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Short title: MC detoxification of bee venom

Abstract

Background

In contrast to their clearly defined roles in allergic diseases, the physiologic functions of Immunoglobulin E antibodies (IgEs) and mast cells (MCs) remain enigmatic. Recent research supports the *toxin hypothesis*, showing that MCs and IgE-related type 2 immune responses can enhance host defense against certain noxious substances, including honeybee venom (BV). However, the mechanisms by which MCs can interfere with BV toxicity are unknown. In this study, we assessed the role of IgE and certain MC products in MC-mediated BV detoxification.

Methods

We applied *in vitro* and *in vivo* fluorescence microscopy imaging, and flow cytometry, fibroblast-based toxicity assays and mass spectrometry to investigate IgE-mediated detoxification of BV cytotoxicity by mouse and human MCs *in vitro*. Pharmacologic strategies to interfere with MC-derived heparin and proteases helped to define the importance of specific detoxification mechanisms.

Results

Venom-specific IgE increased the degranulation and cytokine responses of MCs to BV *in vitro*. Passive serum sensitization enhanced MC degranulation *in vivo*. IgE-activated mouse or human MCs exhibited enhanced potential for detoxifying BV by both proteolytic degradation and heparin-related interference with toxicity. Mediators released by IgE-activated human MCs efficiently degraded multiple BV toxins.

Conclusions

Our results both reveal that IgE sensitization enhances the MC's ability to detoxify BV and also assign efficient toxin-neutralizing activity to MC-derived heparin and proteases. Our study thus highlights the potential importance of IgE, MCs, and particular MC products in defense against BV.

Keywords

Honeybee venom; host defense; IgE; mast cells; toxin hypothesis.

Main Text

Background

Mast cells (MCs) and immunoglobulin E antibodies (IgEs) are generally best known for their adverse functions in allergic diseases¹. Allergic patients elaborate IgEs recognizing epitopes on environmental antigens, which typically represent no obvious danger. However, the exposure of sensitized hosts to antigens recognized by IgEs bound to MCs via the high affinity IgE receptor, FcεRI, result in rapid MC activation with the release of many bioactive compounds, including MC-associated proteases and proteoglycans (e.g., heparin), as well as diverse additional stored and newly synthesized mediators^{1,2}. In concert, such MC-derived mediators cause immediate allergic symptoms and inflammation^{1,2}, in extreme cases leading to potentially fatal anaphylaxis³.

The evolutionary conservation of IgE and MCs despite their life-threatening potential could reflect important protective functions requiring fast and strong immunological and behavioral responses⁴. Traditionally, IgE and MCs have been assigned a protective role in host defense against parasites⁴⁻⁶. However, the importance of such protective functions seems to depend on the respective parasite and model system⁵. In contrast to parasites, toxins and venoms represent acute and highly dangerous threats that require immediate counter-measures in order to avoid extensive damage⁷, and it has been suggested that allergies developed initially as protective responses to noxious substances⁸⁻¹⁰.

Notably, several venoms either contain or are themselves allergens^{8,9}. One example of a highly allergenic venom is honeybee (*Apis mellifera*) venom (BV)^{11,12}. BV is a complex mixture of active substances,

including neurotoxins (such as apamin), enzymes (such as phospholipase A2 [PLA₂]), amines, and the cytolytic peptide melittin¹³. PLA₂, hyaluronidase, acid phosphatase and melittin are considered the main BV allergens (designated Api m1-4, respectively)¹¹. Many BV constituents induce acute pain upon injection into the opponent's tissue¹³. In addition, BV contains compounds which can induce MC degranulation.

Recent evidence shows that mouse MCs can play an important role in innate host defense against an initial challenge with various venoms, including those of different reptiles¹⁴⁻¹⁶, scorpions¹⁶ and the honeybee¹⁴. These studies revealed that detoxification of snake, Gila monster and scorpion venoms was dependent, in large part, on the proteolytic activity of the MC-specific proteases carboxypeptidase A3^{14,15} (CPA3; in case of certain snake venoms) and the chymase MCP4¹⁶ (for Gila monster and scorpion venoms). Moreover, for the venoms of the Russell's viper¹⁷ and the honeybee^{18,19}, we^{17,18} and others¹⁹ showed that subcutaneous sublethal injections of venom induce a type 2 immune response associated with the production of specific IgEs, and that such IgEs and IgE effector cells can play important roles in acquired immunity against subsequent exposure to potentially lethal amounts of the venom^{17,18} or one of its constituents¹⁹. Yet the specific identity of MC-associated defenses against BV remained unclear.

Thus, while it is established that MCs and IgEs can be important in host defense against BV⁵, the mechanisms by which IgE enhances MC-mediated detoxification of BV have not been clarified. In addition, the MC signal transduction response to BV in the absence or presence of IgE is uncharacterized. Here, we performed studies on primary mouse and human MCs to decipher potential mechanisms of BV detoxification.

Methods

Additional methodological details regarding statistics, cellular models, analyses of gene expression, signal transduction, degranulation, viability, degradation of BV, proteomics, PLA₂ activity, imaging, and venom cytotoxicity, as well as more information regarding the mice, reagents and antibodies used in this study, can be found in the online supporting information.

Mice

Animal care and experiments were performed following current guidelines of the National Institutes of Health and with approval of the Stanford University Institutional Animal Care and Use Committee (IACUC protocol #12683). C57BL/6 wildtype (wt) mice were obtained from Jackson Laboratories and housed in the Stanford University animal facility for at least 7 days before starting experiments.

Serum generation

For generation of mouse sera, C57BL/6 wt mice were shaved on the back skin and received two subcutaneous injections of 50 μ l (containing 200 μ g) BV (Lot 12071006HB; ALK Abello Source Material) or PBS in the shaved area¹⁸. Three weeks later, mice were sacrificed and sera were collected.

Statistics

Statistical analyses were conducted using GraphPad Prism (GraphPad Software) and tests were applied as indicated in the figure legends. Differences with *P* values equal or below 0.05 were considered significant. All experiments were performed at least twice.

Results

IgE sensitization increases BV-mediated MC degranulation and signaling

We first tested the ability of BV to cause degranulation and cytotoxicity in mouse fetal skin-derived cultured MCs (FSCMCs). Assessing degranulation by detecting release of the granule enzyme β -hexosaminidase, and monitoring viability by flow cytometry, we found significant BV-related degranulation of FSCMCs at 1 or 10 μ g/ml BV without detectable effects on cell viability (Figure S1A and B). To dissect the effect of sensitization with specific IgE on FSCMC responsiveness to BV, we generated immune serum collected from mice three weeks after two subcutaneous injections of either 200 μ g BV (BV serum) or the respective amount of PBS (PBS serum). This treatment, that approximately mimicked the dose of venom injected by two bee stings^{18,20}, induced elevated levels of serum IgE, IgG1 and IgG2b (Figure S1C). Sensitization with BV serum significantly increased FSCMC degranulation at all tested sub-cytotoxic doses (1, 5 or 10 μ g/ml), and pre-treatment of BV serum with anti-IgE almost completely attenuated this effect (Figure 1A).

We then used flow-cytometry to monitor FSCMC degranulation in real-time, assessing degranulation by fluorescence emitted upon binding of Alexa Fluor 488-tagged avidin [avidin^{AF488}] to granular heparin exposed on the cell surface upon granule exocytosis²¹. We found that degranulation of BV serum-sensitized FSCMCs, which began less than 5 minutes after addition of BV, reached a peak plateau after 10-15 minutes (Figure 1B). Anti-IgE pre-treatment again abrogated degranulation to a level indistinguishable to that of cells mock-sensitized with PBS serum (Figure 1B).

These results indicate that subcutaneous injections of BV induce specific IgE antibodies that strongly amplified the innate degranulation response of FSCMCs to BV. To investigate this further, we analyzed the effect of serum sensitization on BV-related FSCMC signaling. Kinetic analysis of major signaling pathways transducing MC activation signals²² revealed that BV exposure increased phosphorylation of AKT, ERK and

PLC γ in FSCMCs, indicating IgE-independent transduction of activation in these PBS serum-sensitized FSCMCs (Figure 1C). Interestingly, sensitization with BV serum markedly increased BV-mediated ERK phosphorylation (Figure 1C).

The mediator release response of MCs can be tuned depending on the stimulation signals^{23,24}. For instance, IgE/antigen-mediated activation via Fc ϵ RI leads to degranulation and extensive cytokine production, whereas stimulation via G protein-coupled receptors, such as Mrgprb2, induces predominantly degranulation with minor *de novo* cytokine production^{24,25}. We therefore wanted to test whether IgE sensitization affected mediator synthesis of BV-stimulated MCs. We observed that BV exposure induced increased transcription of *il1b*, *il4*, *il5*, *il6*, *il13*, *mip1a*, *ccl1*, *ccl2*, and *ccl4* (Figure 1D). However, many elements of this innate response were profoundly amplified by sensitization with untreated BV serum, reflected by increased levels of *il1b*, *il6*, *il13*, *tnf*, *mip1a*, *ccl1* and *ccl2*. Interference with IgE function, either by serum pre-treatment with anti-IgE or heating the serum to 56°C¹⁸, largely abolished the increased cytokine and chemokine transcription, indicating the important contribution of IgE (Figure 1D).

We next used *in vitro* and *in vivo* imaging approaches to characterize the effects of IgE-sensitization on MC degranulation at the single cell level. Stimulation with BV induced a strong and rapid increase in avidin^{SR} (sulforhodamine 101-tagged avidin) signal in individual FSCMCs sensitized with BV serum (Figure 2A – C and Videos S1 and S2). Anti-IgE pre-treatment abrogated this response down to levels comparable to PBS serum-sensitized cells (Figure 2A to C and Video S3), confirming the essential role of IgE in the FSCMC venom response.

To investigate the *in vivo* relevance of IgE-enhanced MC responsiveness to BV, we injected mice intradermally with either BV serum or PBS serum. Subsequent intradermal BV challenge of PBS serum-mock-sensitized ears led to sustained ear swelling for at least 3 hours (Figure 2D). Interestingly, BV serum significantly increased the immediate tissue response detected 30 minutes after venom injection. Based on the *in vitro* observations described above, we suspected that the increased swelling induced by BV at sites injected with BV serum might be related to increased MC degranulation. We therefore used *Mcpt5-Cre; R26YFP* reporter mice (which express eYFP specifically in mMCP5⁺ connective tissue MCs) and intradermally injected avidin^{SR} to specifically label MC granules²³. One week later, the mice were injected in the ear with either PBS serum or BV serum. Intravital 2 photon microscopy performed immediately after BV challenge on the next day revealed increased skin MC degranulation in BV serum-sensitized ears (Figure 2E and F).

Taken together, our results indicate that anti-BV IgE has significant potential for increasing the MC's response to BV *in vitro* and *in vivo*.

BV and IgE-mediated signals together enhance FSCMC activation

We next investigated whether stimulation with BV and IgE directed against a distinct antigen also could enhance MC activation. To do this, we stimulated anti-dinitrophenyl (DNP) IgE-sensitized FSCMCs with different doses of antigen (DNP-coupled to human serum albumin [DNP-HSA]) in combination with a sub-cytotoxic dose of BV (10 μ g/ml). We observed that at each tested dose of DNP-HSA, BV significantly increased the release of β hexosaminidase (Figure 3A). Similarly, BV exposure increased the realtime degranulation kinetics of IgE-sensitized FSCMCs stimulated with 5 ng/ml DNP-HSA (Figure 3B). At this dose of DNP-HSA, BV stimulation exhibited AKT, ERK and PLC γ phosphorylation (Figure 3C) and a greater effect on ERK phosphorylation than stimulation with BV alone at 1 minute (Figure 3D) and 5 minutes (Figure S2A) after exposure. We conclude from these results that simultaneous stimulation with BV and IgE and antigen can induce a higher activation response in mast cells than either of the individual stimuli on its own.

IgE-mediated activation increases the detoxification potential of mouse and human MCs

Having observed the effects of IgE (directed against either BV or DNP-HSA) sensitization on MC responses to BV, we next wanted to address the potential biologic relevance of this phenomenon. Higginbotham and Karnella²⁶ suggested that MCs might play an important role in host defense against BV-induced cytotoxicity, and this later was proven by tests employing MC-deficient mice¹⁴. However, the exact mechanisms by which MCs mediated detoxification of BV were unclear. Phospholipase A₂ (PLA₂) is an important enzyme found in a wide variety of venoms⁷. Together with the cytolytic agent melittin, PLA₂ is considered a major BV constituent (approximately 50% and 12% of dry BV, respectively) and both compounds contribute to the diverse toxic effects of the venom¹³. Accordingly, either PLA₂ or melittin might be potential targets of MC-related detoxification mechanisms.

Using a chromogenic assay, we found that heparin, an abundant glycosaminoglycan with scaffolding functions in MC granules², can efficiently interfere with the phospholipase activity of BV (Figure 4A; of note, the PLA₂ concentration in this assay with complete BV should be approximately 60 μ g/ml). Similarly, anti-DNP IgE-sensitized MCs that were activated by DNP-HSA for 1 hour profoundly decreased BV phospholipase activity (Figure 4B). In addition to heparin, activated MCs also rapidly release an array of MC-specific and non-specific proteases (such as, carboxypeptidase A3 [CPA3], a chymase [mMCP4], tryptases, and other serine peptidases). Some of these have been shown to provide benefit by mediating

the degradation of endogenous toxic substances, such as endothelin^{15,27} or vasoactive intestinal peptide¹⁶, or exogenous compounds contained in scorpion¹⁶ or reptile¹⁴⁻¹⁶ venoms.

We performed PAGE (poly acrylamide gel electrophoresis) to assess the potential proteolytic effects of FSCMC compounds on the BV proteome. Exposure of BV to supernatants of IgE-activated FSCMCs resulted in a shift in the low molecular weight venom fraction (below 10 kDa), presumably caused by degradation of toxins into smaller fragments (Figure 4C). This effect could be partially rescued by pre-treatment of the FSCMC supernatant with a broad-spectrum protease inhibitor (Figure 4C). The predominant peptide in this low molecular weight BV fraction that was visibly modified by MC proteases is melittin (which has a molecular weight of approximately 2.8 kDa; Figure S3A), the main BV toxin with high cytolytic activity¹³.

To assess the effects of the FSCMC supernatant on BV cytotoxicity, we exposed mouse 3T3 fibroblasts to BV and monitored the cell death kinetics over 1 hour using real-time confocal fluorescence microscopy. The venom exhibited high cytotoxic potency, with a substantial proportion of the cells dying or dead (i.e. viability dye-positive) within a few minutes after starting the recording (Figure 4D to Gand Video S4). Remarkably, pre-exposure of BV with supernatant of IgE/antigen-activated FSCMCs almost completely abrogated its cytotoxicity (Figure 4D to Gand Video S5).

To test the contributions of released heparin and proteases to the observed BV detoxification, we pre-treated FSCMC supernatant with protamine, a heparin-antagonist²⁸, or with a cocktail of protease inhibitors which do not mediate cytotoxicity (Figure S4A and B). Interestingly, while either treatment delayed the kinetics of BV-induced cytotoxicity, the fibroblasts eventually died. This suggests that both heparin- and protease-mediated mechanisms can contribute to efficient BV detoxification in this model (Figure 4D to Gand Videos S6 and S7).

