IgG-aggregates rapidly up-regulate FcgRI expression at the surface of human neutrophils in a FcgRII-dependent fashion: A crucial role for FcgRI in cellular responsiveness.

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Abstract

Auto-immune complexes are an important feature of several auto-immune diseases such as lupus, as they contribute to tissue damage through the activation of immune cells. Neutrophils, key players in lupus, interact with immune complexes through Fc gamma receptors (FcgR). Incubation of neutrophils with aggregated-IgGs increased the surface expression of FcgRI within minutes, in a concentration-dependent fashion. After 30 min, IgG aggregates (1 mg/ml) up-regulated FcgRI by 4.95 ± 0.45-fold increase. FcgRI-positive neutrophils reached 67.24% ± 6.88% on HA-IgGs stimulated neutrophils, from 3.12% ± 1.62% in non-stimulated cells. Compared to factors reported to up-regulate FcgRI in neutrophils, IgG-aggregates were most potent. FcgRII, and possibly FcgRIIIa, appeared to mediate this up-regulation. Syk and PI3K were involved in this process. Moreover, FcgRI-dependent signaling was found to be necessary for ROS production in response to IgG-aggregates. Finally, combinations of aggregates with LPS or fMLP dramatically boosted ROS production. Co-engaging FcgRI and other signaling pathways can impact neutrophil inflammatory responses in a major fashion. This work reveals FcgRI as an essential component in the response of human neutrophils to immune complexes and may help explain how they contribute to tissue damage associated with immune-complex diseases, such as lupus.

Introduction

Immune complex diseases encompass a diverse group of inflammatory conditions characterized by antigen–antibody deposition, such as rheumatoid arthritis and systemic lupus erythematosus. Typical manifestations are glomerulonephritis, synovitis, and dermal vasculitis[1]. Activation of neutrophils, resulting from the interaction with immune complexes, leads to the release of oxygen-free radicals and lysosomal enzymes[2]. The localized release of these toxic substances may bring about tissue inflammation and organ damage.

Phagocytes, including neutrophils, bind to immune complexes with their Fc gamma receptors (FcgRs), serving as molecular links between antibody and immunological responses[3]. Neutrophils express the transmembrane FcgRIIa (CD32a) and the glycosyl-phosphatidylinositol (GPI)-anchored FcgRIIIb (CD16b), which is expressed at 10-fold higher density than FcgRIIa (Reviewed here[4]). FcgRIIb is also present, albeit to a lesser degree[4]. FcgRIIIb and FcgRIIa, both exclusive to higher primates, display specialized context-dependent functions in neutrophil recruitment[5,6]. Unique to neutrophils, FcgRIIIb is the only Fc receptor anchored by a GPI-linker to the plasma membrane[7]; it lacks a cytoplasmic domain and has no signaling capacity by itself. FcgRIIa, with its integrated signaling ITAM, is an important mediator of destructive antibody-based inflammation in autoimmunity[5] and is necessary for neutrophil recruitment within glomerular capillaries following IgG deposition[8,9]. Recently, the expression of FcgRIIIa (CD16a) on neutrophils was demonstrated[10]. FcgRIIIa associates with ITAM-containing signaling molecules. Immune complex (IC) binding to the FcgRIIIa complex leads to calcium flux, phosphatidylinositol 4,5 biphosphate hydrolysis, and an intracellular phosphorylation cascade, suggesting that FcgRIIIa, rather than FcgRIIIb, mediates neutrophil activation by IgG antibodies, as well as phagocytosis of antibody-opsonized beads.

FcgRI (CD64) is the single high-affinity receptor of immunoglobulin G (IgG) in humans[11]. Like FcgRIIIa, FcgRI is an 'activator' using an intracellular ITAM-mediated signaling system to promote phagocytosis, respiratory burst, antibody-dependent cell-mediated cytotoxicity, cytokine production, and NETosis[3,12,13]. Nonetheless, FcgRI surface expression is negligible on circulating neutrophils of healthy individuals and is stored in intracellular vesicles[14]. The up-regulation of FcgRI by cytokines or growth factors was reported more than three decades ago. Interferon-gamma increased FcgRI on neutrophils after 18 hrs of incubation[15]. *In vivo*, but not *in vitro* application of G-CSF increased FcgRI expression on neutrophils within days, and potentiated their tumor cell killing capabilities[16,17]. Also, neutrophil FcgRI expression increases in complications associated with bacterial infections[18-20]; neutrophil FcgRI serves as a sensitive diagnostic marker for early-onset neonatal infection[21].

While monocyte-FcgRI expression is positively associated with immune inflammation and lupus [22-24], neutrophil FcgRI is not significantly elevated on neutrophils in circulation from patients with lupus, unless an infection is also present[25]. Moreover, the implication of neutrophil-FcgRI in responses to immune complexes is not documented.

In this paper, we investigate the impact of aggregated-IgGs, used as a model of immune complexes, on neutrophil activation. We show that the engagement of FcgRs by IgG-aggregates up-regulate FcgRI at the surface of human neutrophils from intracellular stores. Moreover, we assess the necessity for FcgRI activation for downstream cellular responses. This work reveals FcgRI as an essential mediator of neutrophil activation following the engagement of FcgRs.

