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Supplemental Information

A Beneficial Role for Immunoglobulin E in Host Defense against Honeybee Venom

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Supplemental Inventory

Figure S1

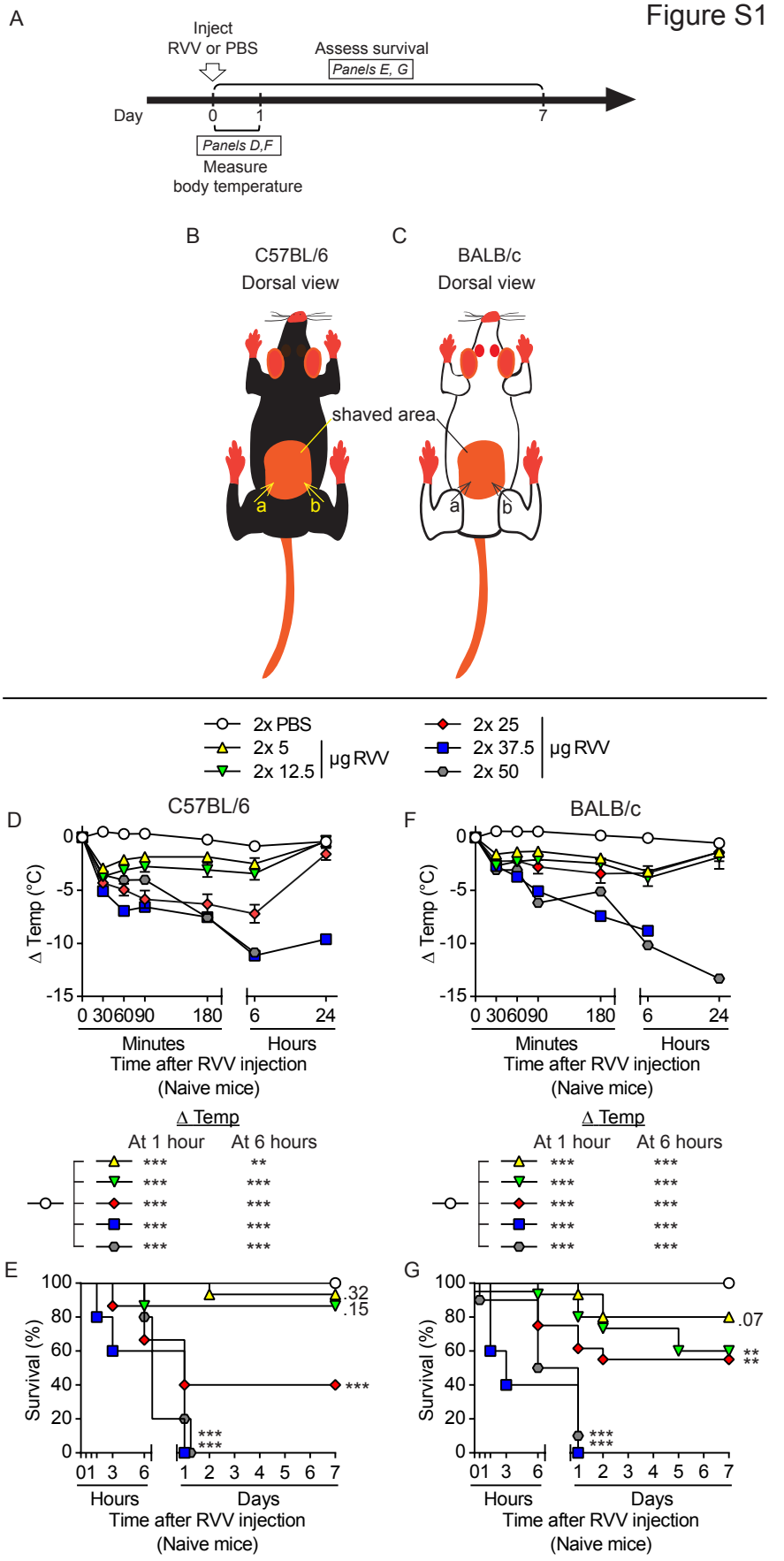
A
Inject
RVV or PBS
Assess survival
Panels E, G
Day
0
1
7
Panels D,F
Measure
body temperature
B
C57BL/6
Dorsal view
C
BALB/c
Dorsal view
shaved area
a
b
2x PBS
2x 5
2x 12.5
µg RVV
2x 25
2x 37.5
2x 50
µg RVV
D
C57BL/6
F
BALB/c
Δ Temp (°C)
0
-5
-10
-15
0 30 60 90
180
6
24
Minutes
Hours
Time after RVV injection
(Naive mice)
Δ Temp
At 1 hour
At 6 hours
***
**
E
G
Survival (%)
100
80
60
40
20
0
.32
.15
.07
01 3 6
1 2 3 4 5 6 7
Hours
Days
Time after RVV injection
(Naive mice)

Figure S1. Acute Systemic Responses of Naive C57BL/6 and BALB/c Mice to Increasing Doses of Russell's Viper Venom (RVV), Related to Figure 1

(A) Experimental outline. (B and C) Schematic showing sites of s.c. injection of RVV (or PBS) in (B) C57BL/6 and (C) BALB/c mice. Arrowheads indicate the injection sites in the shaved area. The two injections of RVV or PBS were carried out in alphabetical order (see description for D-G below). Female (D and E) C57BL/6 and (F and G) BALB/c WT mice received 2 subcutaneous injections (in the back; see B and C, arrows a and b) of 50 µl PBS containing increasing amounts of RVV (5-50 µg of RVV per injection) for a total of 10 to 100 µg. Mock-treated control mice were injected with PBS only. (D and F) Changes in body temperature after challenge (Δ Temp [mean ± SEM]) and (E and G) survival (% of live animals) were monitored at the indicated times. *P* values shown are *versus* PBS-treated mice and were calculated by (D and F) Student's *t* test or (E and G) Mantel-Cox test. (D-G) Data are from one experiment with 6 animals (for groups receiving 2x 37.5 µg or 2x 50 µg RVV) or pooled from 3 independent experiments (n=15/group). **, $P < 0.01$; ***, $P < 0.001$ *versus* PBS; numbers in E and G are *P* values for comparisons to the PBS group that were not significant ($P > 0.05$).