We next addressed translational aspects of our findings, using human peripheral blood-derived cultured MCs (hu PBCMCs). Like FSCMCs, hu PBCMCs also exhibited enhanced degranulation when challenged with 1 or 10 µg/ml BV (reflected by increased supernatant β -hexosaminidase and tryptase activity as well as avidin^{AF488} binding) without detectable cytotoxicity (Figure 5A-B and Figure S5A-B). We then used PAGE to assess the potential of hu PBCMC-released mediators to degrade BV and observed a profound effect of supernatants from hu PBCMCs stimulated for 1 hour with either BV (10 µg/ml) or IgE and anti-IgE (Figure 5C and Figure S5C). Importantly, such treatment substantially decreased the immunorecognition of BV, reflected by a strongly diminished signal mediated by BV-specific serum IgG antibodies in Western blots (Figure 5D). Mass spectrometry analysis revealed that treatment of BV with supernatant collected from IgE-activated hu PBCMCs significantly decreased the abundance of BV toxins, including the major

allergens^{29,30} melittin (also known as Api m 4) and venom dipeptidyl peptidase 4 (DPP4; also known as Api m 5), as well as carboxypeptidase (also known as Api m 9) and vitellogenin (also known as Api m 12) (Figure 5E and F; Table S1). Of note, while not reaching statistical significance, many other BV components, such as Icarapin-like (also known as Api m 10) and venom carboxylesterase-6 (also known as Api m 8) also showed lower abundance after supernatant exposure (Figure S5D; Table S1). Finally, detoxification experiments with supernatant collected from IgE and anti-IgE activated hu PBCMCs recapitulated our observations with FSCMCs: hu PBCMC supernatant counteracted BV cytotoxicity in a predominantly heparin- but also partially protease-dependent manner (Figure 6A-D and Videos S8-11).

We conclude from this series of experiments that IgE-activated human PBCMCs and mouse FSCMCs share the potential of detoxifying BV, and that this reflects, at least in part, their release of both heparin and proteases.

Discussion

MCs are found in most vascularized tissues of mammals, as well as in fish, reptiles and urochordates, but their biological function has remained unclear⁴. As one of just a few cell types, MCs express the high affinity IgE receptor, FcεRI, in a hetero-tetrameric configuration consisting of one IgE-binding α-chain, one transmembrane β-chain and two signal-transducing γ-chains (the γ-chain is also shared with several IgG Fc receptors)³¹. This FcεRI configuration allows antigen-specific priming of MCs, with dramatic cellular responses upon antigen exposure¹.

As this mechanism is central for allergies (with IgEs directed against seemingly innocuous environmental compounds), the physiologic functions of IgEs and MCs have been an important focus of allergy research for decades. One well accepted function of this *allergy module* (IgEs and MCs) of immunity has been host defense against parasites^{4,6,32}. Indeed, many parasites invade host tissues throughout their life cycle, a process often associated with severe tissue damage, which is considered a major signal initiating (T helper cell) type 2 responses³³. Type 2 immune responses and the production of associated cytokines like IL-4 and IL-13 are considered the pre-requisite for the process of antibody class switch required for IgE production by B cells³⁴. While this adaptive immune response can be beneficial, and is required for clearing of certain parasite infections^{33,35}, the experimental evidence regarding IgE and MC functions in such settings is controversial⁵.

Initial evidence for an alternative function of MCs in host defense against bee venom has been provided by R. D. Higginbotham and colleagues²⁶. They reported that BV injection induced MC degranulation and that heparin treatment of BV reduced the lethality of the venom upon its intravenous application in mice²⁶.

More than thirty years later, our lab confirmed that MC-deficient mice exhibited increased susceptibility to subcutaneous BV injections^{14,18}. In subsequent studies, we found that honeybee venom and Russell's viper venom are potent inducers of type 2 immune responses and IgE production^{17,18} in mice. While venom-specific IgE has been commonly associated with the risk of allergy and anaphylaxis, the alternative toxin hypothesis (originally postulated by Margie Profet) suggested a beneficial function of IgEs in host defense against noxious substances, for instance by increasing the innate detoxifying potential of MCs^{8,9}. The results of our lab^{17,18} and the Medzhitov¹⁹ lab provided evidence for this idea, however the specific molecular consequences of IgE sensitization on MC responses to venoms have not been fully addressed.

In the current study, we found that while certain concentrations of BV can trigger active degranulation (independent of cell lysis) of non-sensitized MCs, sensitization with serum from BV-immunized mice strongly enhanced the MC response in an IgE-dependent manner. The presence of anti-BV IgE increased BV-induced MC granule release on a population and single cell level *in vitro* and *in vivo*. Furthermore, the MAP kinase-related ERK signaling kinetics were profoundly extended, and gene expression for diverse cytokines and chemokines were increased in an IgE-dependent manner. These findings indicated that venom-specific IgE can efficiently increase the magnitude of BV-induced MC responses, including the release of preformed and *de novo* produced compounds.

While it has been previously reported that MC CPA3 and mMCP4 can contribute to the degradation of snake venom sarafotoxin^{14,15} and the Gila monster venom constituent helodermin¹⁶, the molecular mechanisms of BV detoxification by MC compounds were unclear¹⁴. Our data show that heparin and other MC compounds can interfere with the venom's phospholipase activity, which is thought to mediate cytotoxicity via production of lysophospholipids¹³. In addition, we observed degradation of several important venom allergens by pre-formed proteases released by IgE/antigen-activated MCs, including melittin (Api m 4), Api m 5, Api m 9 and Api m 12. However, treatment of MC supernatants with a pan protease inhibitor seemed to not fully restore the PAGE migration pattern of untreated BV, possibly indicating incomplete interference with protease activity. Also, the partial BV digestion due to limited protease inhibition was not sufficient to fully detoxify the venom. In summary, our findings suggest that both heparin and proteases can have important roles in BV detoxification.

In humans, population studies show that healthy individuals (i.e., without diagnosed atopic disease) can express IgE specific for hymenoptera venom toxins^{36,37}. This may indicate that toxin-specific IgE has some non-pathologic function(s) in such people. While occasional bee stings are common in the general population¹¹, beekeepers represent a subpopulation that is regularly exposed to BV. Up to ~30% of beekeepers respond with large local or systemic reactions to bee stings and up to 60% exhibit positive

skin tests and detectable BV-specific IgE³⁸. Remarkably, systemic allergic reactions seem to be more frequent in beekeepers who are infrequently stung³⁸. This might be due to the higher levels of venom-specific IgG in such frequently exposed individuals, which outcompetes MC-bound IgE with the same epitope specificity. However, the precise function of BV-specific IgE in the increased venom tolerance of beekeepers³⁸ is still not fully understood.

Overall, our study has identified the contribution of two classes of pre-formed MC compounds, the proteoglycan heparin, as well as proteases, to resistance against BV toxicity. Importantly, we also show that specific serum IgE can substantially increase expression of the detoxifying potential of MCs. These findings thus reveal the significant benefit of this specialized anti-venom module of the adaptive immune system.

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Figure Legends

Figure 1: IgE sensitization modulates FSCMC degranulation, signaling and gene expression responses to honeybee venom

FSCMCs were sensitized overnight with serum derived either from mock (PBS)-immunized or BV-immunized mice (PBS serum or BV serum, respectively) that was either untreated or pre-treated (before

cell incubation) with (A, B, D) an anti-IgE (or isotype control) antibody or (D) by heating as indicated. (A) FSCMCs were unstimulated or exposed to BV at 1, 5 or 10 $\mu\text{g/ml}$ for 1 hour and analyzed for β hexosaminidase (β -hex) release (data are from one of three independent experiments, each of which gave similar results). (B) FSCMCs were stimulated with 10 $\mu\text{g/ml}$ BV and fluorescent signal in live cells due to heparin/avidin^{AF488} interaction was recorded over 20 minutes by flow cytometry. The avidin^{AF488} mean fluorescence intensity (MFI) kinetics are shown. The right panel represents a y-axis magnification of the data as indicated by the dashed lines in the left graph in order to allow identification of minor fluorescence differences (data are from one of three independent experiments, each of which gave similar results). (C) Phosphorylation of AKT, MAPK/ERK and PLC γ 1 and membrane-localized Lamp-1 was analyzed in FSCMCs without stimulation (0 min) and after exposure to 10 μg BV for 1, 5, 10 or 30 minutes by (phospho-) flow cytometry (representative of three independent experiments). (D) Heatmap representing gene expression data (mean of triplicates) from realtime PCR analysis of cytokines and chemokines in FSCMCs after 1 hour exposure to 10 $\mu\text{g/ml}$ BV (fold change compared to non-stimulated cells; data are from one of two independent experiments, each of which gave similar results). (A and B) Two-way ANOVA with Tukey's test for multiple comparisons; (A) * indicates comparisons to the respective unstimulated ctrl in the group; ° indicates comparisons with the same concentration of the BV serum group; P values are adjusted for multiple testing; graphs represent mean + SD; (D) * (or n.s. – not significant) indicates comparisons of the fold changes of PBS serum/isotype-treated vs. BV serum/isotype-treated groups by t test (neither BV serum/anti-IgE-treated nor BV serum/heat-treated groups were significantly different compared to the PBS serum/isotype-treated samples for any of the investigated genes); ns: not significant; */°P $\leq$ 0.05; **/°°P $\leq$ 0.01; ***/°°°P $\leq$ 0.001; numbers in (A) show the actual P value.

Figure 2: Serum sensitization increases FSCMC activation at the single cell level

(A to C) FSCMCs were incubated overnight with isotype control-pre-treated PBS serum or isotype control- or anti-IgE-pre-treated BV serum. Next day, cells were seeded in chamber slides and exposed to 10 $\mu\text{g/ml}$ BV. The avidin^{SR}-mediated signal of degranulating MCs was recorded over 60 minutes by realtime confocal fluorescence microscopy. (A) Snap shots of brightfield (outermost left) 1 minute and fluorescence signals (merge of To-Pro-3 [viability, in turquoise] and avidin^{SR} [in red]) at the indicated time points after BV addition are shown. The white squares in the 1 min brightfield picture indicate the individual cells presented in (B). (B) Brightfield and immunofluorescence pictures of individual representative cells from (A) (as indicated by the white squares) at 1, 15, 30, 45 and 60 minutes after BV exposure are shown. The scale bars in (A) and (B) represent 10 μm . (C) quantification of the mean avidin^{SR} signal kinetics of cells in

field of views shown in (A). (D-F) C57BL/6 mice were intradermally injected with either PBS serum or BV serum into both ears. Next day, one ear of each mouse was injected with either 200 ng BV or PBS. (D) Ear thickness measured directly before and at indicated timepoints after injection. (E and F) *Mcpt5-Cre; R26Y^{EYFP}* mice were pre-treated with immune sera as described for (D). The next day, the serum-treated ear of each mouse was intradermally injected with 200 ng BV containing 8 μ g avidin^{SR}. Thirty minutes after injection, the fluorescent avidin^{SR} signal within a circular area of 60 μ m surrounding single EYFP⁺ cells was determined by intravital 2-photon microscopy. (E) The large picture shows a representative intradermal area of the ear, with EYFP (green, dermal MCs) and avidin^{SR} signal (red, exteriorized MC cytoplasmic granules) indicated (scale bars represent 30 μ m). The smaller pictures on the right represent magnifications of individual MCs as indicated in the large picture by squares with dashed lines. HF indicates hair follicles in the elliptic areas with dashed lines. (F) Avidin^{SR} fluorescent signal from EYFP⁺ MCs in PBS- or BV-serum sensitized ears 30 minutes after BV injection shown as Mean Fluorescence Intensity (MFI, upper panel) and Mean Integrated Intensity (MII, lower panel). (A-C, E and F) data are from one of two independent experiments, each of which gave similar results). (D) results are pooled from two independent experiments with 9-20 mice per group; symbols represent mean $\pm$ SEM (C and D) Two-way ANOVA with Tukey's test for multiple comparisons; P values are adjusted for multiple testing; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Figure 3: IgE-dependent and BV-mediated signals together increase FSCMC activation

(A-D) FSCMCs were sensitized overnight with anti-DNP IgE. (A) FSCMCs were unstimulated or stimulated with different doses of antigen (DNP-HSA) in the absence or presence of 10 μ g/ml BV. Released β hexosaminidase (β -hex; % of total) was determined 1 hour after stimulation. (B and C) FSCMCs were either not stimulated or treated with either 10 μ g/ml BV or 5 ng/ml DNP-HSA or both simultaneously. (B) Realtime fluorescent signal due to binding of avidin^{AF488} of live cells over 20 minutes recorded by flow cytometry. The avidin^{AF488} mean fluorescence intensity (MFI) kinetics are shown. (C) Phosphorylation of AKT, MAPK/ERK and PLC γ 1 and membrane-localized Lamp-1 before (0 min) and after stimulation for 1, 5, 10 or 30 minutes using (phosphor-) flow cytometry. (D) Selected histogram overlays (upper panels) and respective mean fluorescence intensities (MFIs; lower panels) of phosphorylation data 1 minute after stimulation as shown in (C). (A-D) data are from one of at least two independent experiments, each of which gave similar results. (A) graphs represent mean $\pm$ SD; (A and B) Two-way ANOVA with Tukey's test for multiple comparisons; P values are adjusted for multiple testing; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; numbers in (A) show the actual P value.

Figure 4: IgE-mediated activation increases the detoxification potential of mouse FSCMCs

(A and B) BV (12.5 μ g at 500 μ g/ml final concentration, resulting in a PLA₂ concentration of approximately 60 μ g/ml) was either mixed with (A) indicated amounts of heparin or buffer or (B) supernatant of DNP-HSA-exposed non-sensitized or anti-DNP IgE-sensitized MCs for 10 minutes. After mixing with PLA₂ substrate and incubation, substrate cleavage (as indicator of PLA₂ activity) was measured. (C) BV (10 μ g) was incubated (or not) with supernatant (MC SN) of unstimulated (unstim.) or BV-exposed (10 μ g/ml; for 1 hour) BV serum-sensitized FSCMCs for 1 hour. In some cases, supernatant of activated FSCMCs was pre-incubated with protease inhibitor. After incubation of BV and supernatants, the mixtures were separated by PAGE, followed by Coomassie blue stain. (D-G) 3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green). BV was pre-treated (or not) with protamine (100 μ g)- or protease inhibitor (1x final concentration)-treated MC SN of PBS serum or BV serum-sensitized FSCMCs that were exposed to 10 μ g/ml BV for 1 hour. The pre-treated BV was then transferred onto the fibroblasts and monitoring of cell death, by confocal fluorescence microscopy imaging of TO-PRO-3 (turquoise)-positive nuclei over 60 minutes, was started immediately. (D) Representative pictures (outermost left: merge of brightfield, Fluo-4 and TO-PRO-3 fluorescence; remaining pictures show merges of Fluo-4 and TO-PRO-3 channels) of the field of views at indicated timepoints after addition of MC SN-treated BV are shown. Turquoise stains are nuclei of dead cells. The scale bars represent 10 μ m. (E-G) Quantification of toxicity (measured as TO-PRO-3 signal) kinetics from time lapse microscopy data shown in (D). (E) shows the development of TO-PRO-3 mean fluorescence intensity (MFI) over time. (F) Depicts the TO-PRO-3 MFI 60 minutes after BV addition in all cells in the field of view. (G) Illustrates the proportions of dead (with MFI ≥ 1) and live (with MFI < 1) cells (numbers indicate the respective identified and quantified nuclei) in the field of view. (A-G) data are from one of at least two independent experiments, each of which gave similar results. (A and B) T test for pairwise comparisons in (A) to the control group (no heparin) or (B) as indicated; graphs represent mean $\pm$ SD; (F) One-way ANOVA with Tukey's test for multiple comparisons (P values are adjusted for multiple testing); * (or n.s. – not significant) indicates comparisons to the respective untreated (or PBS serum) group (or as indicated); ° indicates comparisons with the BV serum group; P values are adjusted for multiple testing; */°P $\leq$ 0.05; **/°P $\leq$ 0.01; ***/°°P $\leq$ 0.001.

