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Deficiency of IgE protects mice from experimental abdominal aortic aneurysms

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Abstract

Allergic asthma with high plasma IgE levels is a significant risk factor of human abdominal aortic aneurysm (AAA). This study tests a direct role of IgE in angiotensin-II (Ang-II) perfusion- and peri-aortic CaCl₂ injury-induced AAA in mice. In both models, IgE-deficiency in *Apoe*^{-/-}*Ige*^{-/-} mice blunts AAA growth and reduces lesion accumulation of macrophages, CD4⁺ and CD8⁺ T cells, and lesion MHC class-II expression, CD31⁺ microvessel growth, and media smooth muscle cell loss, compared with those from *Apoe*^{-/-} control mice. Real time-PCR reveals significant reductions in expression of neutrophil chemoattractants MIP-2α and CXCL5 in AAA lesions or macrophages from *Apoe*^{-/-}*Ige*^{-/-} mice, along with reduced lesion Ly6G⁺ neutrophil accumulation. Consistent with reduced lesion inflammatory cell accumulation, we find significant reductions of plasma and AAA lesion IL6 expression in *Apoe*^{-/-}*Ige*^{-/-} mice. Immunofluorescent staining and FACS analysis show that AAA lesion neutrophils express FcεR1. Mechanistic study demonstrates that IgE induces neutrophil FcεR1 expression, activates MAPK signaling, and promotes IL6 production. This study supports a direct role of IgE in AAA by promoting lesion chemokine expression, inflammatory cell accumulation, MAPK signaling, and cytokine expression. IgE inhibition may represent a novel therapeutic approach in AAA management.

Keywords

IgE; abdominal aortic aneurysm; neutrophil; IL6; FcεR1; MAPK

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AUTHOR CONTRIBUTIONS

J. Li and G.-P. Shi designed research; J. Li, Z. Deng, and X. Zhang, performed research; J. Li, Z. Deng, C. Yang, F. Liu, and G.-P. Shi, analyzed data; J. Li and Z. Deng performed statistical analysis; J. Li and G.-P. Shi, wrote the paper.

DISCLOSURES

None.

INTRODUCTION

Abdominal aortic aneurysm (AAA) remains a significant cause of death in adults as a result of aortic rupture.^{1,2} When invasive open or endovascular surgeries remain the only treatment for large (greater than 50 mm), symptomatic, or ruptured AAA, there is no effective medication to prevent or slow the growth of small or asymptomatic AAA.^{2,3} Accumulating studies demonstrate that AAA is an inflammatory disease, in which inflammatory cell activation plays essential roles. Therefore, targeting pro-inflammatory phenotypes holds a potential promise as an efficient therapeutic strategy.⁴⁻⁶ Inflammatory cell recruitment and expression of pro-inflammatory cytokines are key pathological features of human AAA. Among the inflammatory cells, neutrophils are among the most abundant cell types and the first cell population recruited to the site of injured aortic wall.^{7,8} After infiltration and activation, neutrophils release pro-inflammatory molecules such as interleukin-6 (IL6), neutrophil-associated proteases and extracellular traps (NETs), matrix metalloproteinases (MMP) such as MMP-9, cysteinyl cathepsins, and myeloperoxidase to induce aortic wall inflammation, vascular cell apoptosis, and matrix degradation,⁹⁻¹³ leading to aortic rupture. Depletion of circulating neutrophils by using anti-neutrophil antibody or targeting neutrophil recruitment required chemokines or adhesion molecules inhibited AAA development in aortic elastase perfusion or peri-aortic calcium phosphate injury-induced mouse AAA models,^{7,8,14,15} supporting an important role of neutrophils in AAA formation.

Plasma and tissue immunoglobulin E (IgE) is a signature molecule of asthma and allergic responses. Allergic airway inflammation expedited the progression of angiotensin-II (Ang-II) perfusion-induced AAA in mice.¹⁶ Our prior studies found that plasma IgE levels were much higher in patients or animals with both AAA and asthma than those who only suffered from AAA or asthma. A classic role of IgE is to bind to its high affinity receptor FcεR1 on mast cells as the most common mechanism of mast cell activation. Yet, we found that increased plasma and tissue IgE acts as an important pro-inflammatory stimulus to activate not only mast cells, but also CD4⁺ T cells, CD8⁺ T cells, and macrophages, as mechanisms of IgE in promoting AAA formation.¹⁶⁻¹⁸ These inflammatory cells all express FcεR1, although at lower levels than do mast cells. Neutrophils also express FcεR1. IgE-mediated neutrophil activation participates importantly in infectious and allergic diseases, such as cerebral malaria, allergy, and asthma among the others.¹⁹⁻²³ Studies also show that IgE binding to its receptor FcεR1 affects neutrophil survival and cytokine secretion.^{24,25}

Although a direct role of IgE in AAA remains untested, FcεR1-deficiency protected mice from Ang-II perfusion-induced AAA in mice.^{17,26} Accumulating evidences suggest that the pathobiological activities of FcεR1 include more than just mediating IgE activities. We showed that FcεR1 formed complexes with toll-like receptor-4 (TLR4) and Na⁺-H⁺ exchanger-1 (Nhe1). Deficiency of FcεR1, TLR4, or Nhe1 blocked IgE activity in inducing macrophage expressions of cytokines and chemokines and macrophage and vascular cell apoptosis.^{27,28} Fibrinogen ligand or disintegrin rhodostomin activates Nhe1 by forming complexes with integrin α_{IIb}β₃, followed by activating the sodium-calcium exchanger NCX1.²⁹ Activation of Nhe1 promotes the opening of integrin α_vβ₃ headpiece, thereby increasing cell adhesion and migration.^{30,31} Therefore, TLR4 ligand lipopolysaccharide (LPS) and integrins may indirectly affect FcεR1 activity. Here we test a direct role of IgE in

Ang-II perfusion-induced and peri-aortic CaCl₂ injury-induced AAA in mice using IgE-deficient mice. Mechanistic studies reveal a previously unrecognized role of IgE in promoting neutrophil cytokine expression and mitogen-activated protein kinase (MAPK) signaling activation.

MATERIALS AND METHODS

Mouse AAA models

Apolipoprotein E (ApoE)-deficient *ApoE*^{-/-}*IgE*^{-/-} mice, *ApoE*^{-/-}*FcεR1a*^{-/-} mice, and *ApoE*^{-/-} control mice was generated by crossbreeding *IgE*^{-/-} mice (C57BL/6) or *FcεR1*^{-/-} mice (C57BL/6) with *ApoE*^{-/-} mice (C57BL/6). Ang-II perfusion- and peri-aortic CaCl₂ injury-induced experimental AAA was produced as described previously.^{16,17} Briefly, 8~10-week old male mice were either infused with 1000 ng/kg/min Ang-II (A9525, Sigma-Aldrich, St. Louis, MO) subcutaneously delivered by Alzet model 2004 osmotic minipumps (DURECT, Cupertino, CA) or peri-aortic application of 0.5 M CaCl₂ for 28 days to generate AAA. All mice were kept on a 12 hrs/12 hrs light/dark cycle with high fat diet for Ang-II-induced AAA or standard chow diet for CaCl₂-induced AAA. Mice were sacrificed with carbon dioxide narcosis, followed by cardiac puncture blood collection. Plasma IL6 (88-7064-88, Invitrogen, Carlsbad, CA), interferon- γ (IFN- γ) (88-7314-88, Invitrogen), and IL2 (88-7024-88, Invitrogen) levels were determined by ELISA according to the manufacturer's protocols. The maximal suprarenal aortic diameter of each aneurysm was measured from post-mortem mice after the peri-aortic tissue was carefully removed from the aortic wall. Aortic diameters of mice were measured using a surgical microscope (Zeiss Stemi SV11) equipped with a micrometer eyepiece (14 mm/0.1, SG20.T0218c, Motic Instruments, Inc., Vancouver, British Columbia, Canada). All animal procedures conformed with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the Harvard Medical School Standing Committee on Animals (Protocol # 03759).

