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Critical But Overlapping Role of FcγRIII and FcγRIV in Activation of Murine Neutrophils by Immobilized Immune Complexes

Zoltán Jakus[*], **Tamás Németh**[*], **J. Sjef Verbeek**[†], and **Attila Mócsai**[*]

[*]Department of Physiology, Semmelweis University School of Medicine, Budapest, Hungary
[†]Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

Abstract

Immune complex-induced activation of neutrophils through cell surface Fc-receptors plays a central role in the pathogenesis of autoimmune inflammatory diseases. These diseases are often modeled using genetically modified mice. However, in contrast to the number of studies on human cells, the identity of Fc-receptors involved in immune complex activation of murine neutrophils is at present unknown. Furthermore, little is known about the cellular functions mediated by the recently identified murine FcγRIV. Here we tested the identity of Fc-receptors involved in the activation of neutrophils by plate-bound immune complexes, using various knockout mouse strains, function-blocking monoclonal antibodies or the combination of both approaches. Activation of murine neutrophils by immobilized IgG immune complexes was abrogated in Fc-receptor γ-chain-deficient cells, but not by the single or combined deficiency of the γ-chain-associated FcγRI and FcγRIII, or by blocking antibodies against either FcγRIII or FcγRIV alone. However, treatment of FcγRIII-deficient neutrophils with FcγRIV-blocking antibodies or simultaneous blocking of FcγRIII and FcγRIV in wild type cells completely inhibited the immune complex-induced cellular responses. In parallel studies, activation of human neutrophils by immobilized immune complexes was abrogated by blocking antibodies against either FcγRIIA or FcγRIIIB alone. Taken together, neutrophil activation by immobilized immune complexes requires the murine FcγRIII/FcγRIV or the human FcγRIIA/FcγRIIIB molecules. While both of the two human receptors are required for this response, the two murine receptors play overlapping, redundant roles. These results promote our understanding of autoimmune diseases and identify an IgG-dependent cellular function of FcγRIV.

Keywords

Neutrophils; Fc-receptors; Cell activation; Inflammation; Transgenic/Knockout mice

INTRODUCTION

Engagement of Fc-receptors by IgG-opsonized microorganisms is one of the major routes of pathogen recognition by neutrophils, triggering a series of antimicrobial elimination mechanisms such as generation of reactive oxygen species or exocytosis of intracellular

Corresponding author: Attila Mócsai, MD, PhD, Department of Physiology, Semmelweis University School of Medicine, P. O. Box 259, 1444 Budapest, Hungary, Tel: +36-1-266-2755 x4053, Fax: +36-1-266-7480, E-mail: mocsai@puskin.sote.hu.

DISCLOSURES

The authors declare that they have no competing financial interests.

Z. J. and A. M. designed the work, interpreted the results and wrote the paper. Z. J., T. N. and A. M. performed the experiments and analyzed the data. J. S. V. provided experimental tools (FcγRI and FcγRIII-deficient mice). A. M. supervised the project.

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granules. During a normal immune response, this activation only occurs at the site of microbial invasion. However, during autoimmune diseases, generation of autoantibodies against host antigens leads to immune complex deposition and concomitant Fc-receptor-mediated neutrophil activation, which is targeted against the host tissues. Examples of such immune complex-induced, neutrophil-mediated autoimmune inflammatory diseases include rheumatoid arthritis (1, 2) and autoantibody-induced acute glomerulonephritis (3). In both these cases, pathogenic immune complexes are formed on a solid surface: the cartilaginous lining of the articular cavity (4) or the glomerular basement membrane. Surface-bound immune complexes thus appear to provoke strong activation of neutrophils and concomitant tissue damage.

Human neutrophils express a number of receptors for the Fc portion of IgG which may be involved in their activation by immobilized immune complexes. These include the immunoreceptor tyrosine-based activation motif (ITAM)-bearing single-chain FcγRIIA and the GPI-linked FcγRIIIB (human neutrophils do not express FcγRIIIA, a transmembrane receptor associated with the ITAM-bearing FcR γ-chain). Besides these low-affinity Fcγ-receptors, human neutrophils also express low levels of the high-affinity FcγRI under resting conditions, and this expression increases upon activation of the cells by inflammatory stimuli (5-7).

The contribution of the above mentioned Fcγ-receptors (in particular, FcγRIIA and FcγRIIIB) to activation of human neutrophils by immune complexes has been tested by a number of groups (8-19). The overall conclusion from those studies is that both FcγRIIA and FcγRIIIB participate in the activation of human neutrophils by immune complexes (8-19). However, the relative contribution of the two receptors appears to vary between the various experimental conditions since some studies reported a predominant role of FcγRIIA (8, 9), while FcγRIIIB was suggested to be the most important receptor involved under other conditions (10-13), and both receptors appeared to be involved, supposedly in a cooperative manner, in yet other assay systems (11-19). Despite these differences, the receptors involved in immune complex activation of human neutrophils are relatively well characterized.

During the last several years, genetically modified mice have become major tools of dissecting immunological and inflammatory processes at the molecular level. Studies on such animals strongly contributed to our understanding of basic biological mechanisms and disease pathogeneses, and they pointed to novel targets of therapeutic intervention. Genetically engineered animals provide two major advantages over the human system: the introduction of germline genomic mutations allows the functional analysis of any chosen protein, and they can also be subjected to *in vivo* disease models, enabling detailed molecular analysis of disease pathogenesis in the context of the entire organism.

A number of studies have shown that neutrophils play a critical role in mouse models of immune complex-mediated diseases such as autoimmune arthritis (1, 20, 21) or autoantibody-induced glomerulonephritis (22-24). Fc-receptors also likely participate in the development of these diseases since the genetic deficiency of the FcR γ-chain completely protects mice from autoimmune arthritis (25-28) or autoantibody-induced glomerulonephritis (29-36), and these diseases are also attenuated (though not completely abolished) in FcγRIII-deficient mice (26, 27, 37-40). Though it is difficult to directly prove, it is likely that the Fc-receptors on the surface of neutrophils participate in these autoimmune diseases.

Given the likely role of neutrophil Fc-receptors in autoimmune disease models, it would be important to know what receptors are involved in immune complex-induced activation of

mouse neutrophils. In contrast to human cells, murine neutrophils appear to primarily express FcR γ-chain-associated Fcγ-receptors. Traditionally, the most prominent member of this group was thought to be FcγRIII, a low-affinity Fcγ-receptor highly expressed on murine neutrophils. In contrast, the expression of the high-affinity activating FcγRI (which is also an FcR γ-chain-associated molecule) is questionable: while resting murine neutrophils (similar to the human cells) fail to express high levels of this molecule (40), there have been no studies on the expression of FcγRI in activated murine neutrophils. In addition to these conventional Fcγ-receptors, recent reports have also described a novel low-affinity Fcγ-receptor (termed FcγRIV) in mice (41-43). The expression of FcγRIV is restricted to the myeloid lineage with neutrophils being one of the most highly expressing cell types (42). Murine neutrophils also likely express a number of other FcR γ-chain-associated molecules such as PIR-A (44-46), OSCAR (47) and LILRC1 (48). Though no immunoglobulin binding of these receptors have been reported, their direct or indirect contribution to immune complex activation of murine neutrophils cannot be excluded.

Despite the extensive characterization of cell surface expression of Fc-receptors and related molecules on murine neutrophils, and in sharp contrast to the large number of papers on the role of individual Fcγ-receptors in immune complex activation of human neutrophils (8-19), there is practically no information available on the identity of Fc-receptor(s) involved in immune complex-induced activation of murine neutrophils. The only related reports studied the role of the FcR γ-chain in the activation of murine neutrophils by immobilized immune complexes (3) or in the initial tethering of these cells under physiological shear rates (49). While these studies indicate a role for FcR γ-chain-associated receptors in immune complex-induced activation of neutrophils, they do not allow the identification of the receptor(s) involved.

There are very few reports on the functional role of the recently identified FcγRIV molecule and most of those studies deal with the contribution of FcγRIV to autoantibody-induced in vivo processes such as autoimmune thrombocytopenia (42), nephrotoxic nephritis (36) or B-cell depletion triggered by monoclonal anti-CD20 antibodies (50). Unfortunately, no experiments aimed at the identification of the FcγRIV-bearing cell types responsible for the reported findings have been performed in these studies. Furthermore, though a recent study has suggested a functional role of FcγRIV in certain IgE immune complex-induced macrophage functions (43), there are no published reports on the role of FcγRIV in any cellular responses triggered by its principal ligand, IgG. Despite the very high expression of FcγRIV on neutrophils, the function of this receptor on these cells is also entirely unclear.