Materials & Methods

Materials

Dextran-500, Lipopolysaccharide (LPS from *Escherichia coli* 0111: B40), Formyl-methionyl-leucyl phenylalanine (fMLP), Wortmannin (from *Penicillium fumiculosum*), Luminol, Isoluminol, and SB216763 were purchased from Sigma-Aldrich (Oakville, ON, Canada). Lymphocyte separation medium was purchased from Wisent (St-Bruno, QC, Canada). AS605240, U0126, and U-73122 were obtained from Cayman Chemical (Ann Arbor, MI, USA). H89 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). PRT-060318 was obtained from Selleckchem (Houston, TX, USA). Gö6976 and PP-2 were purchased from Calbiochem (San Diego, CA, USA). Recombinant human granulocyte- (G), granulocyte-macrophage (GM) colony-stimulating factor (CSF), IFN-gamma (γ), and tumor necrosis factor (TNF) were purchased from PeproTech (Rocky Hill, NJ, USA). CGS-21680 and Nexinhib20 were obtained from Tocris (Oakville, ON, Canada). IFN-alpha (α) was purchased from PBL Assay Science (Piscataway, NJ, USA). Human immunoglobulins G (IgGs): Lyophilized human IgGs, purified from human plasma or serum by fractionation (purity of greater than 97% as determined by SDS-PAGE analysis) were purchased from Innovative Research, Inc. (Novi, MI, USA). Phosphatidylinositol-Specific Phospholipase C (PI-PLC) and 7-Aminoactinomycin D (7-AAD) were from Molecular Probes (Burlington, ON, Canada). Cytochrome C was obtained from Bio Basic (Markham, ON, Canada). Pierce™ F(ab')2 Micro Preparation kit was from Thermo Scientific (Burlington, ON, Canada).

Antibodies. Purified mouse anti-human FcgRIII (3G8) and Alexa Fluor®-labeled 647 mouse anti-human CD16 (3G8) and PE-labeled mouse anti-human FcgRIIIb (Clone CLB-gran11.5), Purified mouse anti-human FcgRI (10.1) and V450-labeled mouse anti-human FcgRI (10.1), PE-labeled

mouse anti-human FcgRII (Clone FLI8.26, also known as 8.26) were purchased from BD Biosciences (San Jose, CA, USA). The monoclonal anti-human FcgRII (IV.3) was purified from ascites of mice inoculated with the hybridoma HB-217 obtained from the American Type Culture Collection (Manassas, VA, USA). This antibody recognizes a native extracellular epitope of FcgRIIa[26].

Ethics. All experiments involving human tissues received approval from the Université Laval ethics committee.

Isolation of blood neutrophils. Informed consent was obtained in writing from all donors. Data collection and analyses were performed anonymously. PMNs were isolated as originally described [27] with modifications [28]. Briefly, venous blood collected on isocitrate anticoagulant solution from healthy volunteers was centrifuged (250 x *g*, 10 min), and the resulting platelet-rich plasma was discarded. Leukocytes were obtained following sedimentation of erythrocytes in 2% Dextran-500 (Sigma). Neutrophils were then separated from other leukocytes by centrifugation on a 10 ml lymphocyte separation medium (Wisent). Contaminating erythrocytes were removed using 20 s of hypotonic lysis. Purified granulocytes (> 95% neutrophils, < 5% eosinophils) contained less than 0.2% monocytes, as determined by esterase staining. Viability was greater than 98%, as determined by trypan blue dye exclusion. The entire cell isolation procedure was carried out under sterile conditions at room temperature.

Cell incubations. Neutrophils were re-suspended at a concentration of 10 x 10^6 cells/ml at 37°C in Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES pH 7.4, 1.6 mM Ca^{2+} and no Mg^{2+} supplemented with 0.1 U/ml adenosine deaminase (ADA) to prevent accumulation of endogenous adenosine in the medium, thus minimizing the previously demonstrated modulating effects of adenosine on inflammatory factor production by neutrophils[29-31].

Preparation of heat-aggregated IgGs. Heat-aggregated (HA)-IgGs were essentially prepared as originally described [32], with modifications. Briefly, soluble aggregates were prepared daily by resuspending IgGs in HBSS 1X at a concentration of 25 mg/ml, and heating at 63˚C for 75 min.

Flow Cytometry. Following appropriate treatments, cell suspensions were spin and resuspended in HBSS containing human Fc Block (BD Biosciences). Cells were incubated with V450-labeled mouse anti-human FcgRI (CD64), Alexa Fluor®-labeled 647 mouse anti-human FcgRIII (CD16) and PE-labeled mouse anti-human FcgRII (CD32) for 30 min in the dark. HBSS and 7-AAD (viability) were added (400 µl), and samples were analyzed using a FACS Canto II flow cytometer with FACSDiva software, version 6.1.3 (BD Biosciences).

Measure of reactive oxygen species production. ROS production was measured as previously described (Fossati, 2002 #232) with modifications. Briefly, neutrophils were re-suspended at 1 x 10^6 cells/ml in HBSS+ADA (with 10% human serum) in the presence of either 2.5% membrane non-permeable cytochrome c (v/v), 10 µM of membrane-permeable luminol, or 10 µM of membrane non-permeable isoluminol. Neutrophils (200 µl) were placed in 96-well microplates, treated as indicated, and stimulated with 1 mg/ml of free IgGs or 1 mg/ml of HA-IgGs. Suspensions were incubated at 37°C in the microplate reader (Infinite M1000 PRO, Tecan (Morrisville, NC, USA), i-control 2.0 software). Luminescence intensity (luminol and isoluminol) and optical density (cytochrome C; 550 nm with a correction at 540 nm) were monitored every 5 minutes. The amount of superoxide anion produced in the cytochrome C reduction assay was calculated using the formula published by Dahlgren and Karlsson[33].

Statistical analysis. Statistical analysis was performed using GraphPad PRISM version 8 (GraphPad Software, San Diego, CA, USA). Where applicable, values are expressed as the mean

± SEM. Statistical analysis was performed using a two-tailed Student's t-test (paired comparisons). Differences were considered significant (marked by an asterisk*) when $p < 0.05$.

Results

Heat-aggregated IgGs rapidly up-regulate FcgRI expression on neutrophils.