Figure 5: IgE-activated human PBCMCs efficiently degrade BV toxins

(A – B) Human peripheral blood-derived cultured MCs (hu PBCMCs) were stimulated with the indicated concentrations of BV and analyzed after 1 hour. (A) Viability assessed by flow cytometry as percentage of PI⁺ among all c-Kit⁺ Fc ϵ R1 α ⁺ cells. (B) β hexosaminidase (β -hex) released into the supernatant (% of total β -hex-mediated signal). (C and D) BV (10 μ g) was exposed to supernatant (MC SN) collected from either

unstimulated (unstim.), 10 µg/ml BV-stimulated (BV-stim) or IgE/anti-IgE stimulated (for 1 hour) hu PBCMCs and then processed by PAGE. (C) Coomassie blue-stained gel. (D) Western blot showing signals of IgG antibodies after incubation with BV serum. (E and F) Mass spectrometry analysis (of sample triplicates) of BV after 1 h exposure to supernatant of IgE/anti-IgE-stimulated hu PBCMCs (vs. untreated BV). In total, 118 BV proteins were identified. (E) Volcano plot or (F) heat map depicting the abundance of identified BV proteins. (E) BV proteins with significantly different abundance are highlighted (see panel F or Table S1 for full designations). Of the 39 proteins with lower abundance (FDR 0.1), the characterized compounds are indicated as white circles with blue border, the uncharacterized ones are indicated as white circles with grey border. Known allergens are annotated. (F) Heatmap of BV protein abundance of compounds with a statistically significantly different abundance (FDR 0.1). Raw and adjusted P values and fold changes are shown in Table S1. Raw abundances were $\log_2$ and z-score transformed prior to visualization. (A-D) data are from one of at least two independent experiments each of which gave similar results. (A and B) graphs represent mean + SD; One-way ANOVA with Dunnett's test for multiple comparisons (P values are adjusted for multiplicity analysis); * indicates comparisons with the untreated (0 µg/ml BV) group; P values are adjusted for multiple testing; ** $P \leq 0.01$; *** $P \leq 0.001$ (E and F) Mass spec analysis of sample triplicates was performed once.

Figure 6: Heparin and proteases released by IgE-activated human PBCMCs interfere with BV toxicity

(A) 3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green). Untreated BV (20 µg/ml final concentration) or BV pre-treated with supernatants of hu PBCMCs (stimulated for 1 hour by IgE/anti-IgE; hu MC SN) that were either untreated or pre-treated with protamine (100 µg) or protease inhibitor (1x final concentration) were transferred onto the fibroblasts, and monitoring by confocal fluorescence microscopy imaging over 60 minutes was started immediately. Representative pictures (outermost left: merge of brightfield, Fluo-4 and TO-PRO-3; remaining pictures show merges of Fluo-4 and TO-PRO-3 channels) of the field of views at different timepoints after venom addition are shown. Turquoise stains are (TO-PRO-3⁺) nuclei of dead cells. (B-D) Quantification of toxicity (measured as TO-PRO-3 signal) kinetics from time lapse microscopy data shown in (A). (B) shows the development of TO-PRO-3 mean fluorescence intensity (MFI) over time. (C) depicts the TO-PRO-3 signal 60 minutes after BV addition and show (upper panel) MFI all cells and (D) shows the portions of dead (with MFI ≥ 1) and live (with MFI < 1) cells (numbers indicate the respective identified and quantified nuclei per field of view). (A-D) data are representative of at least two independent experiments. (C) One-way ANOVA with Tukey's test for multiple comparisons (P values are adjusted for multiple testing); n.s. (not significant) indicates

comparisons to the respective untreated (or PBS serum) group (or as indicated); ° indicates comparisons with the BV serum group; P values are adjusted for multiple testing; *** $P \leq 0.001$.