Mouse aortic tissue immunohistochemical analyses

Mouse aortic tissues were harvested and embedded in optimal cutting temperature (OCT) compound, and aorta segments at 6 μ m were prepared as described previously.^{16,17} The serial sections were stained with antibodies for macrophages (Mac-3, 1:900, 553322, BD Biosciences, San Jose, CA), T cells (CD4, 1:90; 553043, and CD8, 1:100, 01041D, BD Biosciences), major histocompatibility complex class-II MHC-II) (1:250, 556999, BD Biosciences), smooth muscle cell (SMC) (α -actin, 1:750, F3777, Sigma-Aldrich), CD31 (1:1500, 553370, BD Biosciences) and neutrophils (Ly6G, 1:200, BE0075, BioXcell, West Lebanon, NH). CD4⁺ and CD8⁺ T cells, and CD31⁺ microvessels were counted blindly and presented as numbers of total cells per mm² of lesion area. Ly6G⁺ neutrophils were counted and presented as number of cells per section. MHC-II-positive and Mac-3-positive areas were measured using computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD) and presented as positive area per mm² of lesion area. Media SMC accumulation was graded according to the grading keys described previously.³²

Immunofluorescent staining

For immunofluorescent staining, 6 μm frozen sections of aorta were fixed and permeabilized with cold-acetone for 5 min and blocked in phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS) for 1 hr at room temperature. Sections were co-stained with Fc ϵ R1 (1:50, 06727, Sigma-Aldrich) and Ly6G (1:350, BE0075, BioXcell) in 1% FBS at 4 $^{\circ}\text{C}$ overnight. After incubation, the sections were further incubated with Alexa Fluor 488-conjugated donkey anti-rat IgG (1:300, A21208, Invitrogen) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:300, A21428, Invitrogen) in PBS for 1 hr at room temperature. Nuclei were stained for 5 min at room temperature in PBS containing DAPI (0.5 $\mu\text{g}/\text{ml}$, 4083, Cell Signaling Technology, Danvers, MA). Coverslips were mounted with ProLong Gold antifade reagent (P36930, Invitrogen). Images were acquired using an Olympus confocal microscope.

AAA lesion and spleen single cell preparation and flow cytometry analysis

To determine Fc ϵ R1 expression on neutrophils in AAA lesions, Ang-II perfusion-induced AAA lesions from *ApoE*^{-/-} or *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice were minced into small pieces and digested into single cell suspension in an aorta dissociation enzyme stock solution (125 U/ml collagenase type-XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml collagenase type-I; all enzymes were obtained from Sigma-Aldrich) at 37 $^{\circ}\text{C}$ for 1 hr and filtered through a 70 μm cell strainer (322350, BD Bioscience). Single cell preparation was labeled with fixable viability dye eFluorTM 450 (1:200, 65-0863-14, Invitrogen), PerCP-cyanine5.5-conjugated CD45 (1:200, 45-0451-82, Invitrogen), APC-conjugated CD11b (1:200, 17-0112-82, Invitrogen), APC-eFluorTM 780-conjugated Ly6G (1:200, 47-5931-82, eBioscience, San Diego, CA), and FITC-conjugated Fc ϵ R1 (1:200, 11-5898-85, eBioscience) for subsequent FACS analysis.

To characterize the immune cell in spleen from *ApoE*^{-/-} and *ApoE*^{-/-}*IgE*^{-/-} mice, spleen was removed, placed in cold PBS, and grinded into 5 ml PBS before filtering through a 70 μm cell strainer. Splenocytes were collected after depleting the red blood cells. Cells were then washed with PBS and stained with the fixable viability dye eFluorTM 450 or eFluorTM 660 and cell surface marker antibodies including PerCP-cyanine5.5-conjugated CD45, APC-conjugated CD11b, PE-conjugated Ly6G (1:200, 551461, BD PharMingen, Billerica, MA), FITC-conjugated Ly6C (1:200, 53-5932-82, Invitrogen), PE/Cy7-conjugated F4/80 (1:200, 123114, BioLegend, San Diego, CA), Pacific BlueTM-conjugated CD3e (1:200, 100334, BioLegend), PE-conjugated CD8a (1:200, 100708, BioLegend), FITC-conjugated CD4 (1:200, 11-0042-85, Invitrogen), APC-conjugated CD117 (c-Kit) (1:200, 17-1171-83, Invitrogen), and FITC-conjugated Fc ϵ R1 for subsequent FACS analysis.

Macrophage and neutrophil isolation and IgE treatment

Bone-marrow-derived macrophages (BMDMs) were prepared from 8 weeks old *ApoE*^{-/-} and *ApoE*^{-/-}*IgE*^{-/-} mice. Fresh bone-marrow cells were isolated from the femurs and tibias bone and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 20 ng/ml of macrophage-colony stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ) for 9 days. Cells were then stimulated with LPS (Sigma-Aldrich) (100 ng/ml, 24 hrs) and collected for RNA extraction.

Bone-marrow-derived neutrophils were isolated from 8~10 weeks old *Apoe*^{-/-} and *Apoe*^{-/-}*Fcrl1a*^{-/-} mice according to established protocol.³³ Briefly, femur and tibia bones were flushed with RPMI 1640, and cells were collected and treated with ammonium-chloride-potassium lysis buffer (A10492-01, Fisher Scientific, Hampton, NH) to lyse red blood cells. Cell pellet was suspended in HBSS with phenol red and neutrophils were separated and collected by Percoll density gradient centrifugation. Neutrophil purity was confirmed by FACS analysis using APC-conjugated CD11b (1:200, 17-0112-83, eBioscience) and APC-eFluor™ 780-conjugated Ly6G (1:200, 47-5931-82, eBioscience), and Giemsa staining according to manufacturer's protocol (GS500, Sigma-Aldrich). After isolation, neutrophils were plated (200,000/well) on a 6-well plate in RPMI 1640 supplemented with 10% FBS and stimulated with 25 µg/ml aggregated and cytokinergic IgE SPE-7 (D8406, Sigma-Aldrich), monomeric and poorly cytokinergic IgE (H1-DNP-e-206),^{27,34} or AAA lesion lysate for 6 hrs. After stimulation, neutrophil pellet and supernatant were separated by centrifuging at 1000 rpm for 5 min. Cell pellet was collected for RNA and protein extraction and the supernatant was used for measuring IL6 levels using a commercial ELISA kit (88-7064-88, Invitrogen). AAA lysates was prepared from Ang-II perfusion-induced *Apoe*^{-/-}*Apoe*^{-/-} mice. Briefly, AAA was carefully removed and homogenized into RPMI 1640 using TissueLyser-II (Qiagen, Germantown, MD) for 5 min with a frequency of 30/s. The supernatant was separated and collected by centrifugation at 1,000 g for 15 min and the protein concentration was determined using Pierce BCA assay (PI23223, Fisher Scientific).

RNA isolation and real-time-PCR

Mouse aortic tissues were homogenized using the TissueLyser II (Qiagen) according to manufacturer's instructions. Total RNA from mouse aortas and cultured neutrophils were extracted using the TRIzol reagent (15596018, Invitrogen) according to the manufacturer. Reverse transcriptions were performed by using high-capacity cDNA reverse transcription kit (4368813, Applied Biosystems, Foster City, CA). PCR was performed using the SyberGreen GoTaq qPCR master mix (A6001, Promega, Madison, WI) according to the manufacturer. mRNAs levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Changes in expression were calculated using the Ct method. Primers used for real-time-PCR include: C-X-C motif chemokine ligand 2 (CXCL2), forward: CCAACCACCAGGCTACAGG, reverse: GCGTCACACTCAAGCTCTG; C-X-C motif chemokine ligand 5 (CXCL5), forward: GTTCCATCTCGCCATTCATGC, reverse: GCGGCTATGACTGAGGAAGG; IL6, forward: CCTTCCTACCCCAATTTCCAA, reverse: AGATGAATTGGATGGTCTTGGTC; and GAPDH, forward: TGTCATACTTGGCAGGTTTCT, reverse: CGTGTCCTACCCCAATGT.

Protein extraction and immunoblot

Total protein from cultured neutrophils was extracted using the RIPA buffer (BP-115, Boston BioProducts, Ashland, MA) with protease inhibitor and phosphatase inhibitor. Protein concentrations were determined using Pierce BCA assay (PI23223, Fisher Scientific). Equal amount of proteins (20 µg per lane) was separated on a 4-20% Mini-PROTEAN TGX Gel (456-1096, Bio-Rad Laboratories, Hercules, CA). Separated proteins were transferred to PVDF membranes using the Transfer Turbo Blot system (Bio-Rad Laboratories) and the Trans-Blot Turbo RTA transfer kit (170-4272, Bio-Rad Laboratories).