In order to gain insights about the implication of neutrophils in inflammatory events resulting from their interaction with immune complexes, we incubated human neutrophils with heat-aggregated (HA)-IgGs and measured the surface expression of activation markers by flow cytometry. Such aggregates are a good initial model of immune complexes, as they cause no bias based on the antigen[34]. HA-IgGs significantly increased maturity, activation, and adhesion markers (CD10, CD15, CD11b, CD11b^{act}), albeit in a relatively modest fashion (**Table 1**). HA-IgGs had no significant effect on specific granule exocytosis (CD35, CD66b). In contrast, HA-IgGs had a strong impact on FcgRI (CD64), and similarly on primary granules (CD63) (4.61±0.79 and 3.99±0.55-fold increases, respectively). Conversely, HA-IgGs decreased the expression of FcgRII and FcgRIII. Such an up-regulation of neutrophil-FcgRI is unprecedented and prompted us for further investigation.

HA-IgGs increased FcgRI in a concentration-dependent and saturable fashion (**Fig. 1A**). At the highest concentration, the proportion of FcgRI-positive neutrophils reached 75%, from 2.5% on resting cells (**Fig. 1C**). HA-IgGs up-regulated the surface expression of FcgRI within minutes and remained elevated for at least 60 min (**Fig. 1B, D**). Free IgGs had little to no effect on FcgRI expression. Conversely, HA-IgGs decreased the signal intensity of FcgRII and FcgRIII, by 63.0% ± 1.2% and 47.1% ± 4.8%, respectively, compared to non-stimulated cells (**Fig. 1E, I**), but did not

affect percentages of positive cells (**Fig. 1G, H, K, L**). Overall, the impact of HA-IgGs on FcgR expression occurred within minutes (**Fig. 1B, F, J**).

HA-IgGs are a strong stimulus for the up-regulation of FcgRI on human neutrophils.

Injections of interferon-gamma or G-CSF have been reported to up-regulate FcgRI on neutrophils, after several hours, or days[15-17]. In order to compare the potency of these factors and other well-characterized neutrophil agonists with that of HA-IgGs, we incubated neutrophils with LPS, IFN-alpha, IFN-gamma, fMLP, TNF, G-CSF, or GM-CSF, alone or in combination with HA-IgGs, and measured FcgR expression. By themselves, none of these factors had any significant effect on FcgRI expression (**Fig. 2A, white bars**). HA-IgGs led to a 4.95 ± 0.45 fold-increase (n=7) in FcgRI mean fluorescence intensity (**Fig. 2A, black bars**); FcgRI-positive neutrophils reached 67.24% ± 6.88% on HA-IgGs stimulated neutrophils, from 3.12% ± 1.62% in non-stimulated cells (**Fig. 2B**). When used in combination with HA-IgGs, only LPS and GM-CSF had a significantly potentiating effect on FcgRI. While all factors tended to increase percentages of FcgRI-positive neutrophils, this was not significant. GM-CSF slightly increased the basal expression of FcgRIII (**Fig. 2 C, D**); LPS and GM-CSF potentiated the decreasing-effect of HA-IgGs on FcgRIII. Overall, in these conditions, HA-IgGs are the strongest stimulus for FcgRI up-regulation.

FcgRII, but not FcgRIIIb, is instrumental in the up-regulation of FgcRI by HA-IgGs.

We initially assessed the possible implication of FcgRIII in the up-regulation of neutrophil-FcgRI by HA-IgGs. First, the FcgRIII-blocking monoclonal antibody 3G8 failed to prevent FcgRI up-regulation. Second, the FcgRIIIb-specific monoclonal antibody 11.5 significantly enhanced FcgRI

up-regulation. Third, FcgRIIIb shedding by PI-PLC[35] also did not prevent FcgRI up-regulation (**Fig. 3A**), all arguing against a significant role for FcgRIII.

Regarding FcgRII, the blocking monoclonal antibody IV.3 significantly prevented the up-regulation of FcgRI, by approximately 75%. Simultaneous blockade of FcgRIII and FcgRII did not yield a more potent blockade. Together, these results clearly confirm a role for FcgRII, but not for FcgRIIIb. The implication of FcgRIIIa cannot, however, be ruled out at this point.

Syk and PI3K mediate the up-regulation of FcgRI by HA-IgGs

Both FcgRIIa and FcgRIIIa signal through an Immunoreceptor Tyrosine-based Activation Motif (ITAM). After crosslinking of FcγRs by IgG-aggregates or immune complexes, tyrosine residues in the ITAM are phosphorylated by Src-family protein kinases, thereby becoming anchoring sites for the spleen tyrosine kinase (SYK), the activation of which leads to stimulation of phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), ERK (mitogen-activated protein kinase; MAPK), and other downstream pathways[36]. In incubations with HA-IgGs, the protein synthesis inhibitor cycloheximide, and the Src-family kinase inhibitor PP-2 had no significant impact (**Fig. 3B**). In contrast, the Syk inhibitor PRT-060318, and two structurally distinct PI3K inhibitors, Wortmannin and AS605240, each prevented the up-regulation of FcgRI by half. Other downstream pathways, including PLC (U73122), PKC (Gö6976), or glycogen-synthase-kinase (GSK)-3 (SB216763), were not involved.

FcgRI mediates intracellular ROS production.

We evaluated the reactive oxygen species (ROS) production by HA-IgG-stimulated neutrophils, by monitoring the overall MPO-dependent production of HOCl with luminol; the extracellular-only MPO-dependent production of HOCl with isoluminol; and the extracellular-only release of O_2-reactive species with cytochrome C, from HA-IgG-stimulated neutrophils. As shown in **Figure 4**, the incubation of neutrophils with HA-IgGs resulted in the intracellular-only generation of reactive oxygen species (ROS). Free-IgGs had little to no effect.

FcgRI signaling is necessary for ROS production by HA-IgG-stimulated neutrophils.