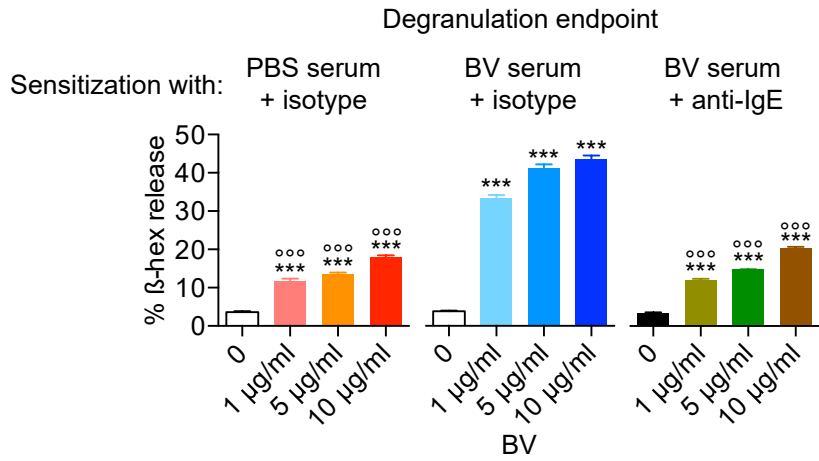
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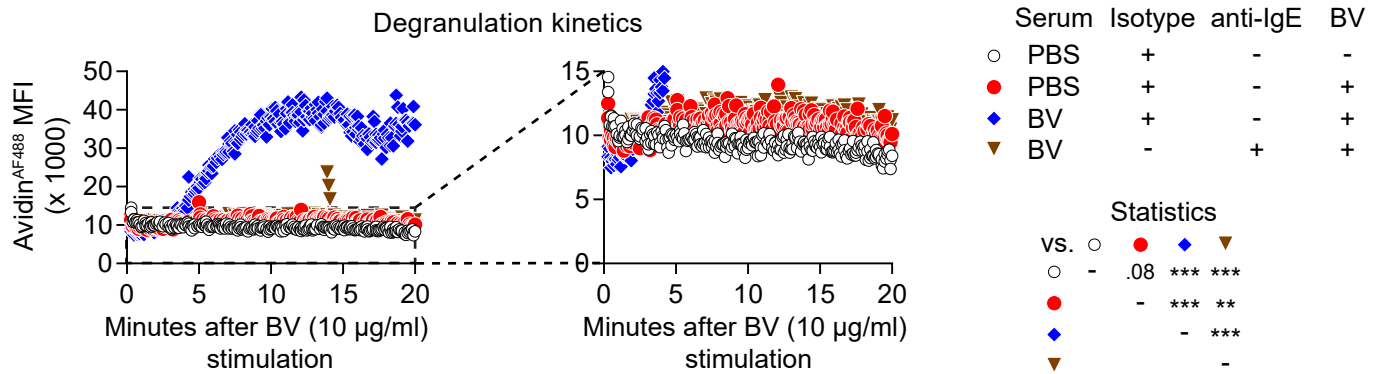
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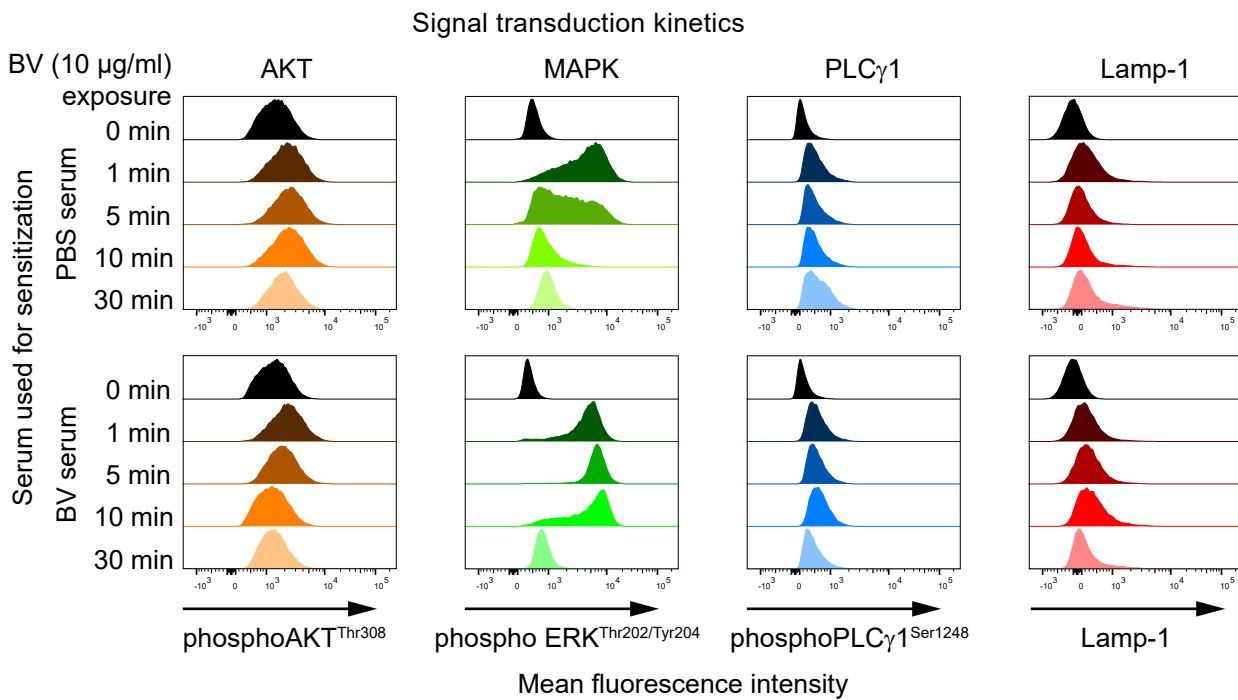
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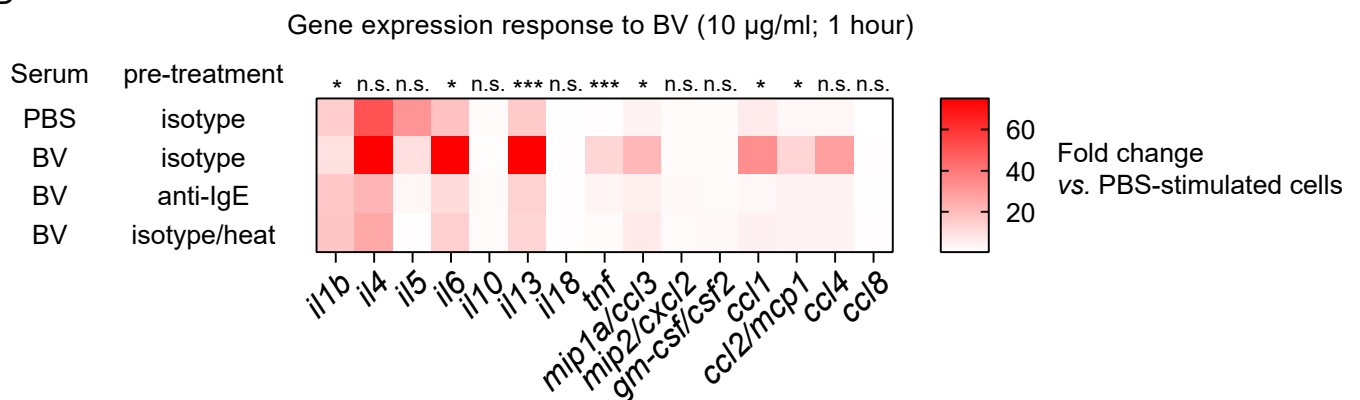
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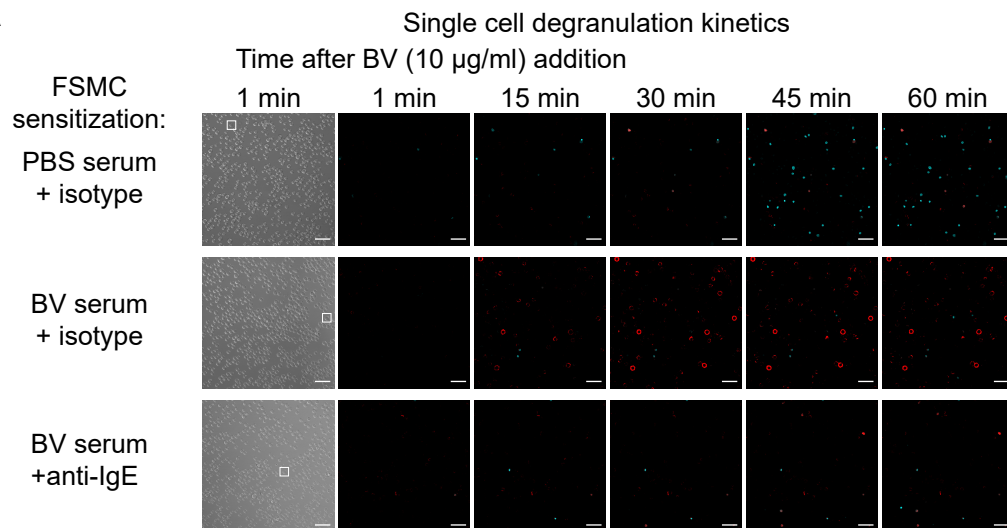
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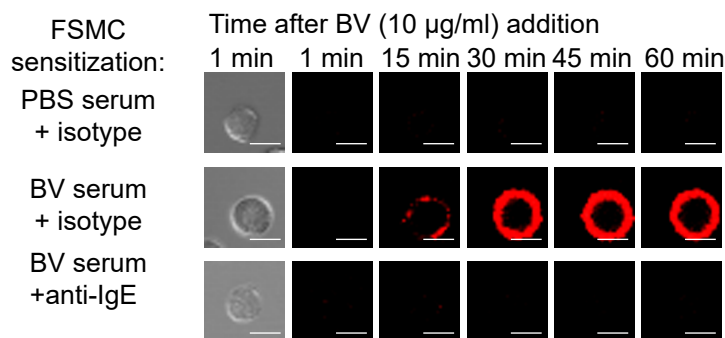
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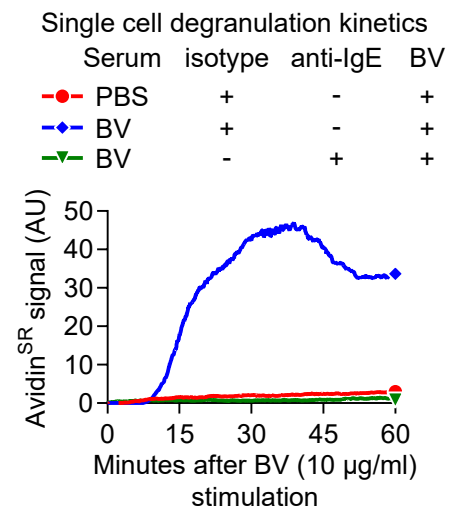
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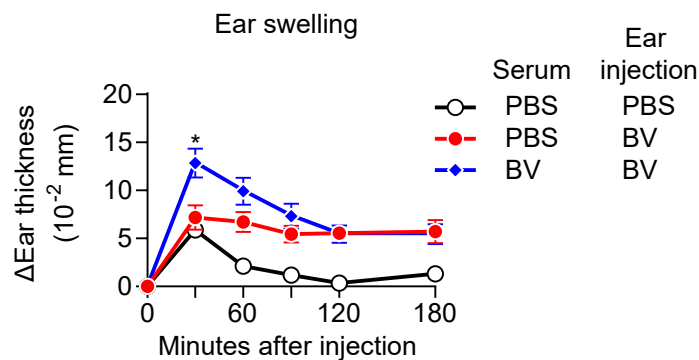
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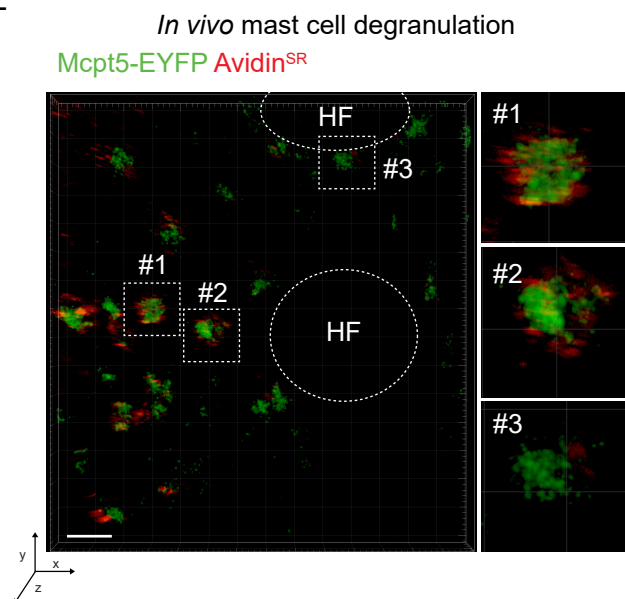
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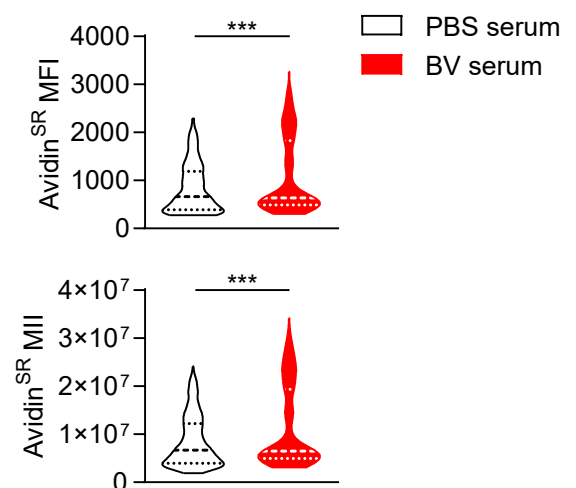
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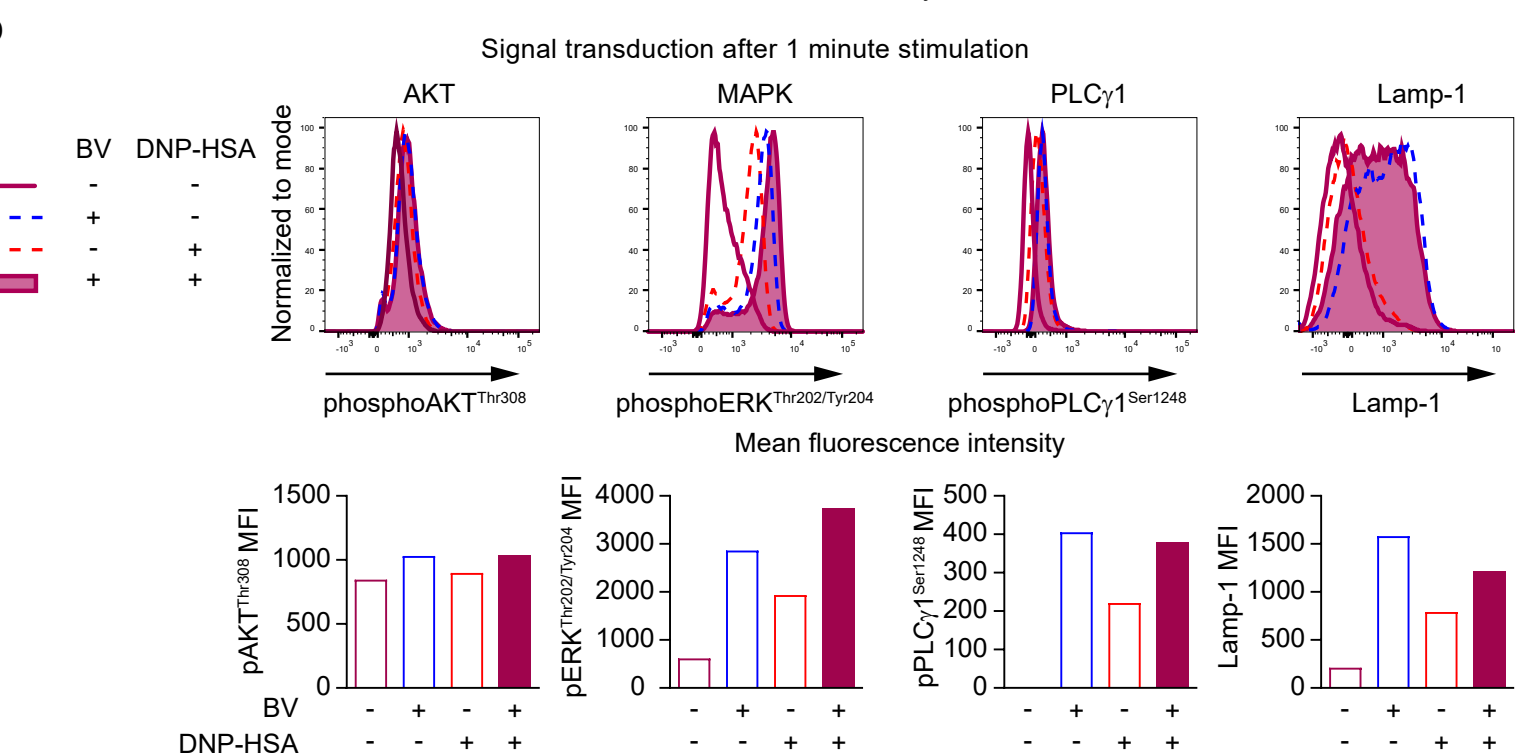
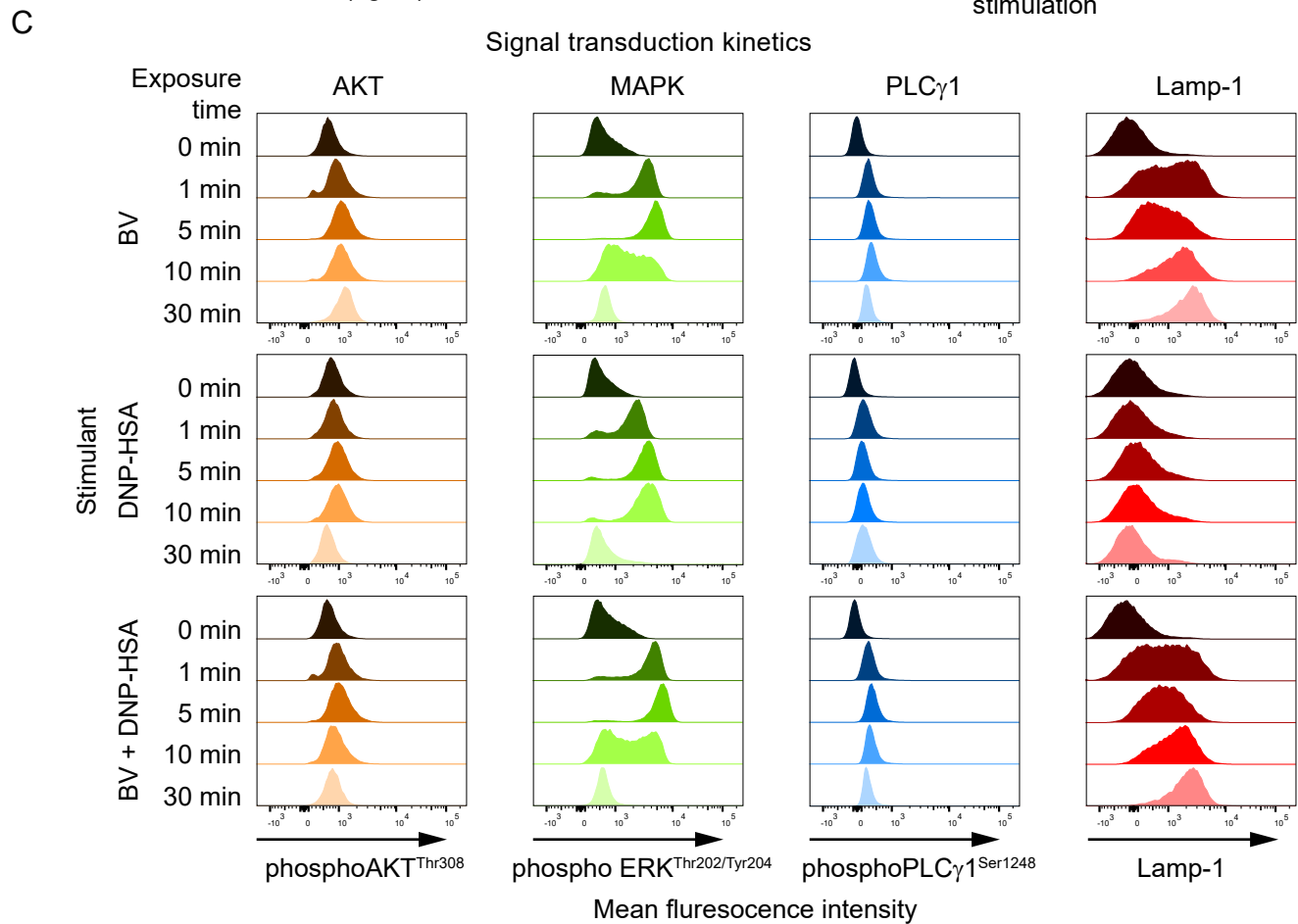
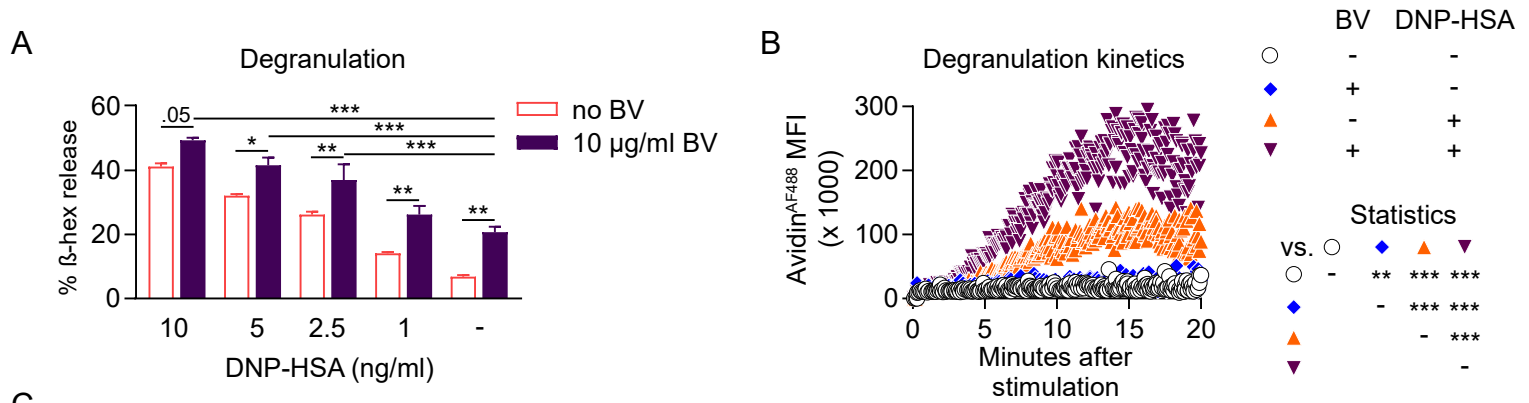


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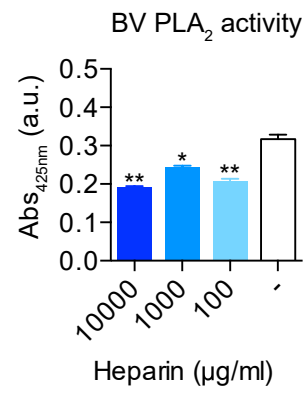


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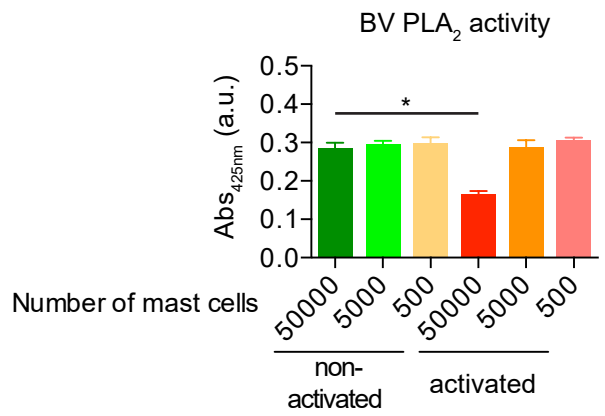