The above issues strongly hinder our understanding of the cellular mechanisms behind immune complex-induced autoimmune inflammatory diseases. This prompted us to test the role of various Fcγ-receptors in neutrophil activation by immobilized IgG immune complexes. Our results indicate that FcγRIII and the recently identified FcγRIV molecule play critical but overlapping roles in immune complex activation of murine neutrophils. Immune complex activation of human neutrophils, on the other hand, requires both FcγRIIA and FcγRIIIB.

MATERIALS AND METHODS

Animals

FcγRI-deficient (*Fcgr1*$^{tm1Sjv/tm1Sjv}$, referred to as *FcγR1*$^{-/-}$) (40) and FcγRIII-deficient (*Fcgr3*$^{tm1Sjv/tm1Sjv}$, referred to as *FcγR3*$^{-/-}$) (51) single mutant mice have been described and were used to generate *FcγR1*$^{-/-}$*FcγR3*$^{-/-}$ double mutant animals. FcR γ-chain-deficient (*Fcer1g*$^{tm1Rav/tm1Rav}$, referred to as *FcRγ*$^{-/-}$) (52) mice were purchased from Taconic Farms (Hudson, NY). DAP12-deficient (*Tyrobp*$^{tm1Lll/tm1Lll}$, referred to as

DAP12$^{-/-}$) (53) mice were generously provided by Lewis Lanier (University of California, San Francisco, CA) and the DNAX Research Institute (Palo Alto, CA). All mice were backcrossed to the C57BL/6 genetic background for six or more generations. Animals were housed in individually sterile ventilated cages in a conventional facility. All animal experiments were approved by the Semmelweis University Animal Experimentation Review Board.

Neutrophil isolation

Mouse neutrophils were isolated from the bone marrow by hypotonic lysis followed by Percoll (GE Healthcare, Chalfont St. Giles, UK) gradient centrifugation as described (54). Human neutrophils were isolated from venous blood of healthy volunteers by Ficoll (GE Healthcare) gradient centrifugation followed by hypotonic lysis of red blood cells as described (55). Neutrophil isolation was performed at room temperature using sterile and endotoxin-free reagents. Cells were kept at room temperature in Ca^{2+}- and Mg^{2+}-free medium until use (usually less than 30 min) and prewarmed to 37 °C prior to activation. Neutrophil assays were performed at 37 °C in Hank's balanced salt solution (Invitrogen, Carlsbad, CA) supplemented with 20 mM HEPES, pH 7.4.

Fc-receptor-blocking antibodies

The monoclonal Fc-receptor blocking antibodies and their isotype controls used in this study are described in Table 1. The antibodies were purchased from BD Biosciences (San Jose, CA) except for the anti-mouse FcγRIV mAb (42) and the anti-human FcγRIIA mAb (56, 57) generously provided by Jeffrey Ravetch (Rockefeller University, New York, NY) and Jeanette Leusen (University Medical Center, Utrecht, The Netherlands), respectively. Wherever possible, isotype controls binding to other neutrophil cell surface receptors were used, or the obtained results were confirmed by Fab preparation of the Fc-receptor-blocking mAbs (see *Results*).

In experiments using Fc-receptor-blocking antibodies, neutrophils were preincubated with the indicated blocking antibodies or their isotype controls at 1 or 4 μg antibody per 10^6 murine or human cells, respectively. After 50 min incubation at room temperature, neutrophils were washed and stimulated in functional assays.

Preparation of plate-bound immune complexes

Unless otherwise stated, immobilized immune complexes were formed using human serum albumin (HSA) antigen and rabbit polyclonal anti-HSA IgG antibodies (both reagents were from Sigma, St. Louis, MO). Immune complex-covered surfaces were prepared by incubating 96-well Maxisorp F96 (Nalge Nunc, Naperville, IL) ELISA plates with 20 μg/ml HSA in 50 mM carbonate/bicarbonate buffer (pH 9.6) for 1 hour, followed by blocking with 10% FCS (Invitrogen) in PBS for 1 hour and a further 1-hour incubation with anti-HSA antibodies at 1:400 dilution (approx. 10 μg/ml HSA-specific IgG) in 10% FCS. Parallel wells prepared without the HSA antigen incubation step served as controls. In some experiments, immune complexes were generated using 20 μg/ml ovalbumin (Ova) or human lactoferrin (Lfr; both from Sigma) as antigens, and 1:400 rabbit polyclonal anti-Ova or anti-Lfr (both from Sigma) as antibodies.

Because of the poor optical features of 96-well ELISA plates, an alternative protocol was used to immobilize immune complexes for microscopic observation. Tissue culture dishes (BD Biosciences) were incubated with 0.1 mg/ml poly-L-lysine (Sigma) for 30 min, washed, then incubated with 2.5% glutaraldehyde (Sigma) for another 15 min and washed again. The dishes were then incubated with 20 μg/ml HSA for 1 hour, then blocked and incubated with anti-HSA antibodies as described above.

$F(ab')_2$ fragments of the anti-HSA antibodies were prepared using the ImmunoPure $F(ab')_2$ preparation kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Optimal conditions for antibody digestion were determined by SDS-PAGE under both reducing and non-reducing conditions. $F(ab')_2$ fragments were then used instead of full IgG to prepare the immune complex-coated surface. The binding of the anti-HSA full IgG or $F(ab')_2$ fragments to the capturing HSA antigen was determined by a direct ELISA using peroxidase-labeled antibodies directed against either the Fc or the Fab portion of rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

Functional assays

Neutrophil activation by immobilized immune complexes was achieved by plating the cells on the immune complex-coated surfaces without any additional stimulus. Other routes of cell activation (CB+fMLP, TNF on fibrinogen, immobilized anti-CD18 antibodies, PMA) were performed as described (54, 55, 58, 59). For respiratory burst and degranulation assays, 1×10^5 human or 4×10^5 murine neutrophils/well were used. Release of superoxide was determined by a cytochrome c reduction test as described (59). Unless otherwise stated, unstimulated control values were subtracted from the stimulated superoxide release. Degranulation of gelatinase (a marker of the specific and gelatinase granules) was determined by an in-gel gelatinase zymography assay. Neutrophil supernatants collected after 30 min stimulation were centrifuged to remove any remaining cells, supplemented with 4× concentrated nonreducing Laemmli sample buffer and run on an 8% SDS-polyacrilamide gel containing 0.1% gelatin (Sigma). Gels were renatured in 2.5% Triton X-100 and incubated overnight at 37 °C in 200 mM NaCl, 5 mM $CaCl_2$, 50 mM Tris, pH 7.4. Digestion of gelatin pre-polymerized into the gel was visualized by Coomassie Blue staining.

For microscopic observations, 2×10^6 human or 5×10^6 murine neutrophils in 1 ml assay medium were plated on immune complex-covered 3.5-cm tissue culture dishes. After 20 min of incubation, the cells were cooled and fixed by the addition of 100 μl formalin (Sigma) to the assay medium. Nonadherent cells were allowed to settle and images were taken on a Leica (Wetzlar, Germany) DMI 6000B inverted microscope with a 20× phase contrast objective, connected to a Leica DFC480 CCD camera.

Presentation of the data

Kinetic curves of superoxide release assays represent three or more independent experiments with similar results. Error bars represent SD of triplicate or quadruplicate readings. Bar graphs of immune complex-induced superoxide release data summarize three or more independent experiments, where stimulus-induced responses have been expressed in percent of that in wild type or isotype control mAb-treated samples. Error bars represent SD from the indicated number of experiments. Statistical analyses of these data have been performed using Student's paired two-population *t*-test. A difference was considered statistically significant at $p < 0.05$. Gelatinase release data and microscopic pictures are representative of three or more independent experiments.

RESULTS

Neutrophil activation by immobilized immune complexes

We first set up an assay system (similar to those used in references (3, 60)) for neutrophil activation by immobilized immune complexes and performed a general characterization of this assay. To this end, 96-well ELISA plates were coated with human serum albumin (HSA), blocked with FCS and then incubated with rabbit polyclonal anti-HSA IgG antibodies. As shown in Fig 1A, human neutrophils plated on such HSA-anti-HSA immune complex surfaces responded with a robust respiratory burst that could not be observed if

either HSA or anti-HSA was omitted from the surface preparation protocol. Neutrophil activation could also be induced by the combination of ovalbumin (Ova) with anti-Ova or lactoferrin (Lfr) with anti-Lfr, but not by non-corresponding antigen-antibody combinations (Fig 1B), confirming the specificity of the assay. As seen in Figs 1A-B, control samples did not induce considerable superoxide release. Hence, and to simplify the presentation of our results, control values (obtained in the absence of the capturing antigen) will be subtracted from the values of immune complex-stimulated samples and only the thus obtained (stimulus-induced) responses will be presented in the following figures.