Figure S2

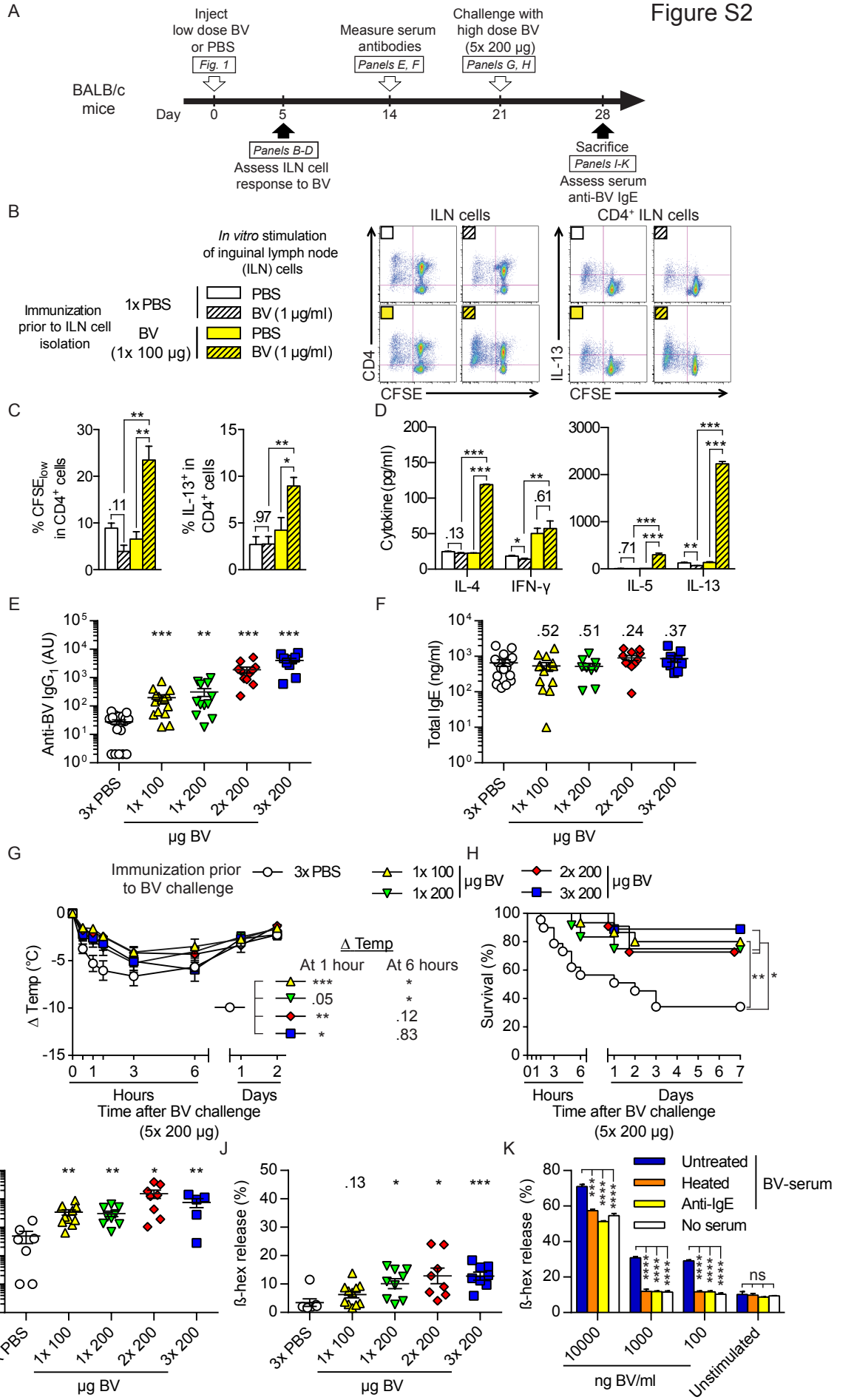
A
Inject low dose BV or PBS
Fig. 1
Measure serum antibodies
Panels E, F
Challenge with high dose BV (5x 200 µg)
Panels G, H
BALB/c mice
Day 0 5 14 21 28
Panels B-D
Assess ILN cell response to BV
Sacrifice
Panels I-K
Assess serum anti-BV IgE
B
In vitro stimulation of inguinal lymph node (ILN) cells
Immunization prior to ILN cell isolation
1x PBS
BV (1x 100 µg)
PBS
BV (1 µg/ml)
ILN cells
CD4+ ILN cells
CD4
IL-13
CFSE
C
% CFSElow in CD4+ cells
% IL-13+ in CD4+ cells
D
Cytokine (pg/ml)
IL-4
IFN-γ
IL-5
IL-13
E
Anti-BV IgG1 (AU)
F
Total IgE (ng/ml)
3x PBS
1x 100
1x 200
2x 200
3x 200
µg BV
G
Immunization prior to BV challenge
3x PBS
1x 100
1x 200
µg BV
2x 200
3x 200
µg BV
Δ Temp (°C)
Δ Temp
At 1 hour
At 6 hours
Hours
Days
Time after BV challenge
(5x 200 µg)
H
Survival (%)
Hours
Days
Time after BV challenge
(5x 200 µg)
I
Anti-BV IgE (AU)
J
ß-hex release (%)
K
ß-hex release (%)
Untreated
Heated
Anti-IgE
No serum
BV-serum
ng BV/ml
Unstimulated
ns

Figure S2. Injection of a Sublethal Dose of BV Induces a Th2 Cell Immune Response that Can Increase the Resistance of BALB/c Mice to the Hypothermia and Mortality Caused by a Subsequent Challenge with a Potentially Lethal Dose of BV, Related to Figure 2

(A) Experimental outline. For assessment of the ILN cell response in B-D, mice were injected s.c. with 1x 100 µg BV or 1x PBS. In panels E-J, mice were injected with 3x PBS, 1x 100, 1x 200, 2x 200 or 3x 200 µg BV and challenged 3 weeks later with 5x 200 µg BV. (B and C) Flow cytometry analysis of CFSE-labeled ILN cells stimulated for 4 days with 1 µg/ml BV or PBS. (B) Representative dot plots and (C) quantification (pooled from 3 independent experiments) of proliferation (% $CFSE_{low}$) and intracellular IL-13 (% IL-13^+) of $CD4^+$ ILN cells. (D) IL-4, -5, -13, and IFN-γ in supernatants of CFSE-labeled ILN cells after 4 days of BV stimulation *in vitro*. (E) BV-specific IgG_1 and (F) total IgE antibody levels in serum obtained two weeks after BV immunization or PBS injection. (G) Changes in body temperature (Δ Temp) and (H) survival (% of live animals) of mice challenged with 5x 200 µg BV three weeks after BV immunization. (I) Titers of BV-specific IgE in sera collected 7 days after venom challenge from all surviving mice whose data are reported in H. (J) BALB/c BMCMCs were sensitized with sera of individual mice, stimulated with 1 µg/ml BV, and the amount of ß-hexosaminidase (ß-hex) in the cell supernatant was measured to assess BMCMC degranulation. (K) Pooled serum of BV-immunized and challenged BALB/c mice (BV-serum, which was either heated to eliminate the ability of IgE to bind to FcεRI [heated], treated with rat anti-mouse IgE [anti-IgE], or mock-treated with PBS [untreated]) was used to sensitize C57BL/6 BMCMCs, which were then stimulated with different concentrations of BV (100 – 10,000 ng/ml) or an equivalent volume of PBS (unstimulated). (C-F) Data are pooled from 3 independent experiments (n=9-19/group). (C to G, I and J) Values are mean ± or + SEM (E, F, I and J also show values for individual mice). (K) Values shown are mean + SD of triplicate values from one representative of 3 independent experiments. *P* values are *versus* (C, D) *in vitro* PBS-treated cells and (E-H, I, J) PBS-injected mice or (K) cells sensitized with untreated BV-serum. (C-G and I-K) Student's *t* test or (H) Mantel-Cox test. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (C, D and K) for the indicated comparisons or (E-J) *versus* PBS; the numbers in C, D, F, G and J are the *P* values for comparisons that were not significant ($P > 0.05$). ns, not significant ($P > 0.05$).