Membranes were blocked with 5% nonfat milk for 1 hr at room temperature. After blocking, the membranes were incubated overnight at 4 °C with antibodies against IL6 (1:1000, 12912), phosphor-c-Jun N-terminal kinase (p-JNK, 1:1000, 9255), total (t)-JNK (1:1000, 9252), p-p38 (1:1000, 9211), t-p38 (1:1000, 9212), phosphor-extracellular-signal-regulated kinase (p-ERK1/2, 1:1000, 9101), t-ERK1/2 (1:1000, 9107), and β -actin (1:4000, 4970), all from Cell Signaling Technology. Proteins on the membranes were visualized by chemiluminescence detection kit (Super signal west femto maximum sensitivity substrate, Fisher Scientific). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to analyze signal abundance.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, Inc., CA). Unpaired 2-tailed Student *t* test was used to determine statistical significance between 2 groups for normally distributed continuous variables. For comparison of multiple groups, ANOVA followed by Tukey multiple comparison analysis or 2-way ANOVA followed by Bonferroni post hoc test were used. For data without normal distribution, nonparametric Mann-Whitney *U* test or Kruskal-Wallis test were applied. All data are presented as mean \pm SEM. $P < 0.05$ was considered statistically significant for all tests.

RESULTS

IgE-deficiency attenuates Ang-II perfusion-induced suprarenal AAA in *Apoe*^{-/-} mice

To examine a direct role of IgE in AAA formation, we generated compound *Apoe*^{-/-}*Ige*^{-/-} mutant mice by crossbreeding IgE-deficient (*Ige*^{-/-}) mice with *Apoe*^{-/-} mice and performed subcutaneous Ang-II (1000 ng·kg⁻¹·min⁻¹) perfusion to produce AAA. Although we did not test whether *Apoe*^{-/-}*Ige*^{-/-} mice differ from *Apoe*^{-/-} mice in lifespan beyond 12 months of age, FACS analysis revealed more CD11b⁺Ly6G⁺ neutrophils, CD11b⁺F4/80⁺ macrophages, and CD11b⁺Ly6C^{int} and CD11b⁺Ly6C^{lo} monocytes in spleens from *Apoe*^{-/-}*Ige*^{-/-} mice than those in age-matched *Apoe*^{-/-} mice (Supplemental Figure S1A–S1C). In contrast, spleens from *Apoe*^{-/-}*Ige*^{-/-} mice had fewer CD8⁺ T cells and CD117⁺FcεR1⁺ mast cells than those from *Apoe*^{-/-} mice (Supplemental Figure S1D/S1E). Splenic CD11b⁺Ly6C^{hi} monocytes and CD4⁺ T cells did not differ between the two types of mice (Supplemental Figure S1C/S1D). Changes in these immune cells may affect AAA development from *Apoe*^{-/-}*Ige*^{-/-} mice. After 4 weeks of Ang-II perfusion, IgE-deficiency markedly blunted the expansion of suprarenal abdominal aortas in *Apoe*^{-/-}*Ige*^{-/-} mice, much smaller than those in IgE-sufficient *Apoe*^{-/-} mice (1.29 \pm 0.18 mm vs. 0.54 \pm 0.07 mm, $P < 0.01$, Figure 1A). In line with the reduction of maximal aortic diameters in *Apoe*^{-/-}*Ige*^{-/-} mice, histological assessment of AAA lesions revealed an 80% reduction of macrophage-positive areas by Mac-3 staining (Figure 1B), a 42% reduction of CD4⁺ T cells and 48% of CD8⁺ T cells by CD4 and CD8 staining (Figure 1C/1D), and a 58% reduction of MHC-II-positive areas (Figure 1E), compared with the lesions from *Apoe*^{-/-} mice. Furthermore, as shown by immunohistochemistry in Figures 1F and 1G, *Apoe*^{-/-}*Ige*^{-/-} mice exhibited a reduction in media SMC loss and microvessel numbers (CD31 staining), compared with those from *Apoe*^{-/-} mice, although we did not test whether IgE-deficiency affected systolic or diastolic blood pressures between *Apoe*^{-/-}*Ige*^{-/-} and *Apoe*^{-/-} mice before and after Ang-II perfusion. These

data indicate that IgE-deficiency alleviates inflammatory response and results in reduced SMC loss, angiogenesis, and AAA lesion progression in Ang-II-perfused *Apoe*^{-/-}*Ige*^{-/-} mice.

IgE-deficiency prevents infrarenal AAA formation in peri-aortic CaCl₂-injured mice

To further establish the protective role of IgE ablation in AAA formation, an independent peri-aortic CaCl₂ injury-induced infrarenal AAA model was introduced in the current study. As we anticipated, IgE-deficient *Apoe*^{-/-}*Ige*^{-/-} mice exhibited a significant reduction of infrarenal maximal outer aortic diameters than those from IgE-sufficient *Apoe*^{-/-} mice (1.04±0.07 mm vs. 0.71±0.07 mm, *P*<0.01, Figure 2A). Consistent with the protective effects found in Ang-II perfusion-induced suprarenal AAA, AAA lesion inflammation (increases in macrophage positive areas, CD4⁺ and CD8⁺ T-cell numbers, and MHC-II-positive areas) (Figures 2B–2E), aortic media SMC loss (Figure 2F), and CD31⁺ microvessel numbers (Figure 2G) were all significantly lower in lesions from *Apoe*^{-/-}*Ige*^{-/-} mice than in lesions from *Apoe*^{-/-} mice. Because CaCl₂ injury-induced AAA did not change systolic or diastolic blood pressure,³⁵ we also did not monitor the blood pressure differences between the groups. Taken together, these observations demonstrate that IgE plays an important role in both chronic and acute injury-induced suprarenal and infrarenal AAA formation.

IgE-deficiency reduces AAA lesion chemokine expression and neutrophil accumulation

Neutrophils are the first cell population recruited to the injured aortic wall and play a critical role in AAA formation and progression.^{36,37} We next asked whether IgE-deficiency also affected neutrophil accumulation during AAA formation. We collected both Ang-II perfusion-induced suprarenal AAA lesion tissues and peri-aortic CaCl₂ injury-induced infrarenal aortic AAA lesion tissues and measured the mRNA levels of macrophage inflammatory protein 2-alpha (MIP-2α) and chemokine CXC motif ligand 5 (CXCL5), both are major neutrophil chemotactic chemokines that are responsible for neutrophil recruitment to the site of inflammation from circulation.^{37,38} As shown in Figure 3A, the expression of MIP-2α repressed by 68% and the expression of CXCL5 reduced by 38% in Ang-II perfusion-induced AAA lesions from *Apoe*^{-/-}*Ige*^{-/-} mice, compared to those from *Apoe*^{-/-} mice. Consistent with these RT-PCR data, immunohistochemical staining showed that the accumulation of Ly6G⁺ neutrophils in lesions from *Apoe*^{-/-}*Ige*^{-/-} mice was significantly blunted compared with those from *Apoe*^{-/-} mice (Figure 3B), although we detected more splenic neutrophils in *Apoe*^{-/-}*Ige*^{-/-} mice than in *Apoe*^{-/-} mice (Supplemental Figure S1A). Ly6G⁺ neutrophils appeared throughout the media and adventitia (Figure 3B).

We obtained similar observations from peri-aortic CaCl₂ injury-induced AAA. Although the expression of MIP-2α did not differ between AAA lesions from *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice, the expression of CXCL5 reduced by 39% in peri-aortic CaCl₂ injury-induced AAA lesions from *Apoe*^{-/-}*Ige*^{-/-} mice (Figure 3C). Again, immunofluorescent staining showed that Ly6G⁺ neutrophils were many fewer in AAA lesion from *Apoe*^{-/-}*Ige*^{-/-} mice than those in lesions from *Apoe*^{-/-} mice (Figure 3D). Ly6G⁺ neutrophils also appeared in the media and adventitia in these AAA lesions (Figure 3D). Macrophages are abundant inflammatory cells in AAA lesions. MIP-2α and CXCL5 expression in macrophages may affect neutrophil

accumulation. In BMDMs from *ApoE*^{-/-} mice, LPS greatly increased the expression of these neutrophil chemokines. Such induction was significantly blocked in cells from *ApoE*^{-/-}*IgE*^{-/-} mice (Figure 3E).