Immune complex activation likely proceeds through cell surface receptors recognizing the Fc portion of the complex-forming antibodies used. To confirm this, we prepared $F(ab')_2$ fragments of the anti-HSA antibodies and used them to generate plate-bound immune complexes. As expected, immune complexes with $F(ab')_2$ fragments failed to induce functional responses of neutrophils (Fig 1C). This defect was not due to the lack of $F(ab')_2$ binding to HSA since similar levels of $F(ab')_2$ and full IgG were detected on the immune complex surfaces with peroxidase-labeled antibodies against the Fab portion of rabbit IgG (Fig 1D). In contrast, peroxidase-labeled antibodies against the Fc portion labeled full IgG but not $F(ab')_2$ immune complexes (Fig 1D), confirming efficient digestion of the full antibodies. These results indicate that the respiratory burst triggered by immobilized full IgG immune complexes indeed proceeds through recognition of the Fc portion of the IgG molecules used.

Comparison of the responses of neutrophils to various routes of cell activation (Fig 1E) revealed that the amount of superoxide production triggered by immobilized immune complexes exceeded that induced by the bacterial tripeptide fMLP in the presence of cytochalasin B (a G_i protein-mediated response (54, 55)), by TNF-stimulation on fibrinogen surface (an integrin-dependent response (58, 61)) or by plate-bound anti-CD18 monoclonal antibodies (which requires both integrins and low-affinity Fcγ-receptors (59, 62)), and approached that induced by PMA, the most robust non-physiological activator of these cells.

We next tested whether neutrophil activation by plate-bound immune complexes activates cellular functions other than the respiratory burst. Immobilized immune complexes were able to trigger the release of gelatinase, a constituent of secondary (specific) and tertiary (gelatinase) granules (63), though this response was slightly lower than that induced by other routes of cell activation tested (Fig 1F). Neutrophils plated on immobilized immune complexes also spread over the activation surface (Fig 1G). On average, approximately 60-80% of human neutrophils spread on immune complex-coated surfaces (not shown), compared to the spreading of 30-50% of TNF-stimulated and 80-95% of PMA-stimulated human neutrophils on fibrinogen surface (64).

All the above experiments were performed on human neutrophils. Since our further studies involved the analysis of genetically modified mice, we also tested the effect of immobilized immune complexes on murine neutrophils. Similar to human cells (Fig 1E), plating murine neutrophils on immobilized immune complexes induced a respiratory burst which exceeded that induced by other physiological stimuli and approached the response triggered by PMA (Fig 1H). Immune complex activation of murine neutrophils also induced gelatinase release, which was comparable to that of TNF-stimulated cells on fibrinogen surface but less than that induced by fMLP stimulation of cytochalasin B-treated cells or by the nonphysiological activator PMA (Fig 1H). Murine neutrophils also spread over the immune complex surface (Fig 1J). The average percentage of spreading under these conditions (60-80%) was significantly higher than the 10-20% spreading of TNF-stimulated murine neutrophils on fibrinogen surface and approached the 80-95% spreading response triggered by PMA (58).

Taken together, plating human or murine neutrophils on immobilized immune complexes induces a robust activation through engagement of cell surface Fc-receptors.

Immune complex activation requires the FcR γ-chain

A number of Fc-receptors and related molecules associate with an ITAM-bearing transmembrane adapter molecule, the FcR γ-chain (52, 65, 66). This adapter may be required for the signal transduction of these receptors because the FcR γ-chain is necessary for the expression of the associated receptors on the cell surface, and/or because the phosphorylation of its ITAM motif may be involved in triggering downstream signal transduction events. As shown in Fig 2A, immune complex stimulation failed to trigger a respiratory burst response from FcR γ-chain-deficient (*FcRγ*$^{-/-}$) neutrophils. In four independent experiments (Fig 2B), the *FcRγ*$^{-/-}$ mutation caused an average inhibition of 99.6 ± 3.7% of the response of wild type cells ($p = 1.4 \times 10^{-5}$). The FcR γ-chain was also required for immune complex-induced gelatinase release (Fig 2C) and spreading of neutrophils over the immobilized immune complex surface (Fig 2D). In contrast, *FcRγ*$^{-/-}$ neutrophils responded normally to the nonphysiological activating agent PMA (Fig 2E).

Neutrophils also express DAP12, an FcR γ-chain-related ITAM-bearing transmembrane adapter molecule (67). However, the respiratory burst of *DAP12*$^{-/-}$ neutrophils plated on immobilized immune complexes was indistinguishable (4.5 ± 9.2% inhibition; p = 0.49; n = 3) from that of wild type cells (Figs 3A-B). Similarly, the immune complex induced degranulation and spreading response (Figs 3C-D), as well as the PMA-induced respiratory burst (Fig 3E) also proceeded normally in the *DAP12*$^{-/-}$ cells. Hence, unlike integrin-mediated neutrophil functions (67), Fc-receptor induced responses do not require DAP12.

Normal immune complex-induced responses in the absence of FcγRI and FcγRIII

Of the best known FcR γ-chain-associated murine Fc-receptors, FcγRIII would be expected to be primarily involved in immune complex activation of murine neutrophils, given its high expression on the surface of these cells and its low IgG-binding affinity, suggesting that it is only activated by high-valency immune complexes. In contrast to our expectations, the FcγRIII-deficient (*FcγR3*$^{-/-}$) murine neutrophils showed nearly normal respiratory burst when plated on immobilized immune complexes (Fig 4A), though statistical analysis of nine independent experiments (Fig 4B) revealed a minor but statistically significant inhibition (14.5 ± 18.1% inhibition; p = 0.032).

Murine neutrophils fail to express the high affinity FcγRI under resting conditions (40) but, similar to the human receptor (5-7), FcγRI may become expressed upon stimulation of the cells. Hence, we tested whether the genetic deficiency of FcγRI would affect immune complex-induced neutrophil responses. As shown in Figs 4A-B, the respiratory burst of FcγRI-deficient (*FcγR1*$^{-/-}$) neutrophils was indistinguishable from that of wild type cells (1.3 ± 12.3% inhibition; p = 0.84; n = 4). We next tested the effect of the combined deficiency of both FcγRI and FcγRIII. The *FcγR1*$^{-/-}$*FcγR3*$^{-/-}$ double mutant neutrophils also released nearly normal amount of superoxide when plated on immune complex surfaces (Fig 4A), though a moderate but statistically significant decrease (21.1 ± 10.0% inhibition; p = 0.0036; n = 6) could be observed (Fig 4B).

We also tested other functional responses in the same mutants. Genetic deficiency of FcγRIII or FcγRI, either alone or in combination, did not abrogate the immune complex-induced gelatinase release (Fig 4C) or spreading (Fig 4D) response, nor did it affect the responses of the cells to the PMA stimulus (Fig 4E).

Taken together, neither FcγRIII nor FcγRI, alone or in combination, play a critical role in neutrophil functions triggered by immobilized IgG immune complexes, though FcγRIII likely plays a partial role in this response.

Blocking FcγRIV abrogates immune complex responses of FcγR3$^{-/-}$ neutrophils

A novel FcR γ-chain-associated murine Fcγ-receptor, named FcγRIV, has recently been identified by several groups (41-43). The fact that this receptor is highly expressed on neutrophils raised the possibility that it may be responsible for the FcγRIII-independent component of neutrophil activation by plate-bound immune complexes. This was tested using wild type or *FcγR3*$^{-/-}$ neutrophils incubated with function-blocking monoclonal antibodies against FcγRIV (Figs 5A-B). As expected from prior experiments, *FcγR3*$^{-/-}$ neutrophils pretreated with an isotype-matched control mAb showed a moderate but statistically significant decrease of immune complex-induced superoxide generation (18.1 ± 7.8% inhibition, p = 0.0024; n = 6). Preincubation of wild type neutrophils with FcγRIV-blocking antibodies (clone 9E9) also led to a moderate but statistically significant decrease (22.9 ± 15.5% inhibition; p = 0.0080; n = 7). Importantly, pretreatment of *FcγR3*$^{-/-}$ neutrophils with the FcγRIV-blocking antibodies completely abolished the immune complex-induced respiratory burst of the cells (99.3 ± 4.3% inhibition; p = 3.4×10^{-8}; n = 6). Similarly, preincubation of *FcγR3*$^{-/-}$ neutrophils with FcγRIV-blocking antibodies also abrogated the degranulation (Fig 5C) and spreading (Fig 5D) responses. Again, the *FcγR3*$^{-/-}$ mutation or the FcγRIV blockade alone had no effect except for a significant but incomplete decrease of gelatinase release in FcγRIV antibody-treated wild type cells (Fig 5C). The responses to PMA were normal in all samples tested (Fig 5E).

