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Brief report

Paraproteins of familial MGUS/multiple myeloma target family-typical antigens: hyperphosphorylation of autoantigens is a consistent finding in familial and sporadic MGUS/MM

Sandra Grass,¹ Klaus-Dieter Preuss,¹ Stephan Thome,² Dennis D. Weisenburger,³ Vinetta Witt,⁴ Jane Lynch,² Florian Zettl,⁵ Lorenz Trümper,⁵ Natalie Fadle,¹ Evi Regitz,¹ *Henry Lynch,² and *Michael Pfreundschuh¹

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Paratarg-7 (P-7) is a frequent paraprotein target in monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), and Waldenström macroglobulinemia. Patients with P-7-specific paraproteins carry a hyperphosphorylated paratarg-7 (pP-7). Because pP-7 carrier state is dominantly inherited, we determined the paraprotein targets in 4 families with familial MGUS/MM. No antigenic target was identified for the

paraproteins from 2 members of one family. Paraproteins from affected members of 2 other families targeted P-7, and paraproteins from 4 affected members of a fourth family targeted P-8, which is encoded by the ATG13 gene. P-8 was hyperphosphorylated in the affected family members (pP-8) and pP-8 carrier state is inherited in a dominant fashion. Six additional autoantigenic nonfamilial paraprotein targets were also hyperphosphory-

lated in the respective patients compared with normal controls. We conclude that paraproteins of affected members with familial MGUS/MM share family-typical hyperphosphorylated antigens and hyperphosphorylation of paraprotein targets might be a general mechanism underlying the pathogenesis of MGUS/MM. (Blood. 2011;118(3):635-637)

Introduction

Using a human fetal brain-derived protein macroarray in a modified SEREX¹ approach, we identified paratarg-7 (P-7) as the target of 15% of IgA or IgG paraproteins² and 11% of IgM paraproteins.³ All patients with P-7 specific paraproteins are carriers of a hyperphosphorylated version of the protein (pP-7), and this hyperphosphorylation is inherited in a dominant fashion.^{2,4} pP-7 carrier state is associated with an increased risk of developing IgA/IgG monoclonal gammopathy of undetermined significance (MGUS)/multiple myeloma (MM) (odds ratio = 7.9)² and IgM-MGUS (odds ratio = 6.5).³ Because pP-7 is the first molecularly defined risk factor for any hematologic neoplasm known to date,^{2,3} we set out to define the antigenic targets of paraproteins from affected members of families with familial MGUS/MM.

Serum screening on high-density protein arrays, immunoblot analyses, absorption studies, IEF, and phosphatase treatment of paraprotein targets

These were performed as described before.^{2,5} Absorption studies using His₆-tagged P-8 protein or erythrocyte lysates were performed as described before for P-7.^{2,5} Similarly, Western blot analyses, isoelectric focusing (IEF), and phosphatase treatment of all paraprotein targets were performed as described before for P-7.^{2,5}

P-7 and P-8 ELISA

The ELISA using full-length recombinant P-7 was performed as described by us previously.^{2,5} For the P-8 ELISA, recombinant P-8 was used as a coat, and the ELISA was performed according to standard protocols using the paraproteins at a dilution of 1:10⁶.

Methods

Patients and controls

This study was approved by the local ethical review board (Ethikkommission der Ärztekammer des Saarlandes) and conducted according to the Declaration of Helsinki. Peripheral blood and serum samples from 31 healthy and 10 affected members of 4 families with familial MGUS/MM were collected after obtaining written informed consent.

Results and discussion

Four families with ≥ 2 cases of MGUS/MM were included in this study. No antigenic target was identified for the paraproteins from a brother and a sister of the first family, both affected by MM. Two sisters diagnosed with MGUS in the second family whose pedigree had been described before² and 2 siblings with MM in the third family (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) all had an anti-P-7 specific paraprotein and were carriers of pP-7.