The FcgRIIIb-specific shedding enzyme PI-PLC diminished ROS production elicited by HA-IgGs, by approximately 35% (**Fig. 5A**). In contrast, Fc-Block, IV.3, or 3G8, each prevented ROS production by more than 80%. Similarly, pre-incubating neutrophils with the FcgRI-specific 10.1 antibody, whole or F(ab')2 fragment, also blocked ROS production, showing the involvement of FcgRI in this cellular function.

Cycloheximide, a protein synthesis inhibitor, had no inhibitory effect on ROS production (**Fig. 5B**), while the inhibition of the Syk, PI3K, and Akt pathways, all but obliterated the cell's response. Blockades of PLC, PKC, or MEK pathways also diminished –although not entirely- ROS production. Nexinhib20, an inhibitor of azurophil granule exocytosis (REF), prevented FcgRI and CD63 up-regulation, and ROS production, in a concentration-dependent fashion and with comparable potencies (**Fig. 5C**). Taken together, these results indicate that FcgRI can readily appear to the cell surface upon exocytosis of intracellular azurophil granules, without the need for

de novo protein synthesis. Most importantly, they show the necessity of FcgRI signaling for optimal ROS production.

Bacterial components markedly amplify the FcgRI-dependent ROS production.

We activated neutrophils with well-characterized agonists and measured the production of ROS. While having no effect by themselves, the formylated peptide fMLP and the TLR4 ligand LPS, each had an enormous amplifying impact on the ROS production elicited by HA-IgGs (**Fig. 6A**) fMLP, in combination with HA-IgGs, potentiated ROS production by more than 9-fold (**Fig. 6A**). For the TLR4 ligand LPS, as little as 0.1 ng/ml was sufficient to increase the production of ROS, eventually increasing it seven-fold at 1 ug/ml (**Fig. 6B**). Under these conditions, blocking FcgRI obliterated the effect of LPS and reduced the production of ROS by 93 ± 4% (n=2), significantly below levels of HA-IgG alone.

Discussion

Lupus is an auto-immune disease with the highest incidence in North America (prevalence of 241/100 000 people)[37], mostly afflicting women of childbearing age. Pathogenic autoantibodies and immune complexes are important factors and contribute to feeding inflammatory flares associated with lupus and a range of human autoimmune diseases from glomerulonephritis, arthritis, and transplantation rejection, to rheumatic fever. Neutrophils recognize immune complexes through the engagement of their Fc receptors, which can lead to the biosynthesis of lipid mediators, antibody-dependent cell phagocytosis, neutrophil extracellular traps (NETs), and production of ROS[38]. While these responses are designed to enhance host defense, they can also amplify tissue damage[39]. Neutrophils binding to deposited immune complexes in the kidneys of patients with lupus nephritis, one of the significant complications of lupus, are likely to contribute to the pathogenesis[38,40-42].

Here, we showed that IgG-aggregates could up-regulate the surface expression of FcgRI on human neutrophils by mobilizing it from intracellular stores. IgG aggregates showed to be one of the most powerful agonists for FcgRI up-regulation, significantly stronger than known agonists[43,44]. Also, FcgRI proved to be instrumental for ROS production. To our knowledge, these observations are unprecedented and provide a key-increment in our understanding of interactions between immune complexes and neutrophils.

The increase in FcgRI elicited by HA-IgGs occurred on human neutrophils within seconds and without the need for *de novo* protein synthesis. This is unlike situations in which patients or neutrophils were exposed to agonists for several hours[43,44]. In the present conditions, the main origin of intracellular FcgRI likely includes azurophil granules. IgG-aggregates equally up-

regulated FcgRI and CD63, a marker of azurophil granules, but not CD66b, a marker of specific granules. Moreover, the azurophil-specific exocytosis inhibitor Nexinhib20[45], efficiently prevented CD63 and FcgRI up-regulation, and ROS production with comparable potencies. Hiding FcgRI in intracellular stores may be a way to keep neutrophils from responding through this high-affinity FcgRI in an untimely fashion, but allowing its rapid exposure when needed.

Results with blocking antibodies clearly point toward a role for FcgRIIa, but not for FcgRIIIb, in the FcgRI rapid exposure. This was also supported by the inefficiency of FcgRIIIb shedding, by PI-PLC, to prevent FcgRI up-regulation. FcgRIIIa and FcgRIIIb share 96% sequence identity in their extracellular immunoglobulin binding regions[7], and the 3G8 antibody does not discriminate between FcgRIIIb and FcgRIIIa. While 3G8 prevents interaction between FcgRIII and immune complexes[46], it also prevents FcgRIIIa-dependent signaling. Thus, it is tempting to think that, instead, FcgRIIIa is involved, but results are inconclusive in this regard. Tools suited for the specific study of FcgRIIIa will be necessary to clarify this issue.

Results from this study have particular relevance in the context of immune complex-mediated inflammatory disorders, where current models include the capture of circulating neutrophils by activated endothelial cells. Immobilized immune complexes support the rapid attachment of neutrophils, under physiologic flow conditions[47]. Our results suggest that such immobilized neutrophils interacting with immune complexes may rapidly express higher levels of FcgRI, enabling inflammatory responses such as ROS production. Within this context, minute concentrations of TLR4 ligands or other bacterial components in the immediate environment could dramatically enhance such responses and lead to uncontrolled inflammatory reactions. Cross-linking of CD11b and CD18 with antibodies also induced a rapid, protein synthesis independent, up-regulation of FcgRI, indicating that events involved in interactions between neutrophils and

endothelial cells also contribute to FcgRI expression[48]. Thus, immune complexes, the presence of microorganism fragments, and adhesion events may profoundly impact neutrophil FcgRI expression and downstream inflammatory functions.

