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, ranking IgG-aggregates among the most potent known agonists. FcgRIIa, and possibly FcgRIIIa, appeared to mediate this up-regulation. Syk and PI3K were involved in this process. Also, FcgRI-dependent signaling was necessary for ROS production in response to IgG-aggregates. Finally, combinations of bacterial materials with aggregates dramatically boosted ROS production. Thus, 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 neutrophils contribute to tissue damage associated with immune complex-associated diseases, such as lupus.

Introduction

Immune complex diseases, such as rheumatoid arthritis and systemic lupus erythematosus, encompass a diverse group of inflammatory conditions characterized by antigen-antibody deposition. Typical manifestations are glomerulonephritis, synovitis, and dermal vasculitis (1). Activation of neutrophils, resulting from the interaction with immune complexes, leads to the production of oxygen-free radicals and the release of lysosomal enzymes (2), which 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 in (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 Immunoreceptor Tyrosine-based Activation Motif (ITAM), is an essential mediator of destructive antibody-based inflammation in autoimmunity (5) and is necessary for neutrophil recruitment within glomerular capillaries following IgG deposition (8, 9). Also, a relatively modest expression of FcgRIIIa (CD16a) on neutrophils was recently demonstrated (10). FcgRIIIa is a classic activating receptor, which, upon stimulation, associates with an ITAM-signaling system.

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 currently serves as a sensitive diagnostic marker for early-onset neonatal infections (21).

While monocyte FcgRI expression is positively associated with immune inflammation and lupus (22-24), expression of FcgRI on circulating neutrophils is barely elevated in 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 investigated 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.

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 interactions 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(26). 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), or on CD62L. In contrast, HA-IgGs had a sizeable and similar impact on FcgRI (CD64) and azurophilic granules (CD63) (4.61±0.79 and 3.99±0.55-fold increases, respectively). Conversely, HA-IgGs decreased the expression of FcgRII (CD32) and FcgRIII (CD16). Such an up-regulation of neutrophil-FcgRI is unprecedented and warrants 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% and 47%, 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 was potent and 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 (IFN)-gamma or G-CSF have been reported to up-regulate FcgRI on neutrophils, after lengthy exposures (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 4.9-fold increase (n=4) in FcgRI mean fluorescence intensity (**Fig. 2A, black bars**); FcgRI-positive neutrophils reached 67% on HA-IgGs stimulated neutrophils, from 3% 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. 2D**); LPS and GM-CSF potentiated the decreasing-effect of HA-IgGs on FcgRIII. Finally, none of these factors affected FcgRII expression (**Fig. 2C**). Overall, in these conditions, HA-IgGs were the most potent stimulus for FcgRI up-regulation.

FcgRIIa, 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. The FcgRIII-blocking monoclonal antibody 3G8 did not prevent FcgRI up-regulation. Moreover, the FcgRIIIb-specific monoclonal antibody 11.5 significantly enhanced FcgRI up-regulation, so did the shedding of FcgRIIIb by PI-PLC(27) (**Fig. 3A**), arguing against a significant role for FcgRIIIb in FcgRI up-regulation, but leaving the possibility open for FcgRIIIa.

As expected, the FcgRIIa-blocking monoclonal antibody IV.3 effectively prevented the up-regulation of FcgRI, by approximately 60%. Simultaneous blockade of FcgRIII and FcgRIIa did not result in more inhibition. Together, these results confirm a role for FcgRIIa, but not for FcgRIIIb, in FcgRI up-regulation.

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

Both FcgRIIa and FcgRIIIa signal through association with ITAM. Tyrosine residues in the ITAM, phosphorylated by Src-family protein kinases, constitute anchoring sites for the spleen tyrosine kinase (Syk), the activation of which leads to stimulation of phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), ERK (mitogen-activated protein kinase; MAPK), and other downstream pathways(28). Despite an inhibitory trend, the Src-family kinase inhibitor PP-2 only had a modest impact on FcgRI up-regulation (**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, approximately by half. Other downstream pathways, including PLC (U73122), PKC (Gö6976), MEK/ERK (U0126), or glycogen-synthase-kinase (GSK)-3 (SB216763), were not involved. Finally, the protein synthesis inhibitor cycloheximide also had no sizeable impact.

FcgRI is pre-formed, stored in intracellular granules.

Parallel up-regulation of FcgRI and CD63 pointed toward an azurophilic granule localization for FcgRI. To ascertain this, we used the neutrophil exocytosis inhibitor Nexinhib20. As shown in **Figure 3C** (black circles), Nexinhib20 effectively reversed FcgRI increase, in a concentration-dependent fashion, and by up to 66±7%, at 10 μM. The effect on CD63 was comparable. Together

with a rapid, protein-synthesis independent up-regulation by HA-IgGs and a profile of expression following that of CD63, these results strongly support an azurophilic granule localization for pre-formed FcgRI, in resting neutrophils.

