

Pathways of immediate hypothermia and leukocyte infiltration in an adjuvant-free mouse model of anaphylaxis

Bianca Balbino, MSc,^{a,b,c} Riccardo Sibilano, PhD,^{d,e,§} Philipp Starkl, PhD,^{d,e,*§} Thomas Marichal, DVM, PhD,^{d,‡} Nicolas Gaudenzio, PhD,^{d,e} Hajime Karasuyama, PhD,^f Pierre Bruhns, PhD,^{a,b} Mindy Tsai, DMSc,^{d,e} Laurent L. Reber, PhD,^{a,b,d,e,||} and Stephen J. Galli, MD^{d,e,g,||}
Paris, France, Stanford, Calif, and Tokyo, Japan

Background: Conflicting results have been obtained regarding the roles of Fc receptors and effector cells in models of active systemic anaphylaxis (ASA). In part, this might reflect the choice of adjuvant used during sensitization because various adjuvants might differentially influence the production of particular antibody isotypes.

Objective: We developed an “adjuvant-free” mouse model of ASA and assessed the contributions of components of the “classical” and “alternative” pathways in this model.

Methods: Mice were sensitized intraperitoneally with ovalbumin at weekly intervals for 6 weeks and challenged intraperitoneally with ovalbumin 2 weeks later.

Results: Wild-type animals had immediate hypothermia and late-phase intraperitoneal inflammation in this model. These features were reduced in mice lacking the IgE receptor FcεRI, the IgG receptor FcγRIII or the common γ-chain FcγR. FcγRIV blockade resulted in a partial reduction of inflammation without any effect on hypothermia. Depletion of monocytes/macrophages with clodronate liposomes significantly reduced the hypothermia response. By contrast, depletion of neutrophils or basophils had no significant effects in this ASA model. Both the hypothermia and inflammation were dependent on platelet-activating factor and histamine and were reduced in 2 types of mast cell (MC)–deficient mice. Finally, engraftment of

MC-deficient mice with bone marrow–derived cultured MCs significantly exacerbated the hypothermia response and restored inflammation to levels similar to those observed in wild-type mice.

Conclusion: Components of the classical and alternative pathways contribute to anaphylaxis in this adjuvant-free model, with key roles for MCs and monocytes/macrophages. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Rodents, mouse model, mast cells/basophils, monocytes/macrophages, neutrophils, antibodies, Fc receptors, allergy, inflammation, anaphylaxis

Anaphylaxis is an acute, life-threatening systemic allergic reaction with a lifetime prevalence of 0.05% to 2.0% in developed countries.¹⁻³ In human subjects it is largely accepted that anaphylaxis can be triggered by histamine and other mediators released in response to antigen cross-linking of IgE bound to its high-affinity receptor, FcεRI, on mast cells (MCs).¹⁻³ However, IgG might also contribute to anaphylaxis in some patients, especially those treated with infused drugs, such as therapeutic mAbs.^{4,5}

Several mouse models of anaphylaxis have been developed and used to assess the contributions of IgE and IgG antibodies and the

From ^aInstitut Pasteur, Department of Immunology, Unit of Antibodies in Therapy and Pathology, Paris; ^bINSERM, U1222, Paris; ^cUniversité Pierre et Marie Curie, Paris; ^dthe Department of Pathology, ^ethe Sean N. Parker Center for Allergy and Asthma Research, and ^fthe Department of Microbiology and Immunology, Stanford University School of Medicine; and ^gthe Department of Immune Regulation, Tokyo Medical and Dental University, Graduate School of Medical and Dental Sciences, Tokyo.

*Dr Starkl is currently affiliated with CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, and the Department of Medicine I, Research Laboratory of Infection Biology, Medical University of Vienna, Vienna, Austria.

‡Dr Marichal is currently affiliated with GIGA-Research and the Faculty of Veterinary Medicine, University of Liege, Liege, Belgium.

§These authors contributed equally to this work.

||These authors were co-senior authors on this work.

B.B. was supported by a stipend from the Pasteur–Paris University (PPU) International PhD Program. R.S. was supported by the Lucile Packard Foundation for Children's Health and the Stanford National Institutes of Health (NIH)/NCRR CTSA award number UL1 RR025744. 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) and is supported by a European Commission Marie Skłodowska-Curie Individual Fellowship (H2020-MSCA-IF-2014 655153). T.M. was supported by the Marie Curie International Outgoing Fellowship for Career Development (299954) and supported by a “Charge de recherches” fellowship of the Belgian National Fund for Scientific Research (F.R.S.-FNRS). N.G. was the recipient of a fellowship from the French “Fondation pour la Recherche Médicale FRM.” P.B. acknowledges support from the European Research Council (ERC)–Seventh Framework Program (ERC-2013-CoG 616050), the Institut National de la Santé et de la Recherche Médicale (INSERM), and the Institut Pasteur.

L.L.R. acknowledges support from the French “Fondation pour la Recherche Médicale FRM,” the Stanford Pediatric Research Fund of the Lucile Packard Foundation for Children's Health and the Stanford CTSA (National Institutes of Health grant UL1 RR025744), NIH grant K99AI110645, the European Commission Marie Skłodowska-Curie Individual Fellowship (H2020-MSCA-IF-2014 656086), and the French “Institut National de la Santé et de la Recherche Médicale” (INSERM). S.J.G. acknowledges support from NIH grants AI023990, CA072074, AI070813, AR067145, U19 AI104209, and NS 080062; the Tobacco-Related Disease Research Program at the University of California; and the Department of Pathology, Stanford University School of Medicine.

Disclosure of potential conflict of interest: R. Sibilano receives grant support from the National Institutes of Health (NIH). P. Starkl receives grant support from the Austrian Science Fund and the Austrian Academy of Science. P. Bruhns receives grant support for the Institut Pasteur and INSERM. M. Tsai receives grant support from the NIH. L. L. Reber receives grant support from INSERM, the European Commission (grant H2020-MSCA65-IF-2014 656086), and the NIH (grant K99AI110645). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication December 21, 2015; revised May 16, 2016; accepted for publication May 31, 2016.

Corresponding authors: Stephen J. Galli, MD, Department of Pathology, CCSR Room 3255b, Stanford University School of Medicine, 269 Campus Dr, Stanford, CA 94305-5176. E-mail: sgalli@stanford.edu. Or: Laurent L. Reber, PhD, Department of Immunology, Unit of Antibodies in Therapy and Pathology, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France. E-mail: laurent.reber@pasteur.fr. 0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2016.05.047>

Abbreviations used

ASA:	Active systemic anaphylaxis
BMCMC:	Bone marrow–derived cultured mast cell
Cpa3:	Carboxypeptidase A3
DT:	Diphtheria toxin
DTR:	Diphtheria toxin receptor
MC:	Mast cell
Mcpt:	Mast cell protease
OVA:	Ovalbumin
PAF:	Platelet-activating factor
PAF-AH:	Platelet-activating factor acetylhydrolase
PCMC:	Peritoneal cell–derived mast cell
PI:	Propidium iodide
PSA:	Passive systemic anaphylaxis
WT:	Wild-type

roles of various effector cells and mediators. The analysis of passive local anaphylaxis or passive systemic anaphylaxis (PSA) models has allowed identification of 2 major pathways of anaphylaxis in mice: a “classical” pathway consisting of IgE, FcεRI, MCs, and histamine^{6–8} and an “alternative” pathway consisting of IgG, FcγRIII and/or FcγRIV,^{5,9–11} platelet-activating factor (PAF),^{12,13} and, depending on the exact model used, macrophages,^{5,14} basophils,^{5,15} and/or neutrophils.^{5,11,15}

Active systemic anaphylaxis (ASA) models, which are arguably more reflective of the clinical situation, have generated more conflicting results. Some ASA models critically depend on IgE, FcεRI, and MCs,^{16–19} whereas some others can develop, at least with respect to the features analyzed, in *IgE*^{−/−}, *FcεRI*^{−/−}, and/or MC-deficient mice.^{9,13,20} Depending on the mouse strain and ASA model used, basophils have been shown to either contribute to the systemic response^{13,19} or play little to no significant role.^{17,21} Similarly, depletion of monocytes/macrophages or antibody-mediated neutrophil depletion reduces anaphylaxis in some but not all ASA models.^{11,17,19,21}

We hypothesize that such conflicting results in ASA models might reflect, at least in part, the choice of adjuvant used during sensitization because various adjuvants might differentially influence the production of individual antibody isotypes. Moreover, in human subjects with anaphylactic reactivity, sensitization to antigen generally occurs in the absence of an artificial adjuvant. Therefore we developed an “adjuvant-free” mouse model of ASA and assessed the contributions of components of the classical and alternative pathways of anaphylaxis in this model.

METHODS**Mouse**

C57BL/6J (wild-type [WT]) mice were purchased from Jackson Laboratories (Bar Harbor, Me) or Charles River (Lyon, France). *FcγRIII*^{−/−} mice (B6.129P2-*Fcgr3*^{tm1Sjv}/J) mice backcrossed to C57BL/6 for 12 generations) were purchased from Jackson Laboratories. *FcRγ*^{−/−} mice (B6.129P2 *Fcer1g*^{tm1Rav} mice backcrossed to C57BL/6 for 12 generations) were from Taconic (New York, NY). C57BL/6-*Kit*^{W-sh/W-sh} mice were originally provided by Peter Besmer (Memorial Sloan-Kettering Cancer Center, New York, NY); we then backcrossed these mice to C57BL/6J mice for more than 11 generations.²² *FcεRI*^{−/−} mice⁷ (FcεRI α chain–deficient mice backcrossed to C57BL/6 for more than 8 generations and kindly provided by Jean-Pierre Kinet, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass), mast cell protease (*Mcpt8*)^{DTR}

mice (backcrossed to C57BL/6 for at least 10 generations),²³ and carboxypeptidase A3 (*Cpa3*)-*Cre*; *Mcl-1*^{fl/fl} mice (backcrossed to C57BL/6 for at least 9 generations)²⁴ were bred and maintained at the Stanford University Research Animal Facility. Age-matched male mice were used in all experiments. Except as otherwise noted, we used littermate controls in all experiments. All animal care and experimentation was conducted in compliance with the guidelines of the National Institutes of Health and specific approval of the Institutional Animal Care and Use Committee of Stanford University and the Animal Ethics Committee CETEA (Institut Pasteur, Paris, France) registered under #C2EA-89.

Ovalbumin-induced adjuvant-free model of active anaphylaxis

Six- to 8-week-old mice were sensitized intraperitoneally with 10 μg of endotoxin-free ovalbumin (OVA; Endograde OVA; BioVendor, Asheville, NC; <0.01 EU endotoxin per injection) in 100 μL of PBS once a week for 6 weeks. Two weeks after the last intraperitoneal sensitization, mice were challenged intraperitoneally with 500 μg of OVA. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge. Mice were killed at various time points after challenge (as indicated) for assessment of inflammatory cell numbers in the peritoneal cavity and histology.

Other methods

See the **Methods** section in this article's Online Repository at www.jacionline.org for the methods for flow cytometry; peritoneal lavage; differential cell counts; depletion of basophils, monocytes/macrophages, and neutrophils; blockade of FcγRIV; histologic analysis; measurement of serum OVA-specific IgG₁ and IgG_{2c} antibodies; IgE-mediated PSA; ASA with adjuvant; treatment with an H₁-antihistamine or a PAF receptor antagonist; quantification of histamine and platelet-activating factor acetylhydrolase (PAF-AH); and generation and adoptive transfer of bone marrow–derived cultured mast cells (BMCMCs).

Statistical analyses

Results represent means ± SEMs or means + SEMs, with values for individual mice represented for quantifications of histamine, PAF-AH, and leukocytes. We used an unpaired Student *t* test (body temperature) or an unpaired Mann-Whitney *U* test (all other data) to assess the significance of differences between 2 sets of data. *P* values of less than .05 are considered statistically significant.

RESULTS**Development of an adjuvant-free model of ASA in C57BL/6 mice**

We developed an adjuvant-free mouse model of ASA consisting of intraperitoneal sensitization with endotoxin-free OVA once a week for 6 weeks and intraperitoneal challenge with OVA 2 weeks after the last sensitization (Fig 1, A). OVA-sensitized C57BL/6 mice had hypothermia after OVA challenge that was maximal at 30 minutes and decreased thereafter (Fig 1, B). Sensitized and challenged mice also had a late-phase inflammatory response with increased numbers of total cells, eosinophils, macrophages, and lymphocytes in the peritoneal cavity, with the highest numbers of leukocytes on day 3 after challenge (Fig 1, C–H). Consistent with the increase in eosinophil numbers, we detected significant amounts of IL-5 in the plasma of 3 of 5 OVA-sensitized mice 18 (but not 72) hours after challenge, whereas levels of IL-4, IL-6, IL-10, TNF-α, and IFN-γ were all less than the detection limit of standard ELISAs at both time points (data not shown).

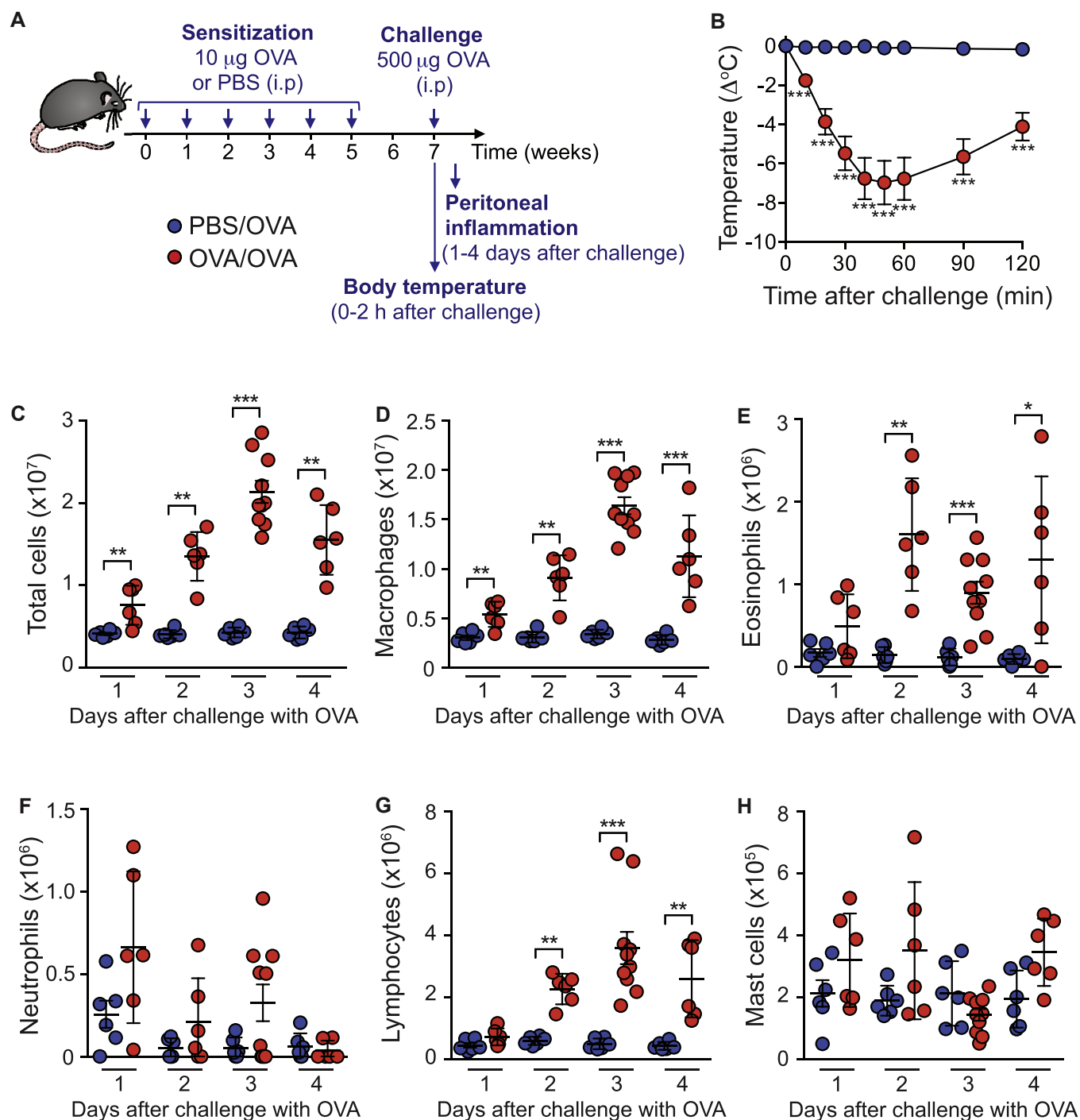


FIG 1. Development of hypothermia and intraperitoneal (*i.p.*) inflammation in mice sensitized and challenged with OVA in the absence of adjuvant. **A**, Experimental outline. **B**, Changes in body temperature after challenge with OVA in OVA-sensitized (OVA/OVA) or PBS-treated (PBS/OVA) mice. **C-H**, Numbers of leukocytes in peritoneal lavage fluid at the indicated time points. Data are pooled from 2 or 3 independent experiments ($n = 6-10$ per group). * $P < .05$, ** $P < .01$, or *** $P < .001$.