Blocking both FcγRIII and FcγRIV abrogates immune complex-induced responses of wild type murine neutrophils

The above experiments showed complete inhibition of neutrophil responses by FcγRIV-blocking antibodies when used on the *FcγR3*$^{-/-}$ genetic background. We next tested whether a similar inhibition can also be attained by antibody-mediated blocking of both FcγRIII and FcγRIV. As shown in Figs 6A-B, an FcγRII/III-blocking antibody (clone 2.4G2) did not affect immune complex-induced respiratory burst of wild type murine neutrophils (3.0 ± 12.5% increase; p = 0.58; n = 6) while the FcγRIV blockade led to a marginal inhibition that did not prove to be statistically significant in this series of experiments (13.2 ± 14.5% inhibition; p = 0.17; n = 4). Combination of both FcγRII/III and FcγRIV-blocking antibodies, however, drastically reduced the respiratory burst triggered by immobilized immune complexes (88.8 ± 3.3% inhibition; p = 1.4×10^{-5}; n = 4). Again, combination of the two antibodies led to complete inhibition of immune complex-induced degranulation (Fig 6C) or spreading (Fig 6D) response while neither of the antibodies alone were effective except for an incomplete inhibition of the gelatinase release in the FcγRIV antibody-treated cells (Fig 6C). The PMA-induced respiratory burst was not affected by any of these interventions (Fig 6E).

Taken together, the experiments presented in Figs 2-6 indicate that the responses of murine neutrophils to immobilized IgG immune complexes (respiratory burst, degranulation and spreading) are mediated by two FcR γ-chain-coupled low-affinity Fcγ-receptors, FcγRIII and FcγRIV. These two proteins play a mostly redundant, overlapping role during the activation of the cells.

FcγRIIA-blocking antibodies abrogate immune complex activation of human neutrophils

In contrast to murine neutrophils that rely on FcR γ-chain-associated low affinity Fcγ-receptors for recognition of IgG immune complexes, human neutrophils do not possess any such receptors but instead express two unique low affinity Fcγ-receptors, FcγRIIA and

FcγRIIIB. Of these, FcγRIIA has an ITAM motif in its cytoplasmic domain and thus is able to be expressed and signal without the FcR γ-chain. FcγRIIIB, on the other hand, is a GPI-linked protein that is anchored to the outer leaflet of the plasma membrane through a glycophosphatidylinositol moiety. Although there have been a number of studies trying to identify the role of these molecules in immune complex-induced activation of human neutrophils (8-19), variable results have been obtained in the different experimental systems (see *Introduction*). For this reason and to facilitate the comparison of experimental results on human and mouse neutrophils, we next attempted to identify the human Fc-receptors involved in the same immobilized immune complex activation assays that was used in our previous mouse studies.

As shown in Fig 7A-B, preincubation of human neutrophils with an FcγRIIA-blocking antibody (clone IV.3) completely abolished the respiratory burst of cells plated on immobilized IgG immune complexes (96.1 ± 4.7% inhibition; $p = 6.1 \times 10^{-8}$; n = 6) relative to cells treated with an isotype-matched control antibody. Similar results were obtained using an Fab fragment (59) of the FcγRIIA-blocking antibody (not shown). Blocking of FcγRIIA also inhibited the degranulation (Fig 7C) and spreading (Fig 7D) response of immune complex-bound human neutrophils. However, the PMA-induced respiratory burst was not affected by the FcγRIIA-blocking antibodies (Fig 7E).

Blockade of FcγRIIIB inhibits immune complex activation of human neutrophils

Next we tested the effect of blocking FcγRIIIB on immune complex-induced responses of human neutrophils. As shown in Fig 8A-B, an FcγRIIIB-blocking monoclonal antibody (clone 3G8) strongly inhibited the respiratory burst of human neutrophils plated on immobilized immune complex surfaces (84.3 ± 19.4% inhibition; $p = 5.3 \times 10^{-6}$; n = 8). The FcγRIIIB-blocking antibody also prevented gelatinase release (Fig 8C) and spreading of the cells (Fig 8D) in response to activation by immobilized immune complexes. However, the PMA-induced respiratory burst was not affected by the antibody treatment (Fig 8E).

Taken together, activation of human neutrophils by immobilized IgG immune complexes is mediated by FcγRIIA and FcγRIIIB. In contrast to the results with murine cells, however, both receptors are required for activation of human neutrophils.

DISCUSSION

Ligation of neutrophil Fc-receptors by immobilized immune complexes is thought to be one of the major triggers of tissue destruction during autoimmune inflammatory diseases. While such diseases are often studied in animal models using genetically modified mice, very little is known about what cell surface receptors are involved in activation of murine neutrophils by immobilized immune complexes. This may be due to the fact that an apparently very important murine Fcγ-receptor, FcγRIV, has only recently been identified (41-43). Our functional studies focusing on the role of this receptor, as well as murine FcγRIII and FcγRI revealed that neutrophil activation by immobilized IgG immune complexes is mediated by FcγRIII and FcγRIV (Figs 5-6) but not by FcγRI (Fig 4). The relative contribution of FcγRIII and FcγRIV appears to be rather balanced, since genetic deficiency or antibody-mediated blocking of either one of them leads to a moderate, 10-20% inhibition, whereas deleting or blocking both receptors completely abrogates the responses of the cells (Figs 4-6). These experiments identify the Fc-receptors involved in activation of murine neutrophils by immobilized immune complexes and provide the first evidence for an IgG-mediated cellular function of the recently described FcγRIV molecule.

Despite the similar role of murine FcγRIII and FcγRIV in our neutrophil assays, other studies indicate that there are significant differences between these molecules. Although

both receptors are highly expressed on neutrophils and macrophages (42, 43), NK-cells and mast cells appear to more specifically express FcγRIII (42, 43) while FcγRIV expression is somewhat higher on dendritic cells (43). There also appears to be a moderate difference between the level of induction of the two proteins upon stimulation of macrophages with proinflammatory agents (43). Sequence analysis of FcγRIV also revealed a YEEP motif in the cytoplasmic tail of FcγRIV but not FcγRIII in all rodent species tested (43). Phosphorylation of the tyrosine residue in this YEEP motif may be involved in initiation of FcγRIV-specific intracellular signals through binding of SH2 domain-containing signaling molecules, as suggested by the crosslinking-induced phosphorylation of the FcγRIV YEEP motif and its association with Crk-L (43).

Besides the differences in their expression and signaling motifs, FcγRIII and FcγRIV also differ in their specificity and affinity towards various Ig classes and subclasses (42, 43). While both receptors are able to bind IgG_{2a} and IgG_{2b} molecules, the affinity of FcγRIV towards these IgG subclasses is 30-50 fold higher than that of FcγRIII (42, 43). In contrast, murine IgG_1 binds to FcγRIII but not FcγRIV (42, 43). Hence, FcγRIV appears to be specific for IgG_2 subclasses whereas FcγRIII is skewed towards recognition of IgG_1. Interestingly, FcγRIV (but not FcγRIII) is able to bind IgE molecules of the “b”, but not the “a” allotype, and IgE^b, but not IgE^a immune complexes are able to activate macrophages in an FcγRIV-dependent manner (43). Hence, FcγRIV, but not FcγRIII, may also be involved in IgE-mediated inflammatory processes in certain mouse strains.