HA-IgGs induce 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. As shown in **Figure 4**, the incubation of neutrophils with HA-IgGs resulted in the intracellular-only generation of reactive oxygen species (ROS), as reported earlier (29). 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**). Antibodies against FcgRIIa (IV.3), FcgRIII (3G8), FcgRIIIb (11.5), or Fc-Block, each prevented ROS production by more than 80%. Similarly, pre-incubating neutrophils with the FcgRI-specific 10.1 antibody blocked ROS production, showing the involvement of FcgRI also in this cellular function. While the up-regulation of FcgRI involved FcgRIIa, the production of ROS appeared to necessitate the engagement of all FcgR monitored: FcgRI, FcgRIIa, FcgRIIIa, and FcgRIIIb.

Inhibition of Syk, PI3K pathways, all but obliterated the cell's response (**Fig. 5B**). Blockades of PLC, PKC, or MEK pathways also diminished –although not entirely- ROS production. Nexinhib20 also prevented ROS production, in a concentration-dependent fashion, but with higher potency than for FcgRI (**Fig. 3C**). Taken together, these results show the necessity of a high number of pathways, including FcgRI signaling, for optimal ROS production.

Bacterial components magnify FcgRI-dependent ROS production.

Given the potentiation of FcgRI expression by fMLP and LPS, we activated neutrophils with these, and other agonists, and measured the production of ROS. While producing no effect by themselves, fMLP and LPS each had an enormous amplifying impact on the ROS production elicited by HA-IgGs by more than 6-fold (**Fig. 6A**). For the TLR4 ligand, LPS, as little as 1 ng/ml was sufficient to increase the production of ROS (**Fig. 6B**). Blocking FcgRI, or addition of Nexinhib20, obliterated the effects of LPS and fMLP, reducing the production of ROS by at least 90%.

Schematics, summarizing the main findings, is presented in **Figure 7**.

Discussion

In the present study, HA-IgGs rapidly up-regulated the surface expression of FcgRI on human neutrophils by mobilizing it from intracellular stores. In this regard, HA-IgGs proved to be more potent than any of the previously reported agonists (16, 17, 30). Furthermore, FcgRI engagement was essential for HA-IgG-induced ROS production; additional stimuli synergized with HA-IgGs, magnifying ROS production. These observations reveal key-elements in our understanding of interactions between immune complexes and neutrophils.

The up-regulation of FcgRI by HA-IgGs was dependent on FcgRIIa engagement as well as on activation of Syk and PI-3K, in line with an FcgR-dependent event (31, 32). PLC, PKC, GSK-3, and MEK pathways, also downstream of ITAM signaling and leading to Ca^{2+} elevation and gene expression (33), were not necessary, indicating that FcgRI exocytosis is regulated by early events, upstream of those pathways.

In the present conditions, FcgRI up-regulation occurred within seconds, without the need for *de novo* protein synthesis. This is different from previous studies in which patients or neutrophils were exposed to agonists for several hours, or days (16, 17, 30). Pre-formed FcgRI appears to be localized intracellular granules, possibly azurophilic, as suggested by the hand-in-hand expressions of FcgRI and CD63, a marker of azurophilic granules. Also, Nexinhib20, an inhibitor of granule exocytosis (34), efficiently prevented the up-regulation of FcgRI, CD63, and CD66b. The concealment of FcgRI inside resting cells may constitute a checkpoint that prevents neutrophils in circulation from getting unduly stimulated; rapid exposure of this high-affinity receptor releases fuller responsiveness when conditions require it.

Complexed-IgGs have long been known to bind to neutrophils through FcgRs and stimulate cellular responses, including the production of ROS (35). Both FcgRIIa and FcgRIIIb have been implicated in this process, often by virtue of using blocking antibodies IV.3 and 3G8, respectively (29). Using similar and additional tools, our results around the expression of FcgRI also supported the involvement of FcgRIIa, but not that of FcgRIIIb. The FcgRIIIb-specific 11.5 blocking antibody, actually enhanced FcgRI up-regulation, as did PI-PLC, a FcgRIIIb-specific shedding enzyme. Moreover, the 3G8 blocking antibody (36), which does not discriminate between the two FcgRIII isoforms, did not affect FcgRI up-regulation, supporting the concept of a decoy role for that receptor (10, 37, 38). Results leave the possibility of a role for FcgRIIIa. This point is relevant because FcgRIIIa, like FcgRI, is a classic activating receptor associating with an ITAM-signaling system; it might mediate a number of events previously attributed to FcgRIIIb (39). Alternatively, the balance in expression between the two isoforms may influence cellular responsiveness (39). Tools better suited for the specific study of FcgRIIIa will help in clarifying this issue.

