IgE Antibodies, FcεRIα and IgE-mediated Local Anaphylaxis Can Limit Snake Venom Toxicity

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P.S. was supported by a Max Kade Fellowship of the Max Kade Foundation and the Austrian Academy of Sciences and a Schroedinger Fellowship of the Austrian Science Fund (FWF): J3399-B21. T.M. was supported by a Marie Curie International Outgoing Fellowship for Career Development (Grant number 299954). N.G was supported by fellowships from the French "Fondation pour la Recherche Médicale FRM". L.L.R. was supported by the Arthritis National Research Foundation (ANRF) and National Institutes of Health grant K99AI110645. R.S. was supported by a fellowship from the Lucile Packard Foundation for Children's Health and the

Stanford NIH/NCRR CTSA award, number UL1 RR025744. This work was supported by National Institutes of Health grants AI023990, CA072074 and AI070813 (to S.J.G.) and by the Department of Pathology of Stanford University.

Online Repository Methods

Mice

All animal care and experiments were carried out in accord with current National Institutes of Health guidelines and with the approval of the Stanford University Institutional Animal Care and Use Committee. For experiments involving WT mice (Figs 1; 3, F-J; 5-7; E1; E2; E3, F-H; E5) and production of serum from WT mice (Figs 3, F-J; 4, F-H), age-matched 5 to 7 week-old C57BL/6J or BALB/cJ female mice were purchased from Jackson Laboratories (Sacramento, CA) and were housed in the Research Animal Facilities at Stanford University for at least 7 days before starting experiments. All transgenic mouse strains were bred and housed with the respective (in house-bred) control mice in the Stanford Animal facilities under specific pathogen free conditions. C57BL/6-*Fcer1a*$^{-/-}$ mice were originally purchased from Jackson Laboratories. *Igh7*$^{-/-}$ and *Igh7*$^{+/+}$ control mice on the BALB/c background were described previously[E1] and were generously provided by Hans C. Oettgen (Harvard Medical School, MA, USA). *Igh7*$^{-/-}$ mice backcrossed 8 generations on the C57BL/6J background and littermate control mice were generously provided by Mitchell Grayson (Medical College of Wisconsin, WI, USA). MC-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and MC- and basophil-deficient C57BL/6-*Cpa3-Cre;Mcl-1*$^{fl/fl}$ mice were previously described[37, 39]. We originally obtained previously described B6.129S4-*Mcpt8*$^{tm1(cre)Lky}$/*J*[E2] (referred to in Figure E3 as *Mcpt8-Cre*) and *B6.129P2-Gt(ROSA)26Sor*$^{tm1(DTA)Lky}$/*J*[E2] (referred to in Fig. E3 as *DTA*^{fl}) from Jackson laboratories.

Homozygous *Mcpt8-Cre* mice were bred at Stanford with $DTA^{fl/-}$ mice in order to generate basophil-deficient $Mcpt8\text{-}Cre^{+/-};DTA^{fl/-}$ and basophil-sufficient $Mcpt8\text{-}Cre^{+/-};DTA^{-/-}$ littermates (blood basophil levels were analyzed for all mice prior to start of the immunization).

Age-matched male and female transgenic and control mice (with comparable gender distribution within the groups) were used for experiments.

Reagents

Russell's viper (*Daboia russelii*) venom (RVV) was obtained from Sigma (Carlsbad, CA) and was resuspended in sterile, endotoxin-free PBS (Gibco, Grand Island, NY) at 10 mg/ml. Two different RVV batches were used throughout this study that appeared to differ in their toxin composition (assessed by PAGE and coomassie blue stain; data not shown) and toxicity: Lot SLBB5602V (country of origin: China) and SLBK7058V (country of origin: Pakistan). Challenge with SLBK7058V resulted in notably lower mortality than injection of the same dose of SLBB5602V. Lot SLBB5602V was used for all experiments except those in Figs. 1, G and H, E2, E3, E6, which employed batch SLBK7058V. Honeybee (*Apis mellifera*) venom (Lot 12071006HB) was obtained from ALK Abello Source Materials, Inc (Spring Mills, PE). Batches of freeze-dried complete BV were resuspended in PBS at 4 mg/ml. BV and RVV were stored in aliquots at -20°C. Dispase was from Gibco. Collagenase A and DNAse I were from Roche. Aqua Dead Cell Stain Kit and propidium iodide were obtained from Life Technologies (Grand Island, NY). V450 (Pacific Blue equivalent)-conjugated anti-mouse CD49b (DX-5), fluorescein-conjugated anti-mouse CD117 (KIT) (clone 2B8) and allophycocyanin-cyanin7-conjugated anti-mouse Ly6G (clone 1A8; used for skin cell analysis) antibodies and phycoerythrin-TexasRed-conjugated streptavidin were from BD Biosciences (San Jose, CA), allophycocyanin-conjugated anti-mouse CD45 (clone 30-F11), anti-mouse Ly6G (Gr-1; fluorescein-conjugated clone RB6-

8C5 used for blood cell analysis in Fig E1); allophycocyanin-conjugated clone 1A8-Ly6g (used for analysis of neutrophil depletion), and allophycocyanin-conjugated anti-mouse F4/80 (clone BM8) antibodies were from eBioscience (San Diego, CA), phycoerythrin-conjugated anti-mouse FcεRIα (clone MAR-1) antibody and fluorescein-conjugated anti-mouse CD11b (clone M1/70; used in combination with 1A8-Ly6g for analysis of neutrophil depletion) were from Biolegend (San Diego, CA), biotin-conjugated anti-mouse Siglec-F (clone ES22-10D8) antibody was from Miltenyi Biotec (Bergisch Gladbach, Germany). Rat anti-mouse IgE (clone R35-92) and rat IgG_1 isotype control (clone R3-34) (used for experiments involving serum pre-treatment) were obtained from BD Pharmingen (San Jose, CA) and dialyzed twice against PBS prior to use in *in vivo* experiments. Clones and sources of antibodies used in ELISA experiments are stated in the respective section below. Dinitrophenyl (DNP)-specific IgE (α-DNP clone ε26[46]) was kindly provided by Dr. Fu-Tong Liu (University of California-Davis). DNP-specific mouse IgG_1 (clone 109.3) and IgG_{2b} (clone 10.12) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and dialyzed twice against PBS before use in *in vivo* experiments. The specificity of dialyzed anti-DNP IgG antibodies for DNP-HSA (see below) was verified by ELISA (data not shown). Anti-ovalbumin (OVA) IgE (clone E-C1) was from Chondrex (Redmond, WA). Platelet activating factor (PAF) receptor antagonist CV-6209 was obtained from Santa Cruz Biotechnology, resuspended at 3.3 mg/ml in sterile saline and stored in aliquots at -20°C until use. Triprolidine hydrochloride, $Dinitrophenyl_{30-40}$-conjugated human serum albumin (DNP-HSA) and other chemicals were obtained from Sigma.