In light of these differences, the apparently similar functions of FcγRIII and FcγRIV in our experiments may be somewhat surprising. There are, however, indications in the literature that these molecules do not perform uniquely specific functions *in vivo* either. Genetic deficiency of FcγRIII attenuates the development of autoantibody-mediated arthritis, though the published effects of the $Fc\gamma R3^{-/-}$ mutation range from nearly complete (37) through considerable (26, 38, 39) to moderate inhibition (27). Interestingly, genetic deficiency of the FcR γ-chain leads to complete inhibition of arthritis development in all reported studies (25-28). $Fc\gamma R1^{-/-}$ animals are protected from arthritis-induced cartilage destruction (39, 40) but not from the inflammatory reaction itself (26, 39). The overall impression from these studies is that an FcR γ-chain-associated receptor other than FcγRI is able to compensate for the lack of FcγRIII during the development of autoimmune arthritis. Genetic deficiency of FcγRIII also attenuates autoantibody-induced glomerulonephritis but again, the different studies report variable phenotypes ranging from nearly complete (31) through intermediate (30) to negligible (36) level of protection from disease. However, deletion of the FcR γ-chain completely eliminated autoantibody-induced glomerulonephritis in all reported studies (29-36). Hence, also in the case of autoantibody-induced glomerulonephritis, an FcR γ-chain-associated receptor other than FcγRIII appears to play an important pathogenetic role, though currently available studies provide conflicting data about whether this compensating receptor is FcγRI (30) or FcγRIV (36). Taken together, various autoimmune diseases are likely mediated by both FcγRIII and another FcR γ chain-associated receptor, and it is reasonable to assume that this latter receptor is, at least under certain conditions, the FcγRIV molecule.

Though studies on the *in vivo* role of FcγRIV are scarce at the moment, these reports also support an overlapping role between FcγRIII and FcγRIV. While FcγRIV-blocking mAbs inhibited IgG_2-mediated experimental immune thrombocytopenia *in vivo*, the inhibition was only partial and did not reach the level of protection caused by the genetic deficiency of the FcR γ-chain (42), and a similar disease induced by anti-platelet mAb of the IgG_1 subclass was entirely dependent on FcγRIII rather than FcγRIV (42). Hence, in a “real world” scenario when both IgG_1 and IgG_2 autoantibodies are present, both FcγRIII and FcγRIV would be expected to contribute to disease pathogenesis. Furthermore, while Kaneko *et al.*

(36) suggested FcγRIV rather than FcγRIII to be primarily involved in autoimmune glomerulonephritis, other groups showed considerable protection of *FcγR3*$^{-/-}$ mice from the disease under very similar conditions (30, 36). Again, the overall impression from these glomerulonephritis studies would be that both FcγRIII and FcγRIV play an important role in this disease.

A possible or even likely explanation for the functional overlap between FcγRIII and FcγRIV in the above *in vivo* experiments and our *in vitro* studies would be the different isotype specificity of the two receptors. A spontaneous polyclonal autoimmune reaction likely generates both IgG_1 and IgG_2 autoantibodies, and the polyclonal antibody preparation used in our *in vitro* studies also likely includes both IgG subclasses. It is then possible that IgG_1 molecules primarily activate FcγRIII whereas IgG_2 molecules preferentially activate FcγRIV. While this could be tested *in vitro* using immune complexes prepared with monoclonal antibodies of defined IgG subclasses, we believe that our assay system using polyclonal antibodies much better reflect the situation in a real autoimmune reaction.

While the involvement of various Fc-receptors in *in vivo* autoimmune models has been quite extensively tested, it is mostly unclear what cell types use these receptors to mediate the autoimmune inflammatory reaction. Neutrophils may be one such cell type. This possibility is supported by our findings that immobilized immune complexes strongly activate these cells (Fig 1) and that the role of FcγRIII and FcγRIV in our *in vitro* assays is in agreement with their supposed role in *in vivo* disease pathogenesis.

In the experiments presented in Fig 6, an FcγRII/III-blocking monoclonal antibody (clone 2.4G2) was used to block FcγRIII function. It should be mentioned that this antibody blocks both murine FcγRII and FcγRIII. However, since mice only express the inhibitory (FcγRIIB) but not the activating (FcγRIIA) isoform of FcγRII and the effect of this mAb was very similar to that of the *FcγR3*$^{-/-}$ mutation (compare Figs 5-6), it is unlikely that the inhibitory effect of the 2.4G2 mAb was due to inhibition of an FcγRII isoform. The 2.4G2 mAb has also been shown to bind to FcγRIV in heterologous expression systems. However, the effect of this mAb in our experiments closely mirrored that of the genetic deficiency of FcγRIII, and an additional FcγRIV-blocking mAb was clearly required for a considerable inhibition of the immune complex-induced neutrophil responses. Furthermore, even a four-fold higher concentration (4 μg/10^6 cells) of 2.4G2 failed to affect the immune complex-induced respiratory burst of wild type neutrophils despite a strong inhibition of the FcγRIII-mediated response triggered by plate-bound anti-CD18 mAbs (59) (data not shown). Hence, it is unlikely that the 2.4G2 mAb caused a significant inhibition of FcγRIV function in our experiments. It is interesting to note, however, that the 2.4G2 mAb blocked IgE^b-mediated phagocytosis of macrophages (36) raising the possibility that this antibody interferes with IgE^b but not IgG binding to FcγRIV.

Though neither FcγRIII nor FcγRIV appeared to have any major non-redundant role in the experiments presented in this paper, it should be noted that the immune complex-induced gelatinase release was consistently lower in samples treated with FcγRIV-blocking antibodies alone (Figs 5C and 6C). Though this inhibition ranged from significant to moderate across the number of experiments performed, these results suggest that FcγRIV may be more directly involved in initiating the degranulation response upon neutrophil activation by plate-bound immune complexes. Further studies, such as detailed kinetic analyses and the determination of the release of the various other exocytic compartments (63) will be required to further clarify this issue. Nevertheless, FcγRIII and FcγRIV do appear to play strongly overlapping functions in most assay systems tested.

Taken together, the experiments presented in this paper indicate that FcγRIII and FcγRIV play a redundant, overlapping role in activation of murine neutrophils by IgG immune complexes. This is in contrast with the human situation where both FcγRIIA and FcγRIIIB are required. These results will promote our understanding of the mechanism of autoimmune inflammation in mice and its extrapolation to the pathogenesis of human disease. They also raise novel questions, such as whether human and murine neutrophils utilize similar or different signal transduction mechanism during activation by immobilized immune complexes. Furthermore, this study provides the first evidence for an IgG-mediated cellular function of the recently identified FcγRIV molecule.

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ABBREVIATIONS

Abbreviations used in this paper:

CB	cytochalasin B
DAP12	DNAX activating protein of 12 kDa
Fbg	fibrinogen
FcRγ	Fc-receptor common γ-chain
HSA	human serum albumin
IC	immune complex
Iso Co	isotype control
Lfr	lactoferrin
PMN	neutrophils (polymorphonuclear cells)
PO	peroxidase
WT	wild type

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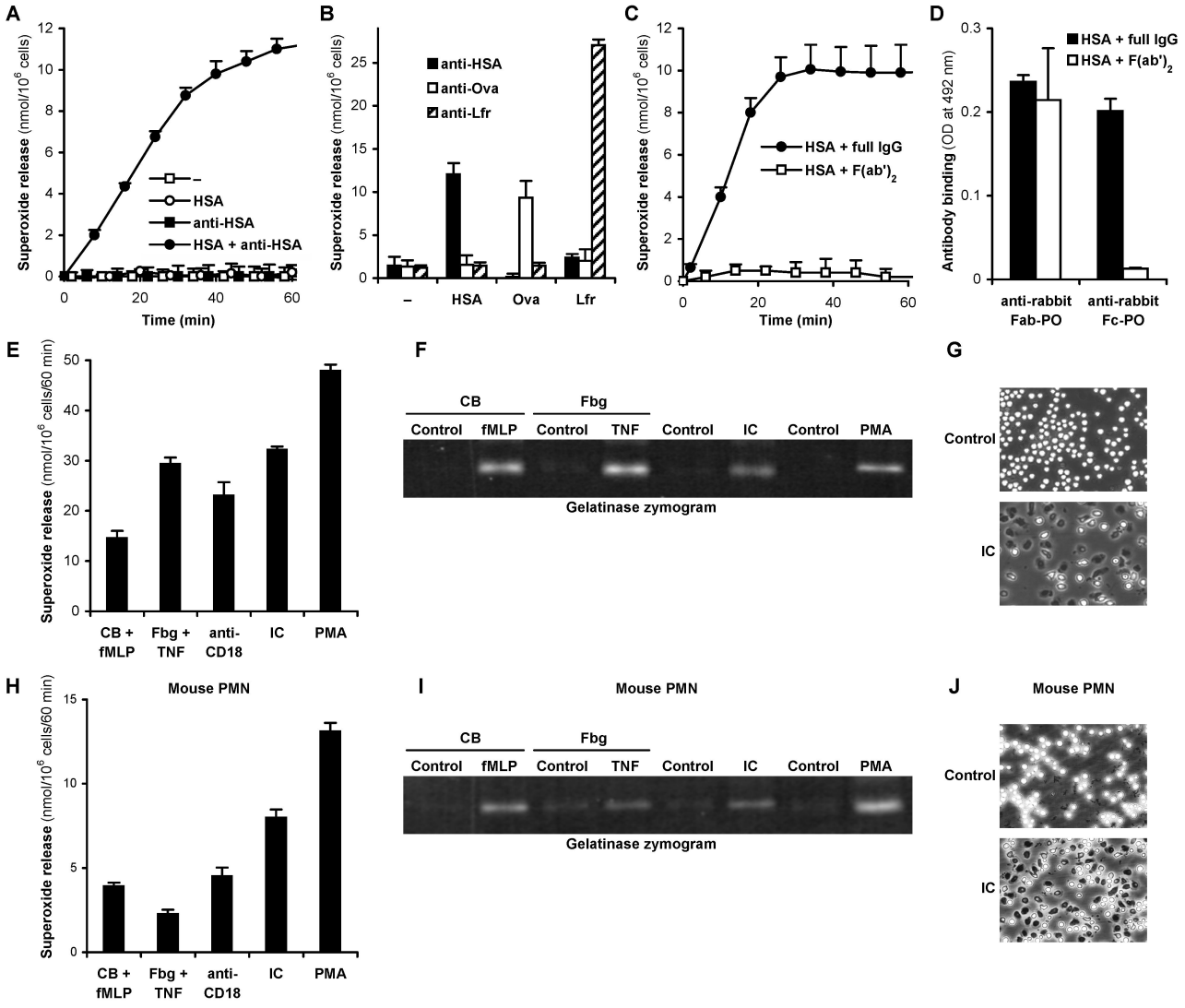