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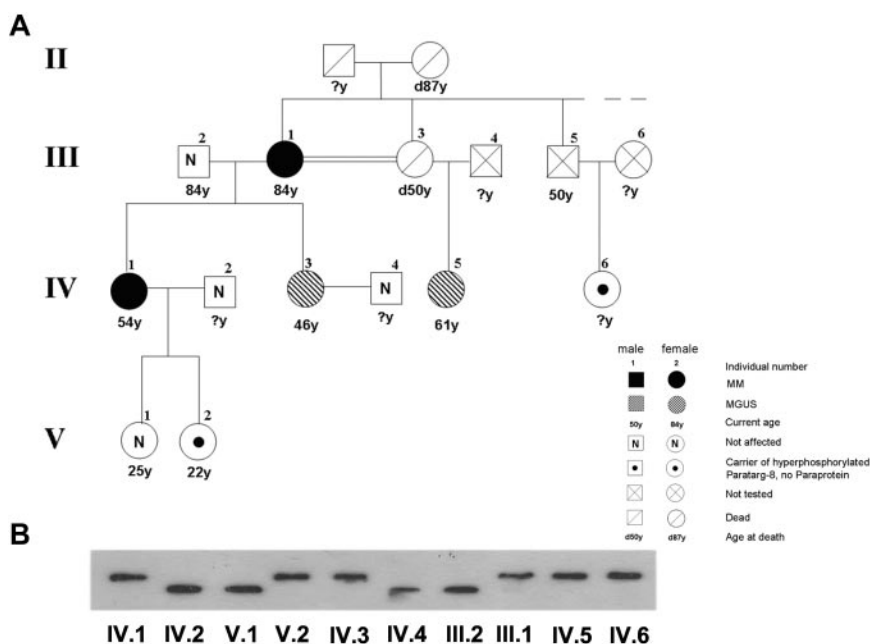


Figure 1. Pedigree of members of family 4, including 2 patients with MM and 2 with MGUS with a P-8 specific paraprotein carrying the hyperphosphorylated P-8. (A) The pedigree shows the family (family 4) of 2 patients with MM (III.1 and IV.1) and 2 patients with MGUS (IV.3 and IV.5), all having P-8 reactive paraproteins and carrying the hyperphosphorylated state of this protein. The pedigree is a part of a pedigree previously published.⁶ (B) Immunostaining of lysate bands derived from whole peripheral blood lysates from family members carrying wild-type (III.2, IV.2, IV.4, and V.1) and hyperphosphorylated P-8 (III.1, IV.1, IV.6, and V.2) after IEF. The numbers indicate family members in different generations.

Identification and characterization of P-8

The paraproteins from 2 MGUS and 2 MM patients of the fourth family (Figure 1) did not react with P-7. Screening of the protein macroarray identified a single protein as the antigenic target of all 4 paraproteins from these family members affected by MGUS/MM. Sequence analysis showed that this protein was coded by the ATG13 gene, a member of the autophagy regulatory complex family of genes.⁷ The specificity and paraprotein-mediated nature of the observed anti-P-8 reaction were shown by the high titer of the reaction (1:10⁸) and by absorption with recombinant P-8 (supplemental Figure 2).

To investigate the prevalence of paraproteins with P-8 specificity, 300 paraprotein-containing sera were tested for reactivity with P-8 by ELISA. Of these, only 1 reacted with P-8 (titer 1:10⁷). Sequence analysis of P-8 derived from patients with a paraprotein-8 specific paraprotein and healthy controls excluded mutations or polymorphisms as a cause of the autoimmunogenicity of P-8. All lysates from patients with a paraprotein with anti-P-8 or non-P-8 specificity and healthy controls showed identical bands in the Western blots (data not shown). However, erythrocyte lysates from healthy controls and from patients with a P-8 specific paraprotein migrated differently in the IEF (supplemental Figure 3). After phosphatase treatment, all lysates showed identical bands in the IEF, regardless of whether they were derived from healthy controls, from patients having a paraprotein with specificity for P-8, or from patients with a paraprotein that did not bind to P-8. Differential banding in the IEF before and after treatment with alkaline phosphatase demonstrated that P-8 is phosphorylated in healthy persons, although to a lesser degree than in MGUS/MM patients with an anti-P-8 reactive paraprotein. All 4 MGUS/MM patients with an anti-P-8 paraprotein expressed hyperphosphorylated P-8, but hyperphosphorylation of P-8 was not observed in MM/MGUS patients whose paraprotein did not bind to P-8.

Only 1 of 200 healthy whites was shown to be carrier of pP-8 as shown by IEF, demonstrating that pP-8 has a much lower prevalence than pP-7 carrier state.

Phosphorylation state of other autoantigenic targets of paraproteins

All other molecularly defined autoantigenic targets of paraproteins from sporadic, nonfamilial cases of MGUS/MM identified by our

group to date, where cells from the respective patients were available, were tested by IEF before and after phosphatase treatment (Table 1). In all these cases, the patients' paraprotein target was hyperphosphorylated compared with normal controls (supplemental Figure 4).