Venom injections and active immunization

Six to 8 week old C57BL/6 and BALB/c WT or transgenic mice were shaved at the injection sites (back skin for RVV, back and belly skin for BV challenge) 24 h before injections

and were consistently treated in the morning (without anesthesia) by administering two s.c. injections of 50 µl PBS alone or containing either 250 µg RVV/ml (i.e., 2x 12.5 µg; used for standard injections of mice prior to high dose RVV exposure), 500 µg RVV/ml (i.e., 2x 25 µg; used for comparing the responses of MC-deficient mice and corresponding MC-sufficient controls [Fig 2] and for testing the effect of immune serum transfer into C57BL/6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice [Fig 4]) or 750 µg RVV/ml (i.e., 2x 37.5 µg; used for challenges with a potentially lethal dose of RVV). Scratching behavior was quantified between 35 and 45 min after RVV injection as the number of scratching attempts with the hind legs per individual mouse. For experiments involving active "immunization" to RVV, mice were challenged 21 days after immunization. Body temperature was measured immediately before challenge and at indicated time intervals after challenge (in surviving mice), using a rectal thermometer.

For experiments investigating the effect of two sequential exposures to RVV on subsequent resistance to high dose RVV challenge (Fig 6), C57BL/6 and BALB/c mice were injected (1) on each of days 0 and 21 with 2 s.c. injections of 50 µl PBS (control mice, referred to as PBS-PBS), (2) on day 0 with 2 s.c. injections of 12.5 µg RVV in 50 µl PBS and on day 21 with 2 s.c. injections of 50 µl PBS (referred to as RVV-PBS mice), or (3) on each of days 0 and 21 with 2 s.c. injections of 12.5 µg RVV in 50 µl PBS (referred to as RVV-RVV mice). At day 42, all mice were challenged with 2x 37.5 µg BV in 50 µl PBS. If in a particular independent challenge experiment shown in Fig 6 more than 50% of the control mice injected only with PBS on day 0 and day 21 exhibited a drop in body temperature of less than 4°C for C57BL/6 mice or less than 3°C for BALB/c mice by one h following injection of 2x 37.5 µg RVV (we occasionally observed such variation in the response, that might be due to the greater age and weight of these mice as compared mice that were challenged 21 days after initial injections), all the mice in that experiment then received a single additional s.c. injection of 25 µg RVV in 50 µl PBS in the back

skin. We used this approach in order to achieve sufficient mortality in the mock-immunized (i.e., PBS-PBS) control animals to allow identification of potential resistance in the two groups of RVV-immunized mice. As in our prior study[21], all venom injections were performed by a team of 2 experimenters working together (to enable better control of the mice during the injections) and experiments were designed and performed to allow the injection of all animals in any single experiment within minutes. For analysis of venom-induced antibody responses, blood was collected at day 14 (in experiments involving challenge after 21 days) or at day 35 (in experiments involving challenge after 42 days) from the retro-orbital vein of anesthetized mice. In experiments investigating the effects of two sequential exposures to BV on subsequent resistance to high dose BV challenge (Fig 7), C57BL/6 and BALB/c mice were injected (1) on each of days 0 and 21 with 2 s.c. injections into the back skin, each consisting of 50 µl PBS (control mice, referred to as PBS-PBS), (2) on day 0 with 2 s.c. injections of 200 µg BV for C57BL/6 mice or 1x 100 µg BV for BALB/c mice, each injection in 50 µl PBS, and on day 21 with 2 s.c. injections into the back skin, each consisting of 50 µl PBS (referred to as BV-PBS mice), or (3) on each of days 0 and 21 with 2 s.c. injections of 200 µg BV for C57BL/6 mice or 1x 100 µg BV for BALB/c mice, each injection in 50 µl PBS (referred to as BV-BV mice). These amounts of BV were selected because we showed previously that they resulted in significantly increased resistance to challenge with a potentially lethal BV dose 21 days later[21]. At day 42, C57BL/6 mice were challenged s.c. with 4x 200 µg BV, each of the 4 injections in 50 µl PBS (3 in back skin, 1 in belly skin) and BALB/c mice were challenged with 5x 200 µg BV, each of the 5 injections in 50 µl PBS (4 in back skin, 1 in belly skin). These challenge protocols caused death in ~70-90% of 6-8 week old animals as determined in previous dose finding experiments[21]. Body temperature was measured immediately before challenge and at indicated time intervals after challenge (in surviving mice), using a rectal thermometer.

Passive immunization – serum preparation and transfer

For experiments presented in Fig 3, F-J, and Fig 4, F-H, 6 to 8 week-old female WT C57BL/6J "donor" mice were immunized by 2 s.c. injections (in the back skin) of 12.5 µg RVV in PBS or with PBS alone. Three weeks later, blood was collected and sera of PBS- and RVV-injected mice were recovered after centrifugation in Serum-Gel Microtubes (Sarstedt, Neumbrecht, Germany). Following quantification of antibody levels (as described above), sera were pooled and aliquoted for later use in treatment groups of 5 mice (~1.3 ml/group) and stored at –20°C. Aliquots of pooled serum derived from "donor" mice which had been injected with either PBS or RVV are referred to as PBS-serum or RVV-serum, respectively. On average, the amount of serum obtained from two "donor" mice at this age (i.e., a total of ~750 µl) was sufficient for i.v. transfer into three "recipient" naïve mice.

For experiments shown in Fig 3, I-J, sera were modified immediately before the transfer as described previously[21]: (1) serum from PBS mock-immunized mice (PBS-serum) was supplemented with 1/10 volume (50 µg/ml final concentration) of the rat IgG_1 isotype control antibody (clone R3-34; BD Pharmingen; dialyzed overnight against 2x 5 L of PBS to remove sodium azide); (2) serum from RVV-immunized mice (RVV-serum) was supplemented with 1/10 volume of dialyzed rat IgG_1 isotype control antibody and not further treated prior to transfer (RVV-serum); (3) RVV-serum was supplemented with 1/10 volume of dialyzed rat IgG_1 isotype control antibody and heated 60 min at 56°C in a water bath (heated BV-serum), in order to specifically ablate IgE function[21, 41, 42, E3, E4]; (4) RVV-serum was supplemented with 1/10 volume of rat anti-mouse IgE antibody (clone R35-92 [this particular clone is commonly used to neutralize cell-mediated IgE function *in vivo*[21, E5, E6]], BD Pharmingen; dialyzed overnight against 2x 5 L of PBS to remove sodium azide) for 30 min at room temperature (Anti-IgE RVV-serum).

Aliquots of these modified sera (250 µl/mouse) were transfused i.v. via the retro-orbital vein into anesthetized 10 to 11 week-old female C57BL/6 "recipient" naïve mice. On the following day, all "recipient" mice were challenged with 2 s.c. injections of 37.5 µg RVV in PBS. We measured body temperature at the indicated times and monitored survival over one week.

For experiments involving serum transfer into MC-deficient recipient mice (Fig 4, F-H), naïve C57BL/6-*Cpa3-Cre;Mcl-1*$^{fl/fl}$ mice received serum generated from C57BL/6J WT mice and were challenged with 2x 25 µg RVV in PBS.

Histology and assessment of MC degranulation

Back skin specimens were removed carefully to avoid stretching or compressing the specimens (that can increase artifactual changes in MC morphology) and were fixed with 10% formalin and embedded in paraffin, and 4-µm sections were stained with 0.1% Toluidine blue or Hematoxylin & Eosin for histologic examination. Images were captured with a Nikon (Belmont, CA) E1000M microscope using a Spotflex camera and Spot version 5.1 software (Diagnostic Instruments, Sterling Heights, MI). MC degranulation is expressed as the % of the skin MCs examined in which >50% ("Extensive" degranulation of that cell), 10-50% ("Moderate" degranulation of that cell) or <10% ("None", indicative of no evidence of significant degranulation of that cell) of the cytoplasmic granules of individual MCs exhibited morphological evidence of degranulation, i.e., alterations in the staining characteristics, size or distribution of the granules[16, 21]. The data are presented as bar graphs of the % of MCs exhibiting the various extents of degranulation (mean+SEM), and the differences between results were examined for statistical significance using the Chi square test.