Figure 1. Neutrophil activation by immobilized immune complexes

A-B, Respiratory burst of human neutrophils plated on immobilized immune complexes formed by various antigen-antibody pairs. C-D, The effect of removal of the Fc portion of the immune complex-forming anti-HSA antibodies on superoxide production of human neutrophils (C) and on antibody binding to the HSA antigen surface detected by a direct ELISA using peroxidase-labeled anti-Fc or anti-Fab antibodies (D). E, comparison of the responses of human neutrophils to immobilized immune complexes and various other routes of cell activation (10 μM cytochalasin B (CB) followed by 1 μM fMLP; 20 ng/ml human TNF on a fibrinogen-coated (Fbg) surface; 20 μg/ml anti-human CD18 antibody-coated surface; 100 nM PMA). F-G, non-oxidative responses (gelatinase release (F) and cell spreading (G)) of human neutrophils plated on immobilized immune complexes. H-J, comparison of the responses of wild type murine neutrophils to immobilized immune complexes and various outher routes of cell activation (10 μM CB followed by 3 μM fMLP; 50 ng/ml murine TNF on a Fbg surface; 20 μg/ml anti-murine CD18 antibody-coated surface; 100 nM PMA). Unstimulated control values were subtracted in C, E and H. HSA, human serum albumin; Ova, ovalbumin; Lfr, lactoferrin; PMN, neutrophils (polymorphonuclear cells); PO, peroxidase.

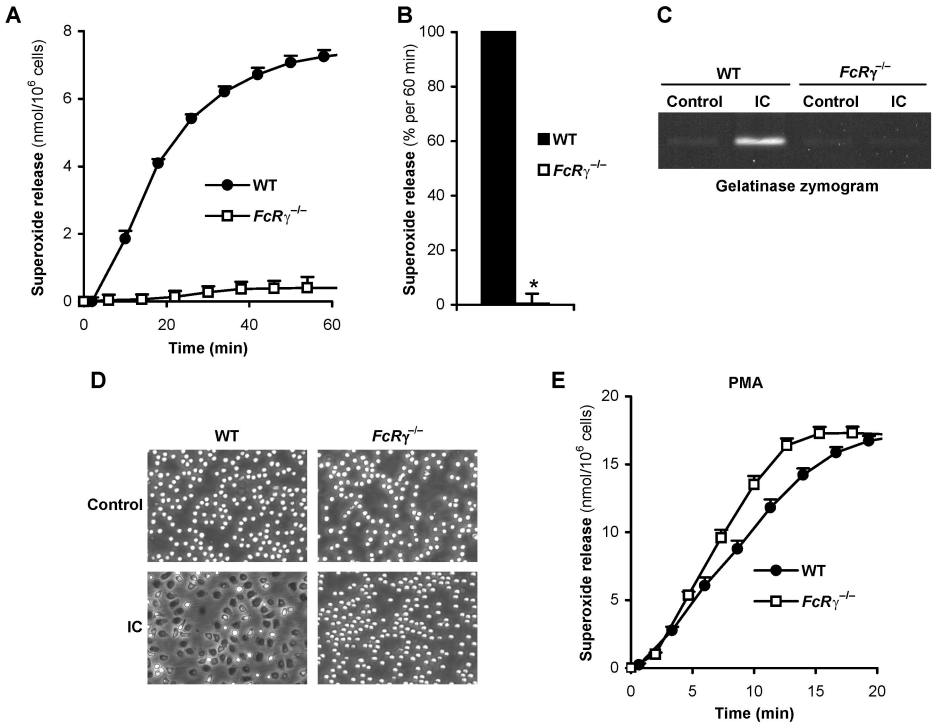

Figure 2. The FcR γ-chain is required for immune complex activation of murine neutrophils

A-B, Superoxide production of wild type (WT) and *FcRγ*$^{-/-}$ murine neutrophils plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 4 independent experiments expressed in % of WT. C-D, Gelatinase release (C) and spreading (D) of *FcRγ*$^{-/-}$ neutrophils plated on IC-covered surfaces. E, Respiratory burst of FcRγ KO neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to WT.

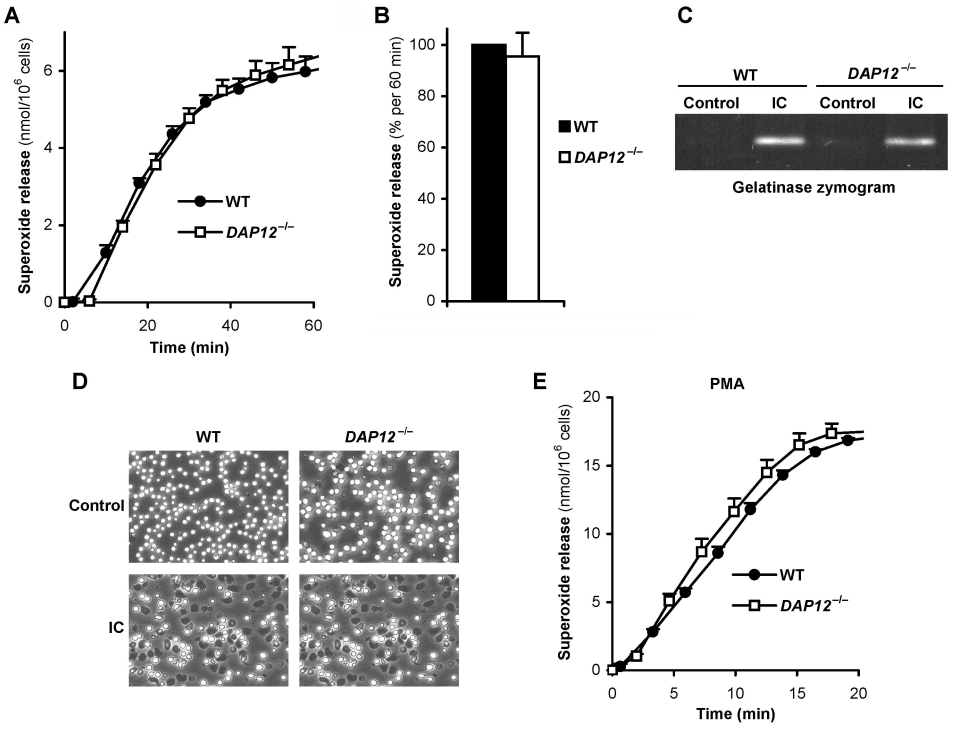

Figure 3. DAP12 is dispensable for immune complex activation

A-B, Superoxide production of wild type (WT) and *DAP12*$^{-/-}$ murine neutrophils plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 3 independent experiments expressed in % of WT. C-D, Gelatinase release (C) and spreading (D) of *DAP12*$^{-/-}$ neutrophils plated on IC-covered surfaces. Unstimulated control values were subtracted in A, B and E.