Paraproteins from patients of families with multiple cases of MGUS/MM are directed against the same autoantigen: whereas no target could be identified for the paraproteins from 2 siblings of family 1, the paraproteins from each of 2 affected family members of family 2 and family 3 (supplemental Figure 1), respectively, recognized P-7, and the paraproteins from family 4 (Figure 1) reacted with P-8. The second intriguing finding of our study is that the autoantigens detected by the autologous paraproteins were hyperphosphorylated in the respective patients compared with normal controls in all (8 of 8) cases where this analysis was possible. The fact that all autoantigenic targets (and possible stimuli) of paraproteins molecularly defined to date are hyperphosphorylated suggests that hyperphosphorylation might be a general mechanism underlying the pathogenesis of MGUS/MM that is operative in many more cases than those with the 8 autoantigenic paraprotein targets described in this study. The elucidation of how this mechanism induces the clonal evolution of a B-cell clone with specificity for the respective autoantigen as well as the question why some carriers of hyperphosphorylated antigens develop MGUS/MM whereas others do not can now be addressed using specific molecular tools.

There have been a number of studies of familial MM, implicating both environmental and inherited factors,^{6,8-11} but results have been inconsistent.¹² pP-7 and pP-8 are the first molecularly characterized structures that provide a plausible explanation for the familial clustering of cases of MGUS/MM, at least in cases with a P-7 (2 families) or P-8 (1 family to date) specific paraprotein. Although a molecular mimicry between these autoantigens and infectious agents cannot be definitely excluded, this mechanism is highly unlikely for 2 reasons: (1) a databank search comparing the autoantigenic targets with sequences from bacteria or viruses did not reveal significant homologies (data not shown); and (2) it would not explain why only the hyperphosphorylated, but not the

Table 1. Hyperphosphorylation of autoantigenic paraprotein targets

Paratarg no.	Antigen	Type of antigen	No. of binding	Subtype*	Phosphorylation state
1	Cylicin 2	Allaantigen†	1/115 nonfamilial cases	1 × IgA-λ	No data available
2	TPP2	Autoantigen‡	1/115 nonfamilial cases	1 × IgG-κ	Hyperphosphorylated
3	IGFBP-2	Autoantigen‡	1/115 nonfamilial cases	1 × IgG-κ	No data available
4	Porcine kinesin	Heteroantigen	1/115 nonfamilial cases	1 × IgG-κ	No data available
5	Microtubule-associated protein	Autoantigen‡	2/103 nonfamilial cases	1 × IgM-κ, 1 × IgG-λ	Hyperphosphorylated
6	LAPTM5	Autoantigen‡	3/103 nonfamilial cases	2 × IgG-λ, 1 × IgG-κ	Hyperphosphorylated
7	SLP-2	Autoantigen‡	4 cases from 2 families; 76/650 nonfamilial cases	See Grass et al ²	Hyperphosphorylated
8	ATG13	Autoantigen‡	4 cases from 1 family; 1/300 nonfamilial cases	3 × IgG-λ, 1 × IgG-κ	Hyperphosphorylated
9	RSP16	Autoantigen‡	1/300 nonfamilial cases	1 × IgG-κ	Hyperphosphorylated
10	SPAG7	Autoantigen‡	2/300 nonfamilial cases	2 × IgG-λ	Hyperphosphorylated
11	SIVA	Autoantigen‡	2/300 nonfamilial cases	1 × IgG-κ, 1 × IgA-λ	Hyperphosphorylated

*The number of paraproteins with the respective specificity among all paraproteins tested for this specificity.

†Cylicin 2 is sperm-specific; hence, it functioned as an alloantigen in the respective female patient.

‡Autoantigen, because it is expressed in the autologous patient's tissues.

wild-type, protein acts as an autoantigen. The hyperphosphorylation of the autoantigens appears to be the most obvious likely reason for their autoimmunogenicity. Indeed, “phosphoepitopes” were reported to induce stronger CD8⁺¹³ and CD4⁺¹⁴ T-cell responses than their nonphosphorylated counterparts. Whether the hyperphosphorylated autoantigenic targets of paraproteins induce the development of MGUS/MM by chronic antigenic stimulation or whether they are only a marker or an epiphenomenon of another susceptibility to develop MGUS/MM can now be investigated in the respective patients and their (not yet) affected relatives. Logical next steps are the identification of the kinases and phosphatases responsible for maintenance of the hyperphosphorylated state and the identification of the mutation or polymorphism(s) underlying the posttranslational modification of the autoantigenic targets of paraproteins.