OVA-induced hypothermia and inflammation depend on the high-affinity IgE receptor FcεRI, the IgG receptor FcγRIII, and their common activating subunit FcRγ

OVA sensitization induced a significant increase in OVA-specific IgG₁ and IgG_{2c} antibody levels in C57BL/6 mice

(see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). We did not detect significant levels of OVA-specific IgE in sera by using a standard ELISA (data not shown). However, we observed degranulation of peritoneal cell-derived mast cells (PCMCs) incubated *in vitro* with sera from OVA-sensitized mice, followed by stimulation with OVA

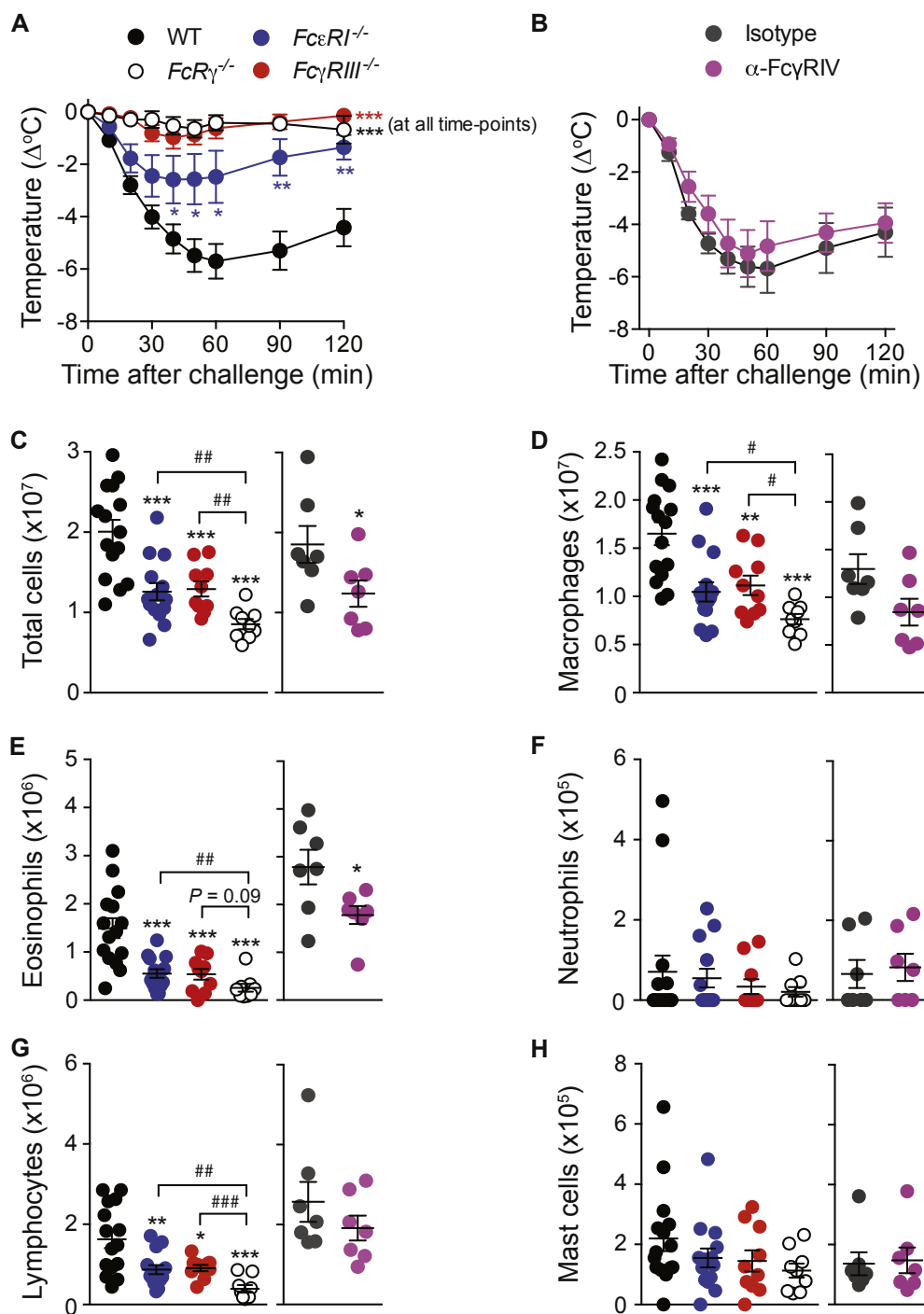


FIG 2. Roles of Fc receptors in OVA-induced ASA. **A** and **B**, OVA-induced hypothermia in OVA-sensitized WT (C57BL/6J), $Fc\epsilon RI^{-/-}$, $Fc\gamma RIII^{-/-}$, and $Fc\gamma RI^{-/-}$ mice (Fig 2, A) or WT mice treated with an anti-Fc γ RIV antibody or an isotype control (Fig 2, B). **C-H**, Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 2 (Isotype and α -Fc γ RIV, $n = 7$ per group) or 3 (all other groups, $n = 9-15$ per group) independent experiments. * $P < .05$, ** $P < .01$, or *** $P < .001$. # $P < .05$, ## $P < .01$, or ### $P < .001$.

(see Fig E1, C and D). Such degranulation was not observed with PCMCs incubated with sera from PBS-treated mice or with sera from OVA-sensitized mice, which had been preincubated with anti-IgE antibodies (see Fig E1, C and D), demonstrating the presence of functional OVA-specific IgE in the sera of sensitized mice.

We then assessed responses of C57BL/6 mice lacking the IgG receptor Fc γ RIII, the high-affinity IgE receptor Fc ϵ RI, or their common activating subunit Fc γ . OVA-induced hypothermia was abolished in $Fc\gamma RI^{-/-}$ and $Fc\gamma RIII^{-/-}$ mice and partially reduced in $Fc\epsilon RI^{-/-}$ mice compared with that seen in WT mice (Fig 2, A). Antibody-mediated blockade of Fc γ RIV had no effect

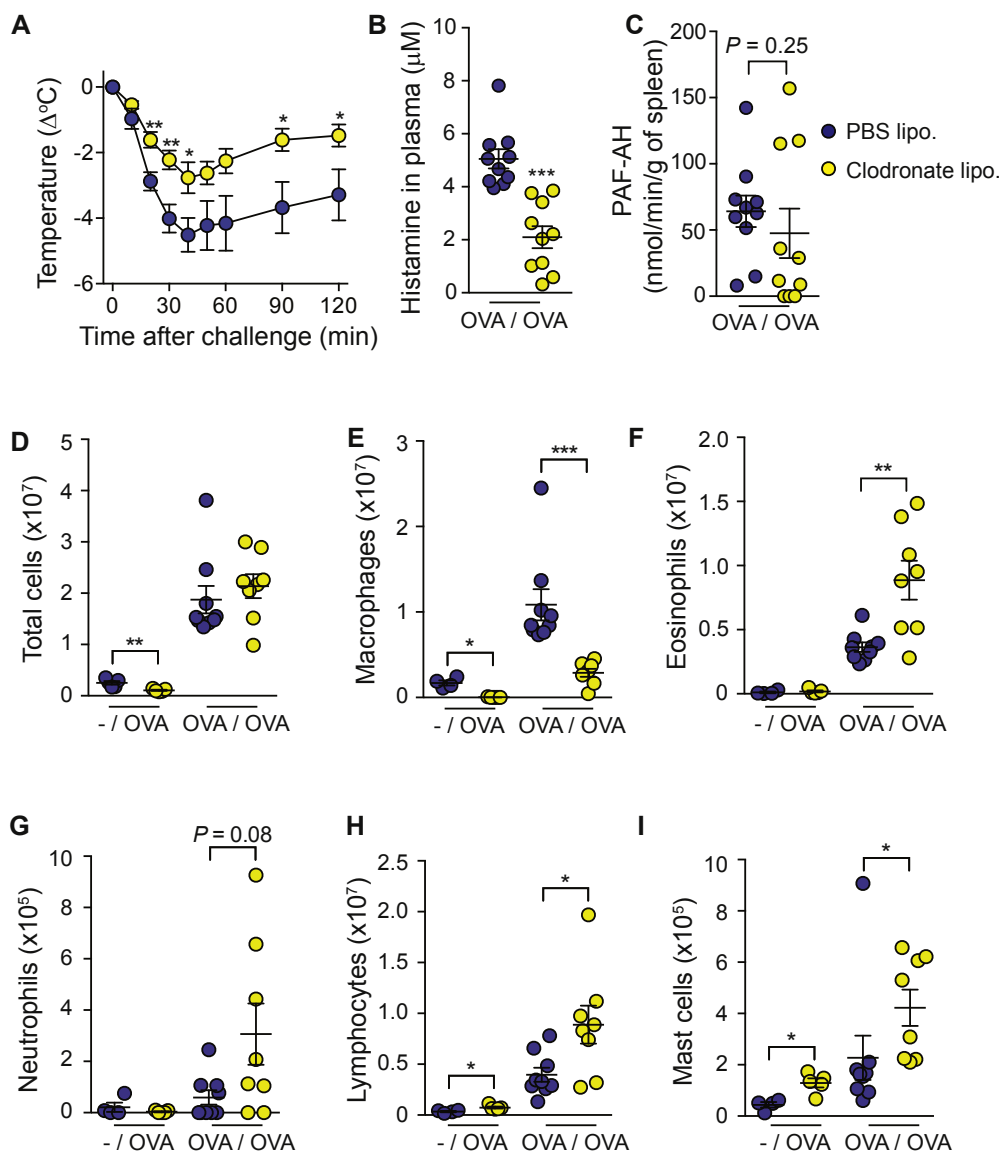


FIG 3. Assessment of the effect of monocyte/macrophage depletion on OVA-induced ASA. Mice were treated with clodronate liposomes (*Clodronate lipo.*) or PBS liposomes (*PBS lipo.*) 24 hours before challenge. **A**, OVA-induced hypothermia. **B**, Levels of histamine in the plasma 20 minutes after challenge. **C**, PAF-AH activity in the spleen 20 minutes after challenge. **D-I**, Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge in nonsensitized mice ($-/\text{OVA}$) or OVA-sensitized mice (OVA/OVA). Data are pooled from 3 independent experiments for all OVA-sensitized groups (total $n = 8-9$ per group) and from 1 experiment for nonsensitized control mice ($n = 4-5$ per group). * $P < .05$, ** $P < .01$, or *** $P < .001$.

on immediate hypothermia (Fig 2, B). WT, *FcεRI*^{−/−}, and *FcγRIII*^{−/−} mice had similar levels of intraperitoneal leukocytes at baseline (except for a small increase in macrophages and lymphocytes in *FcγRIII*^{−/−} mice, see Fig E2 in this article's Online Repository at www.jacionline.org). However, we observed decreased numbers of total cells, macrophages, eosinophils, and lymphocytes in the peritoneal cavities of *FcεRI*^{−/−} and *FcγRIII*^{−/−} mice compared with WT mice 3 days after challenge (Fig 2, C-G). These numbers were further decreased in mice lacking FcRγ (Fig 2, C-G). Antibody-mediated blockade of FcγRIV induced only a moderate decrease of peritoneal leukocyte numbers at day 3, with reduced numbers of total cells and eosinophils compared with those seen in mice treated with an isotype control antibody (Fig 2, C-G).

Monocyte/macrophage depletion with clodronate liposomes decreases immediate hypothermia and enhances late-phase intraperitoneal inflammation

We injected OVA-sensitized mice with clodronate liposomes (or PBS liposomes as a control) 24 hours before challenge with OVA to deplete monocytes/macrophages and assess their potential contribution to anaphylaxis through the alternative pathway. Clodronate liposome-treated mice exhibited depletion of circulating CD11b^{high} monocytes at the time of OVA challenge, with no effect on blood Ly6G⁺ neutrophils (see Fig E3, A, C, and D, in this article's Online Repository at www.jacionline.org). OVA challenge led to a decrease in CD11b expression in blood monocytes in PBS liposome-treated mice at day 3 (see Fig E3, A). Consistent with a previous report,²⁵ we found similar

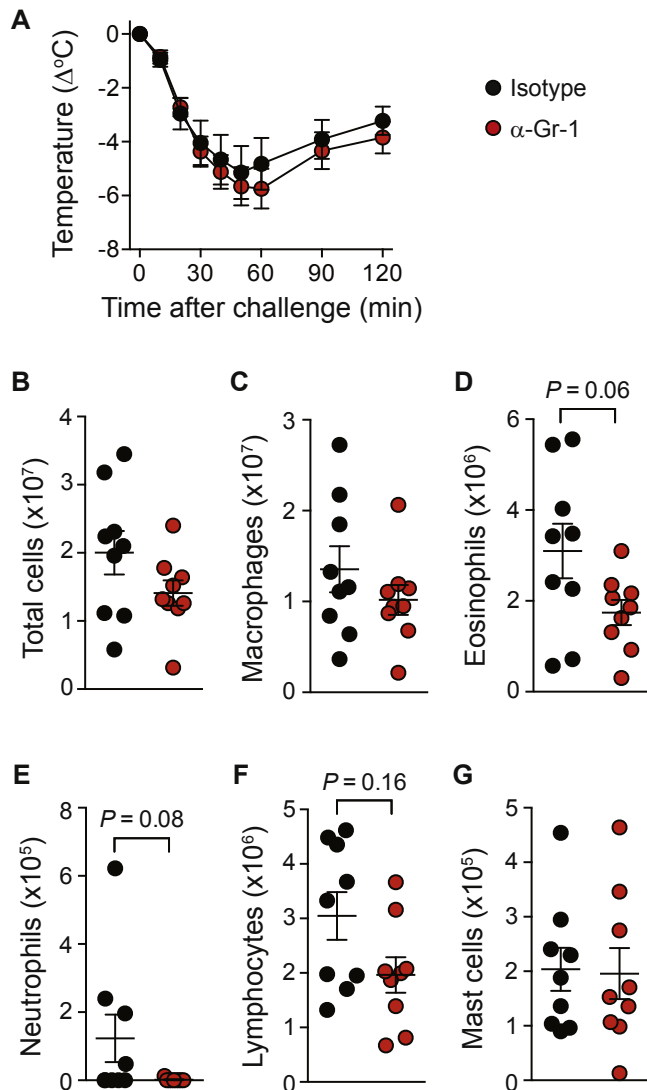


FIG 4. Assessment of antibody-mediated depletion of neutrophils on OVA-induced ASA. OVA-sensitized mice were treated with an anti-Gr-1 neutrophil-depleting antibody (α -Gr-1) or an isotype control (*Isotype*) 40 hours before and 24 hours after challenge with OVA. **A**, OVA-induced hypothermia. **B–G**, Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 independent experiments (total $n = 9$ per group).

percentages of blood monocytes in both PBS liposome- and clodronate liposome-treated mice at day 3 (ie, 4 days after the injection of liposomes; see Fig E3, A, D, and E). However, F4/80⁺ macrophages were depleted in the spleens of clodronate liposome-treated mice at this time point, with no effect on neutrophils (see Fig E3, B, F, and G). Confirming the efficiency of depletion, we also found that levels of peritoneal macrophages were reduced by 73% in clodronate liposome-treated mice compared with those in control mice at day 3 (Fig 3, E).