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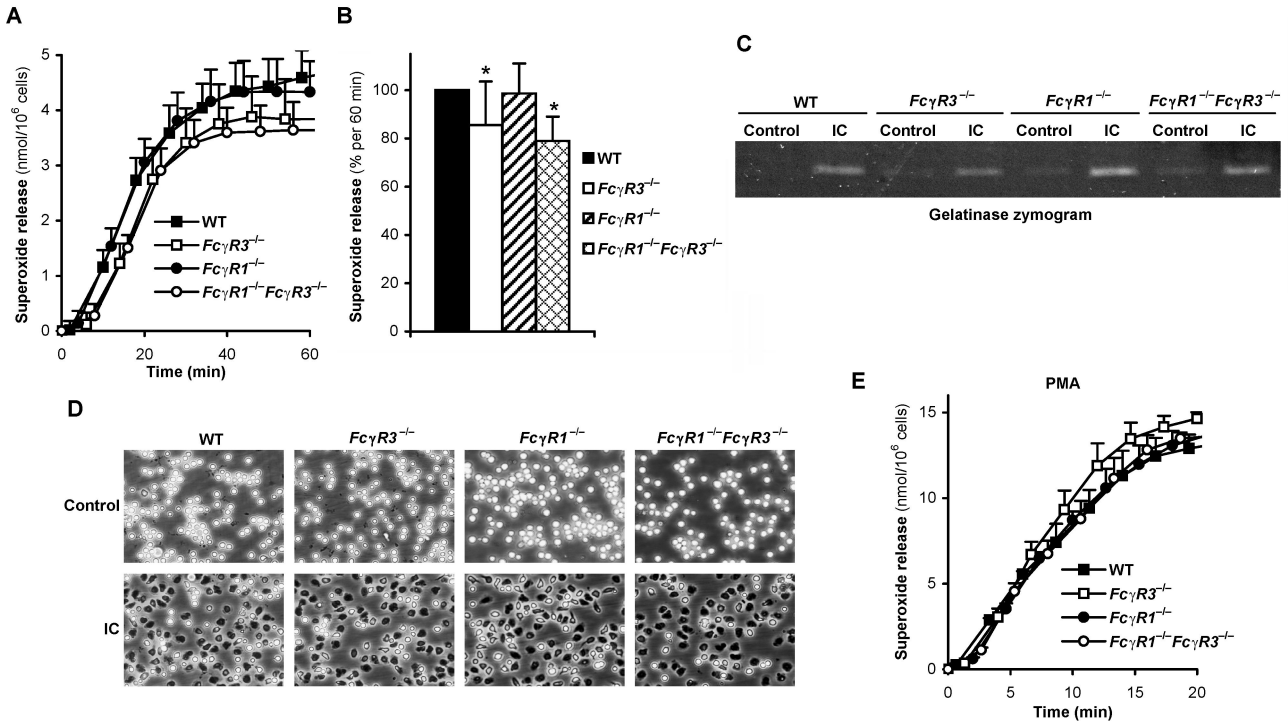

Figure 4. FcγRI and FcγRIII are dispendable for immune complex activation of murine neutrophils

A-B, Superoxide production of wild type (WT), *FcγR1*$^{-/-}$ and *FcγR3*$^{-/-}$ single mutant and *FcγR1*$^{-/-}$*FcγR3*$^{-/-}$double mutant murine neutrophils plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 4-9 independent experiments expressed in % of WT. C-D, Gelatinase release (C) and spreading (D) of the various mutant neutrophils plated on IC-covered surfaces. E, Respiratory burst of the various mutant neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to WT.

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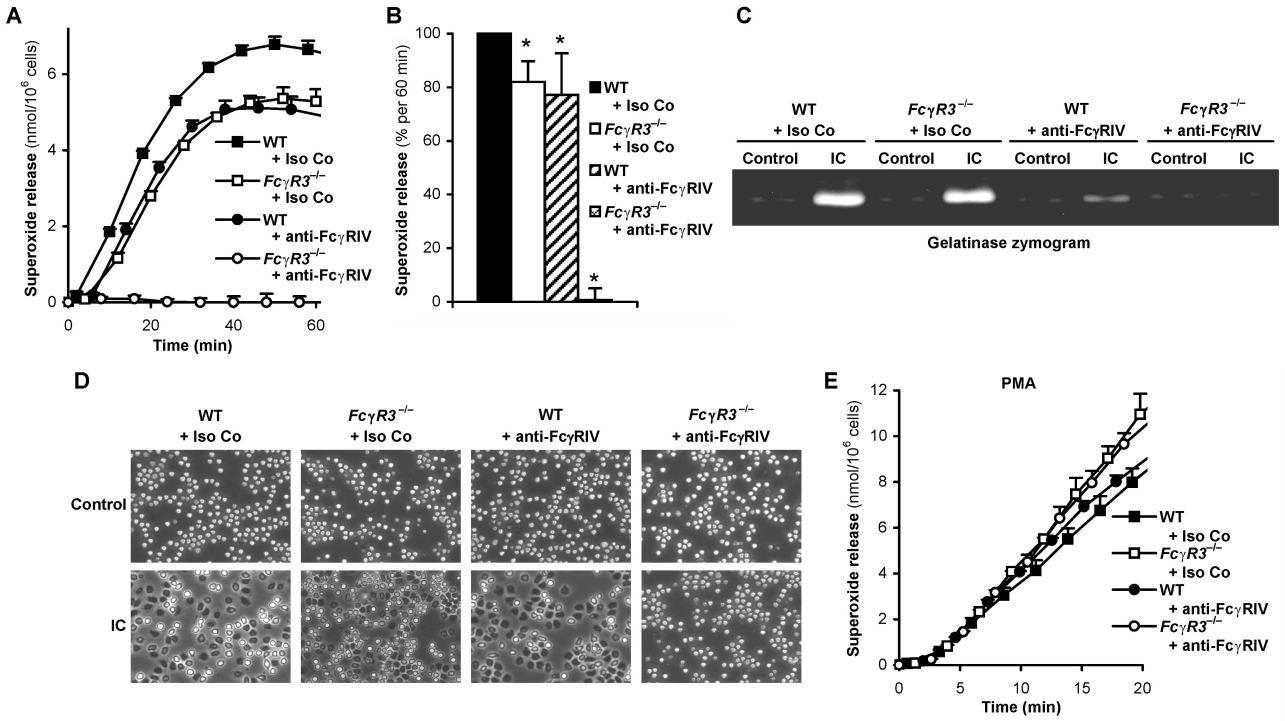

Figure 5. Blocking FcγRIV inhibits immune complex activation of FcγRIII-deficient neutrophils

A-B, Superoxide production of wild type (WT) or *FcγR3*$^{-/-}$ murine neutrophils pretreated with an FcγRIV-blocking mAb or its isotype control (Iso Co) and plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 6-7 independent experiments expressed in % of WT Iso Co. C-D, Gelatinase release (C) and spreading (D) of neutrophils from the indicated sources plated on IC-covered surfaces. E, Respiratory burst of neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to WT Iso Co.