Figure S3

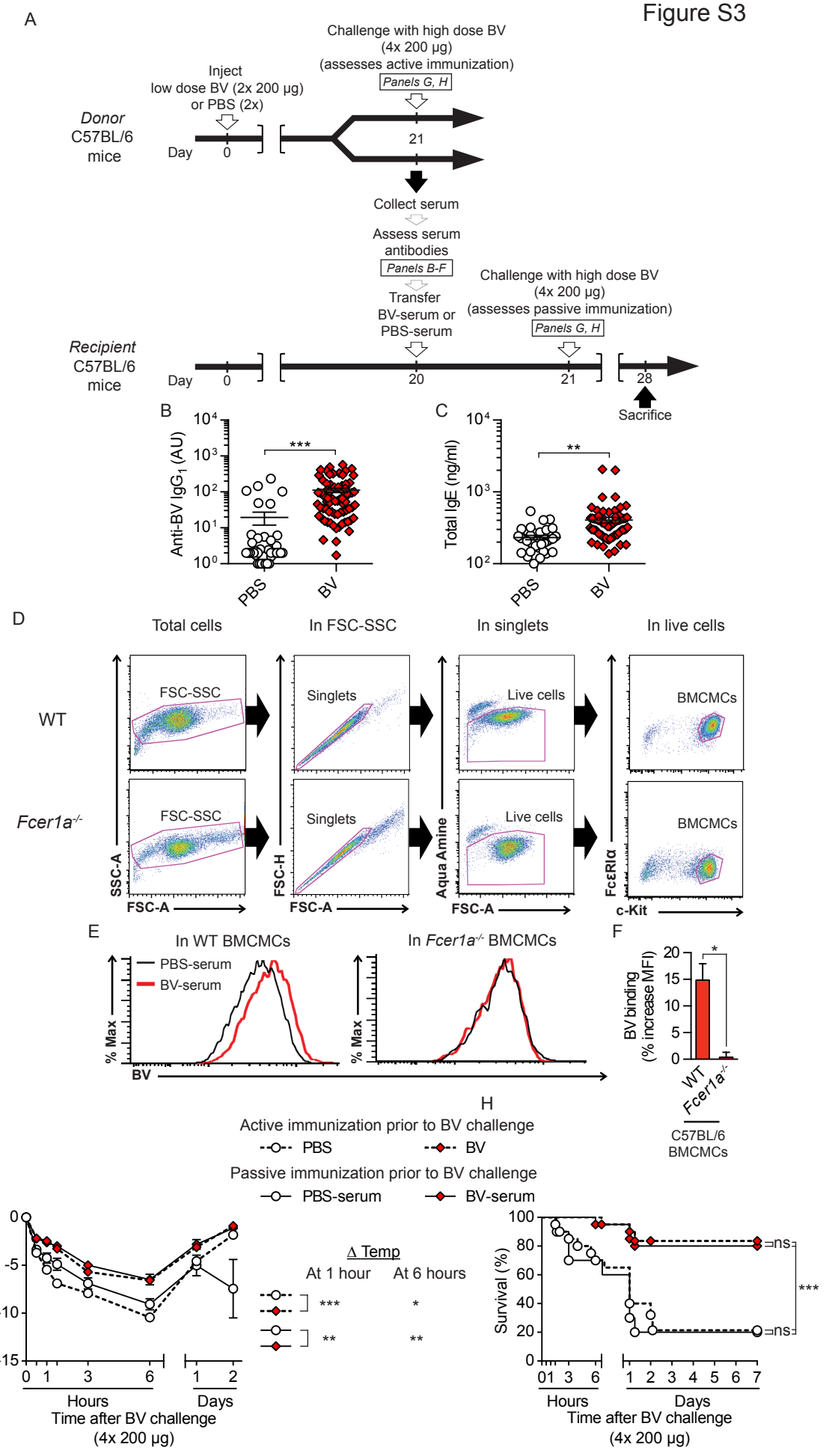
A
Challenge with high dose BV
(4x 200 µg)
(assesses active immunization)
Panels G, H
Inject
low dose BV (2x 200 µg)
or PBS (2x)
Donor
C57BL/6
mice
Day 0
21
Collect serum
Assess serum
antibodies
Panels B-F
Transfer
BV-serum or
PBS-serum
Challenge with high dose BV
(4x 200 µg)
(assesses passive immunization)
Panels G, H
Recipient
C57BL/6
mice
Day 0
20
21
28
Sacrifice
B
Anti-BV IgG1 (AU)
***
PBS
BV
C
Total IgE (ng/ml)
**
PBS
BV
D
Total cells
In FSC-SSC
In singlets
In live cells
WT
Fcer1a-/-
FSC-SSC
Singlets
Live cells
BMCMCs
SSC-A
FSC-A
FSC-H
Aqua Amine
FcεRIα
c-Kit
E
In WT BMCMCs
In Fcer1a-/- BMCMCs
PBS-serum
BV-serum
% Max
BV
F
BV binding
(% increase MFI)
*
WT
Fcer1a-/-
C57BL/6
BMCMCs
G
H
Active immunization prior to BV challenge
PBS
BV
Passive immunization prior to BV challenge
PBS-serum
BV-serum
Δ Temp (°C)
Hours
Days
Time after BV challenge
(4x 200 µg)
Δ Temp
At 1 hour
At 6 hours
***
*
**
**
Survival (%)
ns
ns
***
Hours
Days
Time after BV challenge
(4x 200 µg)

Figure S3. Passive Immunization of Naive C57BL/6 Mice with Serum of BV-Immunized Mice Can Increase Their Resistance to a Subsequent Challenge with a Potentially Lethal Dose of BV, Related to Figure 3

(A) Experimental outline. *Donor* C57BL/6 mice were injected with 2x 200 µg BV or PBS. Three weeks later, sera from PBS- and BV- injected mice were collected and BV-specific (B) IgG_1, and (C) total IgE levels were measured by ELISA. (B and C) Data shown as values from individual mice and as mean ± SEM, and are pooled from 2 independent experiments ([B and C] n=40 or 80 for PBS- or BV- injected mice, respectively). (D-F) C57BL/6 WT or *Fcer1a*$^{-/-}$ BMCMCs were sensitized with pooled serum collected from BV-immunized (BV-serum) or PBS-injected (PBS-serum) mice and then incubated with biotinylated BV and cell-bound biotinylated BV was detected by flow cytometry. (D) Representative gating strategies of WT and *Fcer1a*$^{-/-}$ BMCMCs after elimination of non-singlet and dead (i.e., aqua amine-positive) cells. (E) Representative histograms and (F) quantification of mean fluorescence intensity (MFI, pooled from ≥ 2 independent experiments) of BV binding on BMCMCs (expressed as the % increase in MFI of BV-serum when compared to PBS-serum). (G and H) Naive 10 to 11 week old C57BL/6 mice were passively immunized by i.v. injection of 250 µl pooled serum from PBS-injected (PBS-serum, white circles/solid lines) or BV-immunized mice (BV-serum, red diamonds/solid lines). Twenty-two hours later, all mice were challenged with 4x 200 µg BV. Age-matched C57BL/6 mice that were actively immunized 3 weeks earlier with 2x 200 µg BV (red diamonds/dashed lines) or PBS (white circles/dashed lines) were used as controls. (G) Changes in body temperature (Δ Temp [mean ± SEM]) and (H) survival (% of live animals) were monitored at the indicated times. *P* values shown were calculated by (B, C, F, and G) Student's *t* test or (H) Mantel-Cox test. (G and H) Data shown are pooled from 2-4 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the indicated comparisons. ns, not significant ($P > 0.05$).

Figure S4

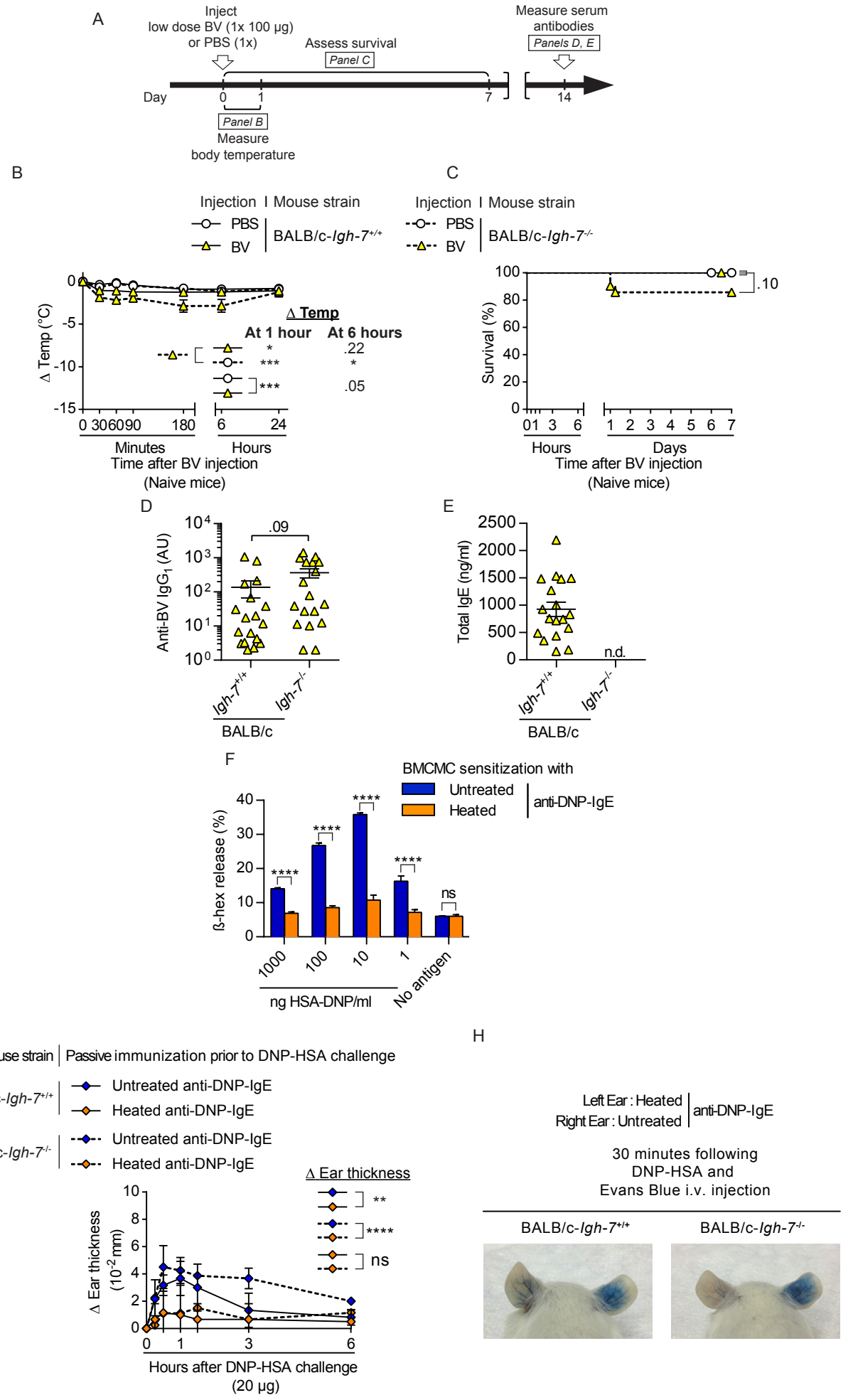
A
Inject
low dose BV (1x 100 µg)
or PBS (1x)
Assess survival
Panel C
Measure serum
antibodies
Panels D, E
Day
0
1
7
14
Panel B
Measure
body temperature
B
Injection | Mouse strain
PBS
BV
BALB/c-Igh-7+/+
Δ Temp
At 1 hour
At 6 hours
Δ Temp (°C)
Minutes
Hours
Time after BV injection
(Naive mice)
C
Injection | Mouse strain
PBS
BV
BALB/c-Igh-7-/-
Survival (%)
Hours
Days
Time after BV injection
(Naive mice)
D
Anti-BV IgG1 (AU)
.09
Igh-7+/+
Igh-7-/-
BALB/c
E
Total IgE (ng/ml)
n.d.
Igh-7+/+
Igh-7-/-
BALB/c
F
BMCMC sensitization with
Untreated
Heated
anti-DNP-IgE
ß-hex release (%)
ns
1000
100
10
1
No antigen
ng HSA-DNP/ml
G
Mouse strain | Passive immunization prior to DNP-HSA challenge
BALB/c-Igh-7+/+
Untreated anti-DNP-IgE
Heated anti-DNP-IgE
BALB/c-Igh-7-/-
Untreated anti-DNP-IgE
Heated anti-DNP-IgE
Δ Ear thickness
Δ Ear thickness (10-2 mm)
ns
Hours after DNP-HSA challenge
(20 µg)
H
Left Ear : Heated
Right Ear : Untreated
anti-DNP-IgE
30 minutes following
DNP-HSA and
Evans Blue i.v. injection
BALB/c-Igh-7+/+
BALB/c-Igh-7-/-

