 300 μ l of saline) resulted in severe hypothermia,

failure to reduce levels of ET-1 in the peritoneal cavity (measured at time of death or at 60 min, whichever was sooner), and death (**D** and **E**), without influencing the extent of PMC degranulation induced by ET-1 (**F**). Although the effects were much less dramatic than those observed in PCI pre-treated mice, chymostatin (150 μ g in 300 μ l of saline) pre-treated mice injected i.p. with either S6b (Fig. 1E) or ET-1 (**D**) also exhibited significant hypothermia compared to values in the corresponding vehicle-treated (saline, 300 μ l) mice; however, all of the chymostatin (or vehicle) pre-treated mice injected with S6b or A.e.v. appeared to recover fully and survive. By contrast, no significant differences were observed in the body temperature responses of mMCP-4^{-/-} versus WT mice to either S6b (Fig. S6A) or ET-1 (**A**) (ANOVA). We suspect that the *in vivo* effects of chymostatin may well reflect the ability of this compound to diminish CPA activity. Indeed, we find that chymostatin can reduce the activity of CPA by up to 40% *in vitro* (**G**), as assessed by comparing the effects of vehicle (Veh), chymostatin (Chy, 120 μ g) or potato carboxypeptidase inhibitor (PCI, 150 μ g) added to lysates from 3×10^6 C57BL/6 peritoneal cells containing 1×10^5 peritoneal MCs at 37° C (total volume 150 μ l) to change the absorbance of a chromogenic substrate specific for CPA (S3). PCI, and to a much lesser albeit significant extent chymostatin, also prevented in part the degradation of ET-1 *in vitro* (**H**). ET-1 (1 nmol in 250 μ l) was incubated *ex vivo* at 37° C for 30 min with vehicle alone (DMEM), or with vehicle containing peritoneal lavage cells (PLCs) from C57BL/6 mice (1×10^6 total cells, containing 2×10^4 PMCs) which were co-incubated with vehicle alone, chymostatin (100 μ M) or PCI (400 μ g/ml). Remaining levels of ET-1 were then measured by ELISA. * $p < 0.05$ versus incubation with vehicle; + $p < 0.05$ versus incubation with PLCs + vehicle; # $p < 0.05$ versus incubation with PLC + chymostatin (Mann-Whitney U test, 2-tailed). Data are the mean + s.e.m. calculated from the average of duplicate values derived from the results of three separate batches of cells, each derived from different mice (n = 3/group). (C and F) Percentage of PMCs exhibiting >50% “Extensive”, 10-50% “Moderate” or <10% “None” degranulation 60 min after injection of ET-1. * $p < 0.01$, *** $p < 0.005$ versus vehicle; n.s. = not significant ($p < 0.05$) versus values for WT (C) or Vehicle-treated (F) mice (Chi-square test) or for values indicated by bracket (B) (Student’s *t* test); † = all mice dead. n = 8 (C57BL/6) or 6 (mMCP4^{-/-}) mice/group (A to C); n = 4 (vehicle) or 5 (chymostatin and PCI) mice/group (D to F); n ≥ 3 /group.



	Survival
C57BL/6	3 / 3
<i>Kit^{W-sh}/Kit^{W-sh}</i>	0 / 3
WT BMCMCs→<i>Kit^{W-sh}/Kit^{W-sh}</i>	3 / 3



	Survival
C57BL/6	3 / 3
<i>Kit^{W-sh}/Kit^{W-sh}</i>	0 / 3
WT BMCMCs→<i>Kit^{W-sh}/Kit^{W-sh}</i>	3 / 3

Fig. S8. Mast cells (MCs) can limit the toxicity of western diamondback rattlesnake and southern copperhead venoms. (A, B), Changes in rectal temperature and 24 h survival after i.p. injection of *Agkistrodon contortrix contortrix* (southern copperhead) venom (A.c.c. venom, 150 µg in 250 µl PBS (A) or *Crotalus atrox* (western diamondback rattlesnake) venom (C.a. venom, 75 µg in 250 µl (B) into C57BL/6 mice, MC-deficient *Kit^{W-sh}/Kit^{W-sh}* mice and *Kit^{W-sh}/Kit^{W-sh}* mice that had been engrafted i.p. with MCs derived *in vitro* from the bone marrow of C57BL/6 mice (WT BMCMCs→*Kit^{W-sh}/Kit^{W-sh}*). * $p < 0.05$ or *** $p < 0.005$ versus C57BL/6 or WT BMCMCs→*Kit^{W-sh}/Kit^{W-sh}* mice (ANOVA).