In conclusion, IgG-aggregates are potent inducers of FcgRI expression on human neutrophils. Moreover, FcgRI proved to be instrumental in ROS production, downstream of interactions with aggregates, identifying FcgRI as a central, limiting factor in such responses. Cross-talks between FcgRI, TLR, GPCR, and integrin signaling pathways may also have a profound impact on cell activation.

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Author's contributions

SH: Performed most experiments, analyzed the data, wrote parts of the manuscript.

CL: Performed experiments, including surface marker measurements.

PF: Designed parts of the study.

EB: Designed parts of the study.

MP: Designed the study, analyzed the data, wrote the manuscript.

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	Relative Intensity Index
CD10	1.69±0.16 *
CD15	1.48±0.13 *
CD11b	1.29±0.08 *
CD11b^{act}	1.61±0.21 *
CD62L	0.97±0.07
CD35	0.82±0.08
CD66b	1.02±0.09
CD63	**3.99±0.55 ***
CD64	**4.61±0.79 ***
CD32	0.65±0.11 *
CD16	0.79±0.14 *

Table 1. Impact of heat-aggregated (HA)-IgGs on neutrophil activation. Human neutrophils were incubated with HA-IgGs (1mg/ml, 30 min, 37˚C), and surface markers were monitored by flow cytometry, as described in *Methods*. Markers of the following parameters were measured: maturity and activation (CD10); adhesion and migration (total, and activated form (^{act}) of CD11b, CD62L); degranulation (CD15, CD63, CD35, CD66b); Fc gamma receptors FcgRI (CD64), FcgRII (CD32), and FcgRIII (CD16). FcgRI and CD63 were most up-regulated (in Bold), both in a comparable fashion. For each marker, results are expressed as the mean fluorescence index after HA-IgG incubations, relative to non-stimulated cells. Mean ± SEM (n≥3 experiments). *$p<0.05$

Figure 1. HA-IgGs rapidly up-regulate FcgRI on human neutrophils, in a concentration-dependent fashion. Neutrophils were incubated with indicated concentrations of heat-aggregated (HA)-IgGs, or free IgGs, for 30 min at 37˚C (left panels), or for indicated times at 1 mg/ml (right panels). Surface expressions of FcgRI (**A-D**), FcgRII, (**E-H**), and FcgRIII (**I-L**) were measured by flow cytometry. Results are expressed as the fluorescence signal intensity relative to non-stimulated cells and are from n=4 experiments, each performed with neutrophils from different healthy donors. *p<0.05, **p<0.01, ***p<0.001

Figure 2. HA-IgGs are a potent stimulus for FcgRI up-regulation in human neutrophils. Cells were incubated with indicated agonists or diluent (dil.) for 15 min (37˚C), then with HA-IgGs for 30 min. FcgR expression was determined by flow cytometry. **A, C, D).** For each of the FcgRs, results are expressed as the fluorescence signal intensity relative to non-stimulated cells (dil., white bars). **B)** Percentages of FcgRI-positive neutrophils. From n=4 experiments, each performed with neutrophils from different donors. Mean ± SEM, n=4. *p<0.05

Figure 3. Identification of signaling pathways surrounding the up-regulation by HA-IgGs. A) Neutrophils were incubated with the following whole antibodies: 3G8 (FcgRIII), 11.5 (FcgRIIIb), IV.3 (FcgRII), or with PI-PLC for 15 minutes (37˚C), then stimulated with HA-IgGs for an additional 30 min. **B)** Neutrophils were incubated with indicated inhibitors before stimulation with HA-IgGs. FcgRI surface expression was measured by flow cytometry. Results are expressed as the fluorescence signal intensity relative to HA-IgGs alone (dil.). Mean ± SEM, n=3. *p<0.05, **p<0.0001

Figure 4. Human neutrophils stimulated with HA-IgGs produce intracellular reactive oxygen species. Neutrophils were incubated in indicated conditions, in the presence of either **A)** 10 uM luminol, **B)** 10 uM isoluminol, or **C)** 2.5% cytochrome C (v/v). ROS production was measured (37˚C) simultaneously by luminescence intensity (luminol and isoluminol) and optical density (cytochrome C) for the indicated times. Results are from one experiment, representative of n=2 experiments performed in identical conditions with neutrophils from different donors. Bar graph: Results are expressed as a percentage of the reactive oxygen species (ROS) production, relative to HA-IgGs alone (HA-IgGs). Mean ± SEM, (n=2). *p<0.05

Figure 5. FcgRI activation contributes to the production of reactive oxygen species. A) Neutrophils were incubated with PI-PLC, Fc-Block, IV.3, 3G8, or 10.1 antibodies, for 15 min before stimulation with HA-IgGs. **B)** Neutrophils were pre-incubated with cycloheximide, PRT-060318, Wortmannin, AS605240, U73122, Gö6976, or U0126, ROS production was measured by luminescence intensity (Luminol). Results are expressed as the percentage of ROS production, relative to HA-IgGs alone (dil.) Mean ± SEM, (n≥4). *p<0.05, ****p<0.0001 **C)** Neutrophils were pre-incubated with indicated concentrations of Nexinhib20. Surface expression of FcgRI, CD63, and production of ROS, were measured. Results are from one experiment representative of two.