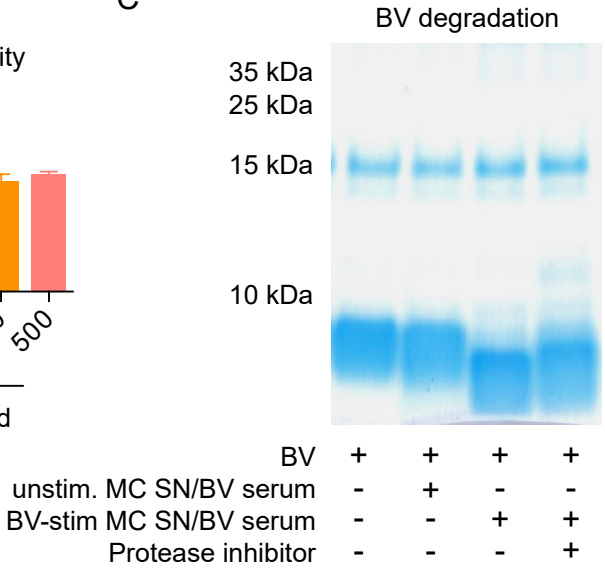
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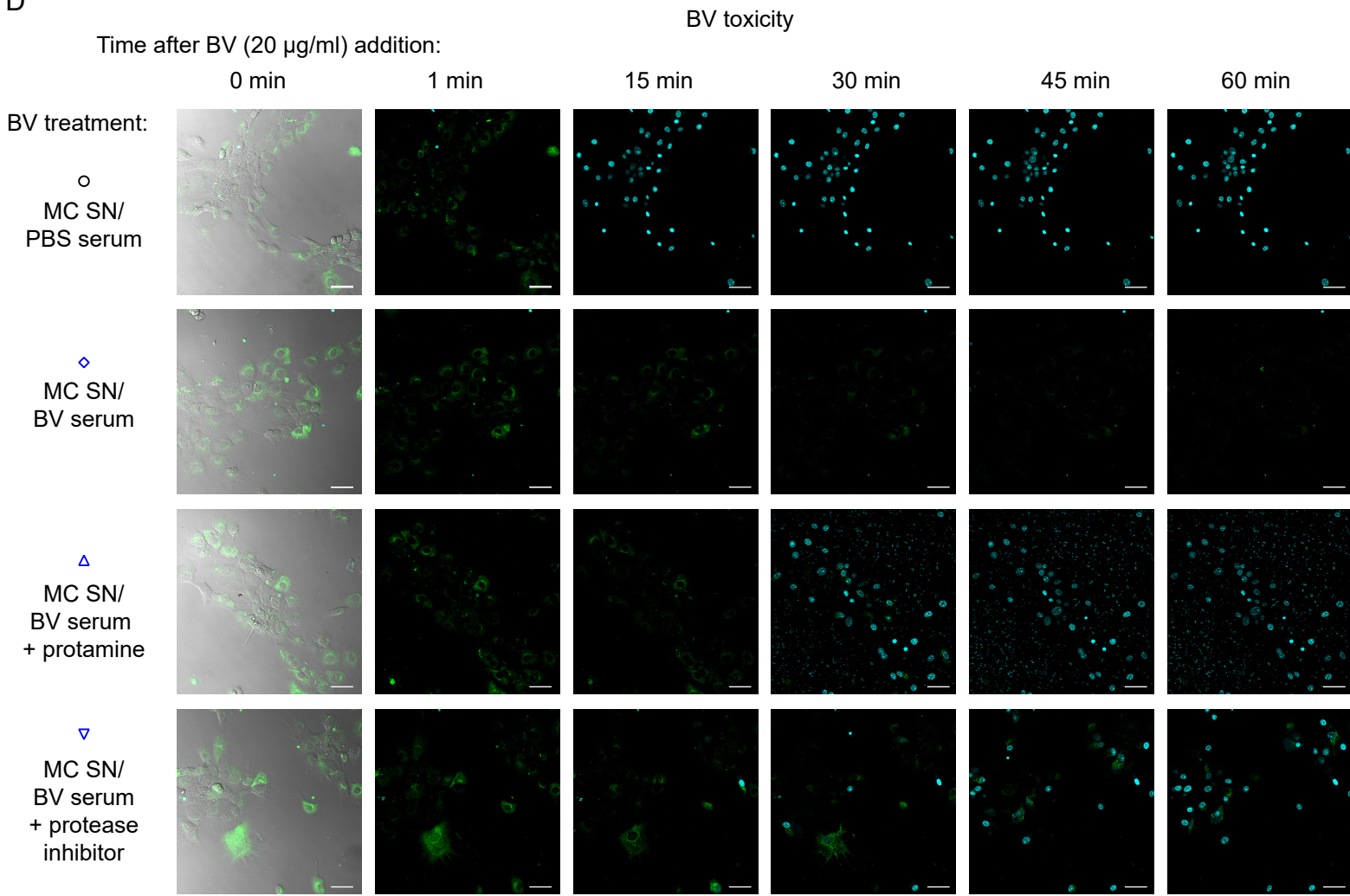
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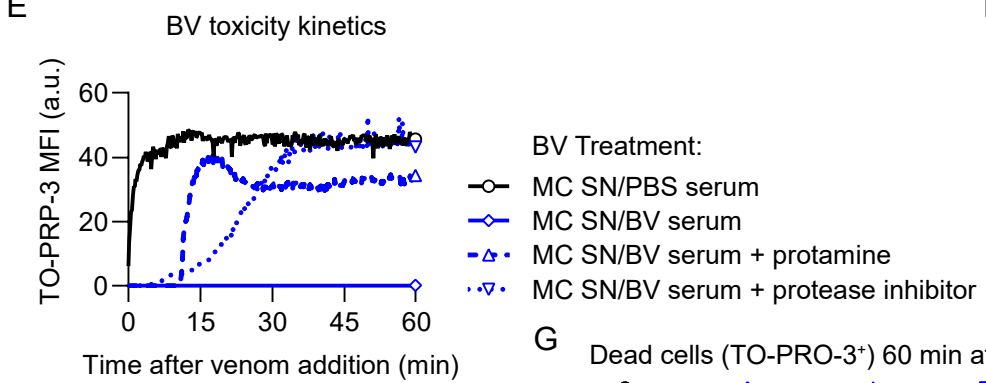
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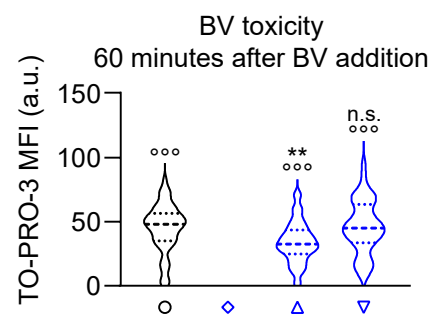
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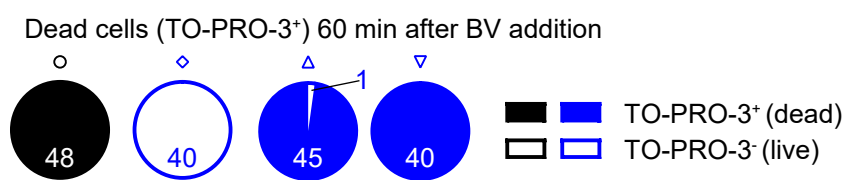
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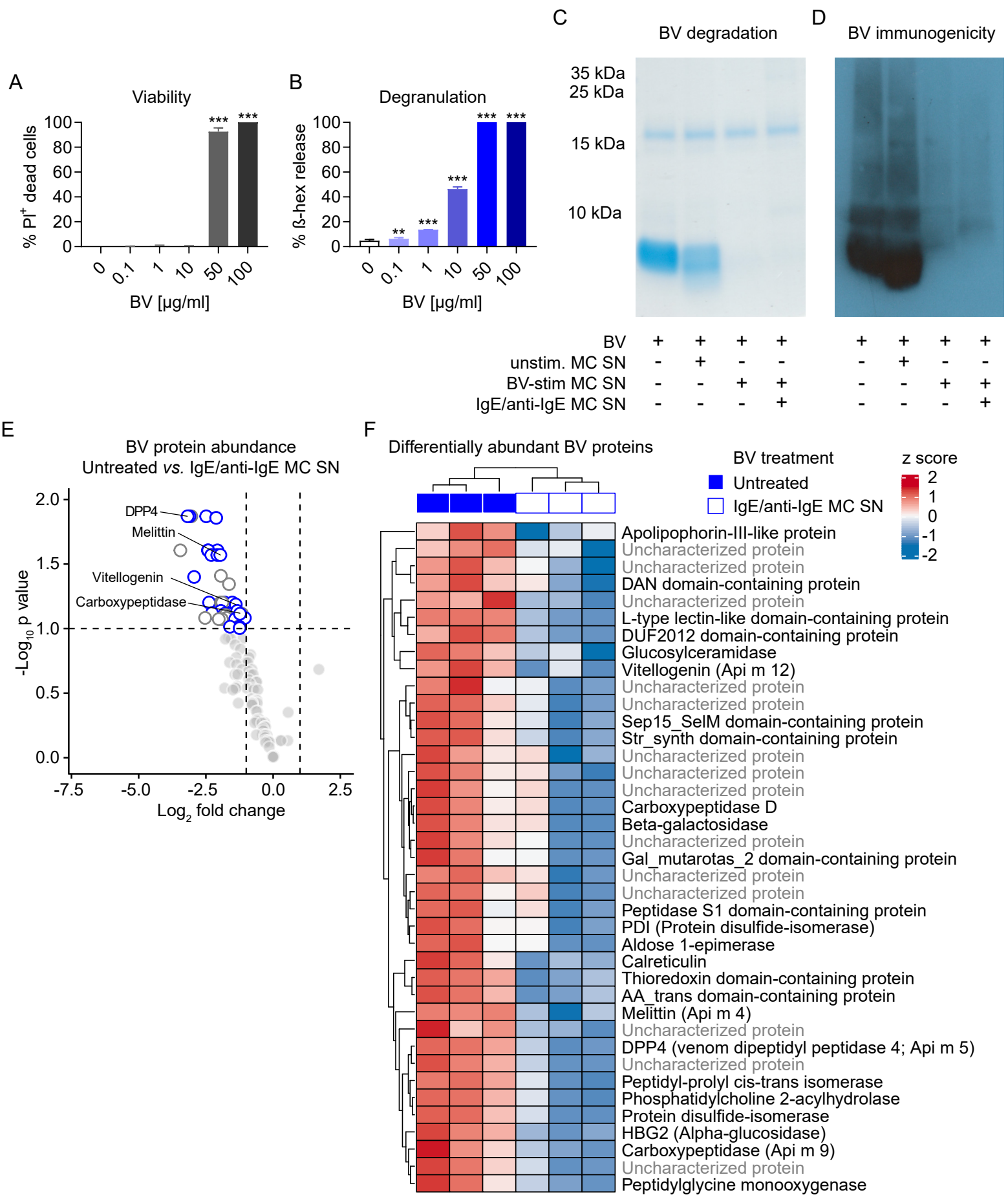


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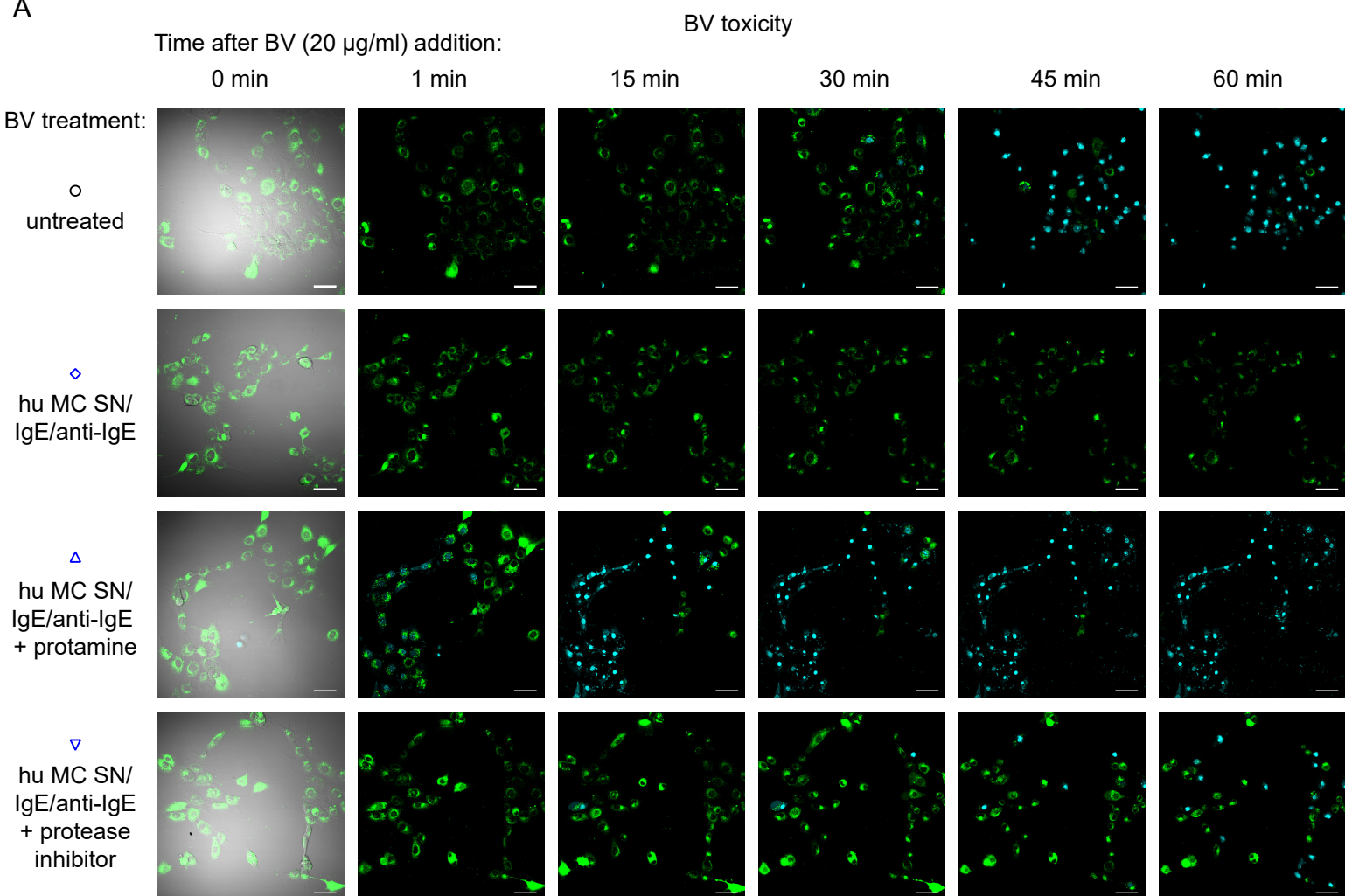


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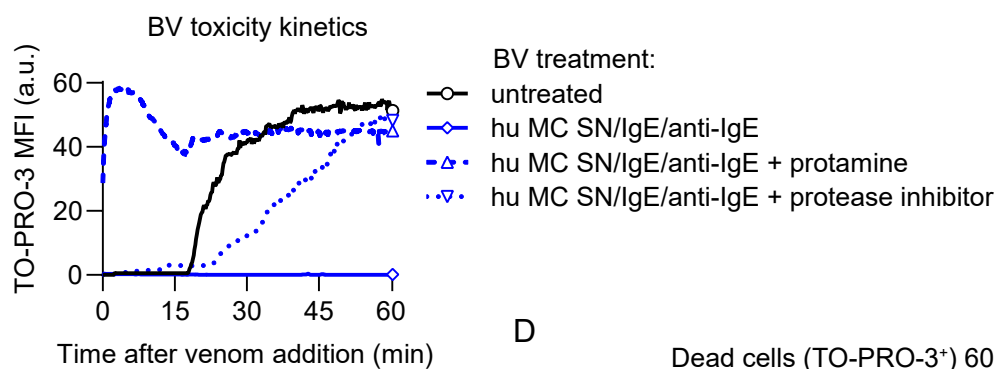




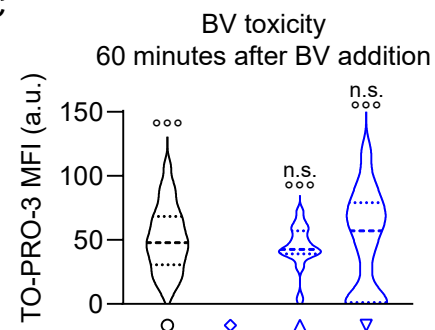
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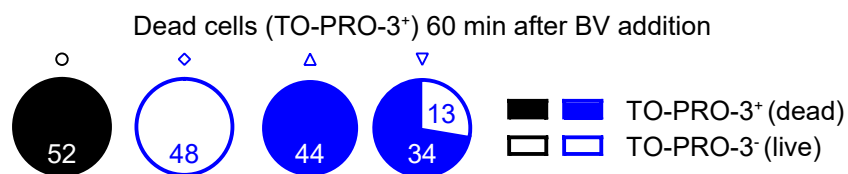
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Supporting Information

IgE antibodies increase honeybee venom responsiveness and detoxification efficiency of mast cells

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Supplementary Methods

Mice

Animal care and experiments were performed following current guidelines of the National Institutes of Health and with approval of the Stanford University Institutional Animal Care and Use Committee (IACUC protocol #12683). Female 6-8 week old mice were used for *in vivo* experiments. C57BL/6 wildtype (wt) mice were obtained from Jackson Laboratories and housed in the Stanford University animal facility for at least 7 days before starting experiments. *Mcpt5-Cre⁺ (Tg^{(Cma1-cre)ARoer})* mice¹ were generously provided by Axel Roers (University of Technology, Dresden, Germany). *R26Y EYFP* (B6.129X1-Gt(*ROSA*)26Sor^{tm1(EYFP)Cos/J}) mice² were originally obtained from Jackson Labs.