Figure S4. Responses of IgE-Deficient (*Igh-7*$^{-/-}$) and Corresponding Wild Type (*Igh-7*$^{+/+}$) Mice to Immunization with a Sublethal Dose of BV and to IgE-Dependent Passive Cutaneous Anaphylaxis, Related to Figure 4

(A) Experimental outline for panels B to E. (B and C) IgE-deficient BALB/c or age-matched wild type controls were immunized with 1x 100 µg BV, or were injected with an equivalent volume of PBS. (B) Changes in body temperature (Δ Temp) and (C) survival (% of live animals) were monitored at the indicated times. Serum was collected 14 days later and (D) BV-specific IgG_1 and (E) total IgE serum antibody levels were analyzed by ELISA. (B-E) Data are pooled from 4 independent experiments (n=17-19/group). (B, D and E) Values are mean ± SEM (D and E also show values for individual mice) (F) *In vitro* assessment of DNP-IgE functionality. C57BL/6 BMCMCs were sensitized with untreated or heated DNP-specific IgE (anti-DNP-IgE), stimulated with DNP-HSA, and the amount of ß-hexosaminidase (ß-hex) in the cell supernatant was measured to assess BMCMC degranulation. Values shown are mean + SD of triplicate values from one representative of 2 independent experiments. (G and H) *In vivo* assessment of anti-DNP-IgE functionality. IgE-deficient BALB/c and wild type control mice were sensitized intradermally in the ear pinnae with untreated or heated anti-DNP-IgE and challenged by intravenous injection of DNP-HSA and Evans Blue. (G) Ear swelling was measured at the indicated time points. (H) Vascular extravasation (indicated by Evans Blue leakage from blood vessels) was assessed by visual evaluation of the ear 30 minutes following challenge. (G) Data shown are means ± SD from one experiment (n=3-4 mice/group). (H) Representative picture of one out of 3-4 mice, each mouse giving similar results. *P* values are calculated by (B, D, E, F) Student's *t* test, (C) Mantel-Cox test, or (G) 2-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ for the indicated comparisons; the numbers in B, C and D are the *P* values for indicated comparisons that were not significant ($P > 0.05$). ns, not significant ($P > 0.05$); n.d., not detectable.

Figure S5

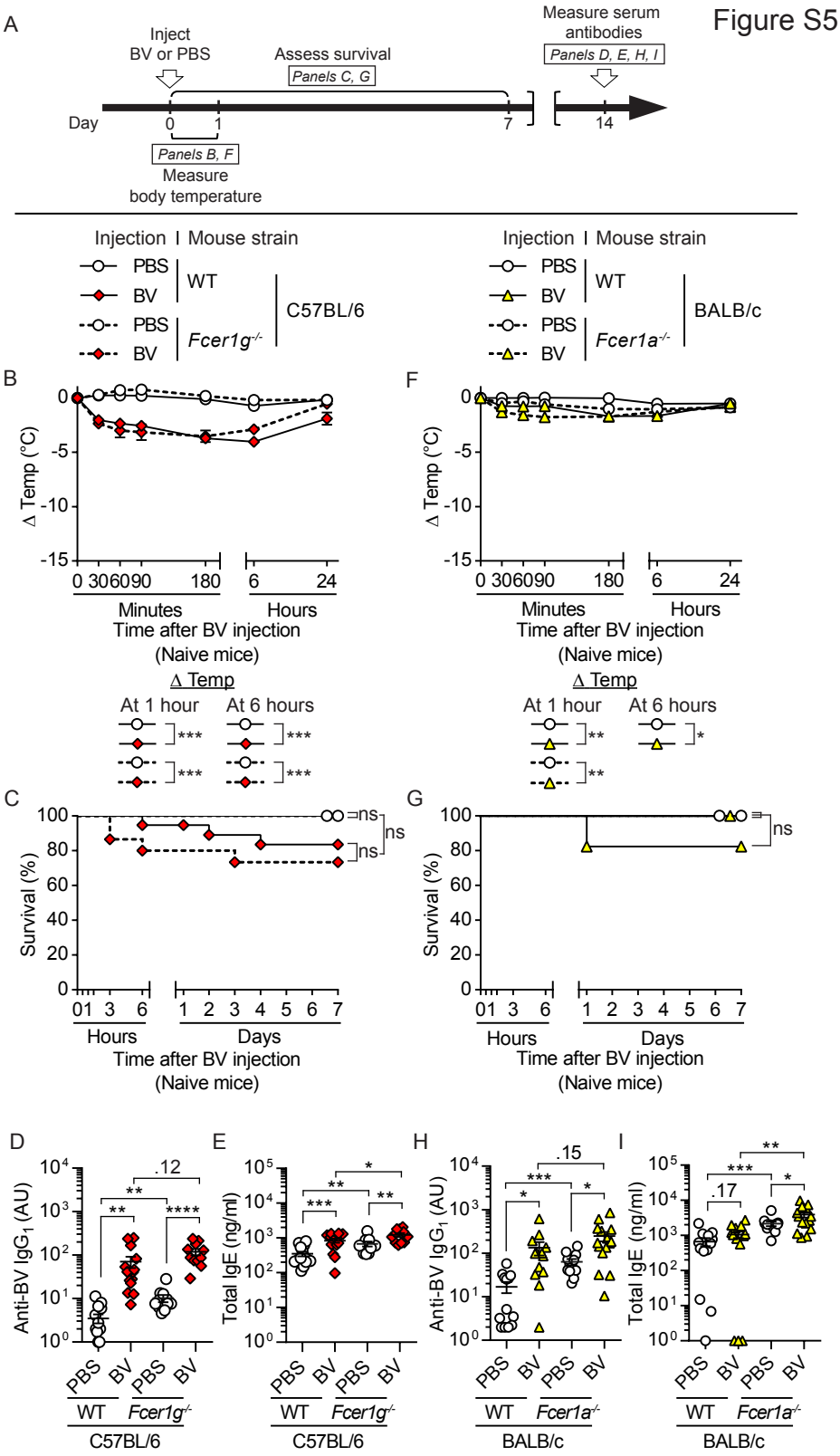
A
Inject
BV or PBS
Assess survival
Panels C, G
Measure serum
antibodies
Panels D, E, H, I
Day
0
1
7
14
Panels B, F
Measure
body temperature
Injection I Mouse strain
PBS
BV
WT
Fcer1g-/-
C57BL/6
Injection I Mouse strain
PBS
BV
WT
Fcer1a-/-
BALB/c
B
F
Δ Temp (°C)
0
-5
-10
-15
0 30 60 90 180 6 24
Minutes
Hours
Time after BV injection
(Naive mice)
Δ Temp
At 1 hour
At 6 hours
***
**
*
C
G
Survival (%)
100
80
60
40
20
0
0 1 3 6
1 2 3 4 5 6 7
Hours
Days
Time after BV injection
(Naive mice)
ns
D
E
H
I
Anti-BV IgG1 (AU)
Total IgE (ng/ml)
.12
.15
.17
PBS
BV
WT
Fcer1g-/-
C57BL/6
Fcer1a-/-
BALB/c