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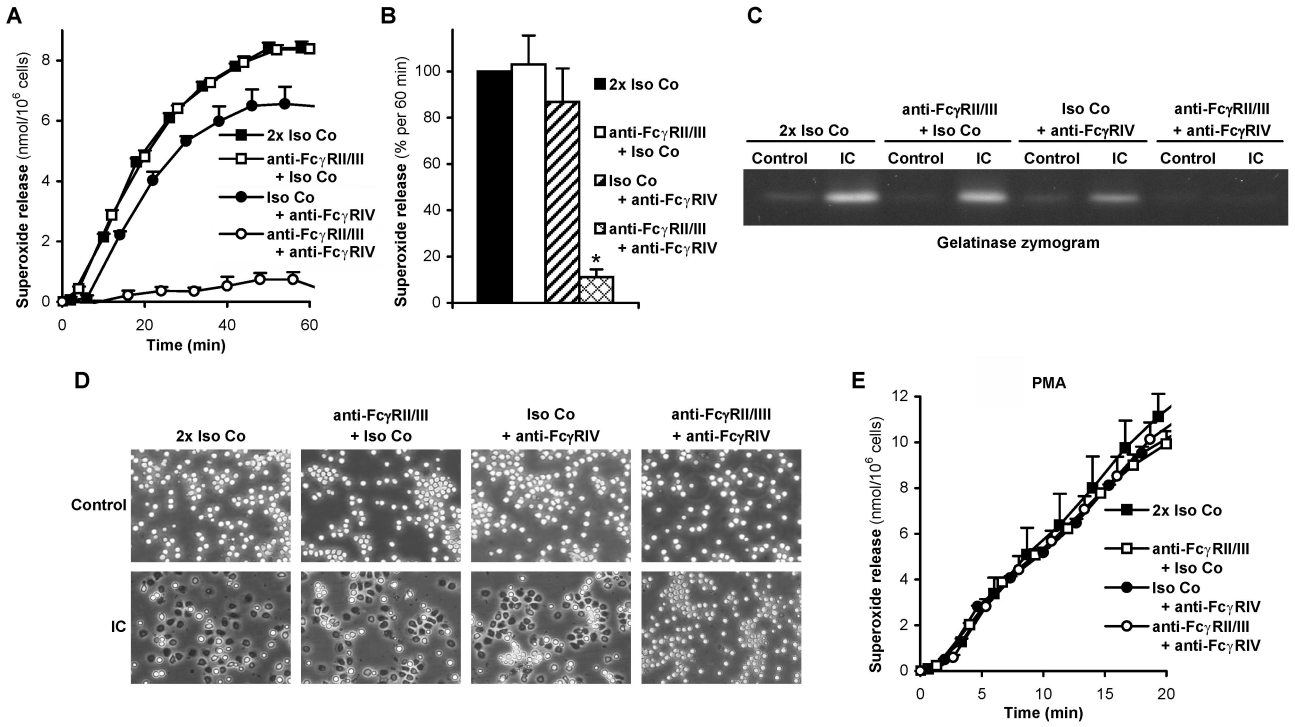

Figure 6. Inhibition of immune complex activation of neutrophils by simultaneously blocking FcγRIII and FcγRIV

A-B, Superoxide production of wild type murine neutrophils pretreated with FcγRII/III-blocking and/or FcγRIV-blocking mAbs or their respective isotype controls (Iso Co) and plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 4-6 independent experiments expressed in % of 2x Iso Co. C-D, Gelatinase release (C) and spreading (D) of neutrophils from the indicated sources plated on IC-covered surfaces. E, Respiratory burst of neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to 2× Iso Co.

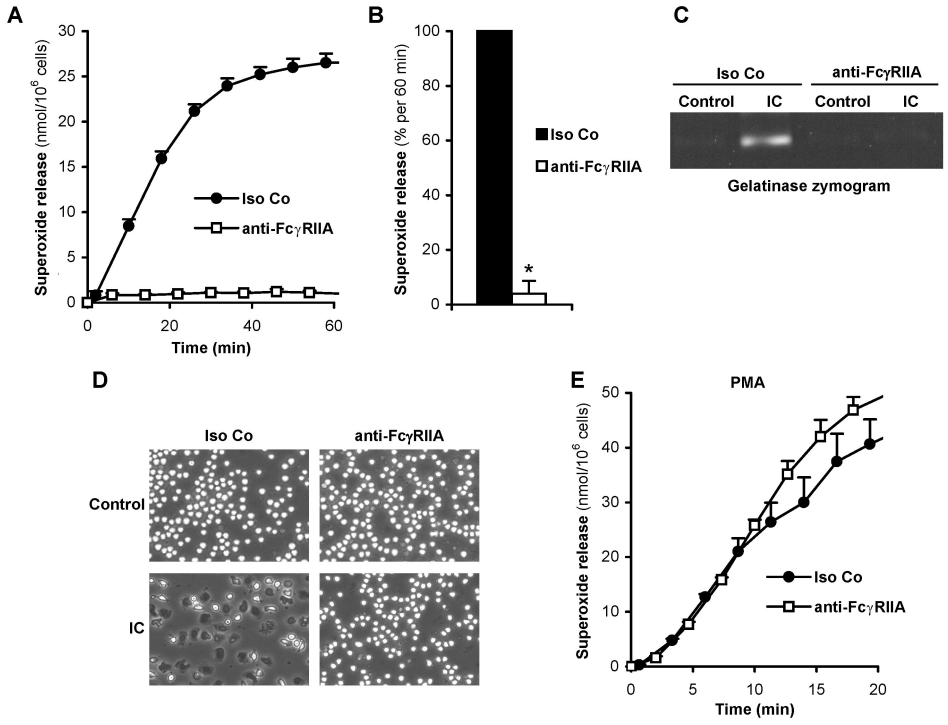

Figure 7. FcγRIIA is required for immune complex activation of human neutrophils

A-B, Superoxide production of human neutrophils pretreated with an FcγRIIA-blocking mAb or its isotype control (Iso Co) and plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 6 independent experiments expressed in % of Iso Co. C-D, Gelatinase release (C) and spreading (D) of antibody-treated neutrophils plated on IC-covered surfaces. E, Respiratory burst of neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to Iso Co.

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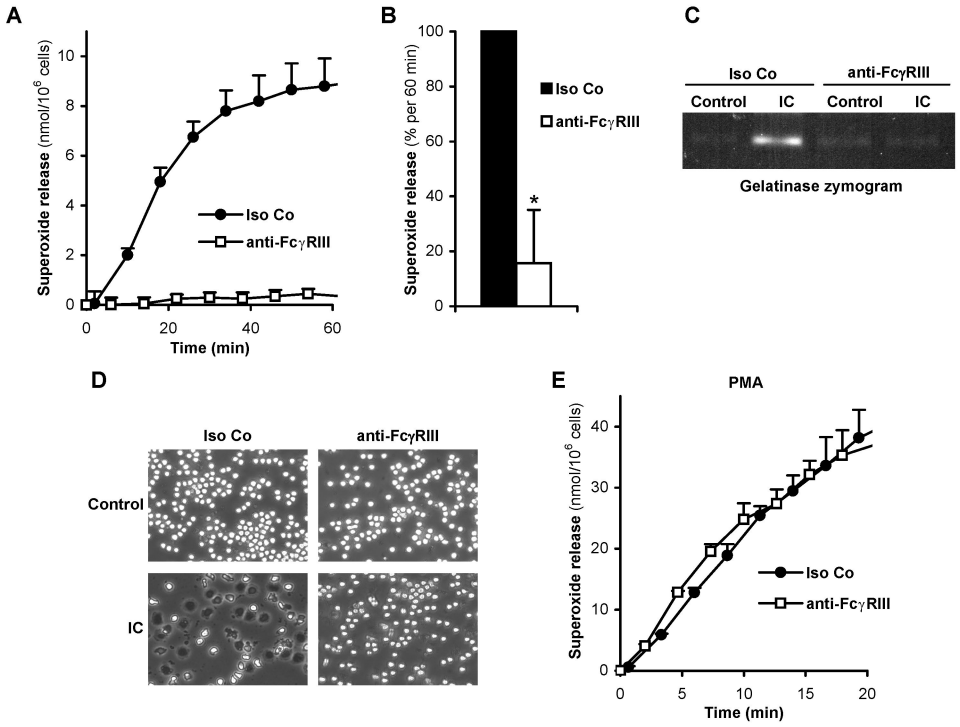

Figure 8. FcγRIIIB is required for immune complex activation of human neutrophils

A-B, Superoxide production of human neutrophils pretreated with an FcγRIII-blocking mAb or its isotype control (Iso Co) and plated on immune complex (IC)-covered surfaces. Panel A shows a representative experiment while panel B summarizes results from 8 independent experiments expressed in % of Iso Co. C-D, Gelatinase release (C) and spreading (D) of antibody-treated neutrophils plated on IC-covered surfaces. E, Respiratory burst of neutrophils triggered by 100 nM PMA. Unstimulated control values were subtracted in A, B and E. *, $p < 0.05$ compared to Iso Co.

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Table 1
Fc-receptor-blocking monoclonal antibodies used in this study

Clone	Specificity	Isotype	Source
RB6-8C5	Mouse Gr1 (Isotype Control)	Rat IgG2b	BD Biosciences
2.4G2	Mouse FcγRII/III	Rat IgG2b	BD Biosciences
A19-3	− (Isotype Control)	Hamster IgG1	BD Biosciences
9E9	Mouse FcγRIV	Hamster IgG1	Jeffrey Ravetch
27-35	− (Isotype Control)	Mouse IgG2b	BD Biosciences
IV.3	Human FcγRIIA	Mouse IgG2b	Jeanette Leusen
HI30	Human CD45 (Isotype Control)	Mouse IgG1	BD Biosciences
3G8	Human FcγRIII	Mouse IgG1	BD Biosciences