In line with previous reports,^{17,26} treatment with clodronate liposomes reduced features of anaphylaxis in an ASA model using sensitization with OVA together with the adjuvant alum and *Bordetella pertussis* toxin (see Fig E4, B and C, in this article's Online Repository at www.jacionline.org). Treatment with clodronate liposomes also significantly diminished the hypothermia response in OVA-sensitized and challenged mice in the

adjuvant-free ASA model (Fig 3, A). Clodronate liposome-treated mice had lower levels of histamine in plasma 20 minutes after challenge compared with those in PBS liposome-treated mice (Fig 3, B). Because such a reduction in histamine levels could reflect a toxic effect of the liposomes on MCs, we also assessed responses of mice treated with clodronate or PBS liposomes in an MC-dependent IgE-mediated PSA model. Hypothermia was slightly but not significantly reduced (except at 10 minutes after challenge) in clodronate liposome-treated mice, which suggests that the clodronate liposomes have only modest effects on MC activity in this model (see Fig E3, H). The activity of PAF-AH (an enzyme that degrades PAF) has been shown to inversely correlate with the severity of anaphylaxis in human subjects.²⁷ However, we found similar PAF-AH activity in the spleens of mice treated with clodronate or PBS liposomes 20 minutes after challenge (Fig 3, C). By contrast, clodronate liposome-treated mice displayed significantly higher levels of eosinophils, lymphocytes, and MCs than PBS liposome-treated mice in the peritoneal cavity 3 days after challenge (Fig 3, F–I). Levels of blood neutrophils were also higher in such clodronate liposome-treated, macrophage-depleted mice compared with those in control mice at day 3 (see Fig E3, A and C). However, we also found a small but significant increase in lymphocyte and MC numbers 3 days after OVA challenge in naive mice treated with clodronate liposomes, which could reflect some proinflammatory effects of the clodronate liposomes (Fig 3, D–I).

Neutrophils are not required for OVA-induced hypothermia and inflammation

We injected OVA-sensitized mice with an anti-Gr-1 antibody (or an isotype control antibody) 40 hours before and 24 hours after challenge with OVA to deplete neutrophils. Such treatment led to complete ablation of circulating neutrophils at the time of challenge (see Fig E5, A and C, in this article's Online Repository at www.jacionline.org), and neutrophils remained absent in the blood and spleens of anti-Gr-1-treated mice 3 days after challenge (see Fig E5, A–C and F). By contrast, treatment with anti-Gr-1 antibodies did not deplete circulating monocytes or spleen macrophages (and the percentages of these cells were even slightly higher than those in mice treated with the isotype control antibody; see Fig E5, A, B, D, E, and G).

In agreement with findings obtained in IgG-mediated PSA or ASA models using adjuvants for the sensitization,^{5,11} we found that anti-Gr-1-treated mice had markedly reduced features of anaphylaxis compared with isotype control-treated mice in an ASA model using sensitization with OVA together with the adjuvants alum and *B pertussis* toxin (see Fig E4, D and E). By contrast, isotype control-treated mice and anti-Gr-1-treated mice had similar levels of immediate hypothermia after OVA challenge (Fig 4, A). Both groups also had similar levels of leukocytes in the peritoneal cavity 3 days after challenge. Although eosinophil and lymphocyte numbers were slightly decreased in the anti-Gr-1-treated group compared with those in the isotype control-treated mice, those differences did not reach statistical significance (Fig 4, B–G).

Cpa3-Cre⁺; Mcl-1^{fl/fl} mice have less OVA-induced hypothermia and inflammation

We next assessed responses of MC-deficient, basophil-depleted Cpa3-Cre⁺; Mcl-1^{fl/fl} mice²⁴ in this adjuvant-free ASA model.

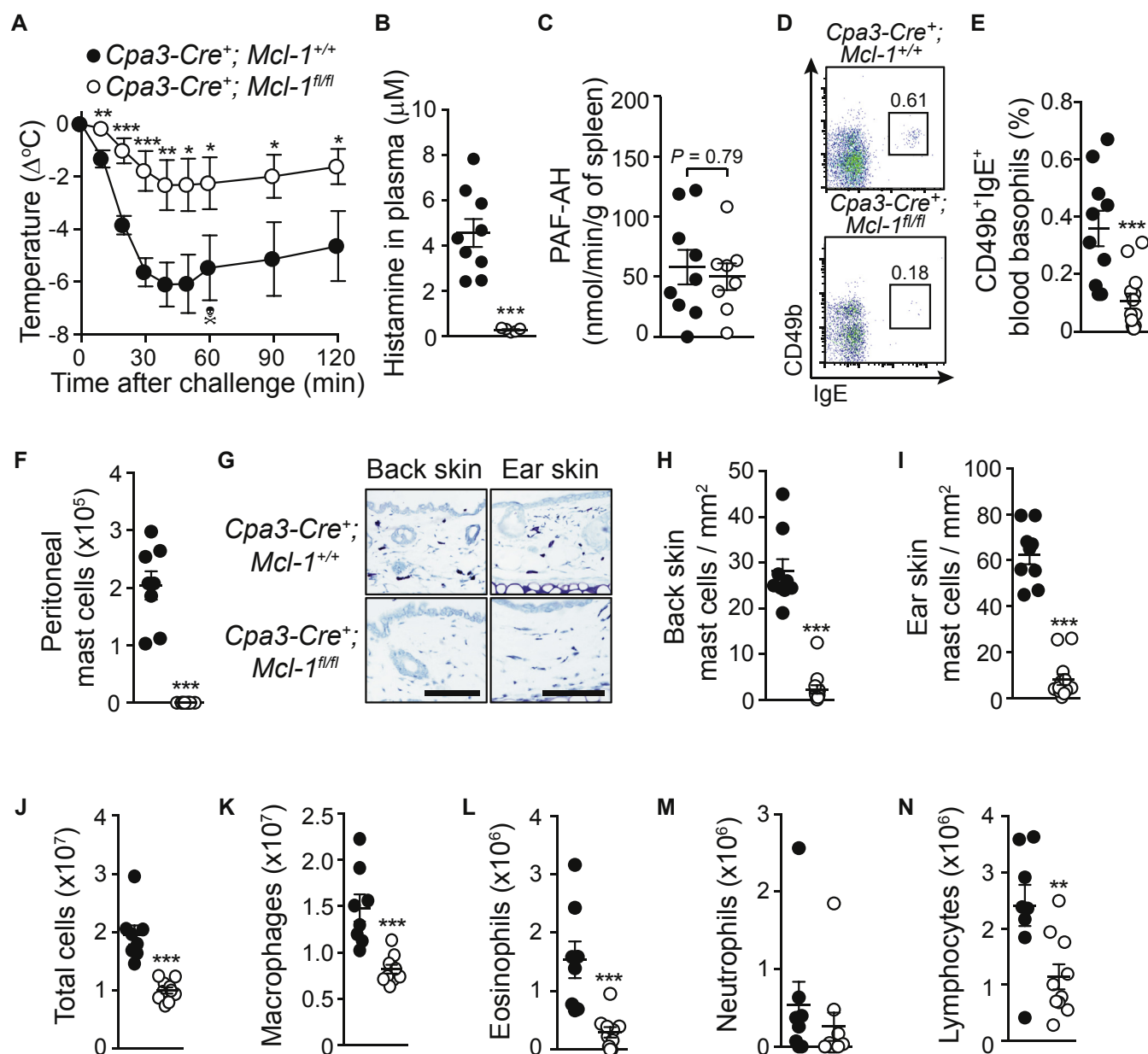


FIG 5. Assessment of OVA-induced ASA in genetically MC-deficient and basophil-depleted *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice. **A**, OVA-induced hypothermia in OVA-sensitized *Cpa3-Cre⁺; Mcl-1^{+/+}* and *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice. **B**, Levels of histamine in the plasma 20 minutes after challenge. **C**, PAF-AH activity in the spleen 20 minutes after challenge. **D** and **E**, Representative fluorescence-activated cell sorting profile (Fig 5, D) and percentage (Fig 5, E) of blood basophils (CD49b⁺; IgE⁺) 24 hours before challenge. **F**, Numbers of MCs in peritoneal lavage fluid 3 days after challenge. **G-I**, Toluidine blue staining for MCs (Fig 5, G) and MC numbers (Fig 5, H and I) in sections of back skin and ear pinna. **J-N**, Numbers of leukocytes in the peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 independent experiments (total n = 9-14 per group). **P* < .05, ***P* < .01, or ****P* < .001. Crossbones symbol indicates the death of 1 mouse. Scale bars = 100 μm.

Cpa3-Cre⁺; Mcl-1^{fl/fl} mice remained highly deficient in MCs and had reduced numbers of basophils, even after repeated sensitizations and challenge with OVA (Fig 5, D-I). These mice had similar levels of OVA-specific IgG₁ and IgG_{2c} antibodies as *Cpa3-Cre⁺; Mcl-1^{+/+}* littermate control mice (see Fig E6, A and B, in this article's Online Repository at www.jacionline.org) and displayed similar amounts of antigen-specific IgE activity in the serum (see Fig E6, C and D), suggesting that

MCs and basophils do not contribute substantially to the sensitization phase in this model. However, sensitized *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice had significantly reduced levels of hypothermia after OVA challenge, suggesting an important role for MCs, basophils, or both in this feature of the model (Fig 5, A). *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice had markedly reduced levels of histamine in the plasma but similar PAF-AH activity in the spleen 20 minutes after challenge (Fig 5, B and C).

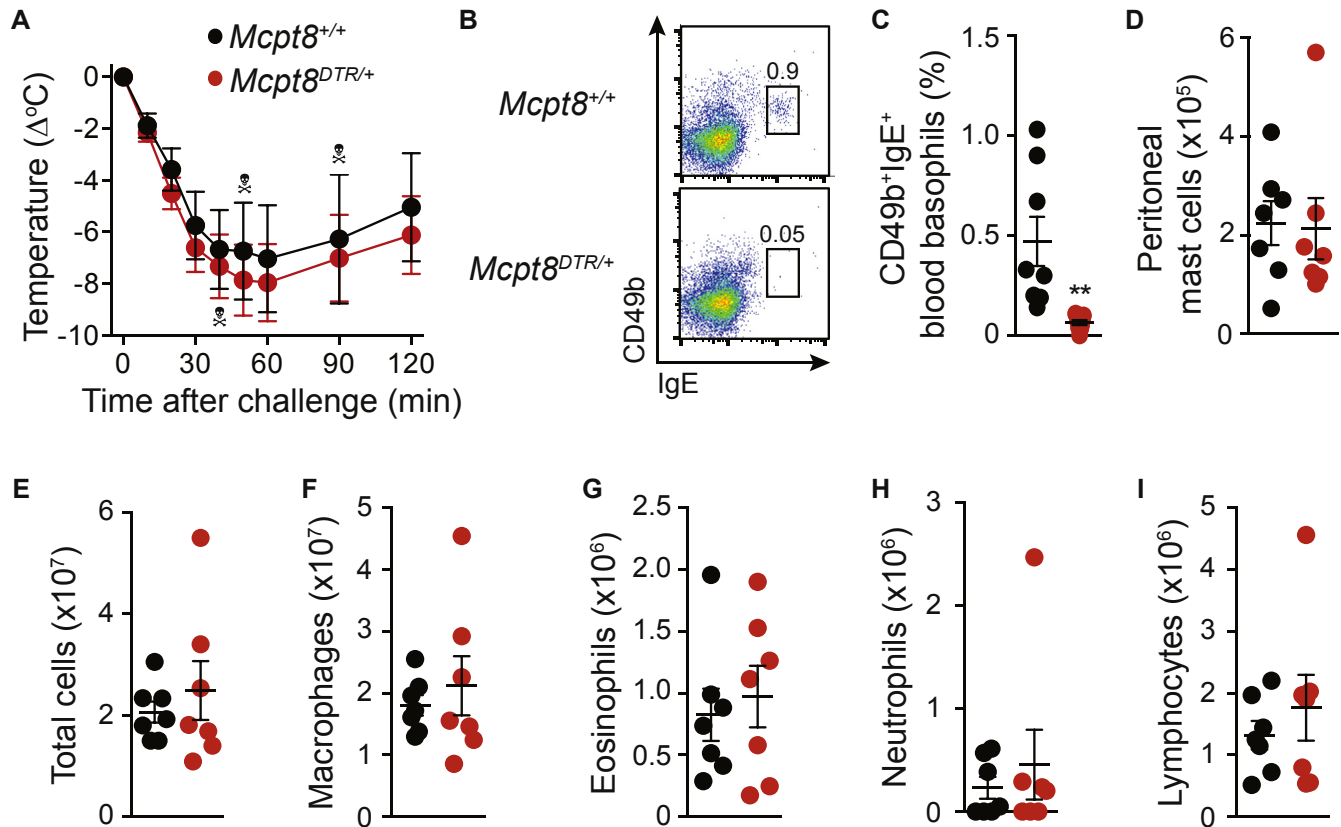


FIG 6. Assessment of the effect of DT-mediated basophil depletion on OVA-induced ASA in *Mcpt8^{DTR}* mice. OVA-sensitized *Mcpt8^{+/+}* and *Mcpt8^{DTR/+}* mice were treated with DT 48 hours before and 24 hours after challenge with OVA. **A**, OVA-induced hypothermia. **B** and **C**, Representative fluorescence-activated cell sorting profile (Fig 6, **B**) and percentage (Fig 6, **C**) of blood basophils (CD49b⁺; IgE⁺) 2 hours before challenge. **D-I**, Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 2 independent experiments (total n = 7-9 per group). ***P* < .01. Each crossbones symbol indicates the death of 1 mouse.

Cpa3-Cre⁺; Mcl-1^{fl/fl} mice also had diminished intraperitoneal inflammation, with decreased numbers of total leukocytes, macrophages, eosinophils, and lymphocytes compared with *Cpa3-Cre⁺; Mcl-1^{+/+}* mice, indicating that MCs, basophils, or both can also contribute importantly to the late-phase leukocyte numbers in this model (Fig 5, *J-N*).

Basophils are not required for OVA-induced hypothermia and inflammation

We assessed the potential role of basophils using *Mcpt8^{DTR}* mice, which express the diphtheria toxin receptor (DTR) in basophils only and in which basophils can be selectively ablated by injection of diphtheria toxin (DT).²³ We first confirmed that treatment with DT induces ablation of basophils, but not MCs, in OVA-sensitized *Mcpt8^{DTR}* mice (Fig 6, *B-D*). We ruled out a significant role for basophils in this model by showing that DT-mediated depletion of basophils in OVA-sensitized *Mcpt8^{DTR}* mice does not affect OVA-induced hypothermia (Fig 6, *A*) or late-phase intraperitoneal leukocyte numbers (Fig 6, *E-I*).