Figure S5. The Innate Acute Systemic Response and the Subsequent Development of the Humoral Response of Naive C57BL/6-*Fcer1g*$^{-/-}$ and BALB/c-*Fcer1a*$^{-/-}$ Mice following Immunization with a Sublethal Dose of BV Are Similar to Those of Their Respective WT Counterparts, Related to Figure 5

(A) Experimental outline. Seven to eight week old (B-E) C57BL/6-*Fcer1g*$^{-/-}$ and (F-I) BALB/c-*Fcer1a*$^{-/-}$ or age-matched control mice were immunized with 2x 200 µg or 1x 100 µg BV, respectively, or were injected with PBS (2x or 1x, respectively). (B and F) Changes in body temperature (Δ Temp) and (C and G) survival (% of live animals) were monitored at the indicated times. Serum (D and H) BV-specific IgG_1 and (E and I) total IgE serum antibody levels of (D and E) C57BL/6-*Fcer1g*$^{-/-}$ and control mice and (H and I) BALB/c-*Fcer1a*$^{-/-}$ and control mice 2 weeks after injection of PBS or BV ([B-E] 2x 200 µg or [F-I] 1x 100 µg BV). (B, D-F, H and I) Values are mean ± SEM (D, E, H and I also show values for individual mice). *P* values were calculated by (B, D-F, H and I) Student's *t* test or (C and G) Mantel-Cox test. All data shown are pooled from three independent experiments (n=12-19/group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ for the indicated comparisons; the numbers in D, H and I are the *P* values for the indicated comparisons that were not significant ($P > 0.05$). ns, not significant ($P > 0.05$).

Figure S6

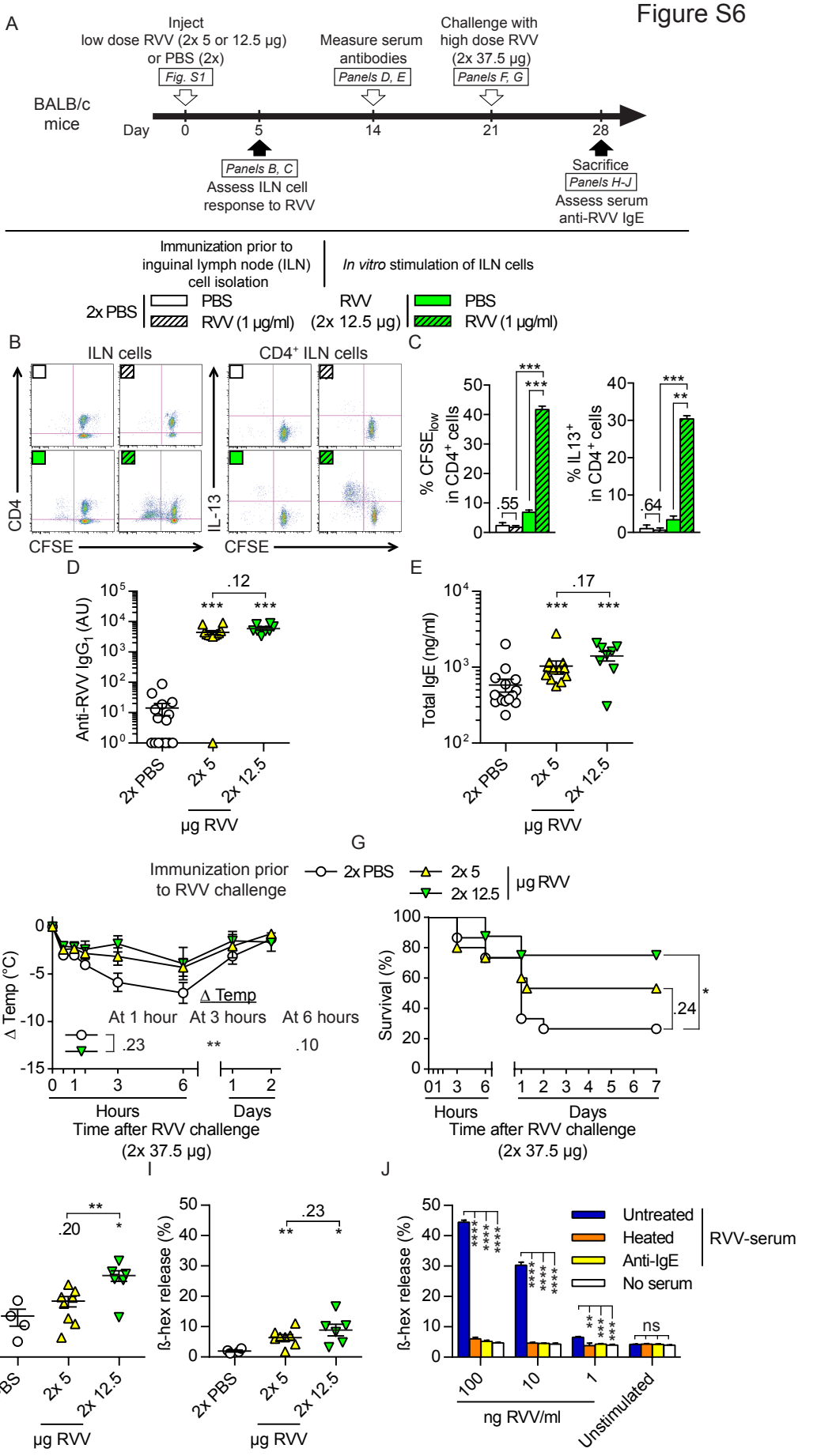
A
Inject
low dose RVV (2x 5 or 12.5 µg)
or PBS (2x)
Fig. S1
Measure serum
antibodies
Panels D, E
Challenge with
high dose RVV
(2x 37.5 µg)
Panels F, G
BALB/c
mice
Day
0
5
14
21
28
Panels B, C
Assess ILN cell
response to RVV
Sacrifice
Panels H-J
Assess serum
anti-RVV IgE
Immunization prior to
inguinal lymph node (ILN)
cell isolation
In vitro stimulation of ILN cells
2x PBS
PBS
RVV (1 µg/ml)
RVV
(2x 12.5 µg)
PBS
RVV (1 µg/ml)
B
ILN cells
CD4⁺ ILN cells
CD4
CFSE
IL-13
CFSE
C
% CFSE$_{low}$ in CD4⁺ cells
% IL13⁺ in CD4⁺ cells
.55
.64
D
Anti-RVV IgG$_1$ (AU)
.12
2x PBS
2x 5
2x 12.5
µg RVV
E
Total IgE (ng/ml)
.17
2x PBS
2x 5
2x 12.5
µg RVV
F
Immunization prior
to RVV challenge
2x PBS
2x 5
2x 12.5
µg RVV
Δ Temp (°C)
Δ Temp
At 1 hour
At 3 hours
At 6 hours
.23
.10
Hours
Days
Time after RVV challenge
(2x 37.5 µg)
G
Survival (%)
.24
Hours
Days
Time after RVV challenge
(2x 37.5 µg)
H
Anti-RVV IgE (AU)
.20
2x PBS
2x 5
2x 12.5
µg RVV
I
ß-hex release (%)
.23
2x PBS
2x 5
2x 12.5
µg RVV
J
ß-hex release (%)
Untreated
Heated
Anti-IgE
No serum
RVV-serum
ns
100
10
1
Unstimulated
ng RVV/ml