In contrast, ROS production could be interrupted by blocking any of the receptors mentioned above. Also, inhibitors of PI3K, PLC, PKC, MEK1/2, each efficiently prevented this cell's response. Moreover, Nexinhib20an agent preventing Rab27a-JFC1 interactions, also inhibited the exocytosis of FcgRI from azurophilic granules, adding to its recognized anti-inflammatory properties (34). While surprising, this profile of results clearly shows that many signaling pathways are to be solicited for the production of ROS, and may indicate a tight, 'all-or-nothing' type of regulation for this critical cellular response.

One of the most salient findings here was the potentiation of HA-IgG-elicited ROS production by bacterial materials. Indeed, fMLP or LPS, enhanced the cells' response, manifold. On the one hand, these results show that minute contaminations can have a strong impact on neutrophil

activation and could profoundly affect the course of an inflammatory reaction, possibly leading to an overt, ill-regulated response. On the other hand, they indicate a crucial for FcgRI in the process, as preventing its engagement all but obliterated ROS production.

The present study finds particular relevance in the context of immune complex-mediated inflammatory disorders where current models include the capture of circulating neutrophils by endothelial cells (40). Immobilized immune complexes elicit the rapid attachment of neutrophils (41). Immune complexes can feed inflammatory flares associated with human autoimmune diseases, such as glomerulonephritis, arthritis, rheumatic fever, and lupus. Neutrophils binding to deposited immune complexes in the kidneys of patients with lupus nephritis, one of the significant complications of lupus, may contribute to its pathogenesis (42-45), through inflammatory responses such as the biosynthesis of lipid mediators, antibody-dependent cell phagocytosis, neutrophil extracellular traps (NETs), ROS production (43). While such functions are designed to contribute in host defense, disordered responses may instead lead to tissue damage (46). In this context, microbial contaminations, even minor, could steer neutrophil reactiveness in harmful directions.

In conclusion, the present study reveals an important role of neutrophil-FcgRI in cellular activation in response to IgG aggregates. Moreover, cross-talk between FcgRI and other signaling pathways may also have a profound impact on cell activation. Together, these results provide molecular insights into how neutrophil inflammatory responses can become deleterious.

Materials & Methods

Materials. Dextran-500, Lipopolysaccharide (LPS from *Escherichia coli* 0111: B40), Formyl-methionyl-leucyl phenylalanine (fMLP), Wortmannin (from *Penicillium fumiculosum*), Luminol, Isoluminol, Cycloheximide, and SB216763 were purchased from Sigma-Aldrich (Oakville, ON, Canada). Lymphocyte separation medium was purchased from Wisent (St-Bruno, QC, Canada). AS605240, U0126, and U73122 were obtained from Cayman Chemical (Ann Arbor, MI, 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). 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). A list of compounds, molecular targets, concentrations, and solvents, is presented in **Table 2**. A list of agonists and concentrations is presented in **Table 3**.

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

mouse anti-human FcgRII (Clone FLI8.26, also known as 8.26), were purchased from BD Biosciences (San Jose, CA, USA). Monoclonal anti-human FcgRIIa (IV.3) was obtained from Bio X Cell (West Lebanon, NH, USA). This antibody recognizes a native extracellular epitope of FcgRIIa (47).

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

Isolation of human neutrophils. Informed consent was obtained in writing from all donors. Data collection and analyses were performed anonymously. Neutrophils were isolated as originally described (48) with modifications (49). 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. Neutrophils were then separated from other leukocytes by centrifugation on a 10 ml lymphocyte separation medium. Contaminating erythrocytes were removed using 20 sec 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 37ºC in Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES pH 7.4, 1.6 mM Ca^{2+} and no Mg^{2+}, 10% human serum, and supplemented with 0.1 U/ml adenosine deaminase (ADA) to prevent the accumulation of endogenous adenosine in the medium, thus minimizing the previously demonstrated modulating effects of adenosine on inflammatory factor production by neutrophils (50-52).

Preparation of heat-aggregated IgGs. Heat-aggregated (HA)-IgGs were essentially prepared as originally described (53), 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 (29) with modifications. Briefly, neutrophils were re-suspended at 1 x 10^6 cells/ml 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 with i-control 2.0 software (Tecan, Morrisville, NC, USA). 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 (54).