MCs exacerbate OVA-induced hypothermia and inflammation

We used *Kit* mutant MC-deficient *Kit^{W-sh/W-sh}* mice to assess further the contributions of MCs in this “adjuvant-free” ASA

model. Because *Kit^{W-sh/W-sh}* mice have many KIT-related phenotypic abnormalities in addition to their MC deficiency,²⁸⁻³³ we also included a group of *Kit^{W-sh/W-sh}* mice that had been engrafted with BMCMCs (BMCMCs → *Kit^{W-sh/W-sh}* mice) both intraperitoneally and intravenously 12 weeks before the first sensitization with OVA.

We found no significant difference in the severity of anaphylaxis between *Kit^{+/+}* and MC-deficient *Kit^{W-sh/W-sh}* mice in an ASA model using sensitization with OVA together with the adjuvants alum and *B pertussis* toxin (see Fig E4, *F* and *G*), confirming previous findings obtained with MC-deficient *Kit^{W/W-v}* mice.⁹ By contrast, MC-deficient *Kit^{W-sh/W-sh}* mice had significantly less hypothermia after challenge with OVA compared with *Kit^{+/+}* mice (Fig 7, *A*). BMCMCs → *Kit^{W-sh/W-sh}* mice had significantly more hypothermia than did *Kit^{W-sh/W-sh}* mice after challenge with OVA, although levels of hypothermia in these mice did not reach those in *Kit^{+/+}* mice (Fig 7, *A*). The intermediate body temperature response in MC-engrafted *Kit^{W-sh/W-sh}* mice compared with WT or MC-deficient *Kit^{W-sh/W-sh}* mice is consistent with our previously reported data in a peanut-induced ASA model.¹⁹ We think it very likely that the technical limitations of such systemic MC engraftment experiments contributed to the intermediate temperature response seen in MC-engrafted *Kit^{W-sh/W-sh}* mice. Specifically, compared with the corresponding

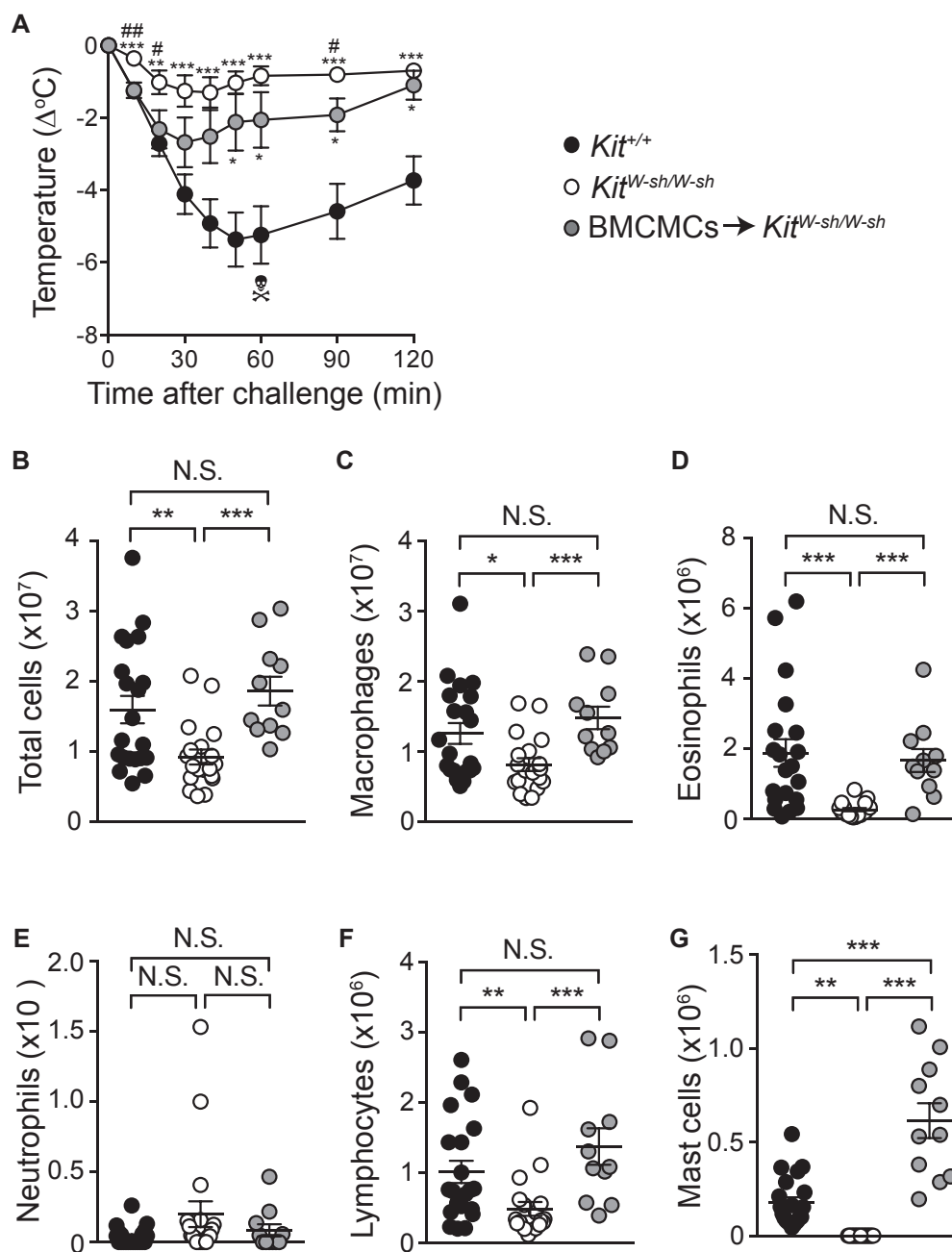


FIG 7. Responses of WT, MC-deficient $\text{Kit}^{W-sh/W-sh}$, and $\text{Kit}^{W-sh/W-sh}$ mice engrafted with BMCMCs in OVA-induced ASA. **A**, OVA-induced hypothermia in $\text{Kit}^{+/+}$, MC-deficient $\text{Kit}^{W-sh/W-sh}$, and $\text{Kit}^{W-sh/W-sh}$ mice engrafted with WT BMCMCs. **B-G**, Numbers of leukocytes in peritoneal lavage fluid 3 days after challenge. Data are pooled from 3 to 5 independent experiments (total $n = 11-25$ per group). * $P < .05$, ** $P < .01$, or *** $P < .001$ versus the $\text{Kit}^{+/+}$ group (Fig 7, A) or indicated group (Fig 7, B-G). # $P < .05$ or ## $P < .01$ versus BMCMCs $\rightarrow \text{Kit}^{W-sh/W-sh}$ group (Fig 7, A). Crossbones symbol indicates the death of 1 mouse.

WT mice, MC-engrafted $\text{Kit}^{W-sh/W-sh}$ mice had similar levels of MCs in the peritoneal cavity and mesenteric windows and even higher numbers of MCs in the spleen. However, these mice had no detectable MCs in the skin, thus eliminating the numerically large population of skin MCs as a potential source of mediators in such mice (Fig 7 and see Fig E7 in this article's Online Repository at www.jacionline.org).

Finally, MC-deficient $\text{Kit}^{W-sh/W-sh}$ mice had significantly diminished numbers of leukocytes during late-phase intraperitoneal

inflammation compared with $\text{Kit}^{+/+}$ mice (Fig 7, B-F). Confirming the role of MCs in this feature of our ASA model, engraftment of $\text{Kit}^{W-sh/W-sh}$ mice with BMCMCs restored levels of total peritoneal leukocytes, neutrophils, eosinophils, macrophages, and lymphocytes quantified 3 days after challenge to those observed in $\text{Kit}^{+/+}$ mice (Fig 7, B-F). Altogether, these results show that MCs can amplify both the immediate hypothermia and the late-phase inflammatory reaction in $\text{Kit}^{W-sh/W-sh}$ mice in this adjuvant-free ASA model.

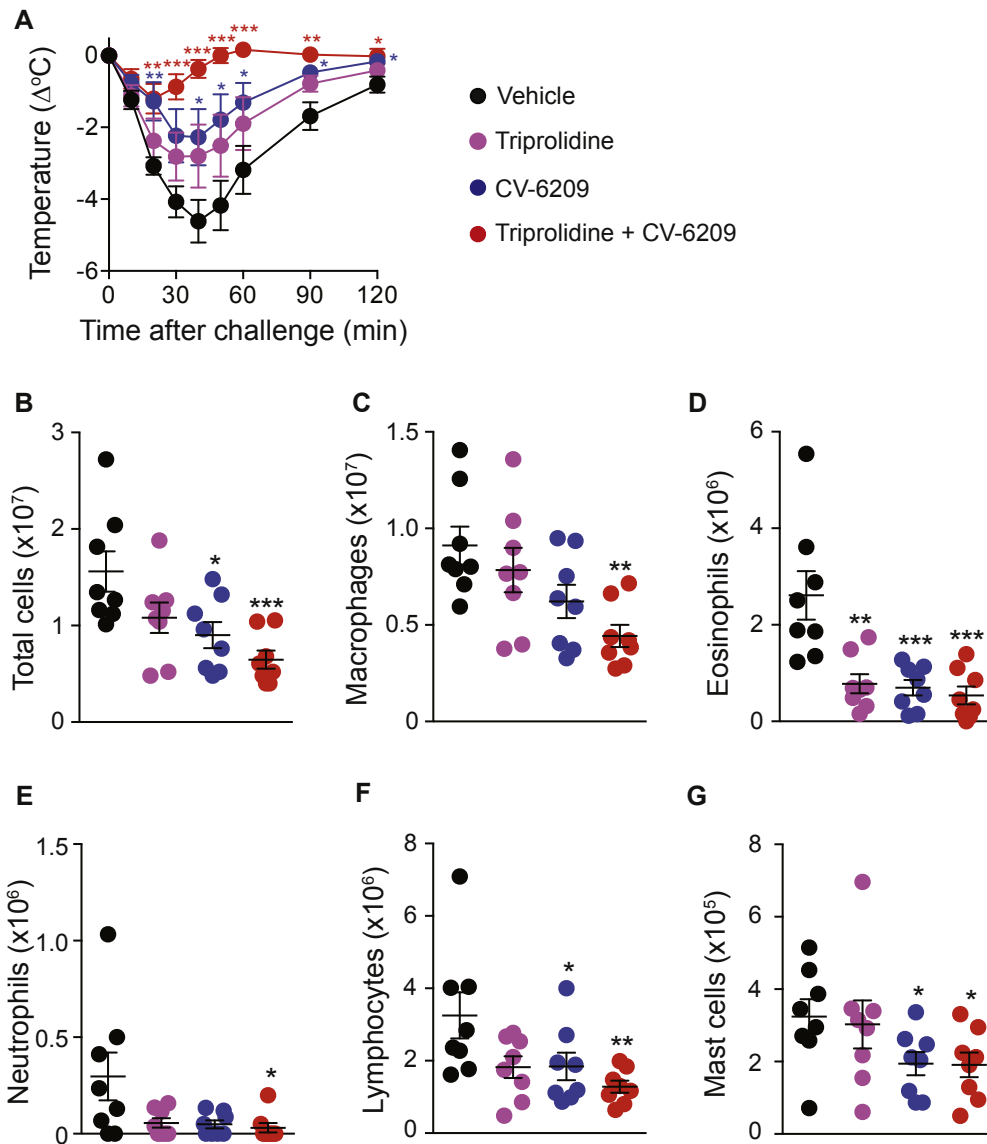


FIG 8. Roles of histamine and PAF in OVA-induced ASA. OVA-sensitized mice were treated with the H_1 -antihistamine tripolidine or with the PAF receptor antagonist CV-6209 alone or in combination 30 minutes before and 1 day after challenge with OVA. Control mice were injected with vehicle (saline) only. **A**, OVA-induced hypothermia. **B-G**, Numbers of leukocytes in peritoneal lavage fluid 3 days after OVA challenge. Data are pooled from 2 independent experiments (total $n = 8$ per group). * $P < .05$, ** $P < .01$, or *** $P < .001$ versus the vehicle group.

Histamine and PAF contribute to OVA-induced hypothermia and inflammation

Pretreatment of mice with the PAF receptor antagonist CV-6209 significantly decreased both the immediate hypothermia and numbers of leukocytes in the late-phase inflammatory response (Fig 8). Pretreatment with the H_1 -antihistamine tripolidine slightly reduced immediate hypothermia and the numbers of leukocytes in the late-phase response (Fig 8), although differences did not reach statistical significance, except for eosinophil numbers (Fig 8, D). However, combined treatment with the antihistamine and PAF receptor antagonist almost entirely blocked both the hypothermia and increased numbers of leukocytes observed during inflammatory responses (Fig 8). By contrast, treatment with tripolidine markedly reduced both

the hypothermia and numbers of intraperitoneal leukocytes in mice pretreated with clodronate liposomes, whereas CV-6209 had no significant effects on these features (see Fig E8 in this article's Online Repository at www.jacionline.org). Collectively, these data demonstrate the involvement of both histamine and PAF in this ASA model in WT mice and suggest that monocytes/macrophages represent the main source of PAF in this model.

DISCUSSION

Most ASA models use adjuvants during the sensitization phase, and such methods typically prime the animals to exhibit allergic reactions that require little or no contribution from MCs and IgE.

We hypothesized that use of adjuvants might boost the production of certain IgG isotypes, favoring activation of alternative pathways of anaphylaxis, which might mask contributions from the classical IgE- and MC-dependent pathway. Therefore we designed a new adjuvant-free model of ASA and assessed the potential roles of various components of the classical and alternative pathways in that model.

Our sensitization protocol resulted in production of OVA-specific IgG₁ and IgG_{2c} antibodies (see Figs E1 and E4), 2 isotypes that can induce anaphylaxis through FcγRIII, FcγRIV, or both.^{5,10,11} Indeed, we found that *FcγRIII*^{-/-} mice had markedly reduced hypothermia and reduced numbers of peritoneal leukocytes compared with WT mice in this model (Fig 2). We used an anti-FcγRIV blocking antibody to assess the contribution of this receptor and found no role for FcγRIV in the hypothermia response and a relatively minor contribution to numbers of intraperitoneal leukocytes (Fig 2). Altogether, our results confirm the important involvement of FcγRIII in allergic shock^{10,11,17,26,34} and reveal that this receptor also has an important role in the development of antigen-induced allergic inflammation.

Although we did not detect significant levels of OVA-specific IgE in the sera of most mice by using a classical ELISA (data not shown), we demonstrated the presence of functionally active specific IgE in sera from OVA-sensitized mice using an *ex vivo* MC activation test (see Figs E1 and E6). These levels of antigen-specific IgE were sufficient to contribute to anaphylaxis and allergic inflammation because mice lacking the high-affinity IgE receptor FcεRI displayed significantly diminished hypothermia and numbers of intraperitoneal leukocytes compared with WT mice (Fig 2).

These results are in agreement with previous findings showing less hypothermia in *FcεRI*^{-/-} or *IgE*^{-/-} mice in models of peanut-induced ASA.^{16,17,35} However, our results are in sharp contrast with reports showing no evidence for involvement of IgE or FcεRI in models of OVA-induced ASA that use alum as an adjuvant during the sensitization phase.^{9,10,20} In line with the strong contributions of both FcγRIII and FcεRI in our model, we found that *FcRγ*^{-/-} mice, which lack the common activating subunit of Fcε and Fcγ receptors,³⁶ were completely protected from hypothermia and developed even lower numbers of intraperitoneal leukocytes than *FcεRI*^{-/-} or *FcγRIII*^{-/-} mice (Fig 2).