Figure S6. Injection of a Sublethal Dose of RVV Induces a Th2 Cell Immune Response that Can Increase the Resistance of BALB/c Mice to the Hypothermia and Mortality Caused by Subsequent Challenge with a Potentially Lethal Dose of RVV, Related to Figure 7

(A) Experimental outline. (B and C) For assessment of the ILN cell response in B and C, mice were injected s.c. with 2x 12.5 µg RVV or PBS. In panels D-I, mice were injected with PBS, 2x 5 µg RVV or 2x 12.5 µg RVV and challenged 3 weeks later with 2x 37.5 µg RVV. (B and C) Flow cytometry analysis of CFSE-labeled ILN cells stimulated for 4 days with 1 µg/ml RVV. (B) Representative dot plots and (C) quantification (pooled from 2 independent experiments) of proliferation (% $CFSE_{low}$) and intracellular IL-13 (% IL-13^{+}) of $CD4^{+}$ ILN cells. (D) RVV-specific IgG_1 and (E) total IgE antibody levels in serum obtained two weeks after RVV immunization or mock immunization with PBS. (F) Changes in body temperature (Δ Temp) and (G) survival (% of live animals) of mice challenged with 2x 37.5 µg RVV three weeks after immunization with RVV or mock immunization with PBS. (H) Titers of RVV-specific IgE in sera collected 7 days after venom challenge from all surviving mice whose data are reported in G. (I) BALB/c BMCMCs were sensitized with sera of individual mice, stimulated with 1 µg/ml RVV, and the amount of ß-hexosaminidase (ß-hex) in the cell supernatant was measured to assess BMCMC degranulation. (J) Pooled serum of RVV-immunized and challenged BALB/c mice (RVV-serum, which was either heated to eliminate the ability of IgE to bind to FcεRI [heated], treated with rat anti-mouse IgE [anti-IgE], or mock-treated with PBS [untreated]) was used to sensitize C57BL/6 BMCMCs, which were then stimulated with different concentrations of RVV (1 – 100 ng/ml) or an equivalent volume of PBS (unstimulated). (C-I) Data are pooled from 3 independent experiments ([C-G] n=8-15/group; [H and I] n=4-8/group). (C-F, H and I) Values are mean ± or + SEM (D, E, H and I also show values for individual mice). (J) Values shown are mean + SD of triplicate values from one representative of 3 independent experiments. *P* values are *versus* (C) *in vitro* PBS-treated cells and (D-F, H, I) PBS-injected mice or (J) cells sensitized with untreated RVV-serum. (C-F and H-J) Student's *t* test or (G) Mantel-Cox test. * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ for the indicated comparisons (C-E and J) or *versus* PBS (E-J); the numbers in C- I are the *P* values for comparisons that were not significant ($P > 0.05$). ns, not significant ($P > 0.05$).

Supplemental Experimental Procedures

Mice

For experiments involving WT mice (Figures 1-3, 7, S1, S2, S3 and S6) and production of serum from WT mice (Figures 4D-F, 5F-H, 6F-H), age-matched 5 to 7 week-old C57BL/6J or BALB/cJ female mice were purchased from Jackson Laboratories and were housed in the Research Animal Facilities at Stanford University for at least 7 days before starting experiments. All the other strains of mice were bred and housed in the Stanford Animal facilities to generate mice for experiments. Transgenic *Fcer1g*$^{-/-}$ mice on the C57BL/6J background (B6.129P2-FcεRIgtm1Rav N12, backcrossed 12 generations to C57BL/6J) were originally purchased from Taconic. WT C57BL/6J controls for *Fcer1g*$^{-/-}$ mice were originally purchased from Jackson Laboratories. Transgenic BALB/c-*Fcer1a*$^{-/-}$ mice were originally obtained from Jean-Pierre Kinet (Harvard Medical School, MA, USA). WT BALB/cJ controls for BALB/c-*Fcer1a*$^{-/-}$ mice were originally purchased from Jackson Laboratories. IgE-deficient mice (BALB/c-*Igh-7*$^{-/-}$) and the corresponding control (i.e., IgE-sufficient) wild type mice (BALB/c-*Igh-7*$^{+/+}$) were received from Hans Oettgen's laboratory (Harvard Medical School, MA, USA) and bred in our facilities. The first generation of pups born in our facilities became the breeders for the mice generated for our experiments. *Fcer1a*$^{-/-}$ mice on a C57BL/6 background (B6.129S2(Cg)-*Fcer1atm1Knt*/J; C57BL/6-*FcecC5*$^{-/-}$) were originally purchased from Jackson. MC-deficient C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were originally provided by Peter Besmer (Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) (Duttlinger et al., 1993); we then backcrossed these mice to C57BL/6J mice for more than 11 generations (Piliponsky et al., 2010). These mice (and C57BL/6-*Kit*$^{+/+}$ controls (Duttlinger et al., 1993)), as well as MC- and basophil-deficient C57BL/6-*Cpa3-Cre*$^{+}$*; Mcl-1*$^{fl/fl}$ mice (and the corresponding C57BL/6-*Cpa3-Cre*$^{+}$*; Mcl-1*$^{+/+}$ control mice (Lilla et al., 2011)) were housed and bred at the local animal facilities. Throughout the experiments involving transgenic mice, some transgenic and control mice were mixed together in the same cage for a duration of 1 to 5 weeks prior to immunization and/or challenge in order to avoid a potential bias due to differences in the microbiota between mice that were born from different breeders. When indicated, mice were anesthetized by inhalation of 3-4% isoflurane using a complete laboratory animal anesthesia system (VetEquip).

Reagents

Honeybee (*Apis mellifera*) venom (BV) was from ALK Abello Source Materials, Inc. Batches of freeze-dried complete BV were resuspended in sterile, endotoxin-free PBS (Gibco) at 4 mg/ml. Russell's viper (*Daboia russelii*) venom (RVV) was obtained from Sigma (Lot SLBB5602V) and was resuspended in PBS at 10 mg/ml. BV and RVV were stored in aliquots at -20°C. Different lots of BV were used. Lot 01271001HB was used for all *in vivo* experiments apart from experiments involving IgE-deficient mice (Figures S4 and 4), C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ and C57BL/6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice (Figures 6C-H) and serum transfer experiments with C57BL/6-*Fcer1g*$^{-/-}$, C57BL/6-*Fcer1a*$^{-/-}$ mice (Figures 5G and 5H), which were conducted using Lot 01271006HB. Lot 06270814HB was used for the *in vitro* experiments shown in Figure 2K and Figure S2K. All 3 lots were comparable in terms of analyzed parameters provided by the supplier, including protein content, ratios of venom constituents, and enzymatic activity. Carboxyfluorescein succinimidyl ester (CFSE) and Aqua Dead Cell Stain Kit (referred to in Figure S3D as Aqua Amine) were obtained from Life Technologies. Ionomycin calcium salt was from Cell Signaling. eFluor450-conjugated anti-mouse CD4 (RM4-5)-, Alexa Fluor 647-

conjugated anti-mouse IL-13 (clone eBio13A)-, allophycocyanain-conjugated anti-mouse CD117 (KIT) (clone 2B8)-specific antibodies and phycoerythrin-conjugated streptavidin were obtained from eBioscience. Pacific Blue-conjugated anti-mouse FcεRIα (clone MAR-1)-specific antibody was obtained from Biolegend. Rat anti-mouse IgE (clone R35-92), rat IgG_1 isotype control (clone R3-34) (used for experiments involving serum pre-treatment) were obtained from BD Pharmingen. Clones and sources of antibodies used in ELISA experiments are stated in the respective section below. Dinitrophenyl (DNP)-specific IgE (α-DNP clone ε26 (Liu et al., 1980)) was kindly provided by Dr Fu-Tong Liu (University of California-Davis)). p-Nitrophenyl-N-acetyl-ß-D-glucosaminide, dinitrophenyl$_{30-40}$-conjugated human serum albumin (DNP-HSA) and other chemicals were obtained from Sigma. Cell culture media and supplements were obtained from Sigma and Life Technologies.