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 $p < 0.05$.

Author's contributions

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

CL: Performed some of the experiments, including all 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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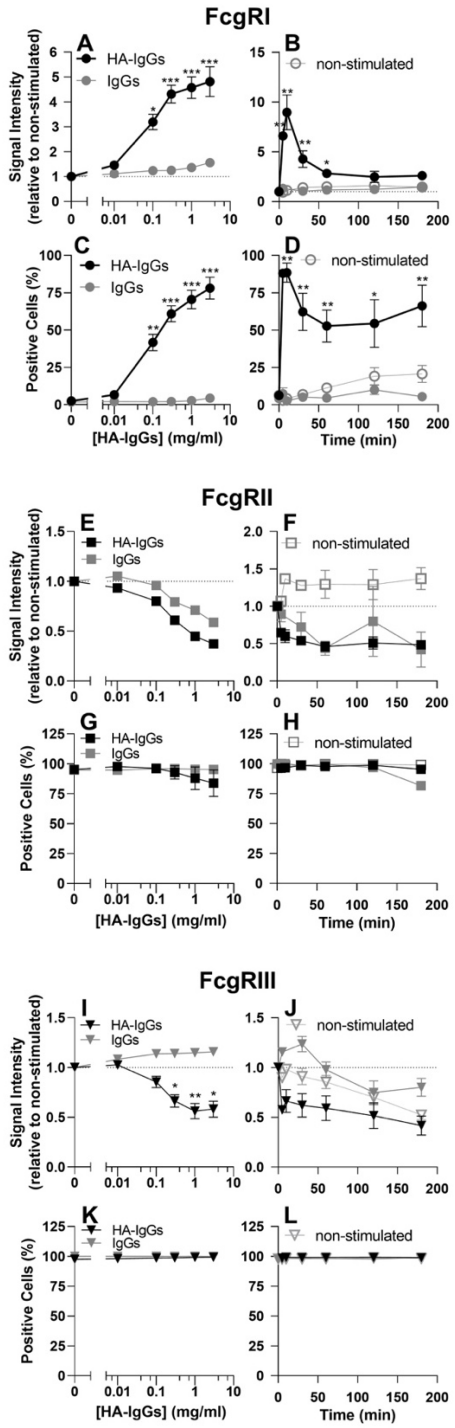

Figure 1. HA-IgGs rapidly up-regulate FcgRI on human neutrophils, in a concentration-dependent fashion. Neutrophils were incubated with indicated concentrations of heat-aggregated (HA)-IgGs, or free IgGs, for 30 min at 37˚C (left panels), or for indicated times at 1 mg/ml (right panels). Surface expressions 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

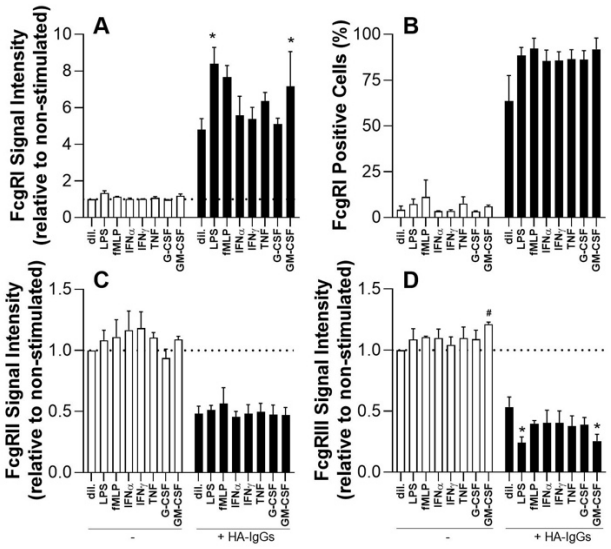

Figure 2. HA-IgGs are a potent stimulus for FcgRI up-regulation in human neutrophils. Cells were incubated with indicated agonists or diluent (dil.) for 15 min (37˚C), then with HA-IgGs for 30 min. FcgR expression was determined by flow cytometry. **A, C, D)** For each of the FcgRs, results are expressed 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. *Statistically different from HA-IgGs alone, or #from resting cells. $p<0.05$