IgE-deficiency reduces IL6 expression in plasma and AAA lesions

Increases of plasma and AAA lesion pro-inflammatory cytokines are signatures of AAA progression and contribute to aortic wall SMC apoptosis, elastin fragmentation, and aortic rupture.^{4,5} As we anticipated, along with reduced AAA formation in *ApoE*^{-/-}*IgE*^{-/-} mice, the increase of plasma IL6 levels in *ApoE*^{-/-} mice was greatly blunted in *ApoE*^{-/-}*IgE*^{-/-} mice from both Ang-II- and CaCl₂-induced AAA models, although plasma IL2 and IFN- γ levels did not differ between the two genotypes of mice from both models (Figure 4A/4B). IgE-deficiency also reduced AAA lesion IL6 expression. RT-PCR analysis of both Ang-II perfusion- and peri-aortic CaCl₂ injury-induced AAA lesions from *ApoE*^{-/-}*IgE*^{-/-} mice demonstrated significant reduction of IL6 mRNA levels, compared with those from *ApoE*^{-/-} mice (Figure 4C/4D).

IgE promotes neutrophil IL6 expression and MAPK activation

IgE-deficiency reduced neutrophil accumulation and IL6 expression in AAA lesions from *ApoE*^{-/-}*IgE*^{-/-} mice (Figures 3 and 4). During AAA development, aortic wall neutrophils are a major source of lesion IL6.³⁹ Based on these prior studies and our own observations, we hypothesized that IgE controls neutrophil IL6 expression and secretion. Using Ang-II perfusion-induced AAA lesion frozen sections prepared from *ApoE*^{-/-} and *ApoE*^{-/-}*IgE*^{-/-} mice, we performed immunofluorescent double staining with Fc ϵ R1 and Ly6G antibodies. Many Ly6G⁺ neutrophils in the adventitia (Figure 5A), media (Figure 5B), and peri-aortic inflammatory cell clusters (Figure 5C) in AAA lesions from *ApoE*^{-/-} mice also expressed Fc ϵ R1. Although there were many fewer Ly6G⁺ neutrophils in AAA lesions from *ApoE*^{-/-}*IgE*^{-/-} mice than those from *ApoE*^{-/-} mice (Figure 3B), we also detected Ly6G⁺Fc ϵ R1⁺ double positive neutrophils in AAA lesions from *ApoE*^{-/-}*IgE*^{-/-} mice (Figure 5D). AAA lesions from age-matched Fc ϵ R1-deficient *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice tested the Fc ϵ R1 antibody specificity. Immunofluorescent staining showed that the Ly6G⁺ neutrophils in these lesions did not express Fc ϵ R1 (Figure 5E). To confirm further that neutrophils in AAA lesions expressed Fc ϵ R1, we prepared AAA lesion single cells from Ang-II perfusion-treated *ApoE*^{-/-} and *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice and performed FACS analysis. We detected Ly6G⁺Fc ϵ R1⁺ neutrophils in AAA lesions from *ApoE*^{-/-} mice, but Ly6G⁺ neutrophils in AAA lesions from *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice did not express Fc ϵ R1 (Figure 5F). Therefore, if not all, some neutrophils in mouse AAA lesions express IgE receptor Fc ϵ R1.

To examine the underlying activity and associated mechanism by which IgE activates neutrophils, we prepared bone-marrow-derived neutrophils from both *ApoE*^{-/-} and IgE receptor Fc ϵ R1-deficient *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice. FACS analysis verified the purity of our purified CD11b⁺Ly6G⁺ neutrophils (Figure 6A) and further confirmed by Giemsa staining (Figure 6B). When neutrophils from *ApoE*^{-/-} mice were treated with 25 μ g/ml of aggregated and cytokinergic IgE (SPE7) for 6 hrs, we detected increased expression of Fc ϵ R1 as determined by FACS analysis. Neutrophils from *ApoE*^{-/-}*Fc ϵ R1*^{-/-} mice were used as negative control and failed to express Fc ϵ R1 after IgE stimulation (Figure 6C/6D). Under

the same IgE treatment condition, neutrophils from *Apoe*^{-/-} mice showed 2-fold increase of IL6 secretion to the culture media as determined by ELISA (Figure 6E) and 3-fold increase of IL6 mRNA levels as determined by RT-PCR (Figure 6F). In contrast, such IgE activities were muted when neutrophils from *Apoe*^{-/-}*Fcer1a*^{-/-} mice were used (Figures 6E/6F), suggesting that IgE activates neutrophil IL6 production *via* cell surface FcεR1.

In response to pro-inflammatory stimuli, MAPK plays an essential role in neutrophil adhesion, migration, and activation.⁴⁰⁻⁴³ Therefore, we examined whether IgE also activated the MAPK signaling pathway as a mechanism to explain increased IL6 expression. When bone-marrow-derived neutrophils from *Apoe*^{-/-} mice were treated with IgE, we detected significant increase of IL6, as determined by immunoblot analysis. From the same samples, we detected concurrent activation of both the phosphor (p)-JNK and p-p38, but not total (t)-JNK or t-p38. Yet, such activities of IgE were muted in neutrophils from *Apoe*^{-/-}*Fcer1a*^{-/-} mice (Figure 6G). In contrast, the activity of IgE in activating p-ERK1/2 remained moderate, regardless whether neutrophils were from *Apoe*^{-/-} or *Apoe*^{-/-}*Fcer1a*^{-/-} mice (Figure 6G).

Neutrophil expression of IL6 in response to IgE stimulation was not limited to autoreactive aggregated and cytokinergic IgE SPE7 (Figure 6C–6G), but also monomeric and poorly cytokinergic IgE H1-DNP-e-206. IgE H1-DNP-e-206 remained active in stimulating neutrophil secretion of IL6 to the culture media and cellular IL6 mRNA levels, as determined by ELISA and RT-PCR (Figure 6H/6I). Same concentration (25 μg/ml) of AAA lysate served as experimental positive control. Although at much lower levels, AAA lysate increased neutrophil IL6 secretion and mRNA levels (Figure 6H/6I). AAA lysate alone without neutrophils was used as ELISA negative control (Figure 6H).

DISCUSSION

Earlier studies demonstrated an essential role of IgE in AAA by activating mast cells, macrophages, and T cells using the IgE receptor FcεR1-deficient *Fcer1a*^{-/-} mice.^{17,26} IgE induces the expression of IL6 and IFN-γ in macrophages, CD4⁺ and CD8⁺ T cells. Such IgE activities were muted when cells from *Fcer1a*^{-/-} mice were used.^{17,27} Yet, studies also showed that IgE induced FcεR1 complex formation with TLR4 and Nhe1 in macrophages,^{27,28} and possibly other inflammatory and vascular cells. It is possible that IgE actions involve not only FcεR1 but also TLR4 or Nhe1. Consistent with this hypothesis, both TLR4 and Nhe1 contribute to AAA growth. Deficiency of TLR4 protected mice from Ang-II perfusion-induced AAA.^{31,44} We recently showed that Nhe1-deficiency also reduced diet-induced atherosclerosis²⁸ and Ang-II perfusion-induced AAA formation in mice (Shi, unpublished observation). We showed that IgE activities in activating foam cell formation and associated signaling transduction (p-AKT, p-PI3K, and p-mTOR), and in inducing protease expression and apoptosis were blocked in macrophages from *Apoe*^{-/-}*Nhe1*^{+/-} mice.²⁸ IgE-induced macrophage expression of IL6 and monocyte chemoattractant protein-1 (MCP-1) and macrophage apoptosis were also muted in cells from TLR4-deficient *Tlr4*^{-/-} mice.²⁷ Therefore, the activities of TLR4 and Nhe1 may also be affected in *Fcer1a*^{-/-} mice, thereby contributing to reduced AAA in these mice.¹⁷ This hypothesis is supported by the observations that both the autoreactive aggregated and cytokinergic IgE SPE7 and the monomeric and poorly cytokinergic IgE H1-DNP-e-206 promoted neutrophil IL6 expression

(Figure 6H/6I) and macrophage extracellular pH reduction, death, and IL6 expression in the absence of antigen dinitrophenyl-human serum albumin (DNP-HSA).²⁷ FcεR1 alone may not explain these IgE activities. IgE-induced FcεR1 complex formation with TLR4²⁷ and Nhe1²⁸ may mediate these IgE activities in the absence of IgE antigens, a possibility that merits further investigation. Nevertheless, the current study tests a direct role of IgE in AAA using IgE-deficient *Ige*^{-/-} mice in both Ang-II perfusion-induced and peri-aortic CaCl₂ injury-induced AAA models. Results demonstrate that IgE-deficiency blunted AAA formation and reduced lesion chemokine expression, inflammatory cell accumulation, and cytokine expression. FcεR1, Nhe1, and TLR4 may all contribute to these IgE activities.