Venom Injections and Active Immunization

A schematic overview of the active immunization protocol is shown in Figure 2A. Six to 8 week old WT or transgenic mice were shaved at the injection sites 24 h before injections and were consistently treated in the morning (without anesthesia) by administering one or several s.c. injections (see Figures 1B and 1C) of 50 µl PBS alone or containing either 2 mg/ml (= 100 µg per injection site, used for mice receiving 1x 100 µg) or 4 mg/ml BV (= 200 µg per injection site, used for all other immunizations; 100 - 200 µg is the amount of venom contained by a single honeybee (Schmidt, 1995)). All venom injections were performed by 2 experimenters and experiments were designed and performed to allow the immunization of all animals in any single experiment within minutes. The day of venom or PBS injection is designated day 0. For experiments using RVV, mice were immunized by two subcutaneous injections of 50 µl PBS containing different amounts of RVV (5 - 50 µg per injection), or were injected only with 2x 50 µl PBS (controls) (see Figures S1B and S1C). As noted above, BV and RVV used in these *in vivo* experiments each were from single lots. For analysis of the inguinal lymph node (ILN) cell response, ILNs were collected 5 days after immunization or PBS injection. For analysis of venom-induced antibody responses, blood was collected at day 14 from the retro-orbital vein of anesthetized mice. On day 20, mice were shaved on the belly and (if required) again on the back skin. On day 21, BV-immunized or PBS-injected (mock immunized; control) C57BL/6 WT or –*Fcer1g*$^{-/-}$ mice were challenged subcutaneously (without anesthesia) by 4 (3 in back skin, 1 in belly skin) injections of 50 µl PBS, each containing 200 µg BV (i.e., a total of 800 µg BV). BALB/c WT, -*Fcer1a*$^{-/-}$, or IgE-deficient mice were challenged by 5 (4 in back skin, 1 in belly skin) injections of 50 µL PBS, each containing 200 µg BV (i.e., a total of 1000 µg BV). For active immunization of transgenic mouse strains (presented in Figures 4 and 5; Figures S4 and S5), C57BL/6-*Fcer1g*$^{-/-}$ mice were immunized on day 0 with 2 injections of 200 µg BV while BALB/c-*Fcer1a*$^{-/-}$ and IgE-deficient mice were immunized with 1 injection of 100 µg BV (these immunization doses did not cause death in a significant number of the WT animals [see Figures 1E and 1G] but conferred significantly increased resistance to challenge with a high dose of BV 3 weeks later [see Figures 2G and 2H; Figures S2G and S2H]). In experiments with RVV, C57BL/6 and BALB/c mice were challenged on day 21 with 2 subcutaneous injections (in back skin), each consisting of 50 µl PBS containing 37.5 µg RVV (i.e., a total of 75 µg RVV). These challenge protocols caused death in ~70-100% of 6-8 week old animals as determined in initial dose finding experiments (see Figure 1 for BV experiments and Figure S1 for RVV experiments). Body temperature was measured immediately before the challenge and at indicated time intervals after challenge (in surviving mice), using a rectal thermometer.

Antigen-Specific Activation of ILN Cells

Five days after subcutaneous injection of 2x 200 µg bee venom (BV) (C57BL/6 mice) or 1x 100 µg BV (BALB/c mice) (for experiments with BV), or 2x 12.5 µg Russell's viper venom (RVV) (C57BL/6 and BALB/c mice, for experiments with RVV) into the back skin, draining inguinal lymph nodes (ILNs) were recovered, minced and ILN cells (5.5 x 10^7 cells/ml PBS) were labeled with CFSE (5 µM in PBS) for 8 minutes at 37°C. Cells were washed in ice-cold PBS containing 5% FCS and then cultured *in vitro* for 4 days with antigen re-stimulation (i.e. 1 µg/ml BV, 1 µg/ml RVV, or with PBS alone as a control) in complete RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, and 1% antibiotic-antimycotic solution at 37°C (4x 10^5 cells per well in a 96-well plate). Supernatants were harvested for analysis of cytokine content by ELISA (for mouse interleukin (IL)-4, IL-5, IL-13, and IFN-γ [R&D Systems] according to the manufacturer's protocol) and the cells then were cultured for 6 hours in complete RPMI 1640 medium containing 10 ng/ml PMA and 3 µM ionomycin in the presence of 2 µM monensin at 37°C. Cells subsequently were stained first for extracellular CD4 and then for intracellular IL-13 expression (using the Cytofix/Cytoperm kit, BD Biosciences) and analyzed by flow cytometry.

Measurement of Serum Antibody Levels

Each incubation step in the ELISAs described below is followed by 3-5 washing steps using PBS containing 0.05% tween. For detection of venom-specific serum antibodies, MaxiSorp ELISAs plates (Nunc) were coated with 5 µg/ml whole BV or RVV at 4°C overnight, followed by blocking with 1% bovine serum albumin (BSA) in PBS for at least 2 h at room temperature. Sera diluted in PBS containing 1% BSA were added and incubated in the blocked wells for 2 h at 37°C. We detected bound IgG_1 and IgE antibodies using biotinylated detection antibodies (rat anti-mouse IgG_1 [clone A85-1, BD Pharmingen; incubated for 1 h at room temperature] and rat anti-mouse IgE [clone R35-118, BD Pharmingen], respectively), followed by incubation with horseradish peroxidase-conjugated streptavidin (BD Pharmingen) for 30 min at room temperature and detection using supersensitive TMB substrate (Sigma). Antibody titers were calculated by plotting the serum dilution that gave half-maximal signal of a reference serum. To determine absolute amounts of total serum IgE, we incubated serial dilutions of purified mouse IgE (BD Pharmingen) as the standard and 1:10 diluted sera for 2 h at 37°C on ELISAs plates (coated with rat anti-mouse IgE [clone R35-72, BD Biosciences, at 2 µg/ml] and blocked as described above). Bound IgE was detected using biotinylated anti-mouse IgE (clone R35-118, BD Pharmingen) and reagents as described above.

Bone Marrow Derived Cultured Mast Cells (BMCMCs) Generation and Analysis of Serum for Functional IgE

BMCMCs were generated from bone marrow cells of female C57BL/6J, C57BL/6-*Fcer1a*$^{-/-}$ (see Figure S3), or BALB/cJ mice in Wehi-3-conditioned, IL-3-containing medium (DMEM containing 20% supernatant of Wehi-3 cells, 10% FCS, 50 µM ß-mercaptoethanol, 2 mM l-glutamine, and 1% antibiotic-antimycotic solution) as previously described (Kalesnikoff and Galli, 2011). BMCMCs were used after at least 4 weeks of culture and confirmation of KIT and FcεRIα expression by flow cytometry (~95 % of cells from C57BL/6J and BALB/cJ were KIT- and FcεRIα- positive, and ~95 % of cells from C57BL/6-*Fcer1a*$^{-/-}$ were KIT positive). To measure the potential of pooled mouse serum to sensitize MCs for antigen-dependent degranulation and the potential contribution of IgE to that process (Figures 2K and 7J; Figures

S2K and S6J), BMCMCs derived from C57BL/6 mice were resuspended at 2x 10^6 cells/ml (in Wehi-3 conditioned medium) and incubated overnight at 37°C with a 1/10 volume of pooled serum (consisting of sera collected one week after mice originally immunized with different amounts of BV or RVV were challenged with 4x 200 µg BV or 2x 37.5 µg RVV [i.e., a total of 800 µg BV or 75 µg RVV, respectively]). Before its incubation with BMCMCs, serum was either used without additional treatment, was heated for 60 minutes at 56°C (which ablates the anaphylactic potential of IgE while the function of other antibody classes, such as IgG_1, is not affected (Ishizaka et al., 1986; Prouvost-Danon et al., 1977; Strait et al., 2006; Wakayama et al., 1998), or was supplemented with rat anti-mouse IgE (clone R35-92, BD Pharmingen) at 50 µg/ml for 30 minutes at room temperature. This clone (R35-92), which is thought to bind to the constant region of free serum IgE without crosslinking FcεRI–bound IgE, has been used previously to neutralize IgE function *in vivo* (Amiri et al., 1994; Haak-Frendscho et al., 1998). Sensitized BMCMCs were washed and resuspended in Tyrode's buffer (100 mM HEPES, 130 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose, 1 g/l bovine serum albumin; pH 7.4) and then stimulated in triplicate with BV or RVV in Tyrode's buffer (50 µl total reaction volume) for 60 minutes at 37°C. We measured ß-hexosaminidase release into supernatant as the indicator of degranulation using the chromogenic substrate 4 mM p-nitrophenyl-N-acetyl-ß-D-glucosaminide as previously described (Akahoshi et al., 2011).