ELISAs

After each incubation step in the ELISAs described below, plates are washed 3-5 times using PBS containing 0.05% tween. For detection of venom-specific IgG_1 serum antibodies, Nunc MaxiSorp ELISAs plates (Thermo Scientific, Waltham, MA) were coated with whole RVV or BV diluted to 5 µg/ml PBS at 4°C overnight, followed by blocking with 1% bovine serum albumin (BSA) in PBS for at least 2 h at room temperature. Sera were diluted in PBS containing 1% BSA and incubated in the blocked wells for 2 h at 37°C or overnight at 4°C. We detected bound IgG_1 using a biotinylated detection antibody (rat anti-mouse IgG_1 [clone A85-1, BD Pharmingen; incubated for 1 h at room temperature]), followed by incubation with horseradish peroxidase-conjugated streptavidin (BD Pharmingen) for 20 min at room temperature and detection of the reaction product after using supersensitive TMB substrate (Sigma). Antibody titers were calculated by plotting the serum dilution that gave half-maximal signal of a reference serum. When the detected signal gave a corresponding titer < or = to 1, an arbitrary value of 1 was assigned.

For determination of absolute amounts of total serum IgE, we coated ELISA plates with rat anti-mouse IgE [clone R35-72, BD Biosciences, at 2 µg/ml], followed by blocking as described above. We incubated coated and blocked wells with serial dilutions of purified mouse IgE (BD Pharmingen) as standard and 1:10 diluted sera (in PBS 1% BSA) for 2 h at 37°C or overnight at 4°C. Bound IgE was detected using biotinylated anti-mouse IgE (clone R35-118, BD Pharmingen) and reagents as described above.

Generation and analysis of degranulation of bone marrow-derived cultured mast cells (BMCMCs)

Mast cells were generated from the bone marrow cells of female age-matched C57BL/6 or BALB/c mice by (simultaneous) culture for 10 weeks in WEHI-conditioned, IL-3 containing

medium until a mast cell purity of ~95 % was reached as previously described[21]. In order to analyze the sensitization potential of immune sera (Fig E6), BMCMCs were sensitized overnight with serum pools derived from three independent experiments (the same serum pools were used in Fig 7, F-G for assessment of PLA_2-specific IgE), followed by 1 h stimulation without or with BV at the indicated concentrations in Tyrode's buffer. The percentage of release of the mast cell granule-stored enzyme β-hexosaminidase in the supernatants of BMCMCs was measured as previously described[21].

Pharmacologic blocking of histamine and platelet activating factor (PAF) in mice exhibiting RVV-mediated hypothermia

Potential contributions of histamine and PAF to the hypothermia observed upon RVV injection were analyzed by specific neutralization of these mediators following a previously described method[E7], with slight modifications: the H1-receptor-specific antihistamine triprolidine was solubilized at 1 mg/ml in sterile saline and filter-sterilized and 200 µl was injected into mice intraperitoneally 1 h prior to venom injections. The PAF receptor antagonist CV-6209 was diluted at 330 µg/ml in saline and 200 µl were injected into the retro-orbital vein of anesthetized mice 30 min prior to venom injections (particular care was taken that all mice were exposed to the isoflurane anesthesia for the same amount of time [i.e., 2.5 – 3 minutes]). All mice were allowed to recover from the anesthesia (they were exposed to an infra-red lamp for 5 min directly after anesthesia) for the same period of time. Recovery from the anesthesia by all mice (i.e., reaching a body temperature of at least 37°C) was verified before injecting 2x 37.5 µg RVV (lot SLBK7058V) and monitoring temperature and survival as described above.

Antibody-mediated neutrophil depletion

For assessment of the potential role of neutrophils in the type 2 immune response against RVV, C57BL/6 mice were injected intraperitoneally with 150 µg anti-GR-1 antibody (clone RB6-8C5, BioXCell, West Lebanon, NH) for neutrophil depletion[E8, E9] or an isotype control antibody (clone LTF-2, BioXCell) in 100 µl PBS or with PBS alone (vehicle). These injections were started 3 d prior to immunization with 2x 12.5 µg RVV and continued every other day until collection of serum 14 d after immunization. Starting with day 1 after immunization, a drop of blood was collected from the tail of the mice and levels of neutrophils were assessed as described above immediately before antibody injection in order to confirm (sustained) neutrophil depletion by flow cytometry using anti-mouse Ly-6G (clone 1A8-Ly6g) combined with anti-mouse CD11b antibodies. In the experiments shown in Fig E3, F and G, levels of neutrophils in the blood of all groups of mice treated with anti-GR-1 antibody ranged from undetectable to <1% of blood cells at all times tested. In the PBS-treated or isotype control antibody-treated mice, blood levels of neutrophils ranged from 2-30% over the time period tested (with the highest levels seen on days 1 or 3 after immunization with RVV).

Biotinylation of RVV and bvPLA$_2$

RVV and bvPLA$_2$ (Sigma) (each at a stock concentration of 4 mg/ml in PBS) were mixed 1:1 with (+)-Biotin N-hydroxysuccinimide ester (stock concentration of 2 mg/ml in dimethyl sulfoxide) and incubated at room temperature for 2 h. One volume of 0.1 M TRIS pH 7 was added for 10 min to quench the reaction. The reaction mixture was dialyzed 2x overnight at 4°C against PBS using Slide-A-Lyzer Dialysis Cassettes (2K MWCO, Pierce, Rockford, IL), followed by desalting using Zeba Spin Desalting Columns (7K MWCO, Pierce). Biotinylated proteins were stored at -20°C. Optimal dilutions (resulting in highest signal-to-noise ratio) of biotinylated proteins and horseradish peroxidase-conjugated streptavidin for detection of specific IgE

antibodies in ELISA experiments (described above) were determined in pilot experiments using control serum from naïve mice and serum pools with high IgE content (derived from mice that had been immunized and challenged with RVV or BV).

DNP-HSA-specific IgE-dependent passive cutaneous anaphylaxis

To test the effects of a local IgE-dependent anaphylactic reaction against an irrelevant antigen on resistance of mice to a potentially lethal challenge with RVV, a model of IgE-dependent passive cutaneous anaphylaxis was established in the back skin of C57BL/6 and BALB/c mice. Six to 8 week-old C57BL/6J and BALB/cJ mice were sensitized with three s.c. injections into the back skin of 50 µl Saline alone or containing 50 ng anti-DNP IgE antibody. For experiments shown in Fig E5, mice were anesthetized 18 h later and injected intravenously with 200 µl PBS containing 1% Evan's blue dye and challenged 30 min later by two s.c. injections of 50 µl PBS containing either 0.1 µg or 0.5 µg DNP-HSA. Changes in body temperature were monitored during the first 30 min. Specimens of 1 cm^2 size of back skin including the s.c. injection sites were collected 1 h after challenge and sacrifice of the mice, minced into 4 equally sized pieces and incubated in 300 µl DMSO in a 48 well plate for 90 min on a shaker at room temperature. The absorption of the supernatant at 620 nm was analyzed to assess levels of extracted Evan's blue dye. For experiments shown in Fig. 5, E5, G-I, and E6, 18 h after sensitization with 3x 50 ng anti-DNP IgE antibody (or, in Fig. 5, with the same amount of an anti-DNP IgG_1 or anti-DNP IgG_{2b} antibody or, in Fig. E6, with 3x 50 ng anti-DNP IgE antibody alone or mixed with 500 ng anti-ovalbumin (OVA) IgE, or with 3x 500 ng anti-OVA IgE alone, or with saline alone as a mock sensitization), mice were challenged with 2 s.c. injections, each consisting of 50 µl PBS containing 37.5 µg RVV and 0.5 µg DNP-HSA.