While IgE-mediated activations of mast cells, macrophages, and T cells are equally important in AAA development,^{17,27} this study focuses on a role of IgE in promoting neutrophil accumulation in AAA lesions, and neutrophil IL6 expression and MAPK activation. Accumulating evidences suggest that neutrophils play pathogenic role in AAA. Patients with AAA demonstrate increased blood neutrophil counts and neutrophil activation marker NGAL (neutrophil gelatinase-associated lipocalin).⁴⁵ Targeting neutrophils with antibodies or blocking chemokine expression necessary for neutrophil recruitment ameliorates experimental AAA formation.^{7,8,14} Here we revealed a role of IgE in regulating neutrophil expression of CXC chemokines CXCL5 and MIP-2α that have been shown essential to neutrophil chemoattraction.⁴⁶⁻⁴⁹ That is probably why we detected many fewer neutrophils in AAA lesions from the *ApoE*^{-/-}*Ige*^{-/-} mice than those in *ApoE*^{-/-} mice (Figure 4B/4D). As a major IL6-producing cell type in AAA lesions,³⁹ neutrophils in AAA lesions may produce IL6 to contribute to lesion inflammation and tissue remodeling. Low numbers of lesion neutrophils in *ApoE*^{-/-}*Ige*^{-/-} mice after Ang-II- and CaCl₂-induced AAA may have contributed to reduced AAA in these mice. Yet, our study did not test a direct role of IgE-mediated neutrophil activation in AAA. One way to test a role of neutrophil-derived IL6 in AAA is to adoptively transfer *in vitro* prepared neutrophils from wild-type and IL6-deficient mice into mice subjected to AAA formation. However, such technique may be limited to studies of acute inflammatory disease.⁵⁰⁻⁵² Because of the short lifespan of donor neutrophils (within 24 hrs),⁵³ daily adoptive transfer of neutrophils to study chronic diseases can be technically challenging in addition to causing unnecessary stress to the subjects that may artificially complicate data interpretation. Therefore, neutrophil-selective IL6-deficiency may be required to test a role of neutrophil IL6 in AAA, a hypothesis that merits further investigation.

MAPK signaling is common in inflamed aortic wall but also contributes to neutrophil activation.^{37,54} Inhibition of the JNK and p38 signaling pathways attenuated AAA growth.^{54,55} FcεR1 expression on neutrophils is required for IgE-induced MAPK activation. Although not tested, IgE may activate the MAPK signaling pathways in other aortic wall inflammatory and vascular cells that express FcεR1.^{17,27,28} This mechanism may involve not only IL6, but also other MAPK signaling-associated pro-inflammatory cytokines, chemokines, and proteases from inflammatory and vascular cells, thereby damaging the aortic wall. For example, IL6 and MAPK signaling stimulate MMP and cysteinyl cathepsin expression in macrophages and fibroblasts.^{56,57} Both MMPs and cysteinyl cathepsins play essential roles in AAA development.^{58,59} Although not tested in this study, reduced numbers

of CD31⁺ microvessel contents in AAA lesions from *ApoE*^{-/-}*IgE*^{-/-} mice (Figure 1G/2G) suggest impaired protease expression and extracellular matrix degradation.

Together, results from this study suggest that in addition to mast cells, macrophages, and T cells, IgE also contribute to neutrophil accumulation and activation in experimental suprarenal and infrarenal AAA lesions. Like what have been tested in the IgE targeting molecules FcεR1, TLR4, and Nhe1 from prior studies, IgE-deficiency protects mice from AAA development. Strategies aiming at decreasing IgE expression or blocking IgE actions may provide novel therapeutic approaches to mitigate AAA formation and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NONSTANDARD ABBREVIATIONS

AAA	abdominal aortic aneurysm
Ang-II	angiotensin-II
IgE	immunoglobulin E
IL6	interleukin-6
NETs	neutrophil extracellular traps
MMP	matrix metalloproteinases
MAPK	mitogen-activated protein kinase
ApoE	apolipoprotein E
OCT	optimal cutting temperature
MHC-II	major histocompatibility complex class-II
SMC	smooth muscle cell
BMDM	bone-marrow-derived macrophage
PBS	phosphate-buffered saline
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

CXCL	C-X-C motif chemokine ligand
p-JNK	phosphor-c-Jun N-terminal kinase
p-ERK1/2	phosphor-extracellular-signal-regulated kinase
MIP-2α	macrophage inflammatory protein 2-alpha
DNP-HSA	dinitrophenyl-human serum albumin
TLR4	Toll-like receptor 4
Nhe1	Na ⁺ -H ⁺ exchanger
NGAL	neutrophil gelatinase-associated lipocalin
MCP-1	monocyte chemoattractant protein-1

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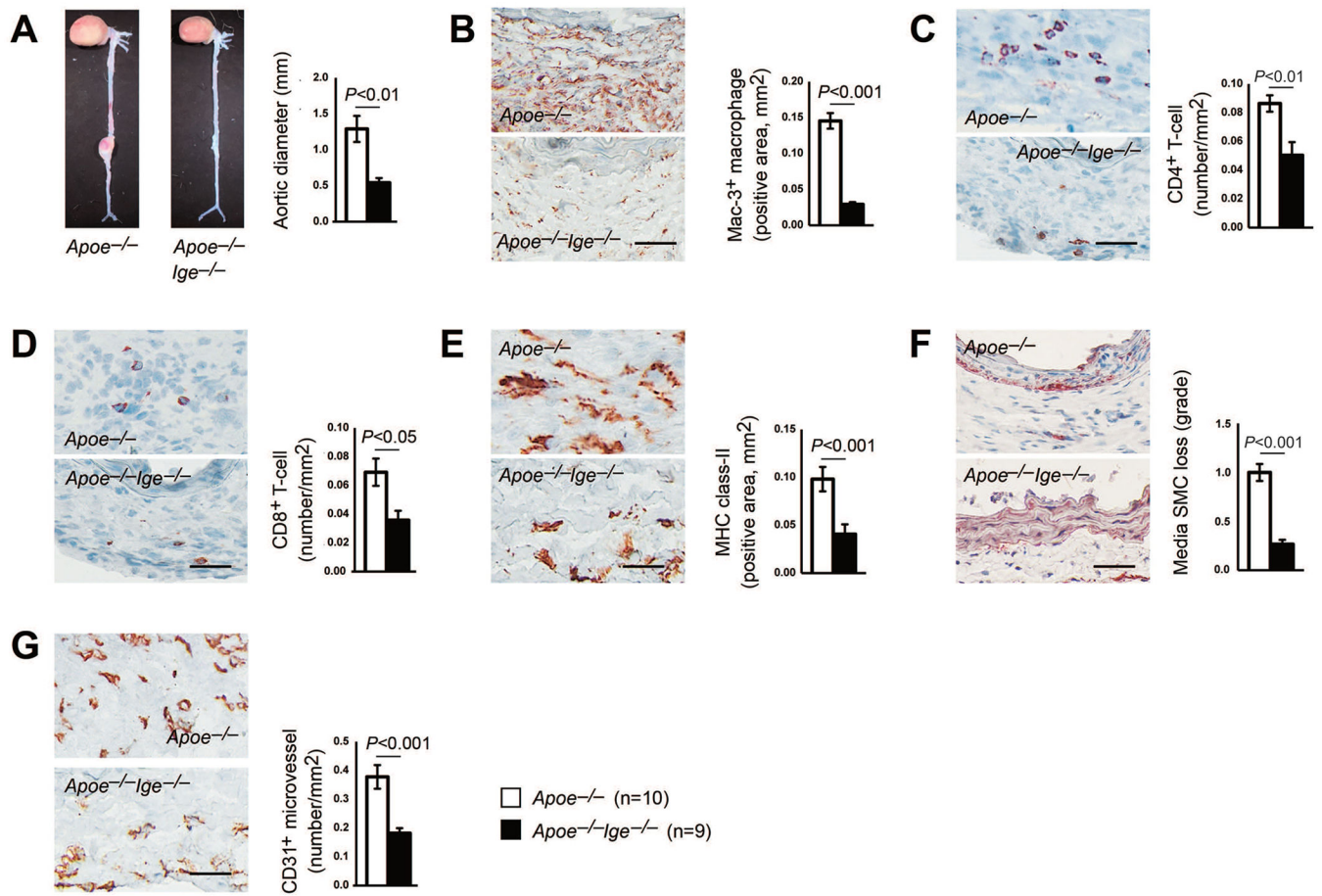


Figure 1.