In mice FcγRIII is expressed on all myeloid cells,³⁷ whereas FcεRI is mainly expressed on MCs and basophils.³⁸ Depending on the model used, MCs, basophils, monocytes/macrophages, and/or neutrophils have been reported to contribute to ASA.^{11,13,14,16-19,21,26,35} Several studies using passive or active models of anaphylaxis have reported an important role for monocytes/macrophages in the immediate hypothermia response after antigen challenge.^{5,14,17,21,26,39-41} We confirmed these findings in an ASA model by using the adjuvants alum and *B pertussis* toxin for sensitization (see Fig E4). Our depletion experiments also demonstrated that monocytes/macrophages contributed to the hypothermia response in our adjuvant-free ASA model (Fig 2). By contrast, little is known about the function of monocytes/macrophages in the late-phase allergic inflammation after anaphylaxis. We found that mice treated with clodronate liposomes to deplete monocytes/macrophages exhibited significantly increased numbers of intraperitoneal leukocytes (Fig 2). This raises the possibility that monocytes/

macrophages can contribute to the resolution phase in this ASA model. However, care should be taken in the interpretation of these findings because such increased leukocyte numbers might also reflect, at least in part, proinflammatory effects of the clodronate liposomes. Indeed, we also found slightly increased numbers of intraperitoneal MCs and lymphocytes in nonsensitized mice treated with clodronate liposomes.

The importance of neutrophils and basophils in anaphylaxis is debated.¹⁵ Some reports indicate that antibody-mediated depletion of neutrophils can reduce IgG₂-mediated PSA^{5,11} and ASA¹¹ in mice, whereas others found no role for neutrophils in the hypothermia reaction after antigen challenge in IgG-mediated PSA^{13,41} or ASA^{19,21} models. In the present study we used anti-Gr-1 antibodies to deplete neutrophils. We found that such treatment markedly reduced anaphylaxis in an ASA model by using the adjuvants alum and *B pertussis* toxin for sensitization (see Fig E4). By contrast, we found no significant contribution for neutrophils in either the immediate hypothermia reaction or the late-phase accumulation of intraperitoneal leukocytes in the adjuvant-free ASA model (Fig 4). Similarly, some reports indicate a contribution of basophils to IgG-mediated PSA^{5,13,34} or ASA,^{11,17,19} whereas others found no significant role for basophils in anaphylaxis models.^{21,41,42} Here we used conditional basophil-deficient *Mcpt8*^{DTR} mice²³ and found no roles for basophils in either the immediate hypothermia reaction or the late-phase allergic inflammation in our adjuvant-free ASA model (Fig 6).

It is largely agreed that MCs play important roles in food allergy and anaphylaxis.^{14,43-45} Although most of the literature on the roles of MCs in experimental anaphylaxis is based on data obtained by using *Kit* mutant genetically MC-deficient mice, several new models have been developed in which the MC deficiency is not dependent on mutations affecting *c-kit* structure or expression.^{24,30-32,46-49} Although discordant findings have been reported in some disease models in newer versus older MC-deficient strains,^{30,46,47,50,51} the importance of MCs to both IgE-mediated PSA^{24,47,52} and peanut-induced ASA¹⁹ has been confirmed by using multiple MC-deficient mouse strains. In agreement with these findings, we demonstrate here that both *Kit*^{W-sh/W-sh} and *Kit*-independent *Cpa3-Cre*; *Mcl-1*^{fl/fl} MC-deficient mice had reduced immediate hypothermia reactions and diminished numbers of late-phase intraperitoneal leukocytes in our adjuvant-free ASA model (Figs 5 and 7). We obtained additional evidence for an important contribution of MCs in this model by showing that engraftment of genetically MC-deficient *Kit*^{W-sh/W-sh} mice with bone marrow-derived cultured MCs partially restored the immediate hypothermia and completely restored the late-phase allergic inflammation induced by antigen challenge (Fig 7).

These results stand in marked contrast with previous reports^{9,13} and our own data (see Fig E4) showing that anaphylaxis can fully develop after challenge with OVA in MC-deficient mice sensitized with OVA together with the adjuvant alum. One potential explanation for such results is that MCs are particularly potent at promoting allergic reactions at low levels of antibodies and/or that increased levels of antibodies (promoted by the use of adjuvants during the sensitization phase) lead to a greater contribution of alternative pathways that mask or render redundant the role of MCs. Indeed, previous reports show that the contribution of MCs to some ASA models is greater when using low doses of adjuvant, antigen, or both for sensitization

and low doses of antigen for challenge.^{17,19} Our results are also in line with previous reports demonstrating that genetically MC-deficient mice exhibit significantly diminished OVA-induced allergic airway inflammation when sensitized without adjuvant⁵³⁻⁵⁶ but can fully develop airway inflammation when alum is used as an adjuvant during the sensitization phase.^{53,57}

Finally, consistent with previous reports, we found that both histamine and PAF contributed to the immediate hypothermia reaction in WT mice.^{14,20,31,58-60} These 2 mediators also contributed to the late-phase inflammatory reaction, with a more pronounced effect of PAF (Fig 8). By contrast, treatment with a PAF receptor antagonist had no effect in clodronate liposome-treated mice, suggesting that monocytes/macrophages represent the major source of PAF in this adjuvant-free ASA model. As expected, we found that plasma histamine levels were markedly reduced in MC- and basophil-deficient *Cpa3-Cre⁺; Mcl-I^{fl/fl}* mice. More surprisingly, however, we also found reduced plasma histamine levels in clodronate liposome-treated mice (although the reduction was not as substantial as that found in *Cpa3-Cre⁺; Mcl-I^{fl/fl}* mice), suggesting that monocytes/macrophages might also directly or indirectly contribute to histamine release in this model.

In conclusion, we demonstrate here that FcεRI- and FcγRIII-dependent signaling, histamine, and PAF are required for the full development of hypothermia and intraperitoneal leukocyte accumulation in an adjuvant-free model of ASA. In this model both MCs and monocytes/macrophages are critically involved in the immediate hypothermia reaction. In addition, MCs are also required for the full development of intraperitoneal inflammation, as assessed based on numbers of intraperitoneal leukocytes. Thus our data strongly support the hypothesis that MCs and monocytes/macrophages are the main effector cells of anaphylaxis in this setting.

We thank Mariola Liebersbach for her help with mouse breeding, Chen Liu for processing slides for histologic analysis, and all members of the Galli and Bruhns laboratories for discussions.

Key messages

- The IgE receptor FcεRI, the IgG receptor FcγRIII, histamine, and PAF contribute to hypothermia and leukocyte numbers in an adjuvant-free ASA model.
- MCs are required for full development of hypothermia and leukocyte numbers in this ASA model, with no significant role for basophils or neutrophils.
- Monocytes/macrophages contribute to hypothermia in this ASA model.

REFERENCES

- Lieberman P, Camargo CA Jr, Bohlke K, Jick H, Miller RL, Sheikh A, et al. Epidemiology of anaphylaxis: findings of the American College of Allergy, Asthma and Immunology Epidemiology of Anaphylaxis Working Group. *Ann Allergy Asthma Immunol* 2006;97:596-602.
- Sampson HA, Munoz-Furlong A, Campbell RL, Adkinson NF Jr, Bock SA, Branum A, et al. Second symposium on the definition and management of anaphylaxis: summary report—Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol* 2006;117:391-7.
- Simons FE. Anaphylaxis. *J Allergy Clin Immunol* 2010;125(suppl):S161-81.
- Khodoun MV, Strait R, Armstrong L, Yanase N, Finkelman FD. Identification of markers that distinguish IgE- from IgG-mediated anaphylaxis. *Proc Natl Acad Sci U S A* 2011;108:12413-8.
- Khodoun MV, Kucuk ZY, Strait RT, Krishnamurthy D, Janek K, Clay CD, et al. Rapid desensitization of mice with anti-FcγRIIb/FcγRIII mAb safely prevents IgG-mediated anaphylaxis. *J Allergy Clin Immunol* 2013;132:1375-87.
- Wershil BK, Mekori YA, Murakami T, Galli SJ. 125I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin. Demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. *J Immunol* 1987;139:2605-14.
- Dombrowicz D, Flamand V, Brigman KK, Koller BH, Kinet JP. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. *Cell* 1993;75:969-76.
- Makabe-Kobayashi Y, Hori Y, Adachi T, Ishigaki-Suzuki S, Kikuchi Y, Kagaya Y, et al. The control effect of histamine on body temperature and respiratory function in IgE-dependent systemic anaphylaxis. *J Allergy Clin Immunol* 2002;110:298-303.
- Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, Galli SJ. Systemic anaphylaxis in the mouse can be mediated largely through IgG₁ and FcγRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG₁-dependent passive anaphylaxis. *J Clin Invest* 1997;99:901-14.
- Dombrowicz D, Flamand V, Miyajima I, Ravetch JV, Galli SJ, Kinet JP. Absence of Fc epsilonRI alpha chain results in upregulation of FcγRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between FcεRI and FcγRIII for limiting amounts of FcεR beta and gamma chains. *J Clin Invest* 1997;99:915-25.
- Jonsson F, Mancardi DA, Kita Y, Karasuyama H, Iannascoli B, Van Rooijen N, et al. Mouse and human neutrophils induce anaphylaxis. *J Clin Invest* 2011;121:1484-96.
- Million M, Fioramonti J, Zajac JM, Bueno L. Effects of neuropeptide FF on intestinal motility and temperature changes induced by endotoxin and platelet-activating factor. *Eur J Pharmacol* 1997;334:67-73.
- Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* 2008;28:581-9.
- Finkelman FD. Anaphylaxis: lessons from mouse models. *J Allergy Clin Immunol* 2007;120:506-17.
- Jonsson F, Mancardi DA, Albanesi M, Bruhns P. Neutrophils in local and systemic antibody-dependent inflammatory and anaphylactic reactions. *J Leukoc Biol* 2013;94:643-56.
- Sun J, Arias K, Alvarez D, Fattouh R, Walker T, Goncharova S, et al. Impact of CD40 ligand, B cells, and mast cells in peanut-induced anaphylactic responses. *J Immunol* 2007;179:6696-703.
- Arias K, Chu DK, Flader K, Botelho F, Walker T, Arias N, et al. Distinct immune effector pathways contribute to the full expression of peanut-induced anaphylactic reactions in mice. *J Allergy Clin Immunol* 2011;127:1552-61.e1.
- Ahrens R, Osterfeld H, Wu D, Chen CY, Arumugam M, Groschwitz K, et al. Intestinal mast cell levels control severity of oral antigen-induced anaphylaxis in mice. *Am J Pathol* 2012;180:1535-46.
- Reber LL, Marichal T, Mukai K, Kita Y, Tokouka SM, Roers A, et al. Selective ablation of mast cells or basophils reduces peanut-induced anaphylaxis in mice. *J Allergy Clin Immunol* 2013;132:881-8, e1-11.
- Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P. Active anaphylaxis in IgE-deficient mice. *Nature* 1994;370:367-70.
- Smit JJ, Willemsen K, Hassing I, Fiechter D, Storm G, van Bloois L, et al. Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. *PLoS One* 2011;6:e28917.
- Piliponsky AM, Chen CC, Grimbaldston MA, Burns-Guydish SM, Hardy J, Kalesnikoff J, et al. Mast cell-derived TNF can exacerbate mortality during severe bacterial infections in C57BL/6-*Ki^{W-shW-sh}* mice. *Am J Pathol* 2010;176:926-38.
- Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J Clin Invest* 2010;120:2867-75.
- Lilla JN, Chen CC, Mukai K, BenBarak MJ, Franco CB, Kalesnikoff J, et al. Reduced mast cell and basophil numbers and function in *Cpa3-Cre⁺; Mcl-I^{fl/fl}* mice. *Blood* 2011;118:6930-8.
- Sunderkotter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 2004;172:4410-7.

26. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD. Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol* 2002;109:658-68.
27. Vadas P, Gold M, Perelman B, Liss GM, Lack G, Blyth T, et al. Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N Engl J Med* 2008;358:28-35.
28. Grimbaldston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant *Kit^{W-sh/W-sh}* mice as a model for investigating mast cell biology *in vivo*. *Am J Pathol* 2005;167:835-48.
29. Nigrovic PA, Gray DH, Jones T, Hallgren J, Kuo FC, Chaletzky B, et al. Genetic inversion in mast cell-deficient (Wsh) mice interrupts *corin* and manifests as hematopoietic and cardiac aberrancy. *Am J Pathol* 2008;173:1693-701.
30. Rodewald HR, Feyerabend TB. Widespread immunological functions of mast cells: fact or fiction? *Immunity* 2012;37:13-24.
31. Reber LL, Marichal T, Galli SJ. New models for analyzing mast cell functions *in vivo*. *Trends Immunol* 2012;33:613-25.
32. Galli SJ, Tsai M, Marichal T, Tchougounova E, Reber LL, Pejler G. Approaches for analyzing the roles of mast cells and their proteases *in vivo*. *Adv Immunol* 2015;126:45-127.
33. Gaudenzio N, Sibilano R, Starkl P, Tsai M, Galli SJ, Reber LL. Analyzing the functions of mast cells *in vivo* using 'mast cell knock-in' mice. *J Vis Exp* 2015;(99):e52753.
34. Beutier H, Gillis CM, Iannascoli B, Godon O, England P, Sibilano R, et al. IgG subclasses determine pathways of anaphylaxis in mice. *J Allergy Clin Immunol* 2016 [Epub ahead of print].
35. Burton OT, Noval Rivas M, Zhou JS, Logsdon SL, Darling AR, Koleoglou KJ, et al. Immunoglobulin E signal inhibition during allergen ingestion leads to reversal of established food allergy and induction of regulatory T cells. *Immunity* 2014;41:141-51.
36. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* 2012;119:5640-9.
37. Bruhns P, Jonsson F. Mouse and human FcR effector functions. *Immunol Rev* 2015;268:25-51.
38. Kinet JP. The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu Rev Immunol* 1999;17:931-72.
39. Lazar G Jr, Lazar G, Kaszaki J, Olah J, Kiss I, Husztik E. Inhibition of anaphylactic shock by gadolinium chloride-induced Kupffer cell blockade. *Agents Actions* 1994;41Spec No:C97-8.
40. Finkelman FD, Rothenberg ME, Brandt EB, Morris SC, Strait RT. Molecular mechanisms of anaphylaxis: lessons from studies with murine models. *J Allergy Clin Immunol* 2005;115:449-58.
41. Jiao D, Liu Y, Lu X, Liu B, Pan Q, Liu Y, et al. Macrophages are the dominant effector cells responsible for IgG-mediated passive systemic anaphylaxis challenged by natural protein antigen in BALB/c and C57BL/6 mice. *Cell Immunol* 2014;289:97-105.
42. Ohnmacht C, Schwartz C, Panzer M, Schiedewitz I, Naumann R, Voehringer D. Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. *Immunity* 2010;33:364-74.
43. Metcalfe DD, Peavy RD, Gilfillan AM. Mechanisms of mast cell signaling in anaphylaxis. *J Allergy Clin Immunol* 2009;124:639-48.
44. Simons FE. Anaphylaxis: recent advances in assessment and treatment. *J Allergy Clin Immunol* 2009;124:625-38.
45. Reber LL, Sibilano R, Mukai K, Galli SJ. Potential effector and immunoregulatory functions of mast cells in mucosal immunity. *Mucosal Immunol* 2015;8:444-63.
46. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 2011;34:973-84.
47. Feyerabend TB, Weiser A, Tietz A, Stassen M, Harris N, Kopf M, et al. Cre-mediated cell ablation contests mast cell contribution in models of antibody- and T cell-mediated autoimmunity. *Immunity* 2011;35:832-44.
48. Otsuka A, Kubo M, Honda T, Egawa G, Nakajima S, Tanizaki H, et al. Requirement of interaction between mast cells and skin dendritic cells to establish contact hypersensitivity. *PLoS One* 2011;6:e25538.
49. Sawaguchi M, Tanaka S, Nakatani Y, Harada Y, Mukai K, Matsunaga Y, et al. Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. *J Immunol* 2012;188:1809-18.
50. Gutierrez DA, Muralidhar S, Feyerabend TB, Herzig S, Rodewald HR. Hematopoietic Kit deficiency, rather than lack of mast cells, protects mice from obesity and insulin resistance. *Cell Metab* 2015;21:678-91.
51. Kasprick A, Yu X, Scholten J, Hartmann K, Pas HH, Zillikens D, et al. Conditional depletion of mast cells has no impact on the severity of experimental epidermolysis bullosa acquisita. *Eur J Immunol* 2015;45:1462-70.
52. Forster A, Blissenbach B, Machova A, Leja S, Rabenhorst A, Wilmschen S, et al. Dicer is indispensable for the development of murine mast cells. *J Allergy Clin Immunol* 2015;135:1077-80.e4.
53. Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med* 2000;192:455-62.
54. Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. Mast cells can promote the development of multiple features of chronic asthma in mice. *J Clin Invest* 2006;116:1633-41.
55. Nakae S, Ho LH, Yu M, Monteforte R, Iikura M, Suto H, et al. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *J Allergy Clin Immunol* 2007;120:48-55.
56. Yu M, Eckart MR, Morgan AA, Mukai K, Butte AJ, Tsai M, et al. Identification of an IFN-gamma/mast cell axis in a mouse model of chronic asthma. *J Clin Invest* 2011;121:3133-43.
57. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449-54.
58. Shibamoto T, Liu W, Cui S, Zhang W, Takano H, Kurata Y. PAF, rather than histamine, participates in mouse anaphylactic hypotension. *Pharmacology* 2008;82:114-20.
59. Arias K, Baig M, Colangelo M, Chu D, Walker T, Goncharova S, et al. Concurrent blockade of platelet-activating factor and histamine prevents life-threatening peanut-induced anaphylactic reactions. *J Allergy Clin Immunol* 2009;124:307-14, e1-2.
60. Wang M, Shibamoto T, Tanida M, Kuda Y, Kurata Y. Mouse anaphylactic shock is caused by reduced cardiac output, but not by systemic vasodilatation or pulmonary vasoconstriction, via PAF and histamine. *Life Sci* 2014;116:98-105.