Treatment of skin and blood samples for flow cytometry analysis

For assessment of skin immune cell infiltration following RVV injection (Fig 1, C,D), skin samples of 1 cm^2 including the injection sites were dissected after sacrifice of the mice and transferred into a dispase solution (2.5 U/ml in Hank's buffered saline solution [HBSS]) for 90 min at 37°C. The epidermis was removed with forceps, and the dermis was cut into small pieces and digested for 60 min at 37°C on a shaker at 200 rpm in 2 ml DMEM medium containing 20 mM HEPES, 396 U/mL DNAse I and 1 mg/ml collagenase A. Cell aggregates were further mechanically disrupted by passing them through a 18G needle, and the cell suspension was filtered through a 70 µm cell strainer.

After red blood cell lysis using ACK buffer, skin dermal cells and white blood cells were stained with Aqua Dead Cell Stain Kit or propidium iodide, respectively, to label dead cells, followed by incubation with an anti-mouse CD16/CD32 (clone 2.4G2; obtained from BD Biosciences) antibody to reduce non-specific binding before staining. Cells were kept at 4°C during processing. We analyzed white blood cells with an Accuri C6 flow cytometer (BD Biosciences) and skin dermal samples with an LSR-II flow cytometer (BD Biosciences) at the Stanford University Flow Cytometry Core Facility. Skin neutrophils were defined as $CD45^+$ $Ly6G^+$ $Siglec\text{-}F^-$ cells, blood neutrophils as $CD45^+$ $Ly6G^{high}$ $F4/80^-$ cells, skin eosinophils as $CD45^+$ $Ly6G^{int}$ $Siglec\text{-}F^+$ cells and skin basophils as $CD45^+$ $Fc\varepsilon RI\alpha^+$ $CD49b^+$ $c\text{-}Kit^-$ cells. Blood monocytes were defined as $CD45^+$ $F4/80^{int}$ $Ly6G^{int}$ cells. Results were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Statistical tests were performed using the software GraphPad PRISM 6 (Graphpad Software, San Diego, CA). Two-tailed Student's *t*-test (unpaired), Mann-Whitney test, Mantel-

Cox or Chi-Square tests were performed as noted in the respective figure legends. ns, not significant (P>0.05); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Unless otherwise specified, data are shown as mean+SEM or mean±SEM.

Online Repository Figure Legends

Fig E1. Subcutaneous RVV induces systemic neutrophilia. **A**, Flow cytometry plots of blood neutrophils ($CD45^+$ $Ly6G^{high}$ $F4/80^-$), and **B**, Percentages (mean+SD, n=3) of blood neutrophils at indicated times following injection. Numbers indicate % of gated cells in total subpopulation. Data in **A** are from one mouse/panel and are representative of three mice per group. *P* values: Student's *t* test.

Fig E2. Effects of triprolidine or CV-6209 pre-treatment on responses to RVV in BALB/c mice. **A**, Experimental outline. **B**, Temperature (right panel magnifies area in dashed box) and **C**, Survival. *P* values: Student's *t* test (*B*); Mantel-Cox test (*C*). Data pooled from 2 experiments. Symbols in (*B*): comparison of group in that color with vehicle-treated mice for that time point.

Fig E3. Assessment of roles of basophils, MCs and neutrophils in type 2 humoral responses to RVV. **A**, Experimental outline (*B*-*E*). **B**-**E**, Serum RVV-specific IgG_1 (*B,D*) and total IgE (*C,E*) in *(B,C)* basophil-deficient *Mcpt8-Cre*$^{+/-}$*;DTA*$^{fl/-}$ and control basophil-sufficient *Mcpt8-Cre*$^{+/-}$*;DTA*$^{-/-}$ mice and in (*D,E*) MC- and basophil-deficient *Cpa3-Cre*$^{+}$*;Mcl-1*$^{fl/fl}$ and corresponding control mice. **F**, Experimental outline (*G,H*). **G,H**, Serum RVV-specific IgG_1 (*G*) and total IgE (*H*) in neutrophil-depleted anti-GR-1-treated (see Methods for extent of neutrophil depletion),

isotype antibody control-treated, and PBS-treated C57BL/6 mice. Data are pooled from 2-4 experiments (n=6-18/group). *P* values: Mann-Whitney test.

Fig E4. IgE contributes to acquired resistance to RVV-induced toxicity in BALB/c mice. **A** Outline of experiments with IgE-deficient (*Igh-7*$^{-/-}$) and *Igh-7*$^{+/+}$ mice. **B,C**, Serum RVV-specific IgG_1 (*B*) and total IgE (*C*). **D**,**E**, body temperature (*D*) and survival (*E*). Data are pooled from 3 experiments (n= 16-19/group). *P* values: Mann-Whitney test (*B*-*C*); Student's *t* test (*D*); Mantel-Cox test (*E*).

Fig E5. Passive cutaneous anaphylaxis to an irrelevant antigen can increase resistance to a potentially lethal challenge with RVV. **A**-**D**, C57BL/6 and BALB/c mice received 3 s.c. injections of 50 µl saline alone or containing 50 ng anti-DNP IgE antibody on the shaved back skin. 18 h later, mice were injected intravenously with 200 µl PBS containing 1% Evan's blue dye and challenged 30 min later by 2 s.c. injections of 50 µl PBS containing 0.5 µg DNP-HSA, administered to the site of prior injection of anti-DNP IgE or saline. **A,C**, body temperature after DNP-HSA challenge. **B,D**, extravasation of intravascular fluid into the skin at the site of DNP-HSA challenge 1 h after challenge as assessed by levels of Evan's blue dye. **E**,**F**, body temperature (*E*) and survival (*F*) of C57BL/6 mice following 2 s.c. injections of 37.5 µg RVV mixed with 0.5 µg DNP-HSA or vehicle. **G**, Experimental outline for (*H,I*). **H,I**, body temperature (*H*) and survival (*I*) of BALB/c mice treated with 3 s.c. injections of 50 µl saline alone or containing 50 ng anti-DNP IgE antibody and challenged 18 h later with 2 s.c. injections, each of 50 µl PBS containing 37.5 µg RVV and 0.5 µg DNP-HSA. Data are pooled from 2-3 experiments (n=6-15/group). *P* values: Student's *t* test (*B, D, H*); Mantel-Cox test (*I*).

Fig E6. Eliciting passive cutaneous anaphylaxis-mediated resistance against RVV depends on employing an antigen-specific IgE. **A**, Experimental outline. **B**,**C**, Body temperature (*B*) and survival (*C*) of IgE-deficient (*Igh-7*$^{-/-}$) C57BL/6 mice treated with 3 s.c. injections of saline alone or containing 50 ng of anti-DNP IgE alone, 50 ng of anti-DNP IgE mixed with 500 ng of anti-OVA IgE, or 500 ng of anti-OVA IgE antibody alone and challenged 18 h later with 2 s.c. injections, each containing 37.5 µg RVV and 0.5 µg DNP-HSA. Data are pooled from 3 independent experiments (n=7/group). *P* values: Student's *t* test (*B*); Mantel-Cox test (*C*).