IgE-deficiency reduces angiotensin-II (Ang-II)-induced AAA in mice. Aortic diameters (A), lesion Mac-3⁺ macrophage contents (B), CD4⁺ T-cell numbers (C), CD8⁺ T-cell numbers (D), MHC class-II levels (E), media SMC loss (F), and CD31⁺ microvessel numbers (G) in AAA lesions from both *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice. Data are mean \pm SEM.

Representative images are shown to the left. Scale: 50 μ m. The number of mice per group and mouse genotypes is indicated.

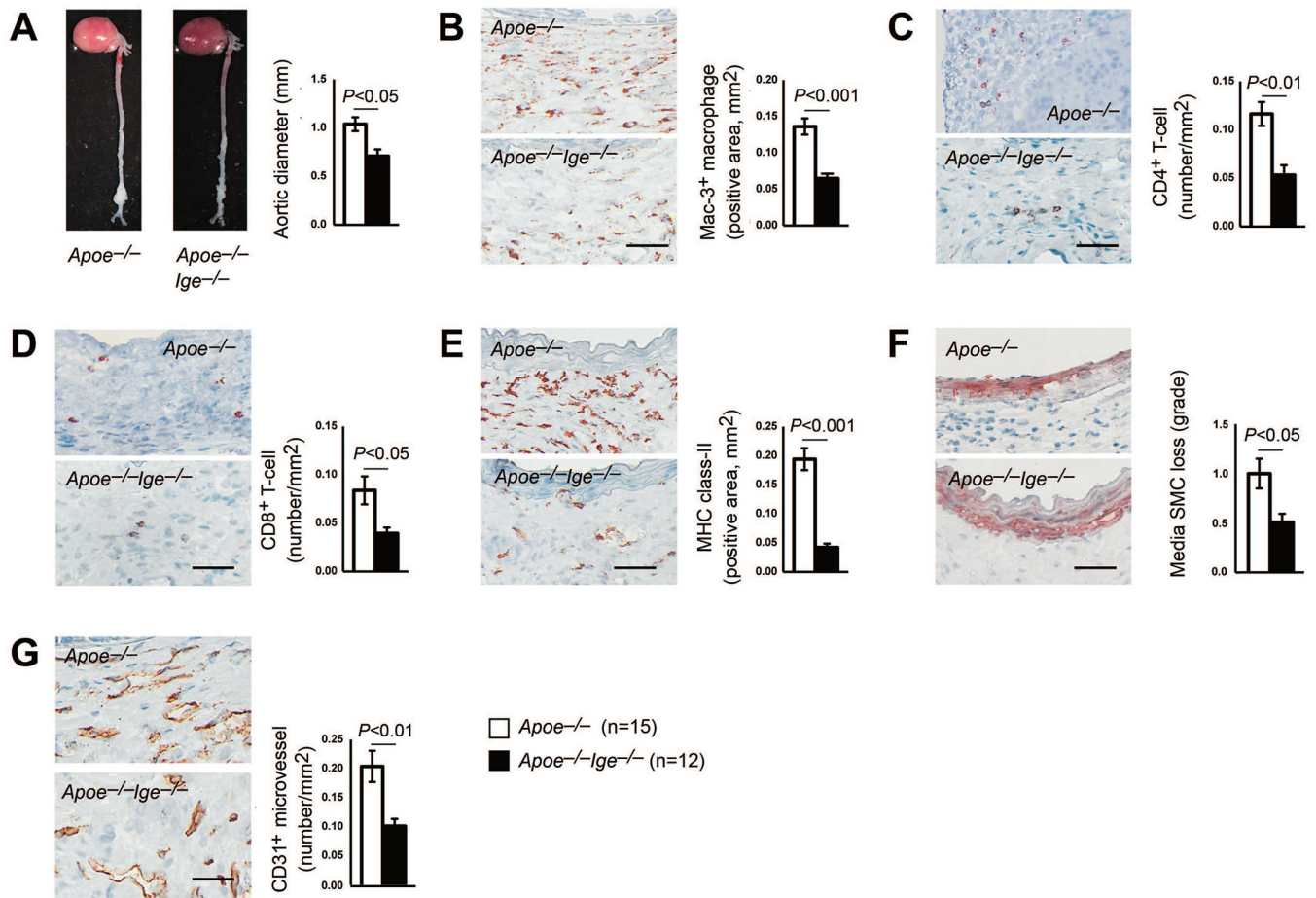
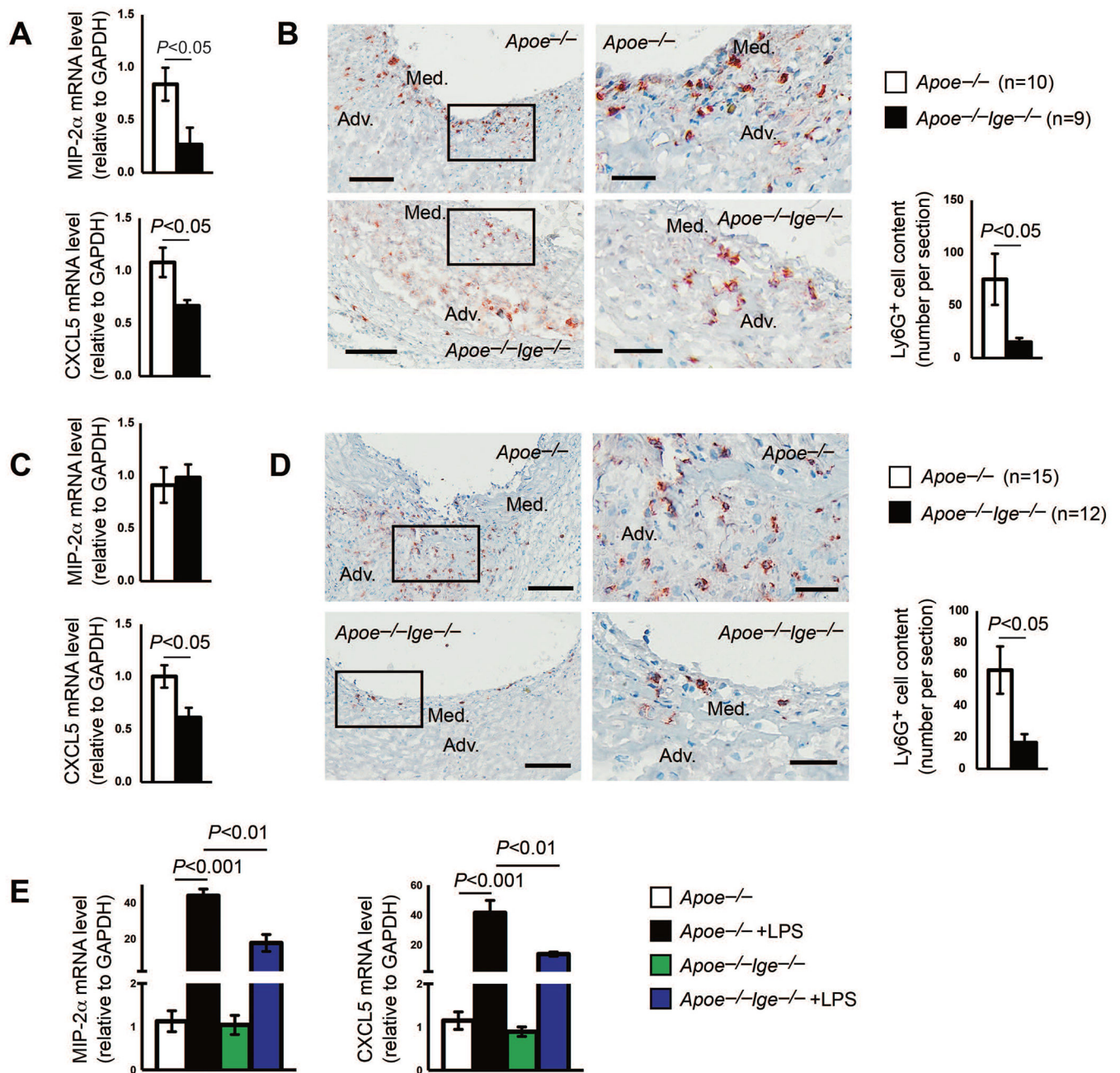


Figure 2. IgE-deficiency reduces peri-aortic CaCl₂ injury-induced AAA in mice. Aortic diameters (A), lesion Mac-3⁺ macrophage contents (B), CD4⁺ T-cell numbers (C), CD8⁺ T-cell numbers (D), MHC class-II levels (E), media SMC loss (F), and CD31⁺ microvessel numbers (G) in AAA lesions from both *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice. Data are mean±SEM. Representative images are shown to the left. Scale: 50 μm. The number of mice per group and mouse genotypes is indicated.