METHODS

Flow cytometry

We used flow cytometry to identify blood basophils ($CD49b^{+}IgE^{+}$), neutrophils ($Ly6G^{+}CD11b^{+}$) and monocytes ($Ly6G^{-}CD11b^{+}$), as well as spleen neutrophils ($Ly6G^{+}F4/80^{-}$) and macrophages ($Ly6G^{-}F4/80^{+}$). Briefly, red blood cells were lysed by means of treatment with pH 7.3 ACK lysis buffer (0.15 mol/L NH_4Cl , 1 mmol/L $KHCO_3$, and 0.1 mmol/L EDTA [pH 8.0]) 2 times for 5 minutes each. Cells were blocked with unconjugated anti-CD16/CD32 antibodies (BioXcell, Beverly, Mass) on ice for 5 minutes and then stained with combinations of anti-CD49b (DX5), anti-IgE (R35-72), anti-Ly6G (1A8), anti-CD11b (M1/70), and anti-F4/80 (BM8; all from eBioscience, San Diego, Calif, or BioLegend, San Diego, Calif) antibodies on ice for 30 minutes. Data were acquired with LSRII and Accuri C6 flow cytometers (all from BD Biosciences, San Jose, Calif). Data were analyzed with FlowJo 9.5.3. software (TreeStar, Ashland, Ore). Dead cells (identified by means of staining with propidium iodide [PI; Gibco, Carlsbad, Calif] or LIVE/DEAD Aqua amine [Invitrogen, Carlsbad, Calif]) were not included in the analysis.

Peritoneal lavage and differential cell counts

To harvest peritoneal cells, 2 mL of ice-cold PBS was injected into the peritoneal cavity 1 to 4 days (3 days for routine experiments) after OVA challenge, and the abdomen was massaged gently for 20 seconds. Fluid containing peritoneal cells was aspirated, and the cells were resuspended in ice-cold PBS. Total cell numbers were measured with a hemocytometer chamber, and cells were cytocentrifuged onto glass slides and stained with May-Grünwald-Giemsa. Differential cell counts on at least 300 cells were obtained by using standard morphologic criteria.

Depletion of basophils, monocytes/macrophages, or neutrophils and blockade of $Fc\gamma RIV$, PAF, and histamine

For depletion of basophils, $Mcpt8^{DTR/+}$ basophil-deficient mice and $Mcpt8^{+/+}$ basophil-sufficient littermates received intraperitoneal injections of 500 ng of DT (Sigma-Aldrich) 2 days before and 1 day after challenge with OVA. The efficiency of depletion was assessed by means of flow cytometric analysis of blood basophils ($CD49b^{+}IgE^{+}$) in blood samples collected by means of retro-orbital bleeding 1 hour before OVA challenge.

For neutrophil depletion, mice were injected intraperitoneally with 150 μ g of an anti-Gr-1 antibody (RB6-8C5; BioXcell) 40 hours before and 24 hours after challenge with OVA.^{E1} Control mice were injected with a rat IgG_{2b} isotype control antibody (LTF-2; BioXcell). The efficiency of neutrophil depletion was assessed by means of flow cytometric analysis of neutrophils in blood samples ($Ly6G^{+}$; $CD11b^{+}$) collected by using retro-orbital bleeding 1 hour before and 3 days after OVA challenge, as well as in spleen samples ($Ly6G^{+}$; $F4/80^{-}$) collected 3 days after challenge.

For depletion of monocytes/macrophages, mice were injected with clodronate liposomes (150 μ g administered intraperitoneally plus 150 μ g administered intravenously; ClodronateLiposomes.com) 24 hours before challenge with OVA.^{E1} Control mice were injected in the same manner with the same amount of PBS liposomes. The efficiency of monocyte/macrophage depletion was assessed by means of flow cytometric analysis of monocytes in blood samples ($Ly6G^{-}$; $CD11b^{+}$) collected by using retro-orbital bleeding 1 hour before and 3 days after OVA challenge, as well as in spleen samples ($Ly6G^{-}$; $F4/80^{+}$) collected 3 days after challenge.

For blockade of $Fc\gamma RIV$, mice were injected intravenously with 200 μ g of an anti- $Fc\gamma RIV$ (9E9) antibody 30 minutes before and 2 days after OVA challenge.^{E1} The hybridoma cell line producing anti- $Fc\gamma RIV$ antibodies (9E9) was kindly provided by J. V. Ravetch (Rockefeller University, New York, NY); control mice were injected with polyclonal Armenian hamster isotype control IgG antibodies (BioXcell).

The potential contributions of histamine and PAF in this model were analyzed based on specific inhibition of the actions of these mediators, according to previously described methods,^{E2} with slight modifications: the H_1 receptor-specific antihistamine triprolidine was solubilized at 1 mg/mL

in sterile saline, and 200 μ L was injected intraperitoneally into mice 30 minutes before and 1 day after OVA challenge. The PAF receptor antagonist CV-6209 (Santa Cruz Biotechnology, Dallas, Tex) was diluted at 330 μ g/mL in saline, and 200 μ L was injected intravenously 30 minutes before and 1 day after OVA challenge.

Evaluation of MCs in mesenteric windows

Evaluation of MCs in mesenteric windows was performed, as previously described.^{E3,E4} Briefly, 4 to 5 mesenteric windows from approximately the same locations in each mouse were arranged onto slides and fixed for 1 hour in Carnoy solution (3:2:1 vol/vol/vol of ethanol, chloroform, and acetic acid). The preparations were stained with Csaba stain, which contains both safranin (red, identifying “mature” MCs) and Alcian blue (blue, identifying “less mature” MCs), which bind to MC cytoplasmic granules.^{E3,E4}

Histologic analysis

Ear skin (ear pinnae), back skin, and spleen specimens were fixed in 10% formalin and embedded in paraffin, and 4- μ m sections were stained with 0.1% Toluidine blue for histologic examination and enumeration of MCs. Images were captured with an Olympus BX60 microscope using a Retiga-2000R QImaging camera run by using Image-Pro Plus Version 6.3 software (Media Cybernetics, Rockville, Md).

Measurement of OVA-specific IgG₁ and IgG_{2c} antibodies

Peripheral blood was collected by mean of retro-orbital bleeding 24 hours before OVA challenge. Serum OVA-specific IgG₁ and IgG_{2c} antibody levels were measured by means of ELISA. Briefly, 96-well EIA/RIA plates (Costar, Cambridge, Mass) were coated with 2 μ g/well of OVA in 50 nmol/L carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich, St Louis, Mo) overnight at 4°C. Coated plates were blocked with 1% BSA for 2 hours at room temperature. Plates were washed 3 times, and diluted serum samples were incubated overnight at 4°C. We diluted samples 1:100,000 for IgG₁ and 1:50 for IgG_{2c}. Plates were then washed 3 times and incubated with biotinylated anti-mouse IgG₁ (A85-1; BD PharMingen) or biotinylated anti-mouse IgG_{2a/c} (R19-15; BD PharMingen) for 1 hour at room temperature. Plates were washed 5 times and incubated with streptavidin-horseradish peroxidase (BD PharMingen) for 30 minutes at room temperature. Plates were washed 3 times and incubated with TMB substrate (Sigma), and the reaction was stopped by addition of 2 mol/L H_2SO_4 . Absorbance was measured at 450 nm. Anti-OVA IgG₁ (4B4E6; Chondrex) and IgG_{2a} (M12E4D5; Chondrex, Redmond, Wash) were used as standard antibodies. C57BL/6 does not express IgG_{2a} but does express IgG_{2c}^{E5} (the R19-15 detection antibody we used in our ELISA recognizes both IgG_{2a} and IgG_{2c}^{E6}), and therefore we expressed levels of OVA-specific IgG_{2c} as arbitrary units.

Generation and adoptive transfer of BMCMCs

Female mouse femoral bone marrow cells were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, penicillin, and streptomycin and 20% WEHI-3 cell-conditioned medium (as a source of IL-3) to generate BMCMCs. Cells were cultured for 6 weeks, with medium changed twice a week until more than 95% were $Fc\epsilon R1\alpha^{+}KIT^{+}$ (assessed by using flow cytometry, data not shown). BMCMCs were transferred by means of intraperitoneal plus intravenous injections (2×10^6 cells administered intraperitoneally plus 10^7 cells administered intravenously) into male $Kir^{W-sh/W-sh}$ mice at 4 to 6 weeks of age. Sensitization with OVA was initiated 12 weeks after BMCMC transfer.

Generation of PCMCs

To generate PCMCs,^{E7} peritoneal cells from C57BL/6J mice were maintained *in vitro* for 4 weeks in medium containing stem cell factor (10 ng/mL; R&D Systems, Minneapolis, Minn) until MCs represented greater

than 95% of the total nonadherent cells, as indicated by May-Grünwald-Giemsa staining.

***In vitro* MC degranulation assay**

We assessed the ability of serum samples (collected 24 hours before the challenge) to sensitize PCMCs for activation in response to OVA *in vitro* as an *ex vivo* method to quantify the presence and functional activity of OVA-specific IgE.^{E8} PCMCs were incubated overnight with sera from PBS-treated or OVA-sensitized mice diluted 1:10 in the PCMC complete culture medium (described above). Cells were washed with PBS and stimulated with 10 ng/mL OVA for 30 minutes at 37°C. PCMCs were washed and incubated with avidin–fluorescein isothiocyanate (a probe that specifically binds to heparin contained in MC granules) and PI for 10 minutes on ice. The extent of MC degranulation was assessed in live (PI[−]) cells by measuring the membrane-bound fraction of granules with flow cytometry, as described previously.^{E9} In some experiments serum samples were incubated with a rat IgG₁ anti-IgE antibody (R35-92; 50 µg/mL; BD PharMingen) or a rat IgG₁ isotype control antibody (R3-34; 50 µg/mL; BD PharMingen) for 30 minutes at 37°C before incubation with BMCMCs.

OVA-induced model of active anaphylaxis using alum and *B pertussis* for sensitization

Mice were sensitized intraperitoneally with 100 µg of endotoxin-free OVA (EndoGrade OVA; BioVendor; <0.01 EU of endotoxin per injection), 300 ng of *B pertussis* toxin (Sigma), and 1 mg of Aluminum Hydroxide Gel Adjuvant (alum; InvivoGen, San Diego, Calif) in 200 µL of PBS. Three weeks after sensitization, mice were challenged intraperitoneally with 500 µg of OVA. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge.

IgE-mediated PSA

Purified mouse IgE anti-TNP antibodies (clone C38-2; BD PharMingen) were administered intraperitoneally at a dose of 20 µg in 200 µL of PBS, followed by an intraperitoneal challenge with 500 µg of TNP₍₂₁₋₃₁₎-BSA (Santa Cruz Biotechnology) in PBS 24 hours later. Rectal measurements of body temperature were performed immediately before (time 0) and at different time points for up to 2 hours after challenge.

Measurement of histamine levels and PAF-AH activity

Histamine levels were measured in plasma collected 20 minutes after challenge with OVA by using a commercially available ELISA kit (Histamine EIA; Beckman Coulter, Fullerton, Calif). For measurement of PAF-AH activity, spleens were collected 20 minutes after OVA challenge, weighed, and dissociated into single cells in 400 µL of cold buffer (0.1 mol/L Tris-HCl, pH 7.2). Cell suspensions were sonicated in ice for 10 seconds and centrifuged (20,000g for 10 minutes at 4°C), and supernatant was collected for the assay. PAF-AH activity was measured in the supernatant by using a commercially available assay, according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, Mich). Results were expressed as enzymatic activity per milligram of spleen.

REFERENCES

- E1. Jonsson F, Mancardi DA, Kita Y, Karasuyama H, Iannascoli B, Van Rooijen N, et al. Mouse and human neutrophils induce anaphylaxis. *J Clin Invest* 2011;121:1484-96.
- E2. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD. Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol* 2002;109:658-68.
- E3. Maurer M, Wedemeyer J, Metz M, Piliponsky AM, Weller K, Chatterjea D, et al. Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. *Nature* 2004;432:512-6.
- E4. Metz M, Piliponsky AM, Chen CC, Lammel V, Abrink M, Pejler G, et al. Mast cells can enhance resistance to snake and honeybee venoms. *Science* 2006;313:526-30.
- E5. Jouvin-Marche E, Morgado MG, Leguern C, Voegtli D, Bonhomme F, Cazenave PA. The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics* 1989;29:92-7.
- E6. Cunningham AF, Khan M, Ball J, Toellner KM, Serre K, Mohr E, et al. Responses to the soluble flagellar protein FliC are Th2, while those to FliC on Salmonella are Th1. *Eur J Immunol* 2004;34:2986-95.
- E7. Malbec O, Roget K, Schiffer C, Iannascoli B, Dumas AR, Arock M, et al. Peritoneal cell-derived mast cells: an *in vitro* model of mature serosal-type mouse mast cells. *J Immunol* 2007;178:6465-75.
- E8. Marichal T, Starkl P, Reber LL, Kalesnikoff J, Oettgen HC, Tsai M, et al. A beneficial role for immunoglobulin E in host defense against honeybee venom. *Immunity* 2013;39:963-75.
- E9. Joulia R, Gaudenzio N, Rodrigues M, Lopez J, Blanchard N, Valitutti S, et al. Mast cells form antibody-dependent degranulatory synapse for dedicated secretion and defence. *Nat Commun* 2015;6:6174.