Fig E7. BV induces similar degranulation responses in bone marrow-derived cultured mast cells (BMCMCs) derived from C57BL/6 or BABL/c mice after their sensitization with BV-immune serum derived from either C57BL/6 or BABL/c mice. **A,B**, BMCMCs were sensitized overnight with pooled immune serum collected on day 42 from PBS-PBS, BV-PBS or BV-BV immunized C57BL/6 or BALB/c mice (these were the same sera used for measurements of PLA_2-specific IgE in Fig 7, F-G). Percentage of β-hexosaminidase released was measured 1 h after exposure to BV. (*B*) Shows selected data from (*A*) to allow side-by-side comparison of results from C57BL/6 and BALB/c BMCMCs (none of the responses to any of the concentrations of BV tested were significantly different in the BMCMCs derived from the two mouse strains). Data pooled from three independent experiments employing the same batch of BMCMCs tested on different days but each of the three experiments using a different batch of serum pooled from 3 independent immunizations. *P* values: Student's *t* test.

Online Repository References

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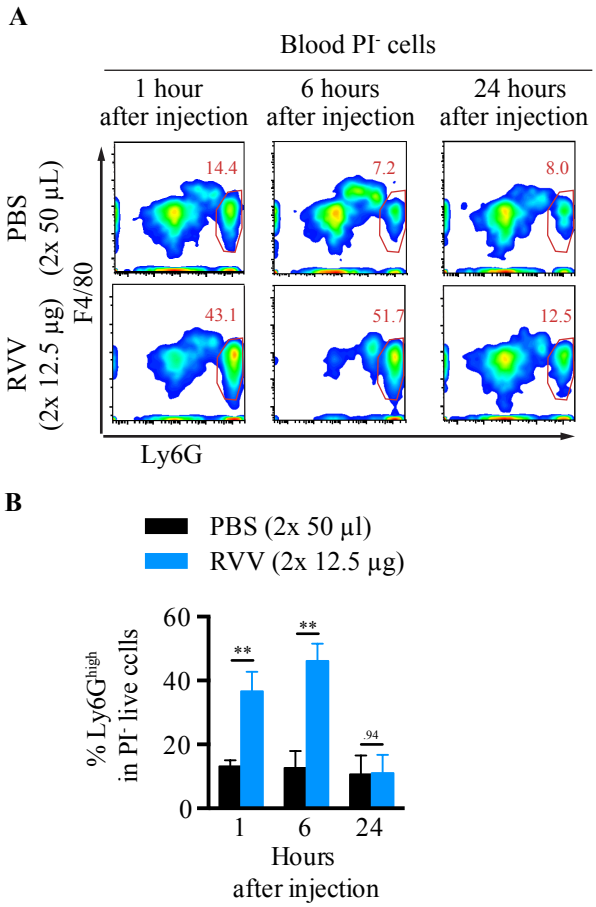
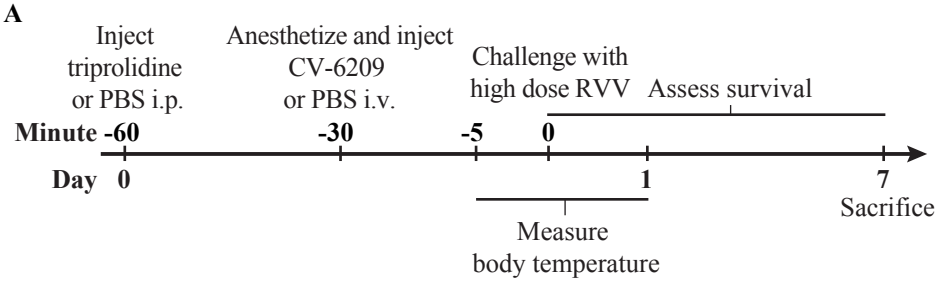
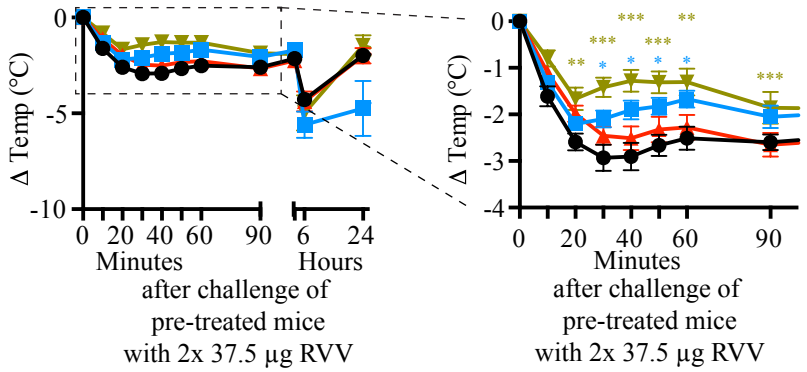

A
Blood PI- cells
1 hour after injection
6 hours after injection
24 hours after injection
PBS (2x 50 µL)
RVV (2x 12.5 µg)
F4/80
Ly6G
14.4
7.2
8.0
43.1
51.7
12.5
B
PBS (2x 50 µl)
RVV (2x 12.5 µg)
% Ly6G^high in PI- live cells
60
40
20
0
**
**
.94
1
6
24
Hours after injection