Figure 6. Combinations of fMLP or LPS with HA-IgGs boost ROS production. Neutrophils were incubated with indicated agonists for 10 min (37°C) before stimulation with HA-IgGs. ROS production was determined. Typical traces of combinations are shown with fMLP (**A**) and with LPS (**B**), and are representative of n=3 experiments. Bar graphs: Results are expressed as the percentage of ROS production relative to HA-IgGs alone (dil., black bar) and are the mean ± SEM, n=3. Hatched bar: Neutrophils were pre-incubated with 10.1 antibody before stimulation. (Mean ± SD, n=2) *$p<0.05$, **$p<0.001$

Figure 7. Schematics: HA-IgGs rapidly up-regulate FcgRI expression at the surface of human neutrophils in a FcgRII-dependent fashion and contribute to ROS production. A) Binding of HA-IgGs to FcgRIIa (and, possibly, FcgRIIIa) up-regulate FcgRI surface expression on human neutrophils, from CD63 positive, azurophilic granules, in a Syk and PI3K-dependent fashion. **B)** Up-regulation and engagement of FcgRI by HA-IgGs is necessary for intracellular ROS production and involves PLC, PKCa/PKCb1, and MEK1/2. **C) In the presence of bacterial components such as LPS or fMLP,** ROS production by HA-IgGs-FcgRI complexes is potentiated. Abbreviations: HA-IgGs; heat-aggregated IgGs, fMLP; formyl-methionyl-leucyl phenylalanine, LPS; lipopolysaccharide, MEK1/2; mitogen-activated protein (MAP)/ERK kinase 1 and 2, PI3K; phosphoinositide 3-kinases, PLC; phospholipase C, PKCa; protein kinase C alpha, PKCb1; protein kinase C beta 1, ROS; reactive oxygen species, Syk; Spleen tyrosine kinase.

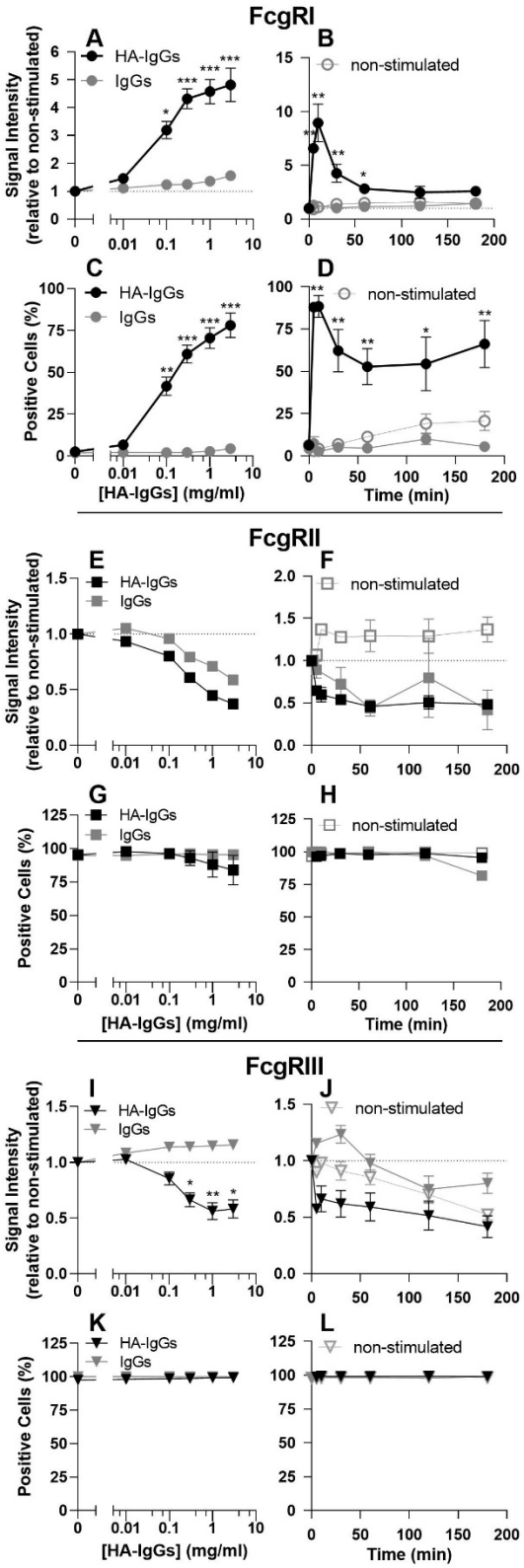
FcgRI
A
B
HA-IgGs
IgGs
non-stimulated
Signal Intensity (relative to non-stimulated)
C
D
Positive Cells (%)
[HA-IgGs] (mg/ml)
Time (min)
FcgRII
E
F
G
H
FcgRIII
I
J
K
L

FIGURE 1

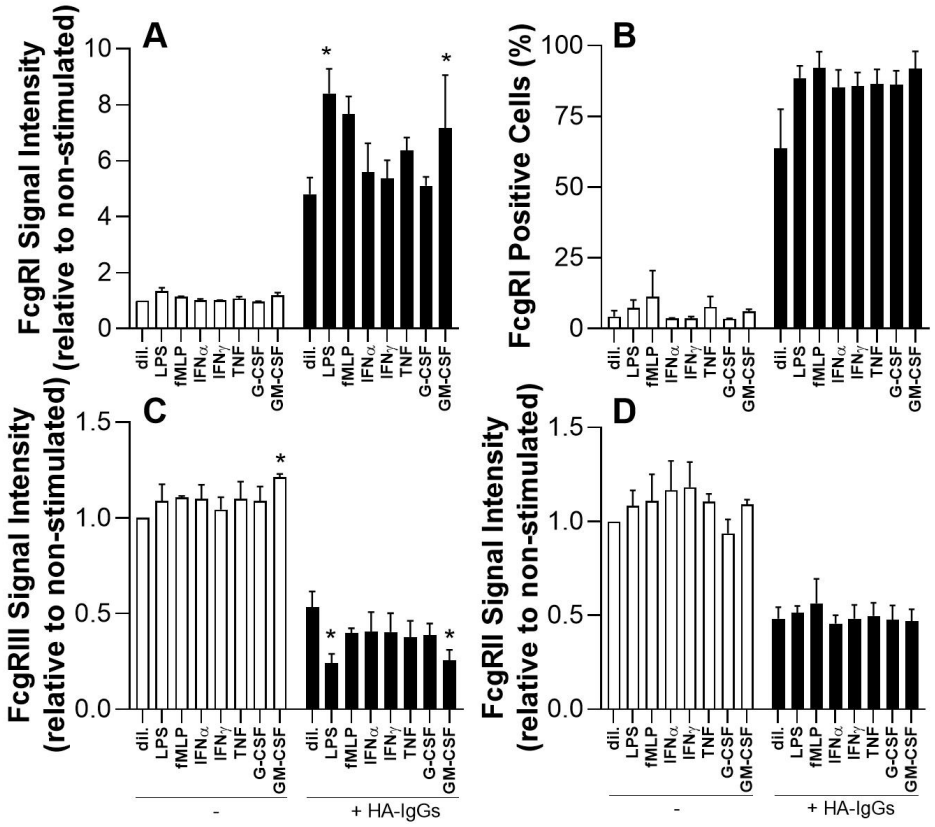
A
FcgRI Signal Intensity
(relative to non-stimulated)
B
FcgRI Positive Cells (%)
C
FcgRIII Signal Intensity
(relative to non-stimulated)
D
FcgRII Signal Intensity
(relative to non-stimulated)
dil.
LPS
fMLP
IFNα
IFNγ
TNF
G-CSF
GM-CSF
-
+ HA-IgGs