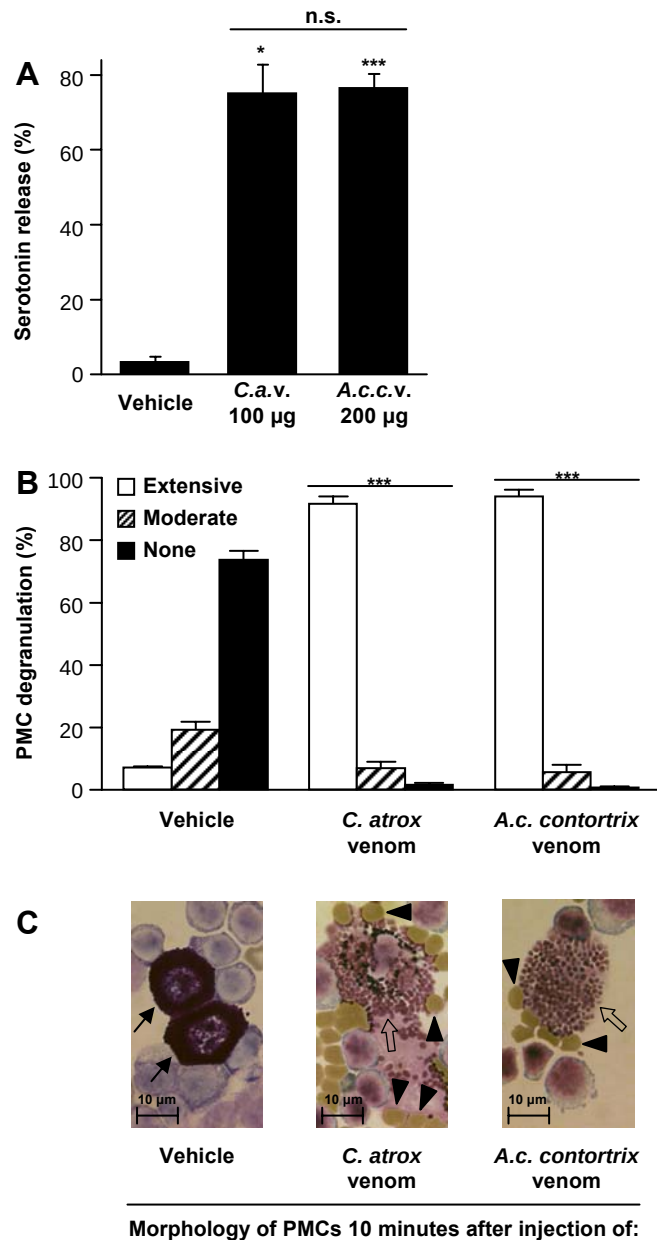


Fig. S9. *Crotalus atrox* (C.a.v.) and *Agkistrodon contortrix contortrix* venom (A.c.c.v.) induce degranulation of peritoneal mast cells (PMCs) *ex vivo* and *in vivo*. (A) Peritoneal lavage cells from C57BL/6 mice were incubated at 37° C for 2 h with ³H-5-hydroxytryptamine (³H-serotonin), washed, and then incubated with vehicle, C.a.v. or A.c.c.v. for 15 min. Results are mean + s.e.m. (n = 4) of values pooled from two independent experiments that gave similar results. * *p*<0.05; *** *p*<0.005 versus vehicle-treated cells; n.s. = not significant (*p*>0.05) for values indicated by bracket (Student's *t* test, 2-tailed). (B) Degranulation of PMCs assessed 10 min after i.p. injection of C.a.v.

(75 μ g in 250 μ l PBS), *A.c.c.v.* (150 μ g in 250 μ l PBS) or PBS (Vehicle) alone. Mast cell degranulation is expressed as % of PMCs exhibiting >50% “Extensive”, 10-50% “Moderate” or <10% “None” degranulation, evaluated in cytopins of peritoneal lavage cells obtained from mice killed 10 min after injection of venom or vehicle (PBS). *** $p < 0.005$ versus vehicle (Chi-square test); $n = 3$ /mice group. **(C)** May-Grünwald-Giemsa stained cytopins of peritoneal lavage cells obtained 10 min after injection of vehicle (PBS), *C.a.v.* or *A.c.c.v.* (shown at x 1,000 magnification; scale bars = 10 μ m). Arrows in the panel illustrating peritoneal cells from a vehicle-injected mouse indicate two PMCs which exhibit no evidence of degranulation (scored as “None”); open arrows in the two other panels indicate PMCs which exhibit the alterations of the staining characteristics, size and distribution of their cytoplasmic granule which are scored as indicative of “Extensive” degranulation; arrowheads indicate red blood cells, evidence of bleeding in the peritoneal cavity of mice injected with *C.a.v.* or *A.c.c.v.*

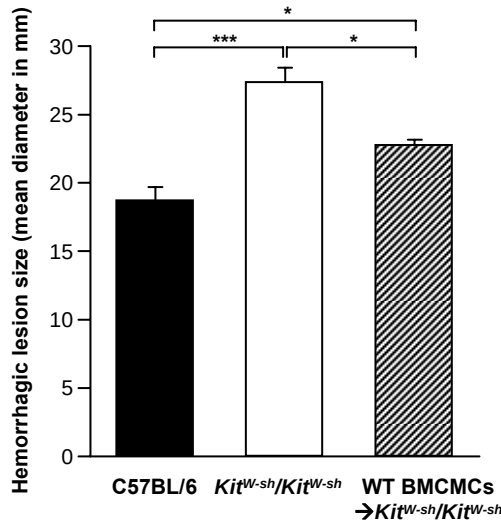


Fig. S10. Mast cells can limit the size of hemorrhagic lesions induced by *Crotalus atrox* venom (C.a.v.) in the skin. C.a.v. (50 μ g in 100 μ l) was injected s.c. in the shaved back skin of age-matched female WT mice, *Kit^{W-sh}/Kit^{W-sh}* mice, or *Kit^{W-sh}/Kit^{W-sh}* mice that had been engrafted i.d., 6 weeks earlier, with 2×10^6 BMCMCs (WT BMCMCs $\rightarrow$ *Kit^{W-sh}/Kit^{W-sh}*) and the size of the hemorrhagic lesions induced was measured 2 h later, by averaging the lengths of the largest diameter and the diameter perpendicular to that. n = 13 (WT or *Kit^{W-sh}/Kit^{W-sh}* mice) or 5 (WT BMCMCs $\rightarrow$ *Kit^{W-sh}/Kit^{W-sh}* mice). * $p < 0.05$; *** $p < 0.005$ for the comparisons indicated by brackets (Student's *t* test, 2-tailed).

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