**Figure 3.**

IgE-deficiency suppresses neutrophil accumulation in Ang-II perfusion-induced AAA lesions. MIP-2 α and CXCL5 mRNA levels (**A**) and Ly6G⁺ neutrophil contents (**B**) in Ang-II perfusion-induced AAA lesions from *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice. MIP-2 α and CXCL5 mRNA levels (**C**) and Ly6G⁺ neutrophil contents (**D**) in peri-aortic CaCl₂ injury-induced AAA lesions from *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice. **E.** MIP-2 α and CXCL5 mRNA levels in BMDMs from *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice treated with and without LPS. Data are mean \pm SEM from three independent experiments. Representative images for panels **B** and **D**

are shown to the left. Scale: 200 μm ; insert: 50 μm . The number of mice per group and mouse genotypes is indicated.

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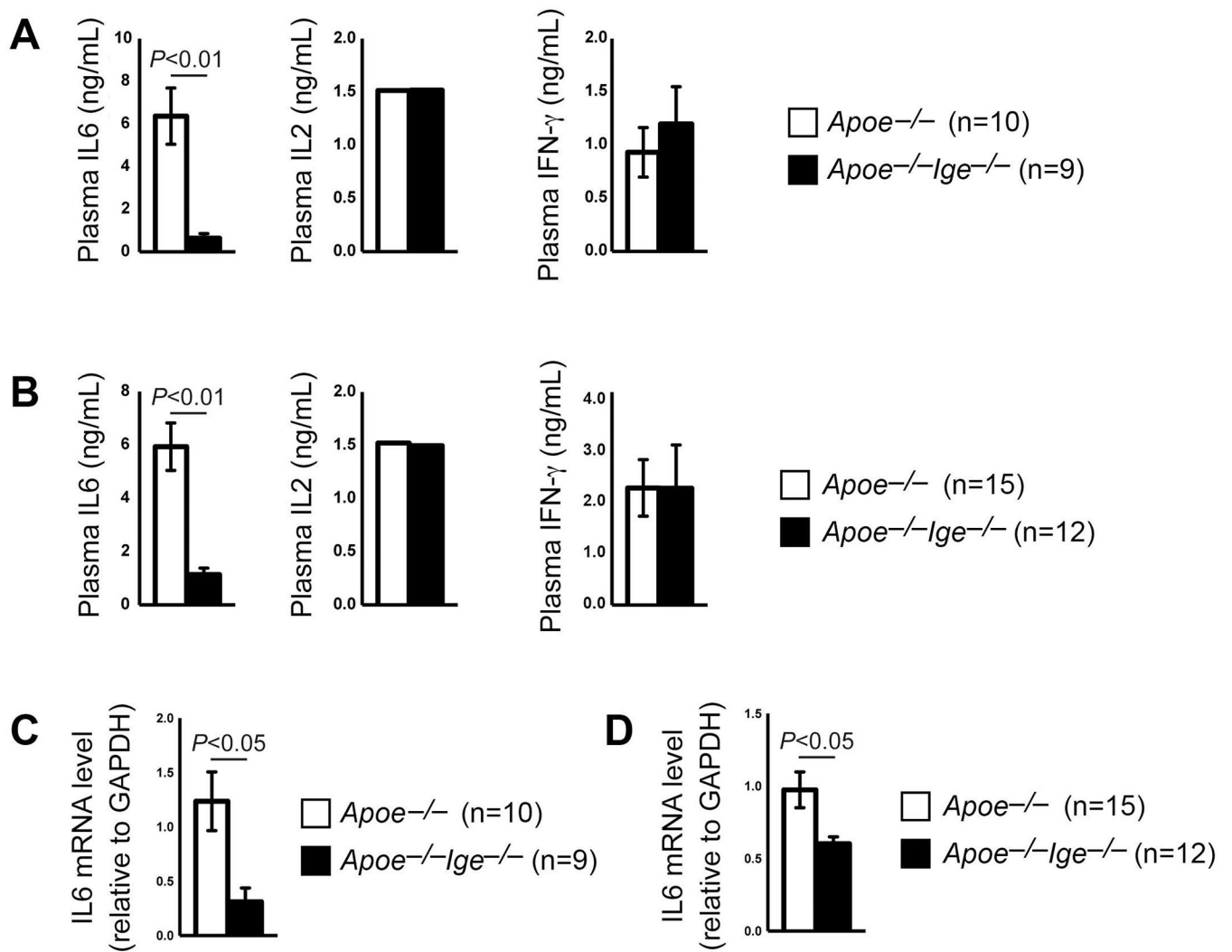
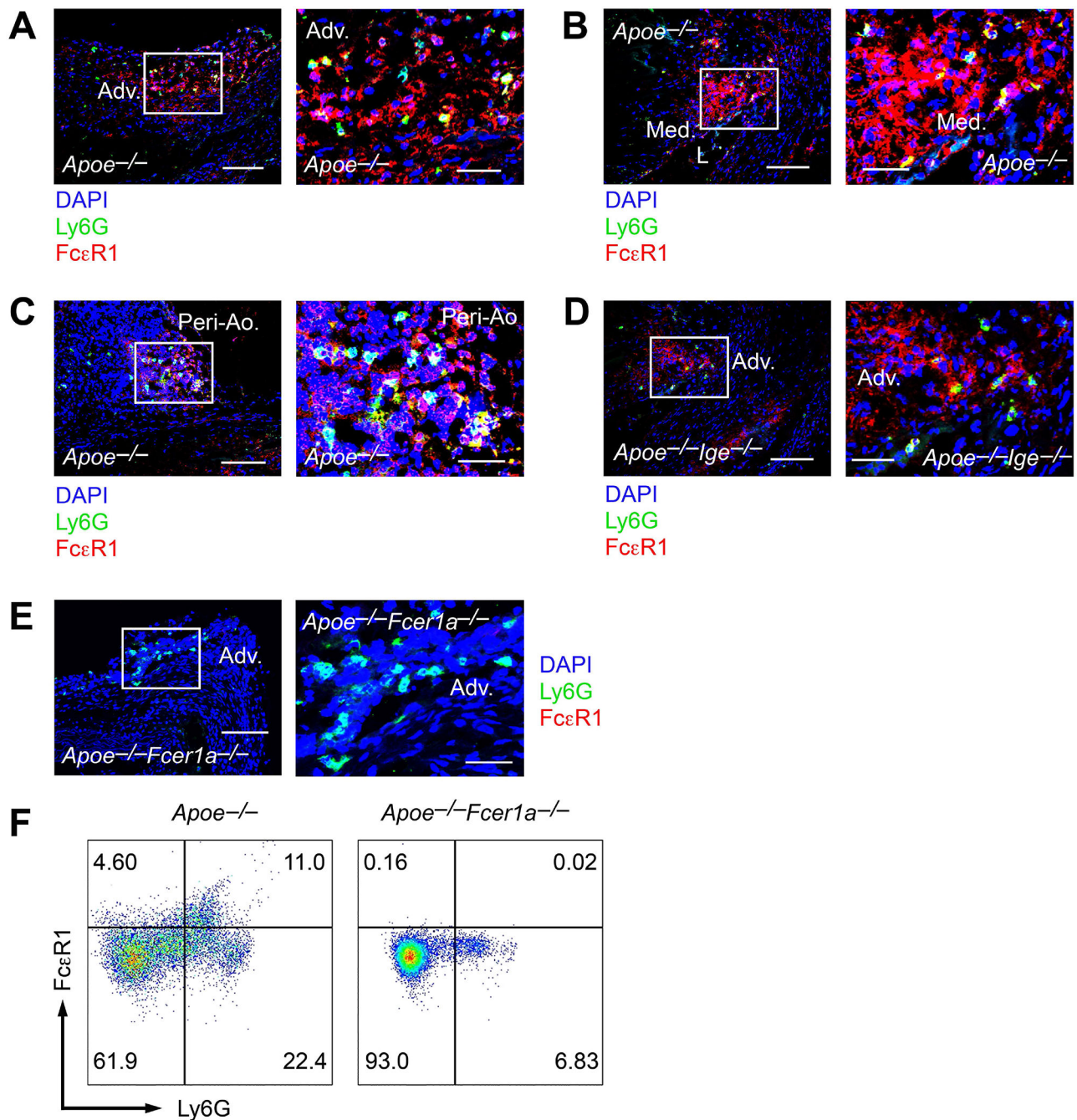


Figure 4.