FIGURE 2

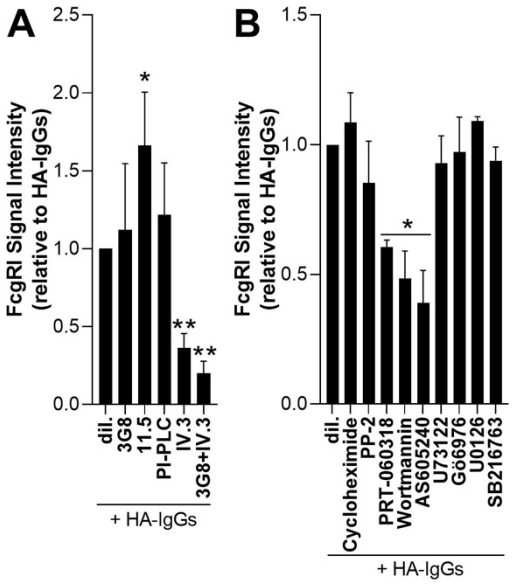
A
B
FcgRI Signal Intensity
(relative to HA-IgGs)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
dil.
3G8
11.5
PI-PLC
IV.3
3G8+IV.3
+ HA-IgGs
1.5
1.0
0.5
0.0
*
dil.
Cycloheximide
PP-2
PRT-060318
Wortmannin
AS605240
U73122
Gö6976
U0126
SB216763
+ HA-IgGs

FIGURE 3

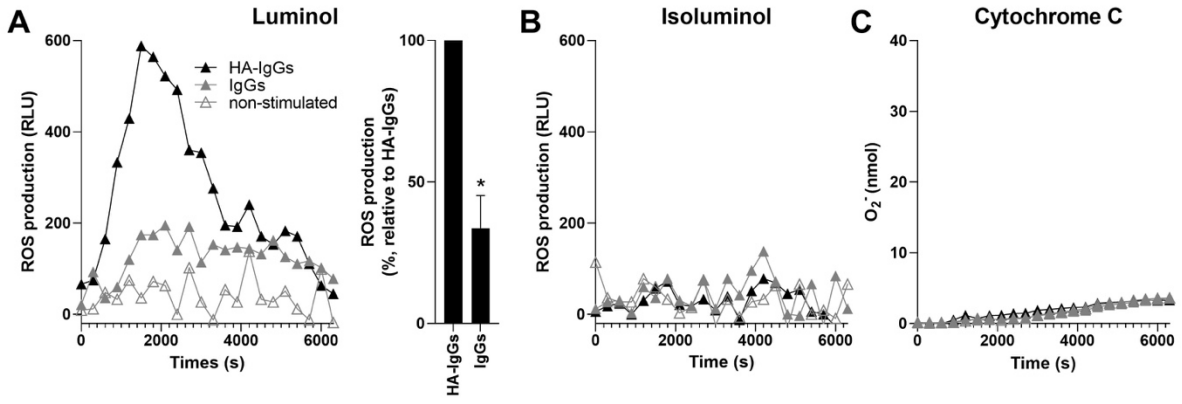
A
Luminol
HA-IgGs
IgGs
non-stimulated
ROS production (RLU)
Times (s)
ROS production
(%, relative to HA-IgGs)
HA-IgGs
IgGs
B
Isoluminol
ROS production (RLU)
Time (s)
C
Cytochrome C
O_2^- (nmol)
Time (s)