Reagents and antibodies

Honeybee venom (all of Lot 12071006HB) was obtained from ALK Abello Source Material. The freeze-dried complete BV was resuspended in sterile, endotoxin-free PBS (Gibco) at 4 mg/ml and stored at -20°C. Mouse anti-dinitrophenyl (DNP) IgE (clone e26) was kindly provided by Fu-Tong Liu³ (then at University of California-Davis). Purified bee venom phospholipase A₂ (PLA₂), purified bee venom melittin, Dinitrophenyl₃₀₋₄₀-conjugated human serum albumin (DNP-HSA), heparan sulfate (heparin), p-nitrophenyl-N-acetyl-β-D-glucosaminide (pNAG), supersensitive TMB substrate, dimethyl sulfoxide (DMSO), DNase I, avidin-sulforhodamine 101 (avidin^{SR}) and protamine sulfate (protamine) were obtained from Sigma. EZ-Link Sulfo-NHS-SS-Biotin, 3.5K molecular weight cutoff Slide-A-Lyzer Cassettes and 2K molecular weight cutoff Zeba Spin Desalting Columns were obtained from Thermo Fisher Scientific. AlexaFluor⁴⁸⁸-labelled avidin (avidin^{AF488}) was obtained from Invitrogen. Protease activity was inhibited using the Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). PLA₂ activity was assessed using 4-nitro-3-(octanoyloxy)-benzoic acid (4N3OBA; Enzo Life Sciences). Rat anti-mouse IgE (clone R35-92; for blocking of IgE binding to FcεRIα) and the respective rat IgG1 isotype control (clone R3-34) were obtained from BD Pharmingen. Human myeloma IgE was from Calbiochem, polyclonal rabbit anti-human IgE were obtained from Bethyl Laboratories. Anti-human FcεRIα (Clone AER-37/CRA1) was obtained from eBioscience, anti-human CD117 (c-Kit; clone 104D2) and anti-human tryptase (clone AA1) were obtained from BioLegend. Anti-mouse FcεRIα (clone Mar-1) and anti-mouse CD117 (c-Kit; clone 2B8) were obtained from BioLegend. Anti-Phospho-Akt (Thr308; clone D25E6), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204; clone 197G2), anti-Phospho-PLCγ1 (Ser1248; clone D25A9) (all from Cell Signaling Technology) were used for phospho-flow cytometry analysis. Serum ELISA reagents rat anti-mouse IgE (clone R35-72), biotinylated rat anti-mouse IgE (clone R35-118), purified mouse IgE (clone C38-2), biotinylated rat anti-mouse IgG1 (clone A85-1), biotinylated rat anti-mouse IgG2b (clone R12-3) and horseradish peroxidase-conjugated streptavidin were all from BD Pharmingen, polyclonal goat anti-mouse IgG2c was obtained from Southern Biotech. Goat anti-mouse

IgG (H+L) Cross-adsorbed horse radish peroxidase-labelled HRP antibody was from Thermo Fisher Scientific.

Serum generation, characterization and processing

For generation of mouse sera, C57BL/6 wt mice were shaved on the back skin and received two subcutaneous injections of 50 μ l (containing 200 μ g) BV or PBS in the shaved area. Three weeks later, mice were sacrificed and blood was collected by cardiac puncture, followed by centrifugation in Serum-Gel Microtubes (Sarstedt), pooling of the sera, and freezing and storage at -20°C.

Mouse sera (either of aliquots from individual animals collected before pooling or from serum pools) were analyzed by ELISA as previously described^{4,5}. Briefly, MaxiSorp ELISA plates (Nunc) were coated with 50 μ l BV (5 μ g/ml in PBS) or anti-mouse IgE (2 μ g/ml in PBS) per well overnight at 4°C. The next day, the plate was washed 3-5 times using PBS containing 0.05% tween (as was done after each subsequent incubation step) and unspecific binding sites were blocked by incubation with 200 μ l of 1% bovine serum albumin (BSA) in PBS for at least 2 hours at room temperature. Fifty μ l serial-diluted individual sera (in PBS containing 1% BSA) and reference immune serum (pool) or IgE isotype (as standard) were added to the blocked wells and incubated 2 h at 37°C. Next, 50 μ l biotinylated detection antibodies specific for mouse IgG1, IgG2b, IgG2c or IgE (all diluted 1:1000 in PBS containing 1% BSA) were added for 1 hour at room temperature, followed by 30 minutes incubation with 50 μ l horseradish peroxidase-conjugated streptavidin (diluted 1:2000 in PBS containing 1% BSA) and detection using 50 μ l supersensitive TMB substrate (and reaction stop by addition of 50 μ l 2 N H₂SO₄). Absorbance at 450 nm (with 620 nm as reference) was detected using a Tecan Sunrise plate reader. IgG1, IgG2b and IgG2c titers were determined by plotting the serum dilution that gave half-maximal signal of the reference serum. Absolute IgE levels were calculated based on the IgE isotype standard curve.

We detected BV PLA₂-specific IgE by ELISA as previously described with minor modifications⁵. Briefly, purified BV PLA₂ (4 mg/ml PBS) was mixed 1:1 with EZ-Link Sulfo-NHS-SS-Biotin (2 mg/ml in DMSO) and incubated for 2 hours at room temperature. After quenching with 1 volume 0.1 M TRIS (pH 7.0) for 10 minutes, the solution was dialyzed twice overnight against PBS at 4°C using Slide-A-Lyzer Dialysis Cassettes, passed over Zeba Spin Desalting Columns (according to the manufacturer's instructions) and eluted again in PBS. The ELISA to detect BV PLA₂-specific IgE was performed as described above for total IgE, replacing the biotinylated anti-IgE antibody with 1:2000-diluted (identified as suitable dilution with lowest signal-to-noise ratio in pilot experiments using mouse immune sera) biotinylated BV PLA₂ and extending the incubation time in this step to 3 hours at 37°C. The mean of all PBS serum pool signals (Absorbance_{450-620nm}) was subtracted as background from all samples.

For neutralization of IgE activity in selected experiments, BV serum was treated for 30 minutes with either 10 µg/ml anti-mouse IgE or with isotype control. PBS serum was treated with isotype control antibody in the respective experiments⁴. Alternatively, IgE was inactivated by heating at 56°C in a water bath for 45 minutes⁴.

Cellular models

Fetal skin-derived cultured mast cells (FSCMCs)

FSCMCs were generated following a published protocol with few modifications⁶. Pregnant C57BL/6 wt mice were euthanized on day 17 or 18 of pregnancy. Fetuses were harvested and rinsed 2 times in sterile endotoxin-free PBS. Whole fetuses were then incubated in 0.05% Trypsin EDTA with phenol red (Gibco, Thermo Fisher Scientific) supplemented with 100 u/ml DNase I for 30 minutes at 37°C in 12 well plates (1 fetus/well). The trypsin suspensions were then pooled and filtered over a 70 µm cell strainer (BD Biosciences) and RPMI 10% FBS was added to stop digestion. After centrifugation for 5 min at 300 g, cells were resuspended in complete culture medium (RPMI 10% fetal bovine serum [FBS], 10 mM HEPES, 1x penicillin/streptomycin, 1x nonessential amino acids, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol; all from Gibco apart from FBS which was from Sigma) supplemented with 10 ng/ml IL-3 and 10 ng/ml SCF (both from Peprotech). Cells from 3 fetuses were seeded in 100 ml medium in 175 cm² tissue culture flasks and maintained for 4 weeks without medium change. Then, cells were harvested and seeded in 50 ml fresh medium (including cytokines) for 2-4 additional weeks before assessment of maturation by flow cytometry (more than 95% FcεRIα/c-Kit double positive cells). Cells were used between 6 – 12 weeks after start of the culture.

Human peripheral blood mononuclear cell-derived mast cells (PBCMCs)

PBCMCs were generated as previously described⁷. Briefly, CD34⁺ precursor cells were isolated from peripheral blood mononuclear cells derived from buffy coats of healthy donors (Stanford Blood Center) using the EasySep Human CD34 Positive Selection Kit (Stemcell Technologies). After culture for one week in StemSpan medium (Stemcell Technologies) supplemented with human SCF (50 ng/ml), IL-3 (10 ng/ml) and IL-6 (50 ng/ml; all cytokines were from Peprotech), the cells were maintained in IMDM Glutamax I, supplemented with 1 mM sodium pyruvate, 50 mM β-mercaptoethanol, 0.5% bovine albumin fraction V, insulin-transferrin selenium (all from Invitrogen/Thermo Fisher Scientific), ciprofloxacin (10 µg/ml; Sigma), IL-6 (50 ng/ml) and SCF (50 ng/ml). Cell maturity was tested by flow cytometry staining for tryptase, c-Kit and FcεRIα expression. PBCMCs were typically used for experiments after approximately 10 weeks of culture.

Gene expression analysis

FSCMCs were seeded at 5×10^5 cells/ml and incubated 1:20 with PBS serum or BV serum (either pre-treated with anti-IgE or isotype control antibody or with heating to 56°C as described above). The next day, the cells were washed and stimulated with $10\ \mu\text{g/ml}$ BV (or the respective amount of PBS) in RPMI 2% BSA for 1 hour in 96 well round bottom plates. Cells were centrifuged 5 minutes at 300 g and pellets were resuspended in Trizol (Invitrogen), followed by RNA isolation. One μg of RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was conducted on a StepOne Plus device (Applied Biosystems) using Power SYBR reagents (Applied Biosystems). Target gene Ct levels were normalized to the housekeeping gene *eef1b2* and relative gene expression compared to non-stimulated samples was calculated using the $2^{-\Delta\Delta\text{Ct}}$ algorithm. Primer sequences:

eef1b2: fwd: AGAGCTACATTGAGGGGTACGT; rev: GACTTGATGTGATTATACCAACGTAG;

il1b: fwd: GCTTCCTTGCAAGTGTCTGAA rev: GAACAGGTCATTCTCATCACTGTCA;

il4: fwd: GTCCTCACAGCAACGAAGAACACCA; rev: CTCATTCATGGTGCAGCTTATCGA;

il5: fwd: TGTTGACAAGCAATGAGACGATGA; rev: GGACAGTTTGATTCTTCAGTATGTC;

il6: fwd: GAAGTTCCTCTCTGCAAGAGAC; rev: GTATCCTCTGTGAAGTCTCCTCT;

il10: fwd: CTTATCGGAAATGATCCAGTTTTACC; rev: ATCACTCTTCACCTGCTCCACTG;

il13: fwd: TATTGAGGAGCTGAGCAACATCAC; rev: TCTGGGTCCTGTAGATGGCA;

il18: fwd: ACACGCTTTACTTTATACCTGAAG; rev: TACTGCGGTTGTACAGTGAAGTC;

tnf: fwd: CTCCCTCTCATCAGTTCTATGG; rev: CTCCACTTGGTGGTTTGCTAC;

mip1a/ccl3: fwd: CTCTGTACCATGACACTCTGCA; rev: CTCTTAGTCAGGAAAATGACACCTG;

mip2/cxcl2: fwd: GTGAACTGCGCTGTCAATGCCTGA; rev: CTTGAGAGTGGCTATGACTTCTGTCT;

gm-csf/csf2: fwd: GATGACATGCCTGTCACGTTGAAT; rev: GTCTGCACACATGTTAGCTTCTTGA;

ccl1: fwd: TCTCCAATAGCTGCTGCTTGAACA; rev: ACCTTTGTTTCAGCCTGAATACCACA;

ccl2/mcp1: fwd: GTGTTGGCTCAGCCAGATGC; rev: ACCTCTCTCTTGAGCTTGGTGACA;

ccl4: fwd: GTTCTCAGCACCAATGGGCTCTGA; rev: AGCAAAGACTGCTGGTCTCATAGTA;

ccl8: fwd: GAAGCTGTGGTTTTCCAGACCA; rev: CTGGTCAAGGATCTCCATGTAC;

Signal transduction analysis by phospho-flow cytometry

FSCMCs were seeded at 5×10^5 /ml in RPMI medium supplemented with 10% FBS and 1x penicillin/streptomycin (without cytokines) and incubated overnight with 1:20 PBS serum or BV serum processed as described above. The next day, cells were washed with RPMI medium without additives (plain RPMI), resuspended at 5×10^6 /ml of pre-warmed plain RPMI and aliquoted at 100 μ l into FACS tubes and incubated at 37°C for at least 5 minutes. Cells were then stimulated by quick addition of 100 μ l pre-warmed plain RPMI or RPMI containing 20 μ g BV/ml and immediately put back to 37°C. At indicated timepoints, 1 ml of 4% paraformaldehyde was added and mixed by pipetting to fix the cells for 15 minutes at room temperature. Fixed cells were then washed with 2 ml ice-cold PBS 1% BSA (FACS buffer) and resuspended in 300 μ l ice-cold (by storage at -20°C) methanol and incubated 30 minutes on ice. Next, cells were washed with 2 ml ice-cold FACS buffer and resuspended in 100 μ l FACS buffer containing 1:100 phospho-flow and surface stain (anti-c-Kit and anti-Fc ϵ RI α) antibodies. After incubation for 2 hours on ice, cells were washed 2x with FACS buffer and analyzed by flow cytometry using an LSR-II flow cytometer (BD Biosciences) at the Stanford University Flow Cytometry Core Facility.

In experiments dissecting the contribution of innate BV stimulation on IgE/antigen (DNP-HSA) mediated signaling, FSCMCs were processed as described above, replacing serum with 1 μ g/ml anti-DNP IgE and stimulated with either 5 ng/ml DNP-HSA, 5 μ g/ml BV, or simultaneously stimulated with both, DNP-HSA and BV.