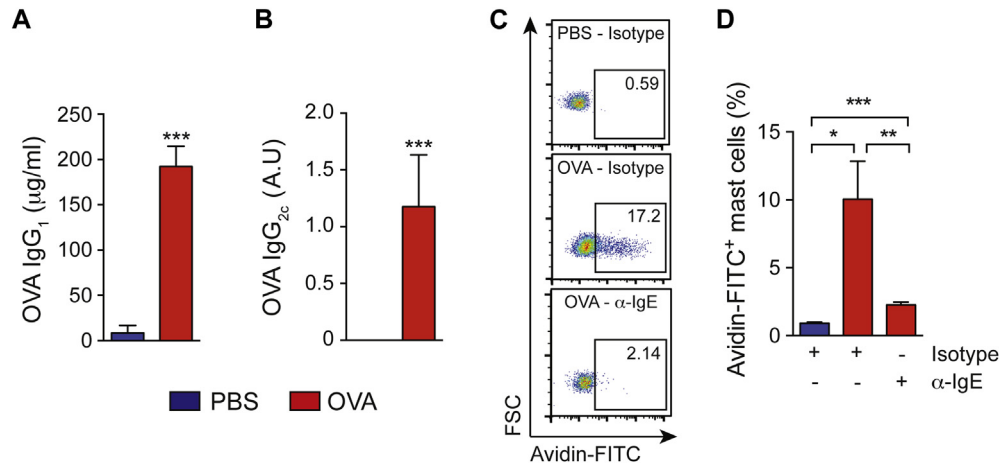


FIG E1. Concentrations of OVA-specific IgG₁ and IgG_{2c} and functional activity of OVA-specific IgE in sera from PBS-treated or OVA-sensitized C57BL/6 mice. **A** and **B**, Concentrations of OVA-specific IgG₁ (Fig E1, A) and OVA-specific IgG_{2c} (Fig E1, B) in serum samples collected 24 hours before challenge in PBS-treated or OVA-sensitized C57BL/6 mice. A.U., Arbitrary units. **C** and **D**, Sera from PBS-treated or OVA-sensitized mice was incubated for 30 minutes with an anti-IgE antibody (to block soluble IgE) or an isotype control antibody, as indicated. Samples were then incubated overnight with PCMCs. PCMCs were washed and stimulated for 30 minutes with OVA. MC degranulation was monitored by means of fluorescence-activated cell sorting analysis with avidin–fluorescein isothiocyanate (*Avidin-FITC*) to stain membrane-bound exocytosed granules. Representative fluorescence-activated cell sorting profiles (Fig E1, C) and percentages of avidin-FITC⁺ degranulated cells (Fig E1, D) are shown. FSC, Forward scatter. Data are pooled from serum samples collected from 2 (PBS group, total n = 5) or 3 (OVA group, total n = 10) independent experiments. **P* < .05, ***P* < .01, and ****P* < .001 versus the corresponding control group or the indicated group.

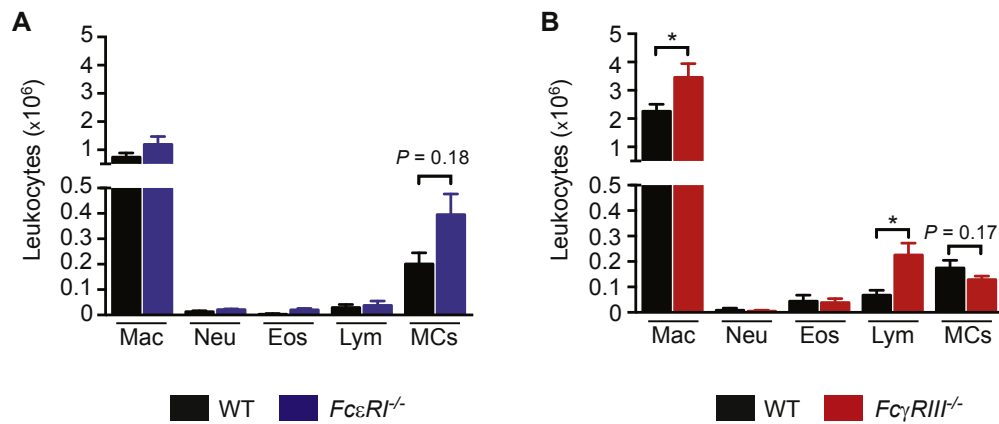


FIG E2. Baseline numbers of intraperitoneal leukocytes in WT, *FcεRI*^{-/-}, and *FcγRIII*^{-/-} mice. Numbers of leukocytes in the peritoneal lavage fluid of naive WT and *FcεRI*^{-/-} mice (**A**) or naive WT and *FcγRIII*^{-/-} mice (**B**; *n* = 6 per group) are shown. **P* < .05. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils.

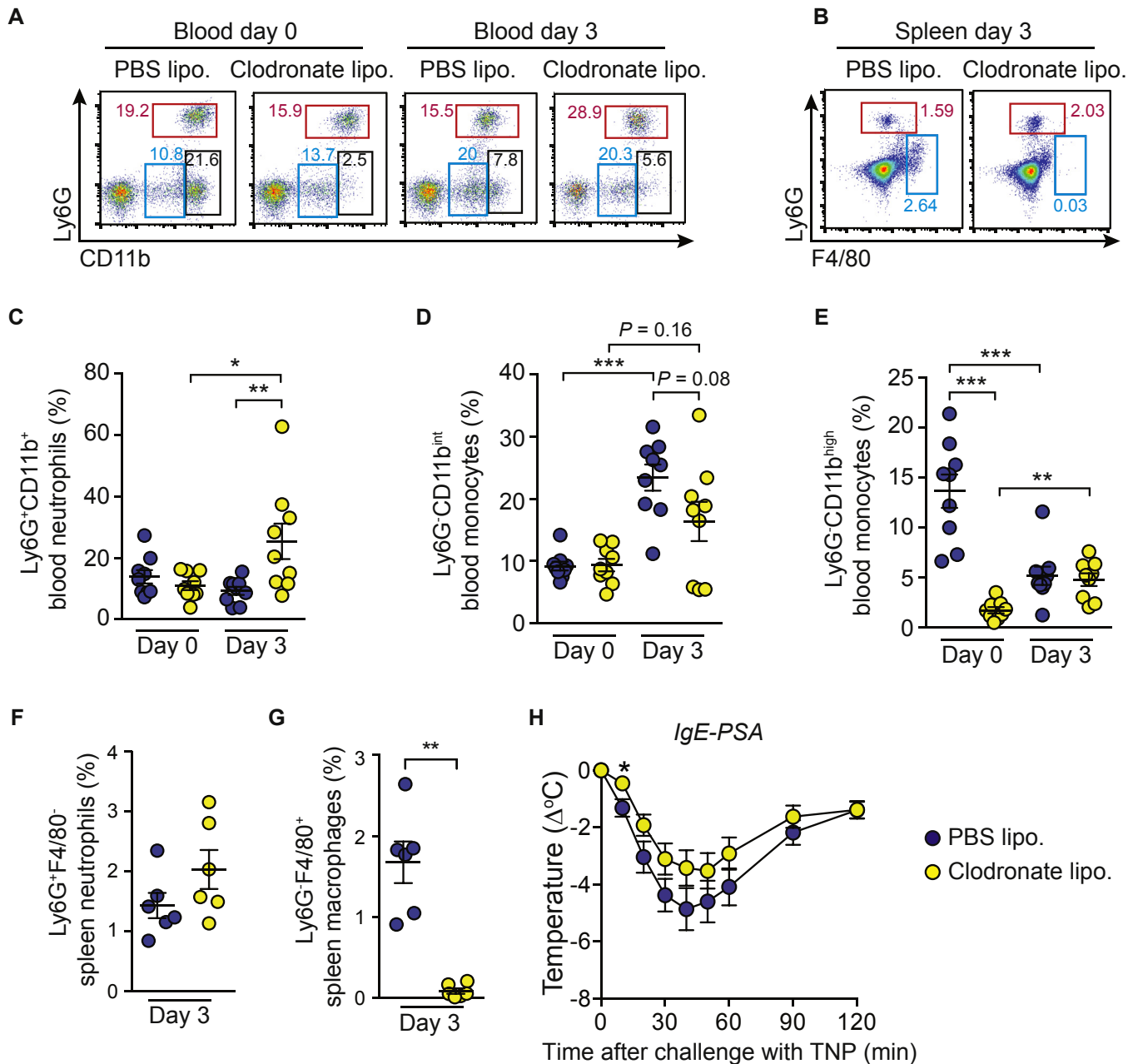


FIG E3. Effect of treatment with clodronate liposomes on macrophages, monocytes, neutrophils, and MCs in C57BL/6 mice. OVA-sensitized mice were treated with clodronate liposomes (150 μ g administered intraperitoneally plus 150 μ g administered intravenously) or the same amount of PBS liposomes (as a control) 24 hours before challenge with OVA. **A**, Representative fluorescence-activated cell sorting profile showing Ly6G⁺CD11b^{high} monocytes (black rectangles), Ly6G⁺CD11b^{int} monocytes (blue rectangles), and Ly6G⁺CD11b⁺ neutrophils (red rectangles) 1 hour before (Blood day 0) and 3 days after (Blood day 3) challenge with OVA. **B**, Representative fluorescence-activated cell sorting profile showing Ly6G⁺F4/80⁺ macrophages (blue rectangles) and Ly6G⁺F4/80⁺ neutrophils (red rectangles) 3 days after challenge with OVA. Numbers in Fig E3, A and B, indicate percentages of each cell population. **C-G**, Percentage of blood neutrophils (Fig E3, C), CD11b^{int} blood monocytes (Fig E3, D), CD11b^{high} blood monocytes (Fig E3, E), spleen neutrophils (Fig E3, F), and spleen macrophages (Fig E3, G) at the indicated time points. **H**, IgE-mediated PSA in mice treated with clodronate liposomes (Clodronate lipo.) or PBS liposomes (PBS lipo.). Fluorescence-activated cell sorting profiles in Fig E3, A and B, are representative of 3 and 2 independent experiments, respectively. Data are pooled from 2 (total n = 6-8 per group; Fig E3, C-E and H) or 3 (total n = 9 per group; Fig E3, F and G) independent experiments. * $P < .05$, ** $P < .01$, or *** $P < .001$ versus the indicated group.

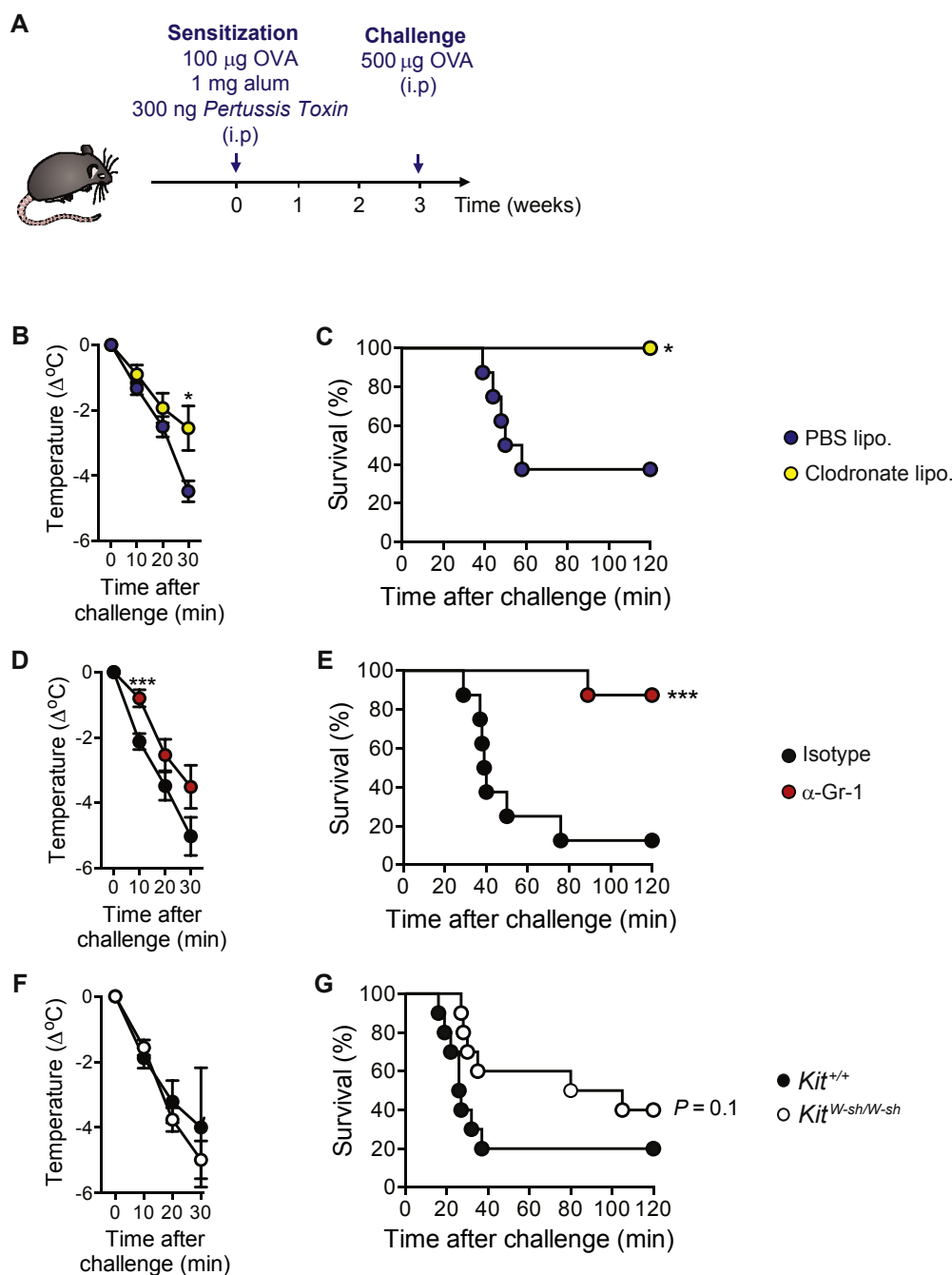


FIG E4. ASA in monocyte/macrophage-depleted mice, neutrophil-depleted mice, and MC-deficient mice sensitized with OVA together with alum and *B pertussis* toxin. **A**, Experimental outline. For monocyte/macrophage depletion, OVA-sensitized mice were treated with clodronate liposomes (*Clodronate lipo.*) or PBS liposomes (*PBS lipo.*; as a control) 24 hours before challenge with OVA. For neutrophil depletion, OVA-sensitized mice were treated with an anti-Gr-1 antibody or an isotype control 40 hours before challenge with OVA. *i.p.*, Intraperitoneal. **B-G**, OVA-induced hypothermia (Fig E4, B, D, and F) and survival (Fig E4, C, E, and G) in the indicated groups of mice. Data are pooled from 2 independent experiments ($n = 8-10$ per group). * $P < .05$ or *** $P < .001$ by using an unpaired Student *t* test (Fig E4, B, D, and F) or a Mantel-Cox log-rank test (Fig E4, C, E, and G).