FIGURE 4

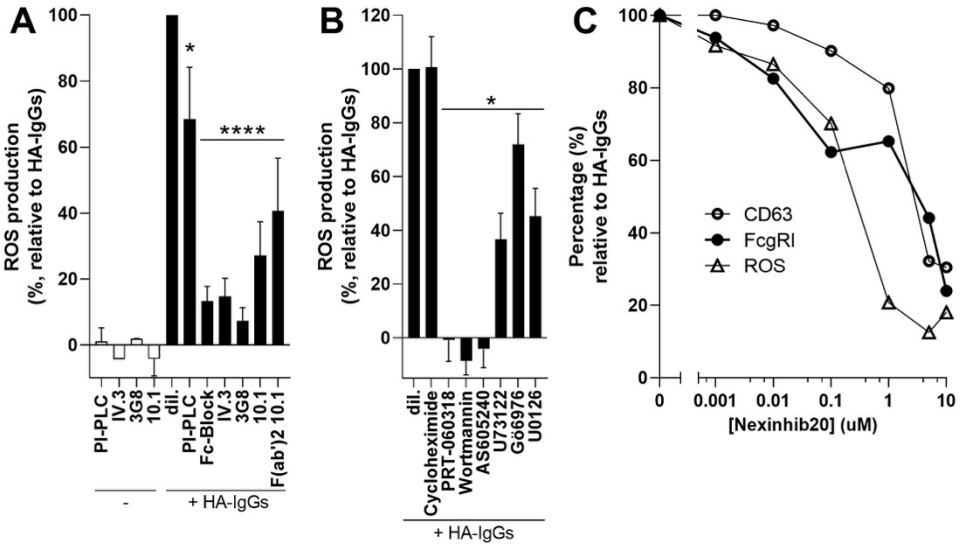
A
ROS production
(%, relative to HA-IgGs)
100
80
60
40
20
0
*
****
PI-PLC
IV.3
3G8
10.1
dil.
PI-PLC
Fc-Block
IV.3
3G8
10.1
F(ab')2 10.1
-
+ HA-IgGs
B
ROS production
(%, relative to HA-IgGs)
120
100
80
60
40
20
0
*
dil.
Cycloheximide
PRT-060318
Wortmannin
AS605240
U73122
Gö6976
U0126
+ HA-IgGs
C
Percentage (%)
relative to HA-IgGs
100
80
60
40
20
0
CD63
FcgRI
ROS
0
0.001
0.01
0.1
1
10
[Nexinhib20] (uM)

FIGURE 5

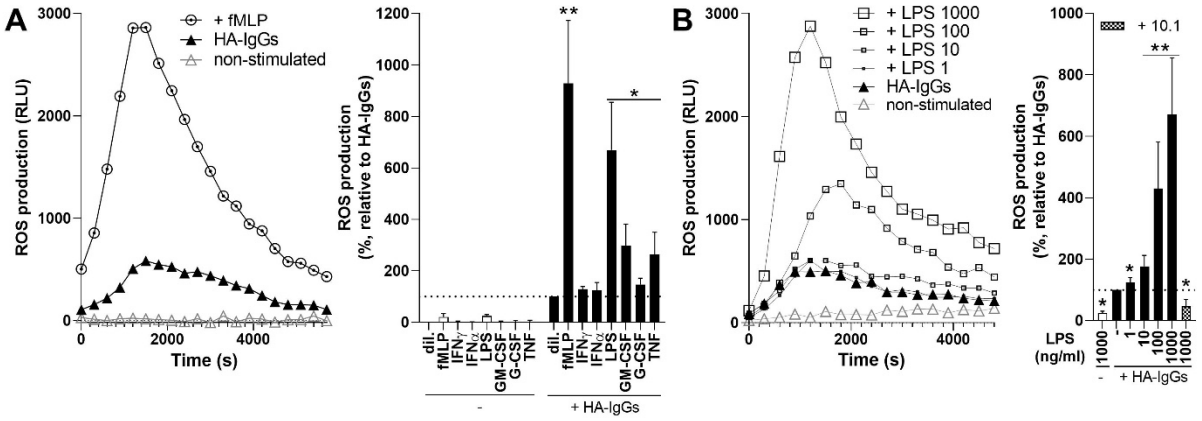
A
+ fMLP
HA-IgGs
non-stimulated
ROS production (RLU)
Time (s)
ROS production (%, relative to HA-IgGs)
dil.
fMLP
IFNγ
IFNα
LPS
GM-CSF
G-CSF
TNF
-
+ HA-IgGs
B
+ LPS 1000
+ LPS 100
+ LPS 10
+ LPS 1
HA-IgGs
non-stimulated
+ 10.1
LPS (ng/ml)

FIGURE 6

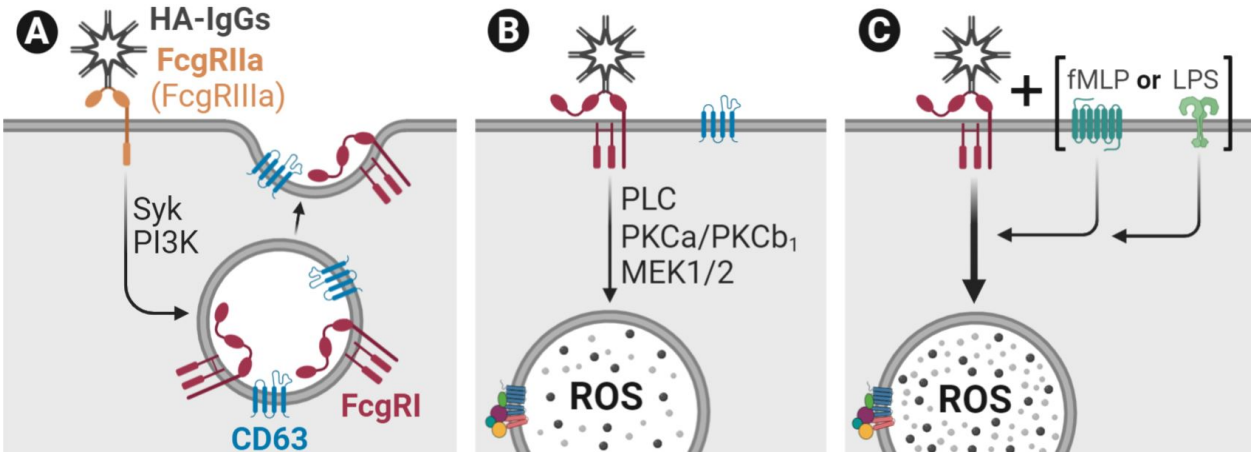
A
HA-IgGs
FcgRIIa
(FcgRIIIa)
Syk
PI3K
FcgRI
CD63
B
PLC
PKCa/PKCb1
MEK1/2
ROS
C
+
fMLP or LPS
ROS

FIGURE 7