Degranulation assays

For analysis of MC degranulation, 5×10^5 FSCMCs/ml were sensitized 1:20 with PBS serum or BV serum (sera processed as described above) overnight. The next day, the cells were centrifuged for 5 minutes at 300 g, washed with Tyrode's buffer (100 mM HEPES, 1.8 mM CaCl₂, 5 mM KCl, 130 mM NaCl, 2 mM MgCl₂, 5.5 mM glucose and 1 g/L BSA; pH 7.4), resuspended at 2×10^6 cells/ml in Tyrode's buffer and aliquoted at 20 μ l in 96 well v-bottom plates. Next, 20 μ l of 2x stimulants in Tyrode's buffer were added and cells were incubated for 1 hour at 37°C. The chromogenic substrate pNAG was used to assess the release of β -hexosaminidase (a granule-stored enzyme) into the supernatant as percentage of the combined signal of cell lysate and supernatant, as previously described⁸.

In experiments dissecting the influence of innate BV stimulation on IgE/antigen (DNP-HSA)-mediated degranulation, FSCMCs were processed as described above, replacing serum with 1 μ g/ml anti-DNP IgE and stimulated with either DNP-HSA or BV, or simultaneously stimulated with both DNP-HSA and BV, at the indicated concentrations.

In experiments assessing the degranulation of human MCs, 2×10^4 hu PBCMCs were stimulated with different venom dosages as indicated for 1 hour at 37°C. Degranulation was assessed as described

above for FSCMCs. For assessment of tryptase activity, cell supernatants and lysates were prepared as described above and 20 μ l of each were incubated with 20 μ l of the chromogenic substrate S-2288⁹ (3 mM final concentration; Chromogenix) for 1 h at 37°C, followed by detection at 405 nm using a Tecan Sunrise plate reader.

MC viability upon BV exposure

FSCMCs and human PBCMCs (10^5 cells/sample) were incubated in Tyrode's buffer with BV at the indicated concentrations for 1 hour at 37°C. Subsequently, cells were centrifuged 5 minutes at 300 g and washed with FACS buffer (see above) and stained for 20 minutes on ice with antibodies for Fc ϵ RI α and c-Kit diluted 1:100 in FACS buffer. After additional washing, cells were resuspended in FACS buffer containing 1:500 propidium iodide (Invitrogen) and analyzed by flow cytometry.

Degradation of BV

For BV digest experiments with FSCMC supernatants, cells were sensitized overnight with PBS or BV serum. The next day, cells were washed and resuspended in Tyrode's buffer at 8×10^6 cells/ml. Cells were then incubated in 1.5 ml microcentrifuge tubes by addition of an equal volume of 20 μ g/ml BV in Tyrode's buffer for 1 hour at 37°C. For digest experiments with hu PBCMC supernatants, cells were sensitized at 5×10^5 cells/ml in culture medium overnight with 2 μ g/ml human myeloma IgE. The next day, hu PBCMCs were washed and resuspended in RPMI without phenol red (and additives; RPMI w/o PR; Gibco) at 10^7 cells/ml and stimulated with an equal volume of 20 μ g/ml BV or 1 μ g/ml anti-human IgE for 1 hour at 37°C. After stimulation, cells were resuspended by pipetting and centrifuged 5 minutes at 300 g. Supernatants were collected and stored at -80°C.

For assessment of digestion capacity, supernatants were thawed and 10 μ l BV (1 mg/ml) was incubated with 20 μ l of MC supernatant in the presence or absence of protease inhibitor (40 μ l total reaction volume) for 1 hour at 37°C. Poly-acrylamide gel electrophoresis (PAGE) of samples was performed under reducing conditions using the Biorad Criterion System and 18 % Tris-HCl Precast (Fig. 4C and 5C) or 15 % Tris-HCL self-made (Fig. S3A) Gels. Gels were stained using the PageBlue Protein Staining Solution (Thermo Fisher Scientific).

In experiments titrating the number of hu PBCMCs required for efficient BV digestion, supernatants of IgE/anti-IgE-activated MCs were diluted to contain the mediators secreted by 10^4 , 5×10^4 or 10^5 cells in 20 μ l.

For analysis of immunorecognition of BV after treatment with SN of hu PBCMCs, PAGE-separated untreated and treated BV (see above) was transferred onto a PVDF membrane (BioRad) using the Trans-Blot SD Semi-Dry Electrophoretic transfer cell (BioRad; 40 V, 2 hours). After at least 2 hours blocking (all incubation and washing steps were performed under gentle shaking) in 5% BSA in Tris-

buffered saline (TBS) at room temperature, the membrane was incubated over night at 4°C with 1:5 BV serum in TBS containing 1% BSA. After washing 3x 10 minutes with TBS containing 0.1 % Tween (Sigma; TBST), the membrane was incubated 1 hour at room temperature with goat anti-mouse IgG (H+L) Cross-adsorbed horse radish peroxidase-labelled HRP antibody (Thermo Fisher Scientific) diluted 1:10000 in TBST containing 1% BSA. After 6x 20 minutes washing in TBST, bound antibody was detected using the SuperSignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific).

Mass spectrometry

For mass spectrometry analysis, triplicates of 20 µg BV (5 µl at 4 mg/ml) were mixed with 10 µl of SN of hu PBCMCs stimulated by IgE/anti-IgE for 1 hour prepared as described above in section “BV digest analysis”. After 60 min incubation at 37°C (with controls containing only BV or hu PBCMC supernatant, respectively, processed in parallel), samples were frozen in liquid nitrogen and stored at -80°C until analysis. For mass spectrometry analysis, samples were processed using adapted Single-Pot solid-phase-enhanced sample preparation (SP3) methodology¹⁰. Briefly, equal volumes (125 µl containing 6250 µg) of two different kind of paramagnetic carboxylate modified particles (SpeedBeads 45152105050250 and 65152105050250; GE Healthcare) were mixed, washed three times with 250 µl water and reconstituted to a final concentration of 50 µg/µl with LC-MS grade water (LiChrosolv; MERCK KgaA). Samples (15 µL) were filled up to 50 µL with LC-MS grade water and mixed with 2x sample buffer (4% SDS, 100mM HEPES, pH 8.0) to a final concentration of 2% SDS, proteins were reduced with a final concentration of 10 mM DTT and incubated at 56°C for 1 hour. After cooling down to room temperature, reduced cysteines were alkylated with iodoacetamide at a final concentration of 55 mM for 30 min in the dark. For tryptic digestion, 400 µg of mixed beads were added to reduced and alkylated samples, vortexed gently and incubated for 5 minutes at room temperature. The formed particles-protein complexes were precipitated by addition of acetonitrile to a final concentration of 70% [V/V], mixed briefly before incubating for 18 minutes at room temperature. Particles were then immobilized using a magnetic rack (DynaMag-2 Magnet; Thermo Fisher Scientific) and supernatant was discarded. SDS was removed by washing two times with 200 µl 70% ethanol and one time with 180 µl 100% acetonitrile. After removal of organic solvent, particles were resuspended in 100 µl of 50 mM NH₄HCO₃ and samples digested by incubating with 1 µg of Trypsin overnight at 37°C. Samples were acidified to a final concentration of 1% Trifluoroacetic acid (Uvasol; MERCK KgaA) prior to immobilizing the beads on the magnetic rack. Forty percent of each recovered digest was then desalted and concentrated using stage tips with two stacked C18 plugs (Empore; MERCK KgaA)¹¹. Stage tips were activated with three times 100 µl acetonitrile and equilibrated with three times 100 µl of 0.4% formic acid, 2% TFA in water before loading the samples. Salts were cleaned up with 100 µl of 0.1% TFA and peptides were eluted using two times 50 µl 90% acetonitrile, 0.4% formic acid. Finally, eluates were dried in a vacuum concentrator and reconstituted in 10 µl of 0.1% TFA.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) via nanoflex ion source interface. Tryptic peptides were loaded onto a trap column (Acclaim PepMap 100 C18, 3 μ m, 5 $\times$ 0.3 mm; Thermo Fisher Scientific) at a flow rate of 10 μ l/min using 0.1% TFA as loading buffer. After loading, the trap column was switched in-line with a 50 cm, 75 μ m inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 μ m; Dr. Maisch). Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nl/min and a 90 min gradient applied (4 to 24% solvent B within 82 min, 24 to 36% solvent B within 8 min and, 36 to 100% solvent B within 1 min, 100% solvent B for 6 min before re-equilibrating at 4% solvent B for 18 min).

For the MS/MS experiment, the Q Exactive MS was operated in a top 10 data-dependent acquisition mode with a MS1 scan range of 375 to 1,650 m/z at a resolution of 70,000 (at 200 m/z). Automatic gain control (AGC) was set to a target of 10^6 and a maximum injection time of 55 ms. MS²-scans were acquired at a resolution of 15,000 (at 200 m/z) with AGC settings of 10^5 and a maximum injection time of 110 ms. Precursor isolation width was set to 1.6 Da and the HCD normalized collision energy to 28%. The threshold for selecting precursor ions for MS2 was set to ~2,000 counts. Dynamic exclusion for selected ions was 90 sec. A single lock mass at m/z 445.120024 was employed¹², XCalibur version 4.3.73.11 and Tune 2.11.3006 were used to operate the instrument.

Acquired raw data files were processed using the Proteome Discoverer 2.4.1.15 platform, utilizing the database search engine Sequest HT. Percolator V3.0 was used for validation and results filtered with a false discovery rate (FDR) of 1% on PSM, peptide and protein level under strict conditions. Searches for bee venom samples were performed with full tryptic digestion against the *Apis mellifera* UniProtKB data base v2020.06 (17,151 sequences and appended known contaminants) and *Apis mellifera* SwissProt database v2020.06 (73 sequences) with up to two miscleavage sites. Hu PBCMC supernatant samples were searched against the human SwissProt database v2020.06 (20,395 sequences and appended known contaminants) or in combination with *Apis mellifera* databases for bee venom treated with hu PBCMC supernatant with up to two miscleavage sites. Oxidation (+15.9949 Da) of methionine and acetylation of protein N-terminus (+42.0110 Da) were set as variable modifications, whilst carbamidomethylation (+57.0214 Da) of cysteine residues was set as a fixed modification. Data was searched with mass tolerances of ± 10 ppm and 0.025 Da on the precursor and fragment ions, respectively. Results were filtered to include peptide spectrum matches (PSMs) with Sequest HT cross-correlation factor (Xcorr) scores of ≥ 1 , proteins with more than 1 unique peptide, and quant values detected in at least all samples. For calculation of protein intensities Minora Feature Detector node

and Precursor Ions Quantifier node, both integrated in Thermo Proteome Discoverer, were used. Automated chromatographic alignment and feature linking mapping were enabled with the total peptide amount used for normalization between individual runs.

The mass spectrometry data (of BV, hu PBCMC supernatant, and the combination of both) have been deposited to the ProteomXchange Consortium via the PRIDE partner repository¹³ with the dataset identifier PXD023851 and 10.6019/PXD023851.

Exported data was then analyzed using R version 4.0.3. Proteins not found in any of the *Apis mellifera* databases were excluded. Additionally, one of the detected peptide sequences matched to *Apis mellifera* superoxide dismutase also matched to *Homo sapiens* superoxide dismutase. Since this protein was detected with higher abundance in MC SN-treated BV, we assume that this represents a false positive match (secreted by activated hu PBCMCs) and excluded it from further analysis. The remaining abundances were log₂-transformed and differential abundance of proteins was analyzed using the *limma* package¹⁴. Features with an absolute log₂ fold change > 1 and FDR < 0.1 were considered differentially abundant.

PLA₂ activity assay

For assessment of PLA₂ activity, 5x 10⁵ FSCMCs were sensitized with 1 µg/ml anti-DNP IgE overnight. The next day, FSCMCs were washed and resuspended in RPMI without phenol red at 4x 10⁶ cells/ml. Cells were then stimulated 1 h at 37°C by addition of an equal volume of 20 ng/ml DNP-HSA, followed by centrifugation and harvesting of the supernatant. Twenty-five µl of undiluted (equivalent to compounds secreted by 50000 cells) or serially 1:10 diluted supernatant or Heparin solution were incubated with 25 µl of 500 µg/ml BV (total of 12.5 µg) and were mixed in a 96 well v-bottom plate and incubated 10 min at 37°C. Next, 100 µl of substrate supernatant (see below) was added, mixed and incubated for 30 minutes at 37°C. The plate then was centrifuged for 2 minutes at 800 g and 100 µl of supernatant was transferred to a 96 well flat bottom plate. Absorbance at 425 nm (with the 600 nm reference value subtracted) was measured using a Sunrise microplate reader (Tecan).

The substrate for analysis of PLA₂ activity^{15,16} was prepared fresh: 4N3OBA (stored 250 mM in DMSO at -20°C) was diluted in 1x substrate buffer (10 mM TRIS HCl, 10 mM CaCl₂, 100 mM NaCl, pH 8.0) to a final concentration of 1 mM, vortexed thoroughly and centrifuged 5 minutes at 18000 g. The substrate supernatant was used for enzyme activity measurement (see above).

Realtime MC degranulation assessment by flow cytometry

FSCMCs were sensitized at 5x 10⁵ cells/ml (2 ml/well/sample in a 12 well tissue culture plate) overnight 1:20 with PBS or BV serum (sera processed as described above). Next day, cells were washed with complete culture medium (without cytokines) and resuspended in 500 µl medium containing 1:500

propidium iodide (Invitrogen) and 8 µg/ml Avidin^{AF488}, transferred to a FACS tube and warmed 15 minutes at 37°C. Next, pre-warmed 20 µg/ml BV in medium (or plain medium) was added to the FACS tube and immediately analyzed over 30 minutes (flowrate 25 µl/min) on an Accuri C6 flow cytometer (BD Biosciences).

Single cell degranulation analysis by confocal fluorescence microscopy

Real-time MC degranulation analysis by confocal fluorescence microscopy was performed as previously described with minor modification⁷. Briefly, eight-well Lab-Tek 1.0 borosilicate cover glass chamber slides (Nunc) were coated with 5 µg/ml poly-D-lysine in PBS. FSCMCs were sensitized at 5x 10⁵ cells/ml (2 ml/well/sample in a 12 well tissue culture plate) overnight 1:20 with PBS or BV serum (sera processed as described above). The next day, cells were washed with complete culture medium (without cytokines) and resuspended in 500 µl medium containing 2 µM Fluo-4 AM (Invitrogen) and incubated for 30 min at 37°C. Cells were then washed twice with medium and resuspended at 2.5x 10⁵/ml in medium containing 25 µg/ml avidin^{SR} and 5 µM TO-PRO-3 (viability stain; Thermo Fisher Scientific) and 190 µl suspension were seeded to a coated-chamber slide well, and transferred to the pre-warmed Zeiss LSM780 inverted confocal laser-scanning microscope. Cells were allowed to settle and adhere for 10 minutes, followed by addition of 10 µl of 200 µg/ml BV (or medium) and immediate time lapse recording of fluorescence for 1 hour. Images were processed and exported using Zen software (Zeiss) and mean fluorescence intensity (MFI) and mean integrated intensity (MII) were quantified as previously described using Image J/FIJI software^{7,17}.