IgE-deficiency reduces plasma and AAA lesion IL6 levels. **A.** ELISA determined plasma IL6, IL2 and INF- γ levels in *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice after Ang-II perfusion-induced AAA. **B.** ELISA determined plasma IL6, IL2 and INF- γ levels in *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice after peri-aortic CaCl₂ injury-induced AAA. RT-PCR determined the IL6 mRNA levels in AAA lesions from *Apoe*^{-/-} and *Apoe*^{-/-}*Ige*^{-/-} mice after Ang-II perfusion-induced (C) and peri-aortic CaCl₂ injury-induced AAA (D). Data are mean \pm SEM. The number of mice per group and mouse genotypes is indicated.

**Figure 5.**

FcεR1 expression on Ly6G⁺ neutrophils in Ang-II perfusion-induced AAA lesions. Immunofluorescent staining of Ly6G (green) and FcεR1 (red) double positive neutrophils in the adventitia (Adv.) (A), media (Med.) next to the lumen (L) (B), and peri-aorta (Peri-Ao.) inflammatory cell clusters (C) in AAA lesions from *Apoe*^{-/-} mice. D. Ly6G (green) and FcεR1 (red) double positive neutrophils the adventitia (Adv.) in AAA lesions from the *Apoe*^{-/-}*Ige*^{-/-} mice. E. AAA lesions from *Apoe*^{-/-}*Fcer1a*^{-/-} mice were used as negative control to detect FcεR1 expression (red) on Ly6G neutrophils (green). Scale: 200 μm; insert: 70 μm.

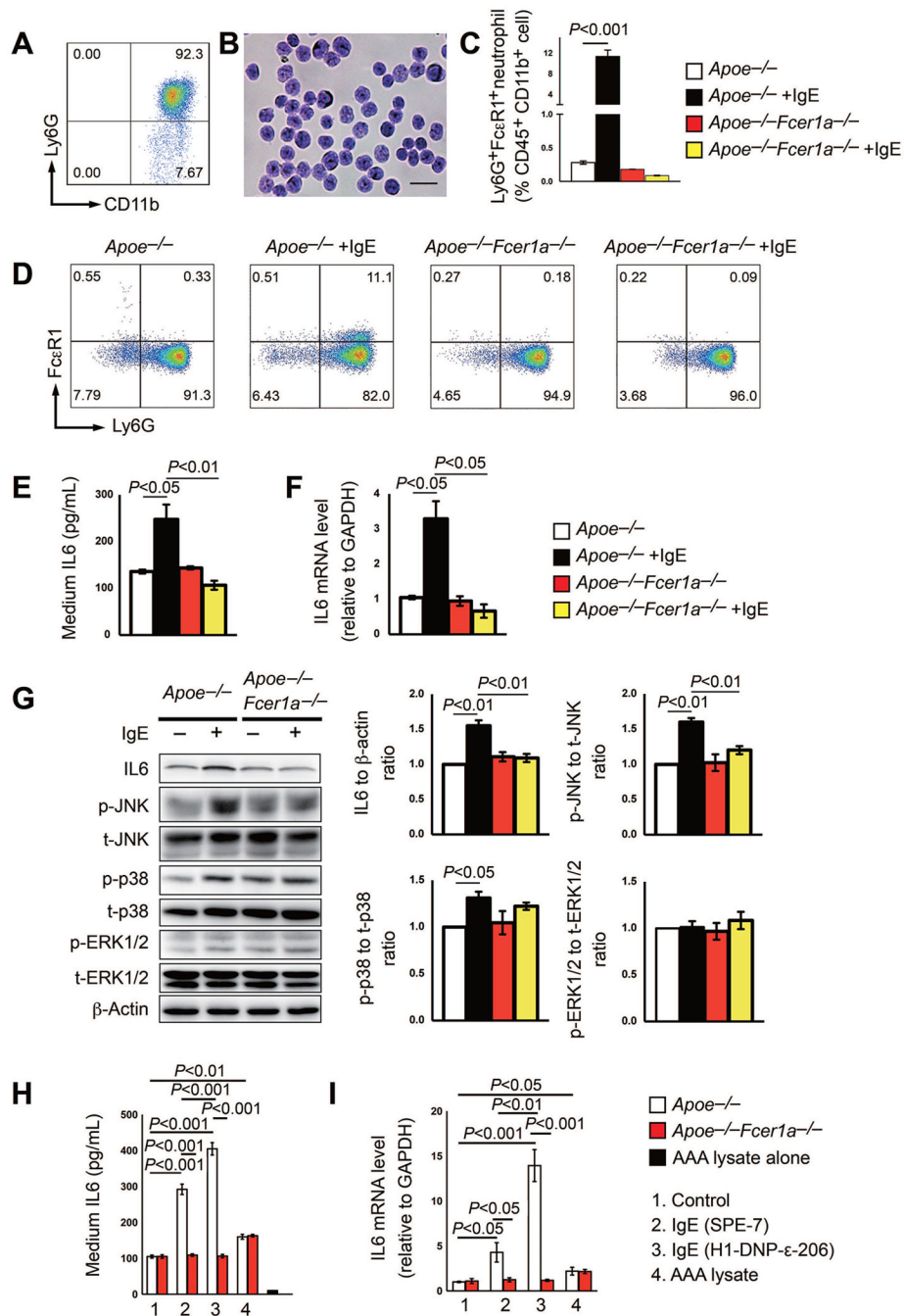
F. FACS analysis of Ly6G⁺FcεR1⁺ neutrophils in AAA lesions from *ApoE*^{-/-} and *ApoE*^{-/-}*FcεR1a*^{-/-} mice (negative control).

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**Figure 6.**

IgE activity in inducing neutrophil IL6 expression and MAPK activation. **A/B**. The purity of neutrophils isolated from *Apoe*^{-/-} and *Apoe*^{-/-}*Fcer1a*^{-/-} mice bone marrow was verified by FACS and Giemsa staining. Scale: 50 μm. **C/D**. FACS analysis to detect FcεR1 expression on Ly6G⁺ neutrophils treated with and without 25 μg/ml of IgE for 6 hrs. Representative images are show in panel **D**. **E/F**. Neutrophil culture media supernatant IL6 levels and IL6 mRNA levels in neutrophils from *Apoe*^{-/-} and *Apoe*^{-/-}*Fcer1a*^{-/-} mice treated with and without IgE as in **C/D**. **G**. Representative Western blot images and quantification of IL6,

phosphor (p)-JNK, total (t)-JNK, p-p38, t-p38, p-ERK1/2, and t-ERK1/2 in cultured neutrophils from *ApoE*^{-/-} and *ApoE*^{-/-}*FcγR1a*^{-/-} mice treated with or without 25 μg/ml IgE for 6 hrs. **H/I.** Neutrophil culture media supernatant IL6 levels and IL6 mRNA levels in neutrophils from *ApoE*^{-/-} and *ApoE*^{-/-}*FcγR1a*^{-/-} mice treated with and without 25 μg/ml aggregated and cytokinergic IgE SPE-7, monomeric and poorly cytokinergic IgE H1-DNP-e-206, and AAA lysate. AAA lysate alone without neutrophils was used as ELISA negative control. Data are mean±SEM from three independent experiments.