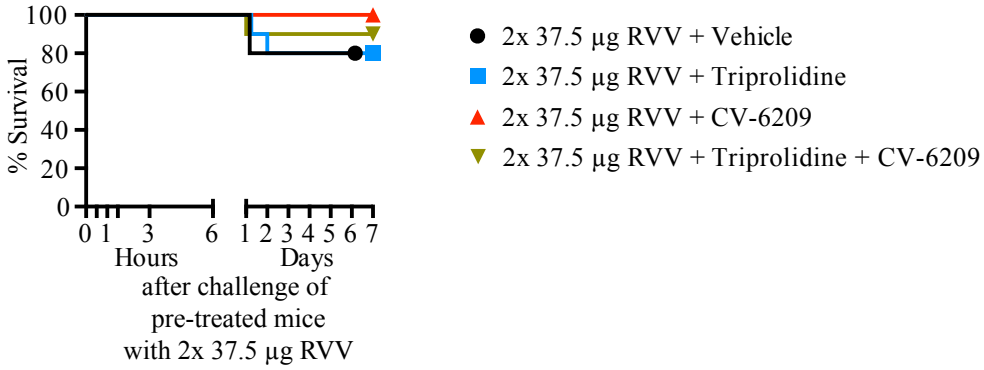
A

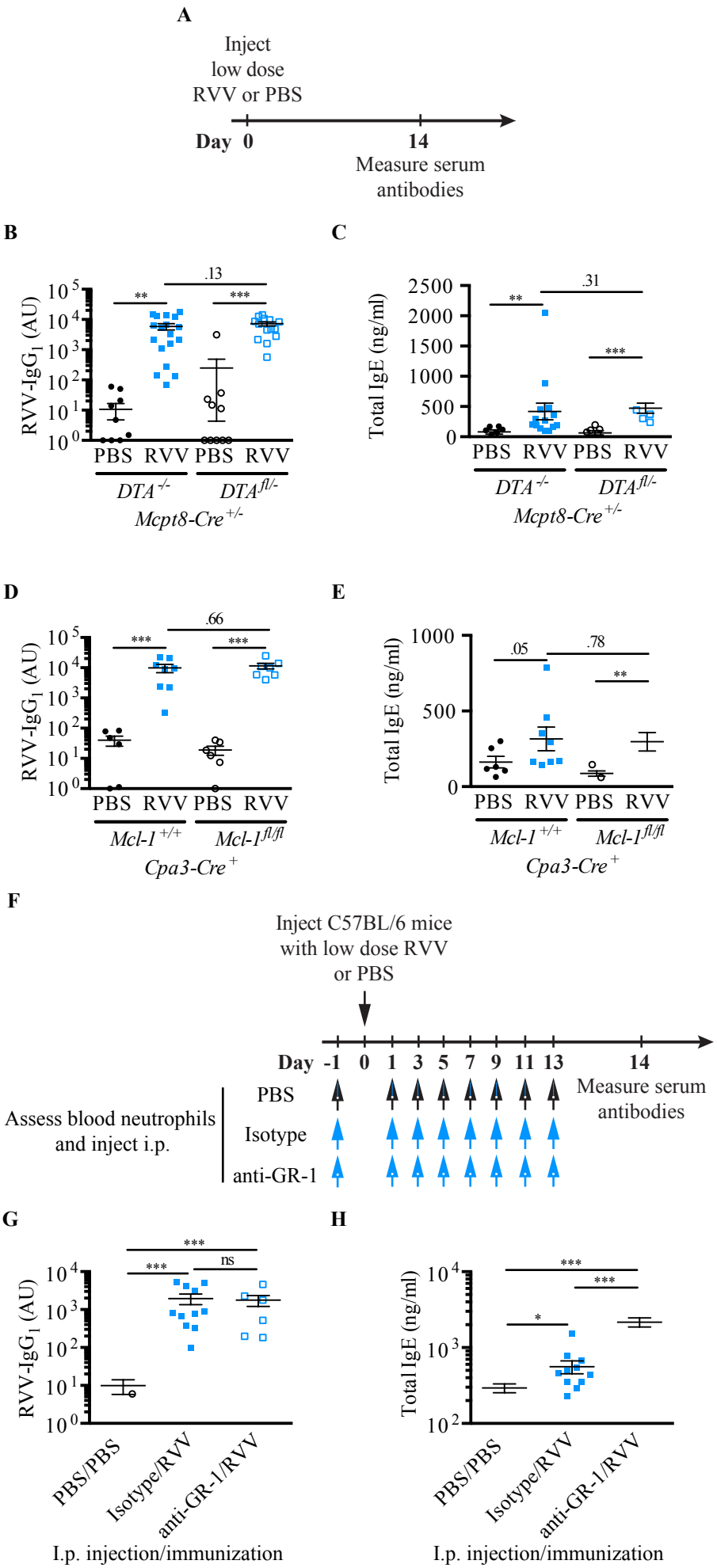

Inject
triprolidine
or PBS i.p.
Anesthetize and inject
CV-6209
or PBS i.v.
Challenge with
high dose RVV
Assess survival
Minute -60
-30
-5
0
Day 0
1
7
Sacrifice
Measure
body temperature