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Authorship

Contribution: S.G. designed the experiments and wrote the manuscript; K.-D.P. designed the experiments; N.F. and E.R. performed and analyzed the experiments; F.Z. and L.T. recruited and helped analyze the family investigation for the study; S.T., D.D.W., V.W., and J.L. recruited and analyzed the American family for the study; and H.L. and M.P. designed the study and wrote the manuscript.

Conflict-of-interest disclosure: K.-D.P. and M.P. have applied for a relevant patent. The remaining authors declare no competing financial interests.

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Antibodies, reagents, and cells

PBS-liposomes and Clodronate-liposomes were prepared as published.²⁶ Alum hydroxide gel, bovine serum albumin (BSA), albumin from chicken egg grade V (ova), DNP₃₀-coupled human serum albumin (DNP₃₀-HSA), rabbit Glucose-6-phosphate Isomerase (GPI), gadolinium(III)-chloride (GdCl₃), Pluronic F-127, complete and incomplete Freund's adjuvant (CFA, IFA), β -hexosaminidase substrate, anti-FLAG mAb, anti-ovalbumin mIgG1 (OVA-14), anti-ova rabbit serum and purified human serum IgG were obtained from Sigma-Aldrich. Anti-mouse CD11b, CD11c, CD3, CD19, Gr1, SiglecF, NK1.1, c-kit, anti-IgE and anti-human Fc γ RI, Fc γ RII, Fc γ RIII, CD3, CD19, CD56, CD24, CD14, CDw125 mAbs were purchased from BD Biosciences (San Diego, CA); isotype controls from BD and Clinisciences (Montrouge, France); anti-mouse DX5, anti-mouse Fc ϵ RI and anti-human Fc ϵ RI mAbs from eBioscience (San Diego, CA); anti-mouse Fc γ RI (290322), anti-mouse Fc γ RIIIA (275003) and anti-human CCR3 from R&D systems; anti-human Fc γ RIIA mAb (IV.3) from StemCell Technologies. The hybridoma producing mAbs anti-mFc γ RIV (9E9) was provided by J.V. Ravetch (Rockefeller University, NY, USA), anti-Gr1 (RB6-8C5) by R. Coffman (DNAX, Palo Alto, CA), anti-human Fc γ RIIA (IV.3) by C.L. Anderson (Heart & Lung Research Institute, Columbus, OH, USA), anti-Ly-6G (NIMP-R14) by C. Leclerc (Institut Pasteur, Paris, France), IgE anti-DNP (2682I) by C.A. Molinaro (Scripps Clinic and Research Foundation, La Jolla, CA, USA). Purified mAbs mouse anti-hFc γ RIIB/C (GB3) were provided by U. Jacob (SuppreMol, Martinsried/München, Germany), anti-CD200R3 (Ba103) by H. Karasuyama (JST, CREST, Tokyo Medical and Dental University Graduate School, Tokyo, Japan), anti-CCR3 by J.J. Lee (Mayo Clinic, AZ, USA).

Rabbit antiserum against the intracellular portion of human Fc γ RIIA was described before.²⁷ ELISA kit for MPO was obtained from HyCult Biotech, for MIP2 from R&D Systems, for LTC₄ and histamine from Neogen and for PDG₂ from Cayman Chemical. CHO-K1 cells stably transfected with FLAG-tagged mouse Fc γ Rs²⁵ or human FLAG-tagged Fc γ Rs¹⁸ were cultured as described.

Flow cytometry analysis

Human blood from healthy donors or murine blood from Fc γ RIIA transgenic mice was incubated with IV.3-FITC or isotype control (mIgG2b-FITC). Human blood cell populations were discriminated based on FSC/SSC properties and staining of CD3 (T cells), CD19 (B cells), CD14 (monocytes), CD56 (NK cells), Fc ϵ RI/CD203c (basophils), CD24 (neutrophils) and CCR3/CDw125 (eosinophils). Mouse blood cell populations were discriminated based on FSC/SSC properties and staining of CD3 (T cells), B220 (B cells), CD11b/CD115 (monocytes), NK1.1/DX5 (NK cells), IgE/DX5 (basophils), CD11b/Gr1^{high} (neutrophils) and Gr1^{int}/SiglecF (eosinophils). Macrophages in the peritoneum were identified as CD11b⁺/Gr1^{neg} cells, mast cells as IgE⁺/c-kit⁺ expressing cells. Cells in BAL were stained with CD11c/Gr1 and enumerated using Counting beads (Caltag). Expression of different Flag-tagged FcRs in CHO-K1 cells was compared using anti-FLAG antibody.