Venom cytotoxicity analysis

Twenty-thousand 3T3-Swiss albino fibroblasts (ATCC CCL-92) were seeded in poly-D-lysine coated 8 well chamber slides (see above) in DMEM medium supplemented with 10% FBS, 1x penicillin/streptomycin and 1x glutamine (all from Gibco). The next day, 20 µl MC supernatant (prepared in RPMI w/o PR as described above in section BV digest analysis) was incubated either with 10 µl protamine chloride (protamine; 400 µg/ml RPMI w/o PR) or 10 µl 4x protease inhibitor (and supplemented with RPMI w/o PR to total 30 µl) for 20 minutes at room temperature. Next, 10 µl of BV (800 µg/ml) was added and incubated for 30 minutes at 37°C. In parallel, fibroblasts were stained with 2 µM Fluo-4 AM in RPMI w/o PR 10% FBS for 30 minutes at 37°C. Cells were washed twice with medium and 225 µl RPMI w/o PR 10% FBS with 5 µM TO-PRO-3 was added and cells were moved to the pre-warmed microscope (see above). After approximately 10 minutes, 25 µl of untreated or treated BV (resulting in a final concentration 20 µg/ml) was added, followed by immediate time lapse recording of fluorescence for 1 hour.

In experiments assessing the toxicity of BV in combination with protamine and protease inhibitor (Fig. S4B), BV was mixed 1:1 with RPMI w/o PR, 400 µg/ml protamine or 4x protease inhibitor, followed by

transfer of 25 μ l of the BV mix to chamber slides with Fluo-4 AM-stained 3T3 fibroblasts in 225 μ l RPMI w/o PR 10% FBS with 5 μ M TO-PRO-3 addition, resulting in final BV concentrations of 20 μ g/ml, 10 μ g/ml or 5 μ g/ml (with constant optional protamine and protease inhibitor concentrations). Images of the same field of view were taken before and 60 minutes after addition of the BV mix.

Images were processed and exported using Zen software (Zeiss) and mean TO-PRO-3 fluorescence intensity (MFI) was quantified as previously described using Image J/FIJI software^{7,17}.

BV-mediated ear swelling

C57BL/6 mice were anesthetized and intradermally injected with either 20 μ l PBS serum or BV serum into both ears. The next day, 20 μ l PBS alone or PBS containing 200 ng BV were injected into one ear, respectively, of each mouse. Ear thickness was measured using a 0.01 mm Dial Thickness Gauge (Peacock Ozaki) directly before and at indicated timepoints after injection.

In vivo imaging

Mcpt5-Cre^{cre}; R26Y^{EYFP} mice were intradermally injected with 20 μ l of PBS or BV serum into the ear. Next day the mice were intradermally injected with 20 μ l PBS containing 10 μ g/ml BV and 8 μ g of avidin^{SR}. Ears were assessed by intravital 2 photon microscopy 30 minutes later and MC degranulation was analyzed as previously described⁷.

Analysis and software

Flow cytometry data were analyzed using FlowJo (FlowJo LLC). Confocal microscopy data were processed and analyzed using Zen software (Zeiss) and Image J/FIJI¹⁷. Two-photon microscopy data were analyzed using Imaris software (Oxford Instruments)⁷. Statistical analyses (apart from that of mass spectrometry results; see above) were conducted using GraphPad Prism (GraphPad Software) and tests were applied as indicated in the figure legends. Differences with *P* values equal or below 0.05 were considered significant. All experiments were performed at least twice.

Supplementary Figure Legends

Figure S1: BV response of FSCMCs and serum characterization

(A and B) FSCMCs were incubated with different concentrations of BV for 1 hour. (A) Cell viability assessed as % of propidium iodide (PI)-positive (dead) cells by flow cytometry (representative of two independent experiments). (B) Degranulation assessed as β hexosaminidase (β -hex) release into the cell supernatant (% of total β -hex signal; representative of two independent experiments). (C) C57BL/6 wt mice were sensitized with 2 subcutaneous injections (on the back) of 50 μ l PBS alone or containing 200 μ g BV. Three weeks later, blood was collected and BV-specific IgG1, IgG2b and IgG2c antibody content and total IgE (in sera of individual mice) as well as BV PLA₂-specific IgE (in serum pools), was

analyzed by ELISA. Each symbol represents either serum from an individual mouse (collected from 2-3 immunization experiments) or a serum pool (collected in 3-4 independent immunization experiments). (A and B) mean +SD; (C) mean +SEM; (A and B) One-way ANOVA with Dunnett's test for multiple comparisons (P values are adjusted for multiple testing); * (or n.s. – not significant) indicates comparisons with the untreated (0 µg/ml BV) group; P values are adjusted for multiple testing; (C) Mann-Whitney test; n = 20-40 (individual animals) or 3-4 (serum pools); * $P \leq 0.05$; *** $P \leq 0.001$

Figure S2: Phospho-flow cytometry profile of IgE and BV-stimulated FSCMCs

(A) FSCMCs were sensitized overnight with anti-DNP IgE and not stimulated or stimulated with either 10 µg/ml BV, 5 ng/ml DNP-HSA, or both. Phosphorylation of AKT, MAPK/ERK and PLCγ1 and membrane-localized Lamp-1 was measured before (0 min) and after stimulation for 1, 5, 10 and 30 minutes by (phospho-) flow cytometry. Selected histogram overlays (upper panels) and respective mean fluorescence intensities (MFIs, lower panels) of phosphorylation data 5 minutes after stimulation, as shown in Figure 3C. Data are from one of three independent experiments, each of which gave similar results.

Figure S3: PAGE analysis of BV and purified BV toxins

(A) Complete BV (40 µg; lane 2), purified BV PLA₂ (20 µg; lane 3) or purified BV melittin (20 µg; lane 4) were separated by PAGE (15% acrylamide), followed by Coomassie blue staining of the gel. Labels on the left of the picture indicate the molecular weight of the protein marker (lane 1) band at the same height (or as indicated by a connecting line). The experiment was performed once.

Figure S4: Viability of 3T3 fibroblasts exposed to FSCMC supernatant, protamine or protease inhibitor

(A) 3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green) and left untreated or exposed to supernatant of IgE/antigen (DNP-HSA)-activated FSCMCs that were either untreated or pre-treated with protamine or protease inhibitor (as shown in Figure 4D). Cell death was monitored by confocal fluorescence microscopy imaging of TO-PRO-3 (turquoise)-positive nuclei over 60 minutes. Representative pictures (outermost left: merge of brightfield, Fluo-4 and TO-PRO-3; remaining pictures show merges of Fluo-4 and TO-PRO-3 channels) of the field of views at different timepoints after treatment are shown. (B) 3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green) and incubated for 60 minutes with 5, 10 or 20 µg/ml BV only or BV in combination with protamine (100 µg/ml)- or protease inhibitor (1x final concentration) in the presence of TO-PRO-3. Cell death was monitored by confocal fluorescence microscopy immediately before (0 min) and 60 minutes after addition of BV (and additives) by imaging of TO-PRO-3 (turquoise)-positive nuclei. Representative pictures (merges of Fluo-4 and TO-PRO-3 channels) of the field of views at the respective timepoints

are shown. The lower panels illustrate the proportions of dead (with MFI ≥ 1) and live (with MFI < 1) cells (numbers indicate the respective identified and quantified nuclei) in the field of view after 60 minutes of BV exposure (cells with a TO-PRO-3-positive nucleus at timepoint 0 min were excluded from the analysis). Turquoise stains label nuclei of dead cells. See also Figure 4D and 6A. (A and B) The scale bars represent 10 μm . Data are representative of 2 independent experiments.

Figure S5: Response of hu PBCMCs BV-mediated activation, and BV degradation

(A and B) Human peripheral blood-derived cultured MCs (hu PBCMCs) were stimulated with the indicated concentrations of BV and analyzed after 1 hour. (A) Tryptase activity in the supernatant (% of total tryptase-mediated signal). (B) Percentage of avidin-positive cells among PI-negative (live) cells. (C) Ten μg BV were exposed for 1 hour to supernatant (SN) collected different numbers of IgE/anti-IgE stimulated (for 1 hour) hu PBCMCs, followed by PAGE and staining of the gel with Coomassie blue. Labels on the left of the picture indicate the molecular weight of the protein marker (not depicted) band at the respective height. (D) Heatmap depicting mass spectrometry analysis results (of sample triplicates) of BV protein abundance after 1 h exposure to SN of IgE/anti-IgE-stimulated (1 hour) hu PBCMCs (vs. untreated BV). All 118 detected BV-specific proteins are shown. Characterized allergens (Api m 1-12) are indicated. Raw abundances were log₂- and z-score-transformed prior to visualization. Raw and adjusted P values and fold changes are shown in Table S1. (A-C) Data are from one of at least two independent experiments, each of which gave similar results. (A and B) graphs depict mean + SD. One-way ANOVA with Dunnett's test for multiple comparisons (P values are adjusted for multiple testing); * (or n.s. – not significant) indicates comparisons with the untreated (0 $\mu\text{g}/\text{ml}$ BV) group; P values are adjusted for multiple testing; *** $P \leq 0.001$ (D) Mass spectrometry analysis of sample triplicates was performed once.

Supplementary Tables

Table S1: Mass spectrometry analysis of BV exposed to supernatant of IgE/anti-IgE-activated hu PBCMCs

Human peripheral blood-derived cultured MCs (hu PBCMCs) were stimulated with IgE/anti-IgE (for 1 hour) and the supernatant (MC SN) was collected. BV was exposed in triplicate (rep_1 – 3) for 1 h to MC SN (BV+MC SN) or PBS (untreated) and analyzed by mass spectrometry. Normalized abundance and P values were calculated as described in the methods section. Data in the table correspond to results shown in Fig. 5E and F and S5D.

Supplementary Videos

Videos S1-3: Time lapse of the IgE-mediated FSCMC degranulation response to BV

FSCMCs were incubated overnight with isotype control-pre-treated PBS serum or isotype control- or anti-IgE-pre-treated BV serum. The next day, cells were seeded in chamber slides stained with Fluo-4 and exposed to 10 µg/ml BV in presence of avidin^{SR} and To-Pro-3. The avidin^{SR}-mediated signal of degranulating MCs was recorded over 60 minutes by realtime confocal fluorescence microscopy. The video shows a time-lapse of merged fluorescence signals of Fluo-4 (calcium, in green) To-Pro-3 (viability, in turquoise) and avidin^{SR} (in red). Turquoise stains are nuclei of dead cells. Video S1: PBS serum, isotype ctrl, 10 µg/ml BV; Video S2: BV serum, isotype ctrl, 10 µg/ml BV; and Video S3: BV serum, anti-IgE, 10 µg/ml BV.

Videos S4-7: Realtime monitoring of FSCMC-mediated BV detoxification

3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green). BV was optionally pre-treated with supernatant of IgE/BV-antigen-activated (BV serum/10 µg/ml BV; stimulated for 1 hour) FSCMCs that were either untreated or pre-treated with protamine or protease inhibitor. The untreated or pre-treated BV was transferred onto the fibroblasts and monitoring of cell death, by confocal fluorescence microscopy imaging of To-Pro-3 (turquoise)-positive nuclei over 60 minutes, was started immediately. The video shows a time-lapse of merged fluorescence signals of Fluo-4 (calcium, in green) and To-Pro-3 (viability, in turquoise). Turquoise stains are nuclei of dead cells. Video S4: 20 µg/ml BV pre-incubated with supernatant of non-stimulated FSCMCs; Video S5: 20 µg/ml BV pre-incubated with untreated supernatant of BV-stimulated FSCMCs; Video S6: 20 µg/ml BV pre-incubated with protamine-treated supernatant of BV-stimulated FSCMCs; and Video S7: 20 µg/ml BV pre-incubated with protease inhibitor-treated supernatant of BV-stimulated FSCMCs.

Videos S8-11: Realtime monitoring of hu PBCMC-mediated BV detoxification

3T3 fibroblasts were seeded in chamber slides and stained with Fluo-4 (green). BV was optionally pre-treated with supernatant of IgE/anti-IgE activated (stimulated for 1 hour) hu PBCMCs that were either untreated or pre-treated with protamine or protease inhibitor. The untreated or pre-treated BV was transferred onto the fibroblasts and monitoring of cell death, by confocal fluorescence microscopy imaging of To-Pro-3 (turquoise)-positive nuclei over 60 minutes, was started immediately. The video shows a time-lapse of merged fluorescence signals of Fluo-4 (calcium, in green) and To-Pro-3 (viability, in turquoise). Turquoise stains are nuclei of dead cells. Video S8: 20 µg/ml BV pre-incubated with supernatant of non-stimulated hu PBCMCs; Video S9: 20 µg/ml BV pre-incubated with untreated supernatant of IgE/anti-IgE-stimulated hu PBCMCs; Video S10: 20 µg/ml BV pre-incubated with

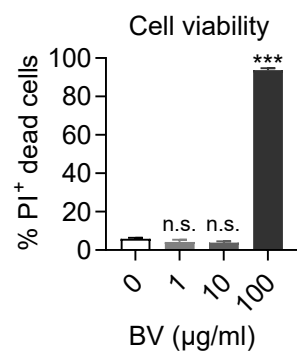
protamine-treated supernatant of IgE/anti-IgE-stimulated hu PBCMCs; and Video S11: 20 µg/ml BV pre-incubated with protease inhibitor-treated supernatant of IgE/anti-IgE-stimulated hu PBCMCs.

Supplementary References

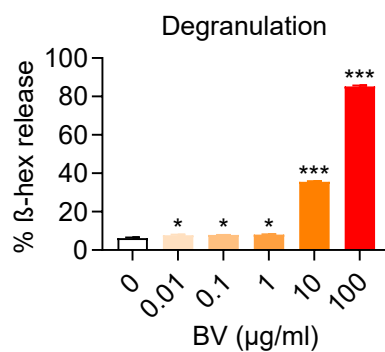
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Figure S1

A



B



C

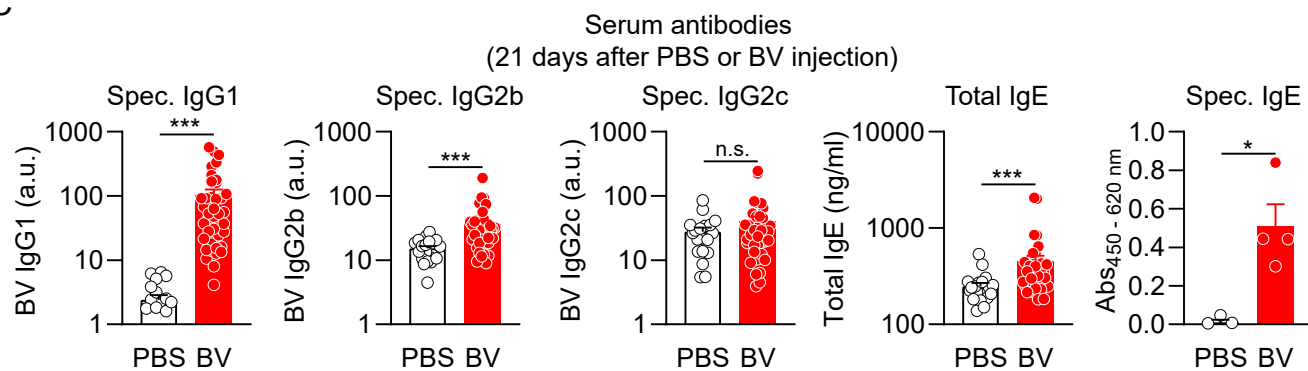


Figure S2

A