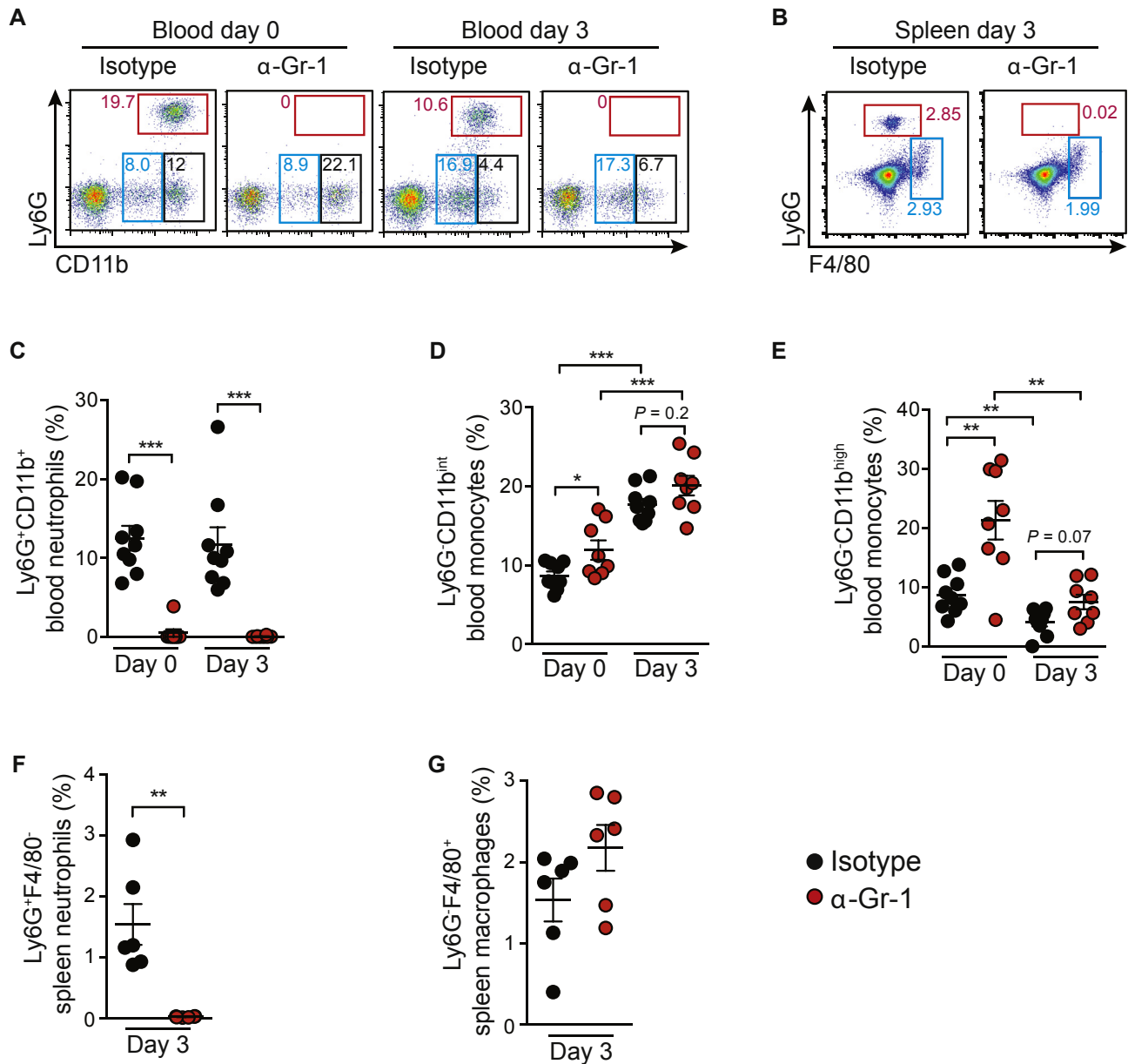


FIG E5. Effect of treatment with an anti-Gr-1 antibody on macrophages, monocytes, and neutrophils in C57BL/6 mice. OVA-sensitized mice were treated with an anti-Gr-1 neutrophil-depleting antibody (150 μ g) or the same amount of an isotype control antibody 40 hours before and 24 hours after challenge with OVA. **A**, Representative fluorescence-activated cell sorting profile showing Ly6G⁺CD11b^{high} monocytes (black rectangles), Ly6G⁺CD11b^{int} monocytes (blue rectangles), and Ly6G⁺CD11b⁺ neutrophils (red rectangles) 1 hour before (Blood day 0) and 3 days after (Blood day 3) challenge with OVA. **B**, Representative fluorescence-activated cell sorting profile showing Ly6G⁺F4/80⁺ macrophages (blue rectangles) and Ly6G⁺F4/80⁻ neutrophils (red rectangles) 3 days after challenge with OVA. Numbers in Fig E5, A and B, indicate percentages of each cell population. **C-G**, Percentage of blood neutrophils (Fig E5, C), CD11b^{int} blood monocytes (Fig E5, D), CD11b^{high} blood monocytes (Fig E5, E), spleen neutrophils (Fig E5, F), and spleen macrophages (Fig E5, G) at the indicated time point. Fluorescence-activated cell sorting profiles in Fig E5, A and B, are representative of 3 and 2 independent experiments, respectively. Data are pooled from 2 (total n = 6 per group; Fig E5, C-E) or 3 (total n = 8-9 per group; Fig E5, F and G) independent experiments. * $P < .05$, ** $P < .01$, or *** $P < .001$ versus indicated group.

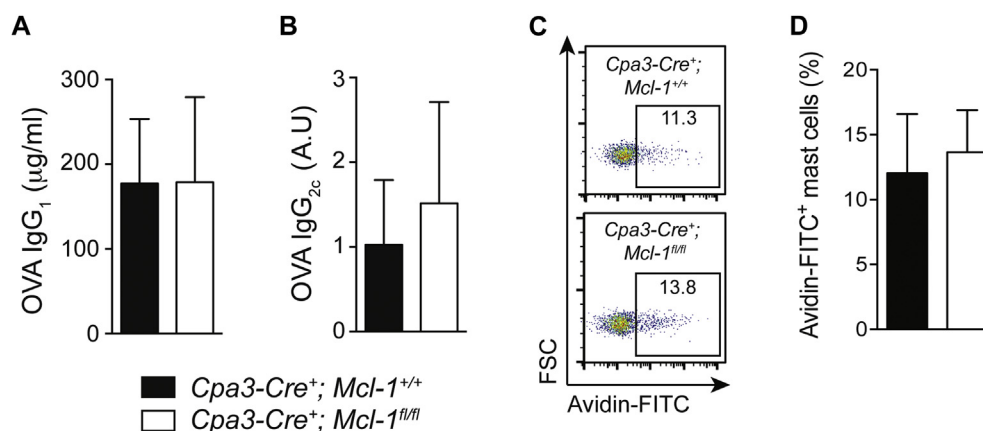


FIG E6. Concentrations of OVA-specific IgG₁ and IgG_{2c} and functional activity of OVA-specific IgE in sera from OVA-sensitized MC- and basophil-deficient *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice. **A** and **B**, Concentrations of OVA-specific IgG₁ (Fig E6, A) and OVA-specific IgG_{2c} (Fig E6, B) in serum samples collected 24 hours before challenge in OVA-sensitized mice. A.U., Arbitrary units. **C** and **D**, PCMCs were incubated overnight with sera from OVA-sensitized *Cpa3-Cre⁺; Mcl-1^{fl/fl}* mice or littermate control (*Cpa3-Cre⁺; Mcl-1^{+/+}*) mice. PCMCs were washed and stimulated for 30 minutes with OVA. MC degranulation was monitored by means of fluorescence-activated cell sorting analysis with avidin-fluorescein isothiocyanate (*Avidin-FITC*) to stain membrane-bound exocytosed granules. Representative fluorescence-activated cell sorting profiles (Fig E6, C) and percentage of avidin-FITC⁺ degranulated cells (Fig E6, D) are shown. Data are pooled from serum samples collected from 3 independent experiments (total n = 9-14 per group). Differences between groups are not significant ($P > .05$).

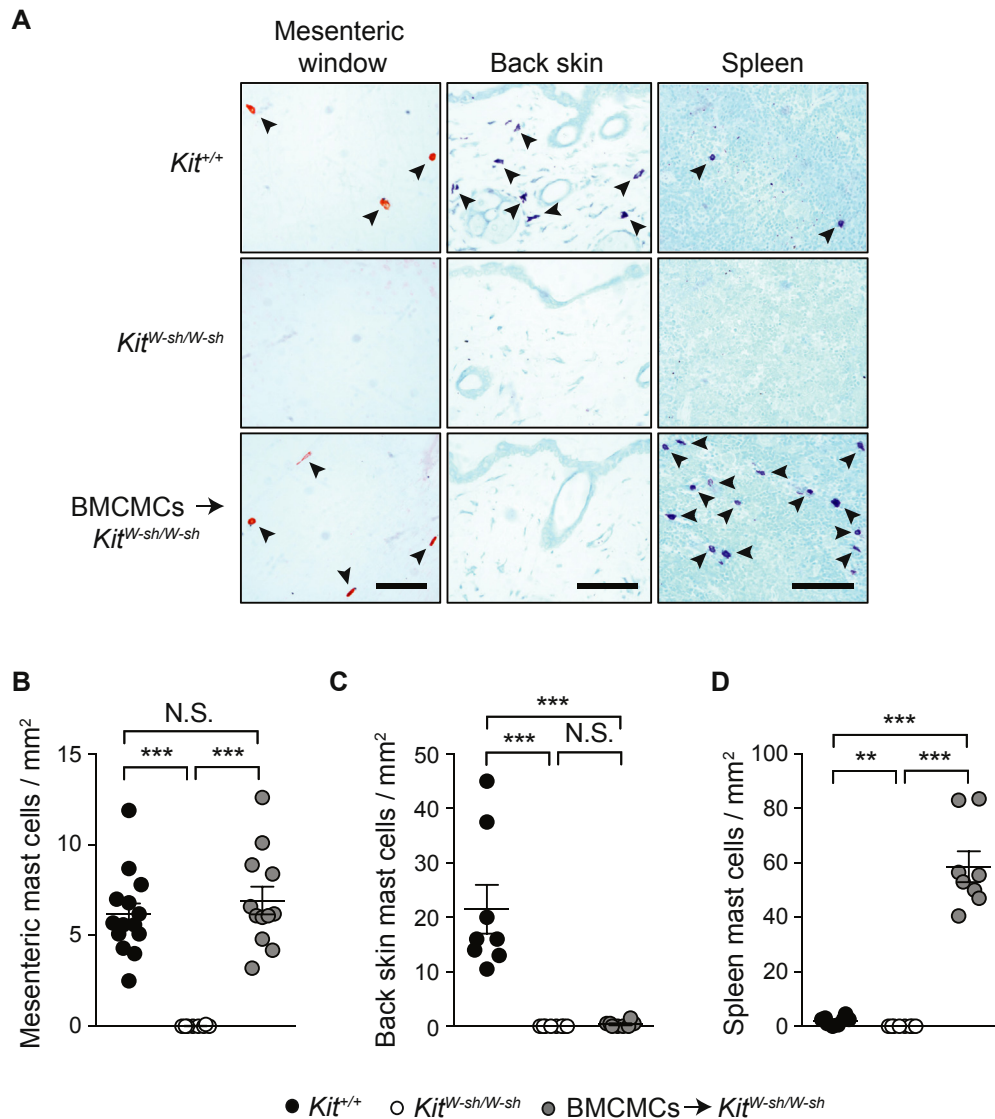


FIG E7. Distribution and numbers of MCs in the skin, spleen, and mesenteric windows of *Kit*^{+/+} WT mice, MC-deficient *Kit*^{W-sh/W-sh} mice, and *Kit*^{W-sh/W-sh} mice engrafted with WT BMCMCs. Tissue samples were collected 3 days after challenge with OVA in OVA-sensitized mice. Representative pictures (**A**) and quantification of MCs in the mesentery (mesenteric window; **B**), back skin (**C**), and spleen (**D**) are shown. MCs in the mesentery (Csaba stain), back skin, and spleen (Toluidine blue stain) are indicated by arrowheads in photomicrographs. Pictures in Fig E7, A, are representative of at least 3 independent experiments. Data in Fig E7, B-D, are pooled from at least 3 independent experiments (total n = 8-14 per group). ***P* < .01 or ****P* < .001 versus the indicated group. N.S., Not significant (*P* > .05).

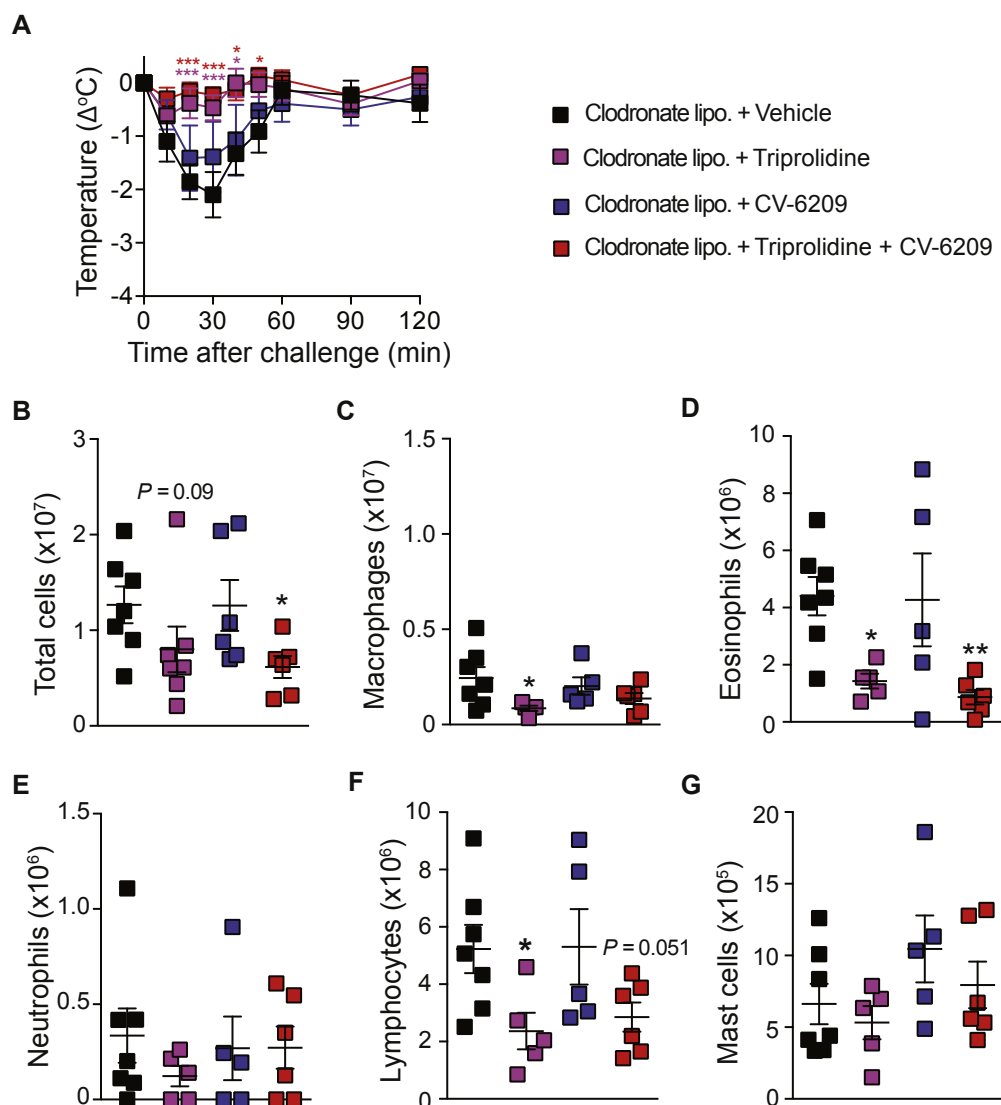


FIG E8. Roles of histamine and PAF in OVA-induced ASA in monocyte/macrophage-depleted mice. OVA-sensitized mice were treated with clodronate liposomes (*Clodronate lipo.*) 24 hours before challenge with OVA. Mice were then treated with the H_1 -antihistamine tripolidine or with the PAF receptor antagonist CV-6209 alone or in combination (or vehicle [saline] as a control) 30 minutes before and 1 day after challenge with OVA. **A**, OVA-induced hypothermia. **B–G**, Numbers of leukocytes in the peritoneal lavage fluid 3 days after OVA challenge. Data are pooled from 2 independent experiments (total $n = 6$ –8 per group). * $P < .05$, ** $P < .01$, or *** $P < .001$ versus the vehicle group.