Immune complex binding: CHO-K1 cells were incubated with preformed ICs made of 0.5 μ g/mL GPI and 1 μ g/mL anti-GPI polyclonal antibodies purified from KRN serum, or of 1 μ g/mL ova and rabbit anti-ova antiserum (1/500 dilution), for 1 hour at 4°C. PCMC were incubated with preformed ICs made of 20 μ g/mL GPI and KRN serum (1/100 dilution) or of 100 μ g/mL ovalbumin and 100 μ g/mL mAb OVA-14, for 30 min at 4°C. Bound ICs were detected using F(ab')₂ GAM-F(ab')₂-PE or DAM-FITC.

Flow cytometric analysis of Ca²⁺ mobilization: 1×10^6 PCMC/ml were preloaded with 50 μ g Fluo-3 AM (Molecular Probes, Eugene, OR) in 0.2% Pluronic F-127 for 30 min and [Ca²⁺]_i was monitored by flow cytometry.

***In vitro* cell activation**

PCMC degranulation and mediator production

PCMC were generated as described²² and challenged with preformed ICs made of 50 μ g/ml GPI either with 1/100 dilution of anti-GPI antiserum or with Protein G-purified IgG anti-GPI (100 μ g/ml), or ICs made of 100 μ g/ml mAb OVA-14 and 100 μ g/ml ova, at 37°C. As a positive control, mast cells were sensitized with IgE anti-DNP 2682I and challenged with 0,01 μ g/ml DNP-HSA. β -hexosaminidase was assayed after 15 min stimulation, and histamine, LTC₄ and PDG₂ after 30 min stimulation.

Human skin-derived mast cells

Mast cells were obtained from human skin as described²³ and resuspended in serum-free X-VIVO 15 medium containing 100 ng/ml of rhSCF (gift of Amgen Inc.). 10^6 /ml 6-16 week-old mature mast cells were incubated at 37°C for 30 min with anti-hFc ϵ RI mAb 22E7 (1 μ g/ml) or with a mix of IV.3 mAb (1 μ g/ml) and F(ab')₂ goat-anti-mouse IgG Fc γ -specific (2 μ g/ml) to cross-link Fc ϵ RI and Fc γ RIIA, respectively. β -hexosaminidase release was determined as described.²⁴

Activation of primary mouse peritoneal mast cells

Mouse peritoneal cells were stimulated *ex vivo* with preformed IC or 10 μ g/ml anti-IgE (R35-72, BD Pharmingen) for 15 min at 37°C, followed by incubation with toluidine blue at 0°C. Degranulation was assessed morphologically under the microscope on at least 200 cells per experimental point.

Western blot analysis

Immediately after stimulation with indicated reagents, PCMC were lysed in 10 mM Tris (pH 7.2) 1% SDS lysis buffer at 95°C. 20 μ g of proteins (quantified by D_c protein assay, Bio-Rad) were separated by electrophoresis, transferred on PDVF membranes. Membranes were incubated with anti-pERK1/2 (Thr202/Tyr204), anti-pSyk (Tyr525/526), anti-pAkt (S473), anti-pI κ B (Ser32), anti-actin (I-19) mAbs (Cell Signaling), anti-pSHIP (Stemcell Technologies), anti-pLat (Tyr191) (Millipore), anti-pPLC γ 1 (Tyr783) (Santa Cruz) or rabbit serum anti-hFc γ RIIA intracellular domain, followed by incubations with HRP-labeled secondary antibodies (Santa Cruz), detected using enhanced chemoluminescence (Amersham Biosciences).

Human monocyte and neutrophil activation

Blood samples from normal human donors were fractionated by density centrifugation on Lympholyte-poly (Cedarlane Labs). Monocytes were purified from PBMCs, and neutrophils from granulocytes, using anti-CD14 or anti-CD15 magnetic beads (Miltenyi), respectively. $0.5-1 \times 10^6$ purified cells were incubated or not with HA-human IgG or -IV.3 mAb (100 μ g/ml) for 20 min at 37°C in HBSS buffer supplemented with 1.26mM CaCl₂ and 0.9mM MgCl₂. A small fraction of the cells was used to assess cell activation using CD62L expression by flow cytometry; the major fraction of the cells was centrifuged and used for lipid mediator measurement.