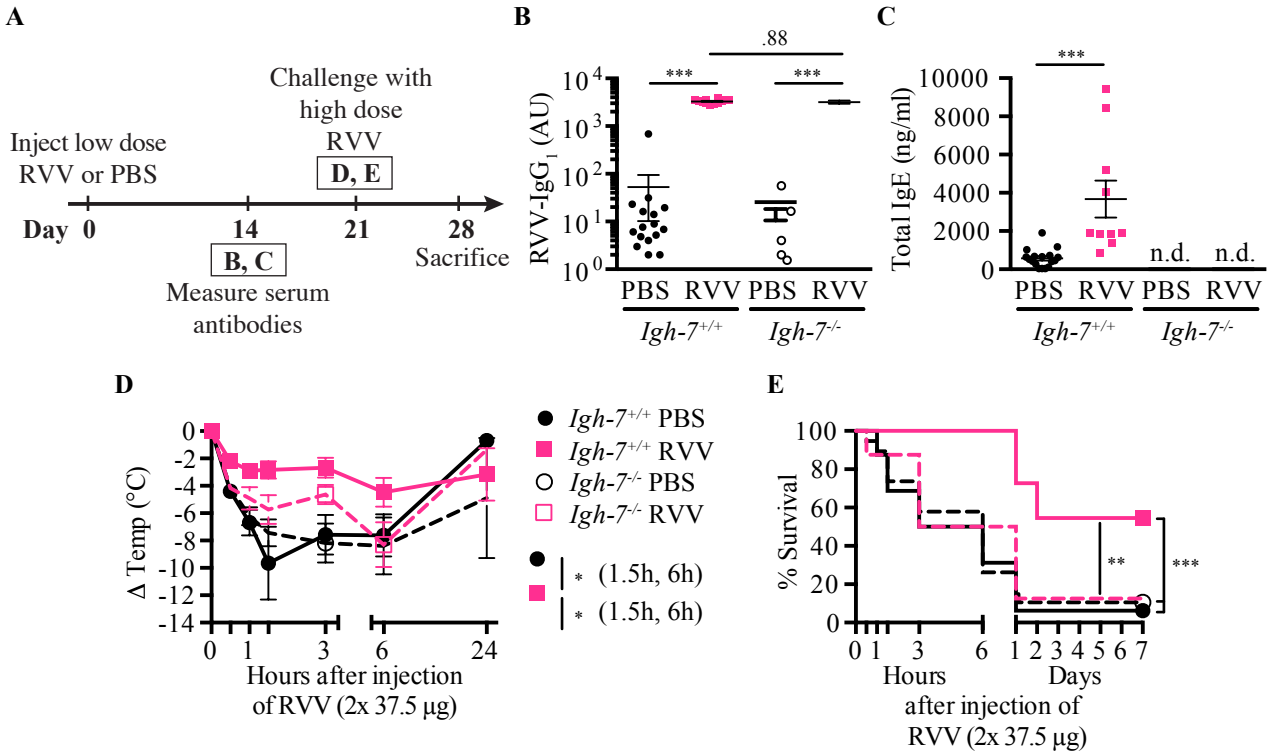
B

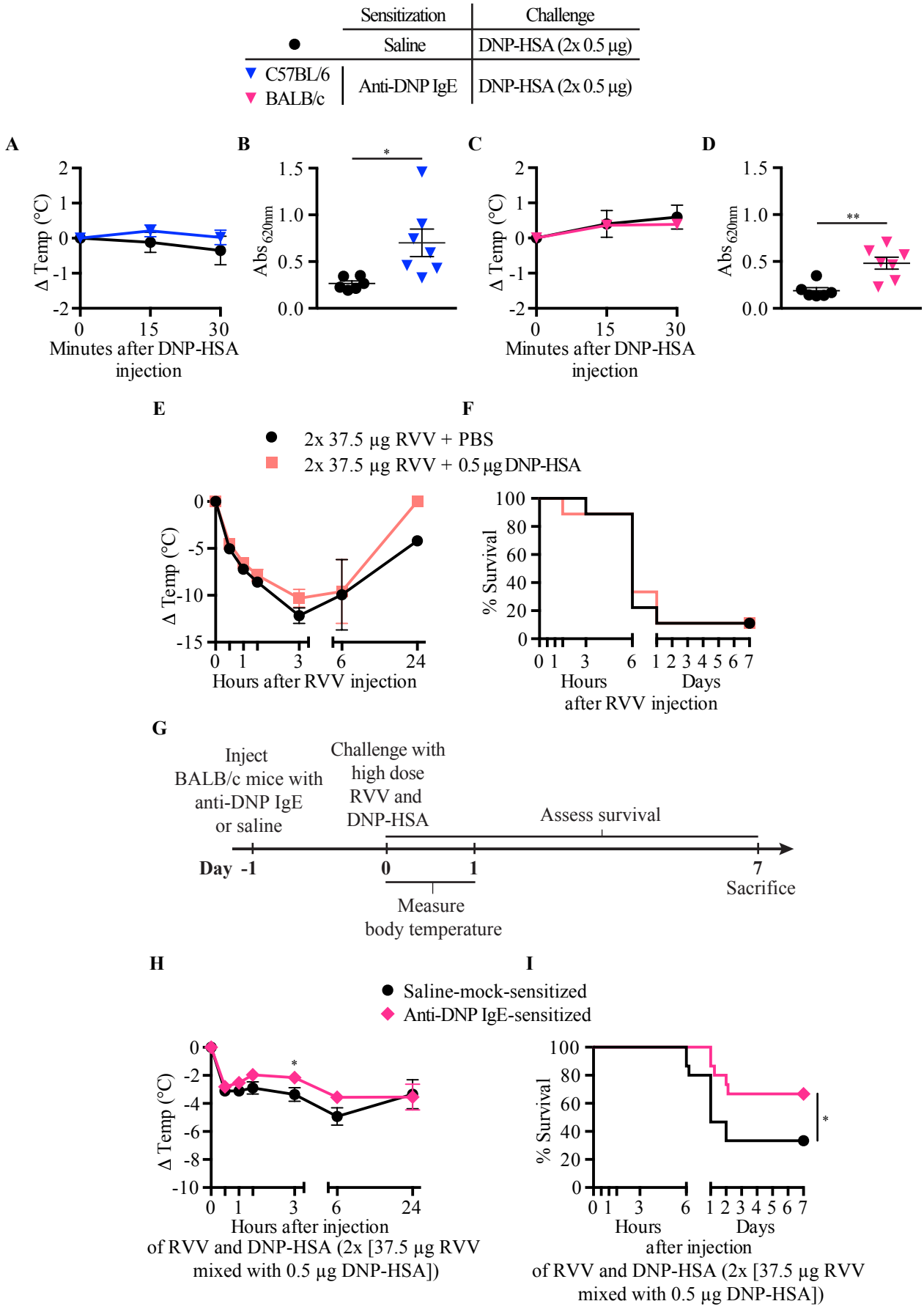

Δ Temp (°C)
0
-5
-10
0 20 40 60 90 6 24
Minutes
Hours
after challenge of
pre-treated mice
with 2x 37.5 µg RVV
Δ Temp (°C)
0
-1
-2
-3
-4
0 20 40 60 90
Minutes
after challenge of
pre-treated mice
with 2x 37.5 µg RVV