To test the functionality of IgE contained in individual sera (collected from surviving mice 7 days after challenge with a high BV [Figure 2J; Figure S2J] or RVV dose [Figure 7I; Figure S6I]), 5x 10^5 BMCMCs (in 500 µl of Wehi-3 conditioned medium; BMCMCs were derived from mice of the same background as the mice whose individual sera were tested) per well were seeded in a 48-well plate and incubated overnight with 1/20 volume of untreated serum of individual mice. Sensitized BMCMCs were washed, resuspended in 100 µl of Tyrode's buffer and seeded in 20 µl duplicates (each containing approximately 10^5 cells) into 96-well v-bottom plates containing either 20 µl of Tyrode's buffer alone or 2 µg/ml BV or RVV. After stimulation for 60 minutes at 37°C, ß-hexosaminidase release was analyzed as described above. The signal detected in sensitized, buffer-stimulated BMCMCs was considered as serum-related background and subtracted from values for the venom-stimulated samples.

To detect BV-specific IgE in sera of BV-immunized mice collected for serum transfer experiments (see protocols in Figure 3A; Figure S3A), WT C57BL/6 or C57BL/6-*Fcer1a*$^{-/-}$ BMCMCs were sensitized (at 10^6 cells/ml) overnight with 1/10 volume serum from BV-immunized (BV-serum) or PBS-injected (PBS-serum) mice. Serum-sensitized BMCMCs were incubated with biotinylated BV (diluted 50 10^{-3} in PBS) and cell-bound BV was detected on live BMCMCs by flow cytometry using a fluorochrome-conjugated streptavidin. We biotinylated BV (at 4 mg/ml in PBS) using EZ-Link Sulfo-NHS-SS-Biotin (Pierce), followed by dialysis against PBS overnight at 4°C using Slide-A-Lyzer cassettes (cut off 3.5 KDa, Pierce) and removal of unbound residual biotin using Zeba Spin Desalting columns (Pierce).

To test the functionality of DNP-specific IgE to induce MC degranulation *in vitro*, BMCMCs derived from C57BL/6 WT mice were sensitized (at 10^6 cells/ml) overnight with either 1 µg/ml of untreated or heated (for 60 min at 56°C) DNP-specific IgE. Sensitized BMCMCs were further processed for assessment of ß-hexosaminidase release after stimulation with different concentrations (as indicated) of DNP-HSA as described above.

Passive Immunization – Serum Preparation

Figure S3A shows a schematic overview of the passive immunization protocol. Six to eight week-old female C57BL/6J "donor" mice were immunized by 2 subcutaneous injections of 50 µl PBS in the back skin, each injection containing 200 µg BV (i.e., a total of 400), or with PBS alone. Three weeks later, ~25% of "donor" C57BL/6 mice were challenged with 4x 200 µg BV (injected 3x in the back skin and 1x in the belly skin) and body temperature and survival were monitored to ensure that successful immunization (defined by increased resistance of BV-immunized mice compared to PBS mock-immunized mice, see Figures S3G and S3H) had been achieved. Blood was collected from the remaining ~75% of the donor mice and sera of PBS- and BV-injected mice were recovered after centrifugation in Serum-Gel Microtubes (Sarstedt). Following quantification of antibody levels (as described above), sera were pooled and aliquoted for later use in treatment groups of 5 mice (~1.3 10^3 µl/group) and stored at –20°C. Typically, the amount of blood collected from two "donor" mice at this age yielded sufficient serum (i.e., a total of ~750 µl) for i.v. transfer into three "recipient" mice (please see section immediately below).

Passive Immunization – Serum Transfer

For experiments involving WT recipient mice (see Figures S3 and 3) aliquots of pooled serum derived from "donor" mice which had been injected with either PBS or BV (see above) were thawed and allowed to reach room temperature on the day of transfer. Immediately before the transfer, sera were modified as follows: (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 BV-immunized mice (BV-serum) was supplemented with 1/10 volume of rat IgG_1 istoype control antibody and not further treated prior to transfer (untreated BV-serum); (3) BV-serum was supplemented with 1/10 volume of rat IgG_1 istoype control antibody and heated 60 min at 56°C in a water bath (heated BV-serum), in order to ablate IgE function specifically (Ishizaka et al., 1986; Prouvost-Danon et al., 1977; Strait et al., 2006; Wakayama et al., 1998) (see also Figure S4F to S4H); (4) BV-serum was supplemented with 1/10 volume of rat anti-mouse IgE antibody (clone R35-92, BD Pharmingen; dialyzed overnight against 2x 5 L of PBS to remove sodium azide [this particular clone has been previously used to neutralize cell-mediated IgE function *in vivo* (Amiri et al., 1994; Haak-Frendscho et al., 1998)]) for 30 minutes at room temperature (anti-IgE BV-serum). Notably, each method to neutralize serum IgE significantly reduced the ability of the immune serum to increase the sensitivity of BMCMCs to degranulate in response to BV (Figure 2K; Figure S2K) or RVV (Figure 7J; Figure S6J) *in vitro.*

Ten- to eleven-week-old female C57BL/6J "recipient" mice (received at 6-7 weeks of age and housed 4 weeks in the local animal facilities) were shaved on the back and belly and were transfused i.v. 22 hours before the challenge with 250 µl of serum (treated as described above and pre-warmed to 37°C) into the retro-orbital vein. On the following day, we challenged all "recipient" mice with 4 s.c. injections (3x back, 1x belly) of 50 µl PBS, each containing 200 µg BV. We measured body temperature at indicated time points and monitored survival over one week.

For experiments involving transgenic recipient mice (see Figures 4, 5 and 6), C57BL/6-*Fcer1g*$^{-/-}$ and C57BL/6-*Fcer1a*$^{-/-}$ mice received serum generated from C57BL/6J WT mice and

were challenged as described above. C57BL/6-*Kit*$^{W\text{-}sh/W\text{-}sh}$ and C57BL/6-*Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice received serum generated from C57BL/6J WT mice and were challenged with 3x 200 µg BV (3x back). IgE-deficient BALB/c mice received serum derived from BALB/cJ WT animals and were challenged with 5x 200 µg BV (4x back, 1x belly). In serum transfer experiments involving transgenic animals, untreated and heated sera did not contain rat IgG_1 isotype control antibody since no serum was treated with rat anti-mouse IgE antibody in these experiments. Certain IgE-deficient BALB/c mice received untreated DNP-specific IgE in 250 µl PBS at concentrations equivalent to total IgE concentrations transferred in 250 µl of BV-serum, so that mice which received either DNP-specific IgE or BV-serum received the same amount of total IgE.

Flow Cytometry

ILN cells and BMCMCs were incubated with 2.4G2 Fc-receptor antibodies to reduce non-specific binding before staining. Cells were kept at 4°C during processing. We analyzed samples with a FACS LSR-II flow cytometer (BD Biosciences) at the Stanford University Flow Cytometry Core Facility and results were analyzed using FlowJo software (Tree Star).

IgE-Dependent Passive Cutaneous Anaphylaxis (PCA)

To test the functionality of DNP-specific IgE *in vivo*, IgE-dependent PCA was induced in the ear pinna of IgE-deficient and corresponding wild type BALB/c mice. Mice under isoflurane anesthesia were passively sensitized by intradermal injection of 20 µl of PBS containing 20 µg untreated DNP–specific IgE in 20 µL of PBS in the right ear pinna and the same amount of heated (60 min at 56°C) DNP-specific IgE in the left ear pinna as a control. The next day, mice were injected intravenously with 20 µg of DNP-HSA and 2 mg Evans Blue in 200 µL of PBS. Immediately before and at intervals after antigen challenge, ear thickness was measured with a dial thickness gauge (G-1A; Ozaki), and vascular extravasation was evaluated visually.

Histology and Assessment of MC Degranulation

Back skin specimens were fixed with 10% formalin and embedded in paraffin, and 4-µm sections were stained with 0.1% Toluidine blue for histologic examination. Images were captured with a Nikon E1000M microscope using a Spotflex camera and Spot version 5.1 software. MC degranulation is expressed as the % of the skin MCs examined (at least 100 MCs per section) 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 that MC exhibited morphological evidence of degranulation, i.e., alterations in the staining characteristics, size or distribution of the granules (see Figures 6A and 6B).

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