Lipid mediator measurement: Culture supernatants were mixed 1:10 v/v in methanol, or cell pellets resuspended in methanol, and centrifuged at 15,000rpm for 10min. Resulting supernatants were frozen at -20°C. Lipid mediators were quantified as described. After adding deuterized PAF and LTB₄ (PAF-d4 and LTB₄-d4, Cayman chemical, Ann Arbor, MI) as internal standards, lipid mediators were extracted with Oasis HLB solid phase extraction cartridge (1cc/10mg, Waters, Milford, MA). PAF, LTB₄ and LTC₄ were quantified using liquid chromatography-tandem mass spectrometry on a TSQ Quantum Ultra mass spectrometer (Thermo Fischer Scientific, Waltham, MA) that was operated in negative electrospray ionization. Selected reaction monitoring (SRM) mode was used to monitor 568.5 to 59 *m/z* transitions for PAF, 572.5 to 59 *m/z* transitions for PAF-d4, 335 to 195 *m/z* transitions for LTB₄, and 624 to 272 *m/z* transitions for LTC₄. Commercial PAF, LTB₄ and LTC₄ (Cayman chemical) mixed with PAF-d4 and LTB₄-d4 were used to draw calibration curves, and data were processed using the Xcalibur 2.0 software (Thermo Fischer Scientific). PAF, LTB₄ and LTC₄ values less than 4pg should be considered under the lower quantification limit (represented by a dashed line in Fig.4).

Lung histology

3µm sections of paraffin-embedded mouse lungs or human lung biopsies were stained for FcγRIIA using rabbit antiserum against the intracellular portion of human FcγRIIA and Histofine (simple stain mouse MAX PO, Nichirei Bioscience Inc.) followed by revelation with AEC (Sigma), and counterstained with Hematoxylin (Merck).

Statistical analyses

Data was analyzed using one-way ANOVA with Bonferroni post-test (Fig. 2A,C-D, and 2E-F lower panels, Fig. 3, 4, 6H, 7H-I), two-way ANOVA with Bonferroni post-test (Fig. 1G, 2E and F upper panels, Fig. 5A-C, 6E-F, 7K-L, S3A-B), Mantel Cox test for all Survival curves, or Student's t-test (all other data).

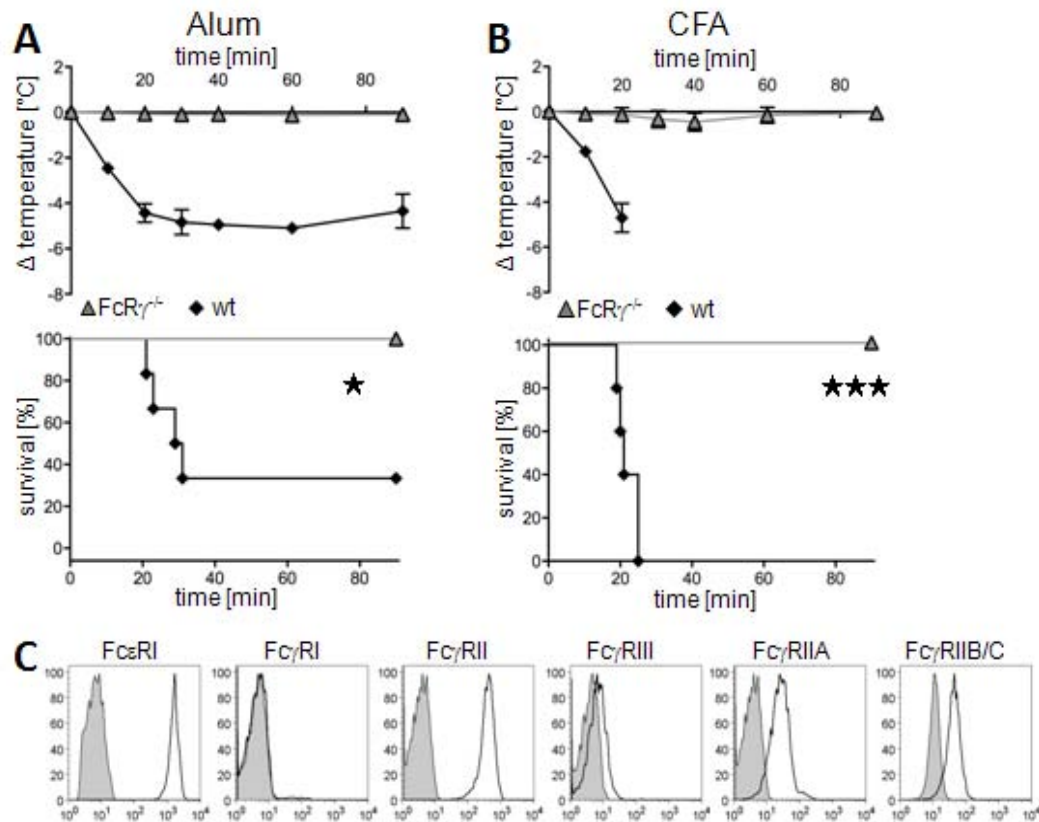


Figure S1

(A–B) Indicated mice were immunized with BSA in alum (A) or in Freund's adjuvant (B) and challenged with BSA. Central body temperatures and survival rates were monitored. ASA in wt (A, n=6; B, n=5) and FcR $\gamma^{-/-}$ mice (A, n=7; B, n=7). (C) Representative histogram plots of human FcR expression on human blood basophils (gated on Fc ϵ RI⁺/CD203c⁺ cells).