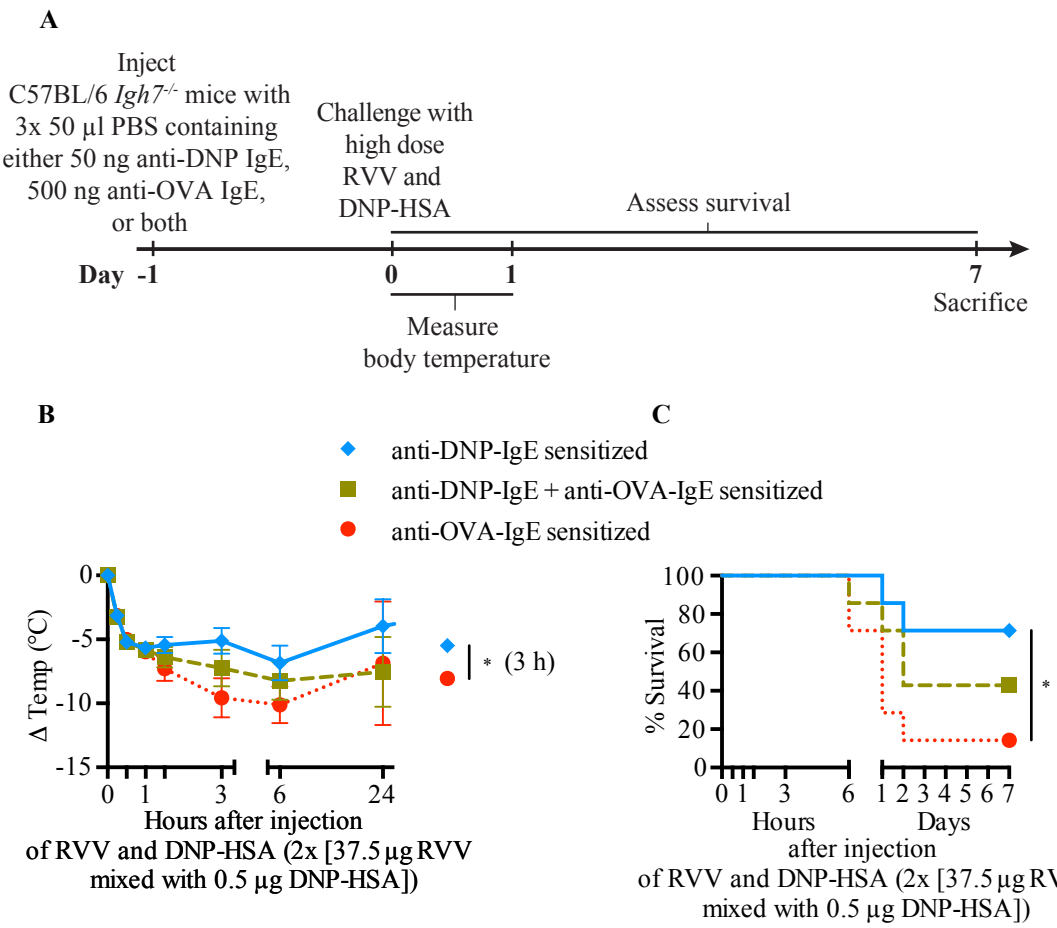
C

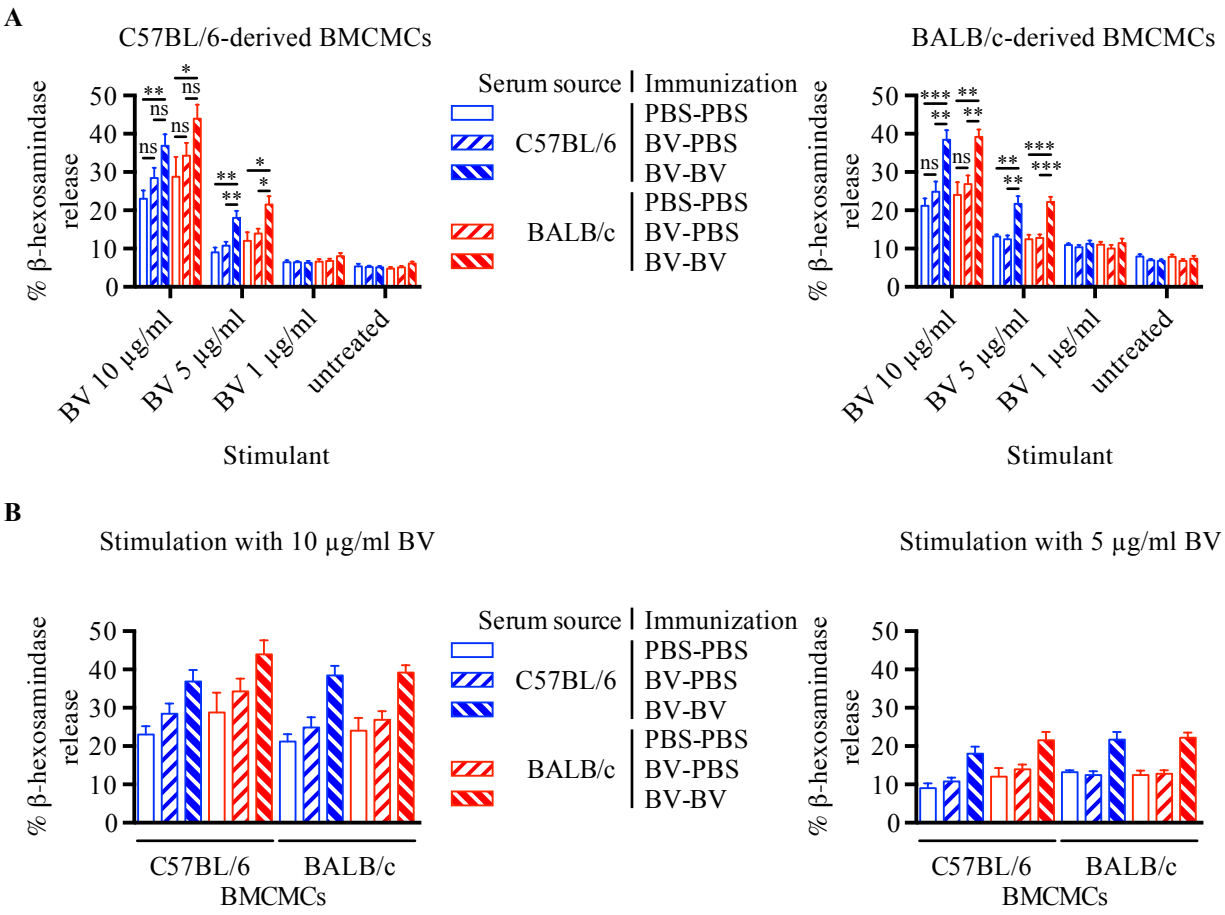

% Survival
100
80
60
40
20
0
0 1 3 6 1 2 3 4 5 6 7
Hours
Days
after challenge of
pre-treated mice
with 2x 37.5 µg RVV
2x 37.5 µg RVV + Vehicle
2x 37.5 µg RVV + Triprolidine
2x 37.5 µg RVV + CV-6209
2x 37.5 µg RVV + Triprolidine + CV-6209


A
Inject
low dose
RVV or PBS
Day 0
14
Measure serum
antibodies
B
RVV-IgG1 (AU)
.13
**
***
PBS RVV PBS RVV
DTA-/-
DTAfl/-
Mcpt8-Cre+/-
C
Total IgE (ng/ml)
.31
**
***
PBS RVV PBS RVV
DTA-/-
DTAfl/-
Mcpt8-Cre+/-
D
RVV-IgG1 (AU)
.66
***
***
PBS RVV PBS RVV
Mcl-1+/+
Mcl-1fl/fl
Cpa3-Cre+
E
Total IgE (ng/ml)
.05
.78
**
PBS RVV PBS RVV
Mcl-1+/+
Mcl-1fl/fl
Cpa3-Cre+
F
Inject C57BL/6 mice
with low dose RVV
or PBS
Day -1 0 1 3 5 7 9 11 13 14
Measure serum
antibodies
Assess blood neutrophils
and inject i.p.
PBS
Isotype
anti-GR-1
G
RVV-IgG1 (AU)
***
***
ns
PBS/PBS
Isotype/RVV
anti-GR-1/RVV
I.p. injection/immunization
H
Total IgE (ng/ml)
***
***
*
PBS/PBS
Isotype/RVV
anti-GR-1/RVV
I.p. injection/immunization


A
Inject low dose RVV or PBS
Challenge with high dose RVV
D, E
Day 0
14
21
28
Sacrifice
B, C
Measure serum antibodies
B
.88
***
***
RVV-IgG1 (AU)
PBS RVV PBS RVV
Igh-7+/+
Igh-7-/-
C
***
Total IgE (ng/ml)
n.d. n.d.
PBS RVV PBS RVV
Igh-7+/+
Igh-7-/-
D
Δ Temp (°C)
Igh-7+/+ PBS
Igh-7+/+ RVV
Igh-7-/- PBS
Igh-7-/- RVV
* (1.5h, 6h)
* (1.5h, 6h)
Hours after injection of RVV (2x 37.5 µg)
E
% Survival
**
***
Hours
Days
after injection of RVV (2x 37.5 µg)


Sensitization
Challenge
Saline
DNP-HSA (2x 0.5 µg)
C57BL/6
BALB/c
Anti-DNP IgE
DNP-HSA (2x 0.5 µg)
A
B
C
D
Δ Temp (°C)
Minutes after DNP-HSA injection
Abs 620nm
*
**
E
F
2x 37.5 µg RVV + PBS
2x 37.5 µg RVV + 0.5 µg DNP-HSA
Hours after RVV injection
% Survival
Hours
Days
after RVV injection
G
Inject BALB/c mice with anti-DNP IgE or saline
Challenge with high dose RVV and DNP-HSA
Assess survival
Day -1
0
1
7
Sacrifice
Measure body temperature
H
I
Saline-mock-sensitized
Anti-DNP IgE-sensitized
Hours after injection of RVV and DNP-HSA (2x [37.5 µg RVV mixed with 0.5 µg DNP-HSA])
Hours after injection of RVV and DNP-HSA (2x [37.5 µg RVV mixed with 0.5 µg DNP-HSA])


A
Inject
C57BL/6 Igh7-/- mice with
3x 50 µl PBS containing
either 50 ng anti-DNP IgE,
500 ng anti-OVA IgE,
or both
Challenge with
high dose
RVV and
DNP-HSA
Assess survival
Day -1
0
1
7
Sacrifice
Measure
body temperature
B
C
anti-DNP-IgE sensitized
anti-DNP-IgE + anti-OVA-IgE sensitized
anti-OVA-IgE sensitized
Δ Temp (°C)
0
-5
-10
-15
0 1 3 6 24
* (3 h)
Hours after injection
of RVV and DNP-HSA (2x [37.5 µg RVV
mixed with 0.5 µg DNP-HSA])
% Survival
100
80
60
40
20
0
0 1 3 6 1 2 3 4 5 6 7
*
Hours
Days
after injection
of RVV and DNP-HSA (2x [37.5 µg RVV
mixed with 0.5 µg DNP-HSA])


A
C57BL/6-derived BMCMCs
BALB/c-derived BMCMCs
% β-hexosamindase release
Serum source
Immunization
C57BL/6
BALB/c
PBS-PBS
BV-PBS
BV-BV
BV 10 µg/ml
BV 5 µg/ml
BV 1 µg/ml
untreated
Stimulant
B
Stimulation with 10 µg/ml BV
Stimulation with 5 µg/ml BV
C57BL/6 BMCMCs
BALB/c BMCMCs