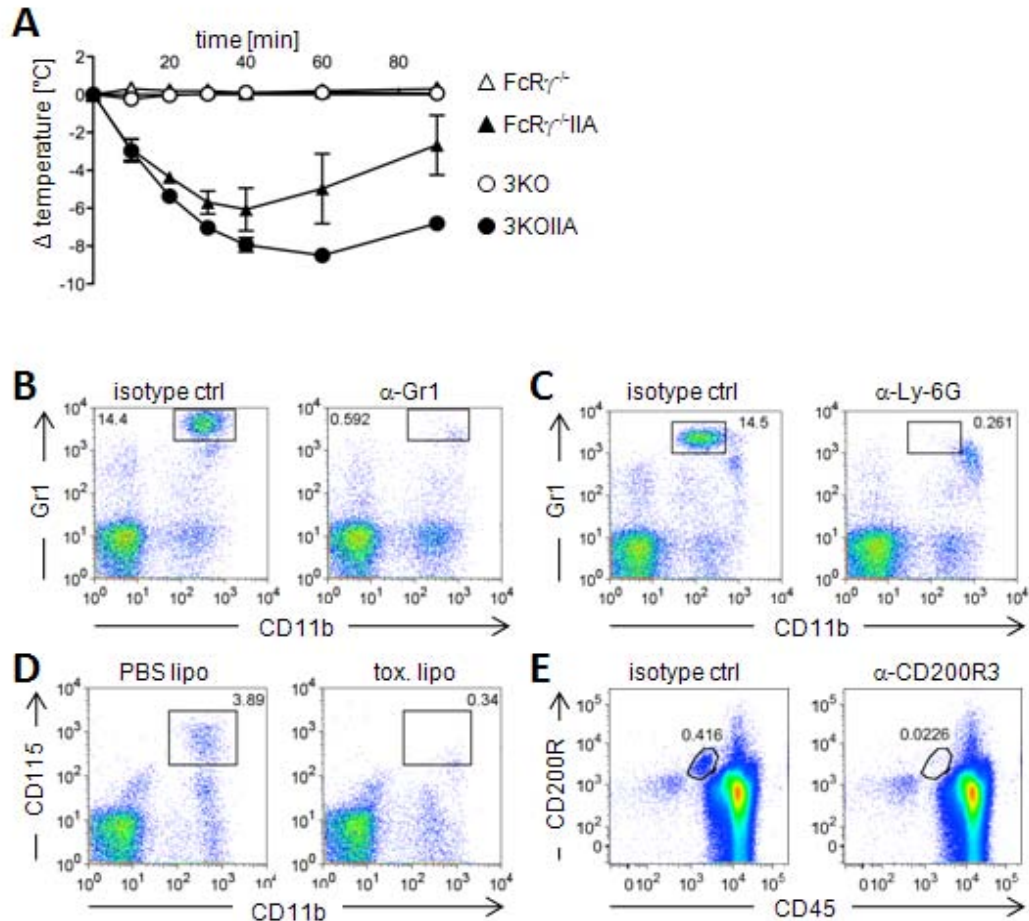


Figure S2

(A) Indicated mice were injected with preformed polyclonal IgG-IC (mouse anti-GPI antiserum plus GPI), and central temperatures were monitored (n=3). (B–E) Representative density plots of mouse blood stained with indicated antibodies upon cell depletion as used in experiments represented in Fig. 3. (B,C) Depletion of mouse neutrophils following injection of anti-Gr1 (B) or anti-Ly6G (C) mAbs. (D) Depletion of monocytes by injection of toxic liposomes. PBS liposomes were used as controls. (E) Depletion of basophils following anti-CD200R3 injection. (B,C,E; left panels) Matched isotype controls are shown for each specific mAb injection. (A) Data are represented as mean $\pm$ SEM. (A–E) Data are representative from at least two independent experiments.

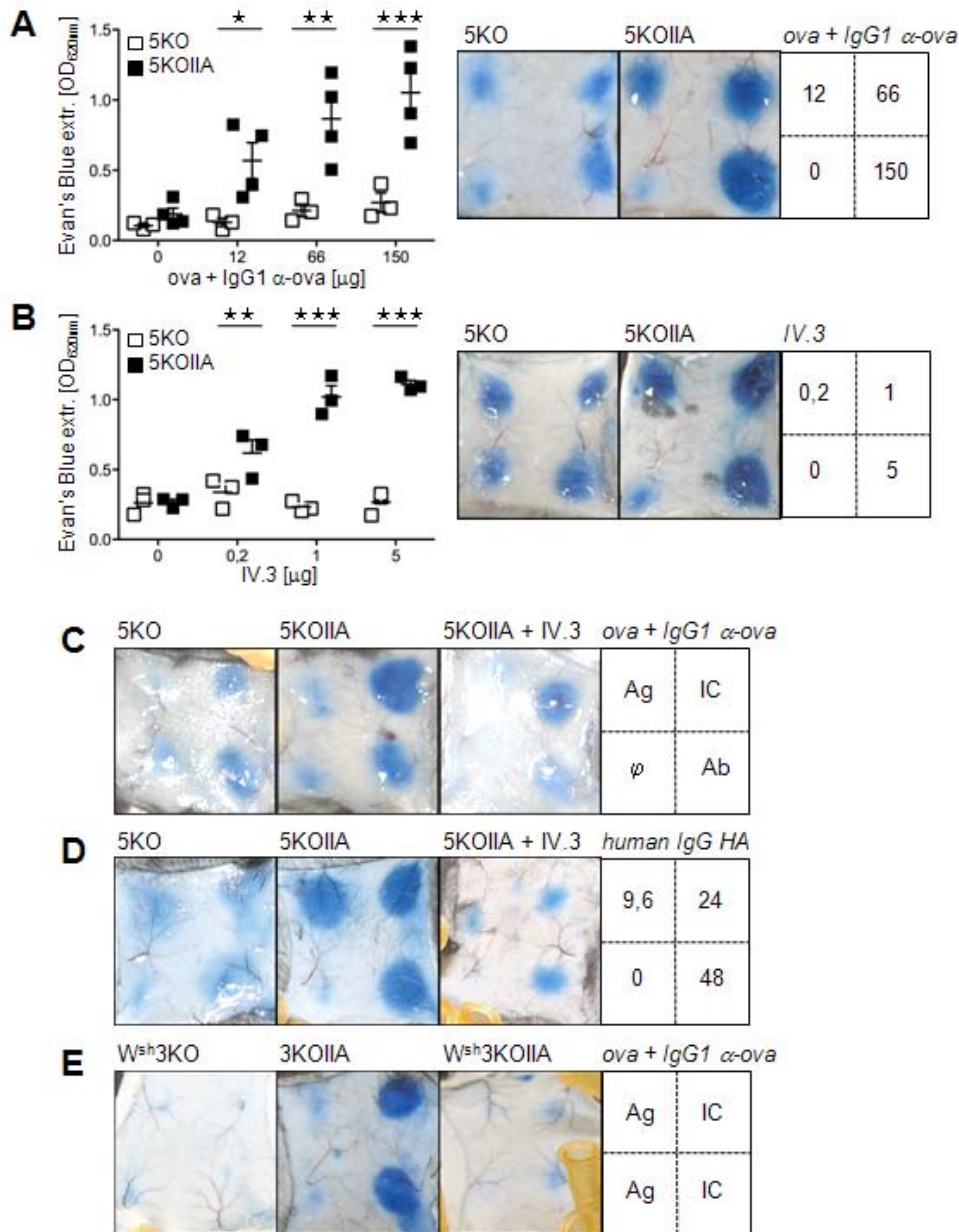


Figure S3

(A–E) Mice were injected intradermally with indicated reagents and i.v. with Evan's blue. (A,B) PCA in 5KO (open symbols: A, n=3; B, n=3), 5KOIIA (black symbols: A, n=4, B n=3). Quantification of Evan's blue extracted from skin tissue are represented (left panel). Data are represented as single measured points, and mean $\pm$ SEM and are representative of at least 2 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. Representative photographs of the four injections sites on the skin (middle panel) and the corresponding doses of reagents (right panel) are indicated. (C–E) Representative photographs of the four injections sites on the skin (left panel) and the corresponding doses of reagents (right panel) corresponding to the PCA reactions from Fig. 5A (C), Fig. 5B (D) and Fig. 5C (E).