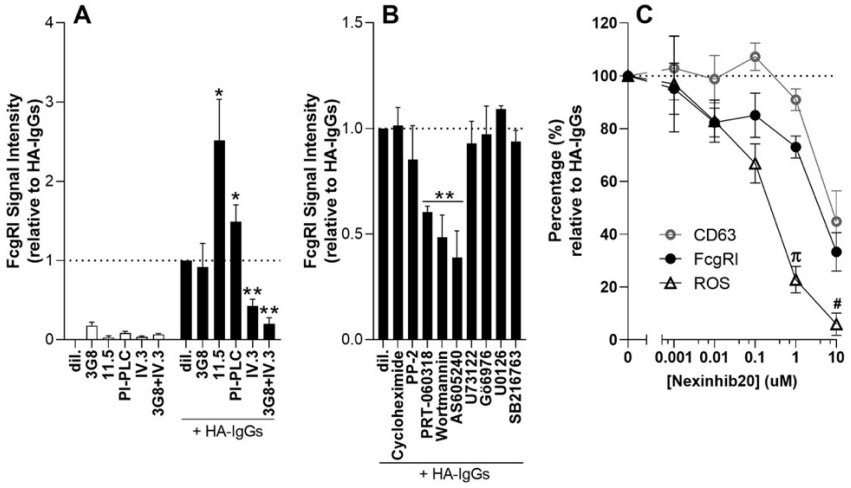

Figure 3. Signaling pathways involved in the up-regulation by HA-IgGs. A) Neutrophils were incubated with the following whole antibodies: 3G8 (FcgRIII), 11.5 (FcgRIIIb), IV.3 (FcgRII), or with PI-PLC for 15 min (37˚C), then stimulated with HA-IgGs for an additional 30 min. **B)** Neutrophils were incubated with indicated pharmacological 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.). **C)** Neutrophils were incubated with indicated concentrations of Nexinhib20 for 15 min before stimulation with HA-IgGs. Mean ± SEM, n=3. *Significantly higher, and **significantly lower than HA-IgGs alone. $p<0.05$. $^{\#,\pi}$Significantly lower than FcgRI. $^{\#}p<0.05$, $^{\pi}p<0.001$

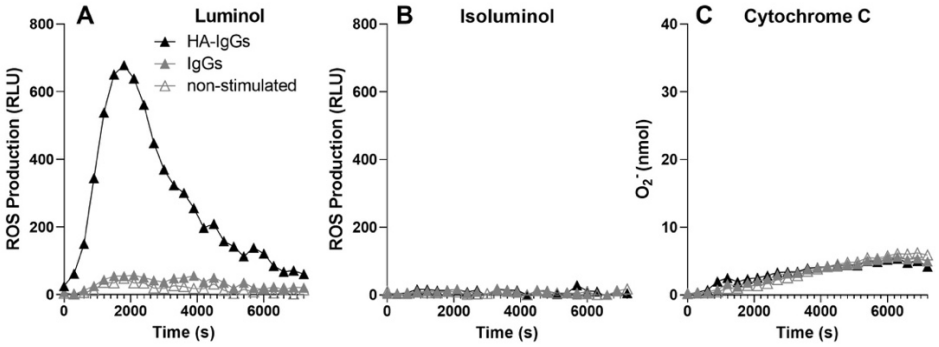

Figure 4. Human neutrophils stimulated with HA-IgGs produce intracellular reactive oxygen species. Neutrophils were incubated in indicated conditions, in the presence of either **A)** 10 µM luminol, **B)** 10 µM isoluminol, or **C)** 2.5% cytochrome C (v/v). ROS production was measured (37˚C) simultaneously by luminescence intensity (luminol and isoluminol) and optical density (cytochrome C) every 5 min and for the indicated times. Results are from one experiment, representative of n=4 experiments performed in identical conditions with neutrophils from different donors.

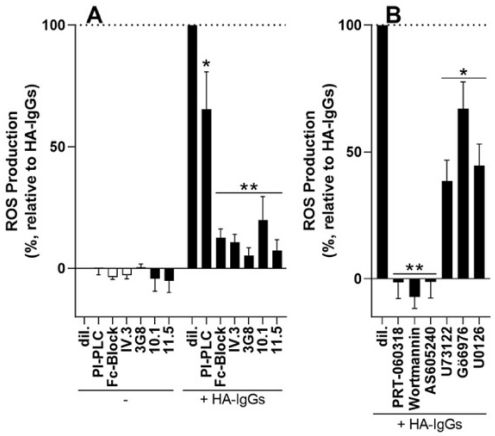

Figure 5. FcgRI activation contributes to the production of reactive oxygen species. A) Neutrophils were incubated with **(A)** PI-PLC, Fc-Block, indicated antibodies, or **(B)** with indicated pharmacological inhibitors for 15 min before stimulation with HA-IgGs. ROS production was measured by luminescence intensity (Luminol). Results are expressed as the percentage of production, relative to HA-IgGs alone (dil.) Mean ± SEM, (n=4). *$p<0.05$, **$p<0.0001$

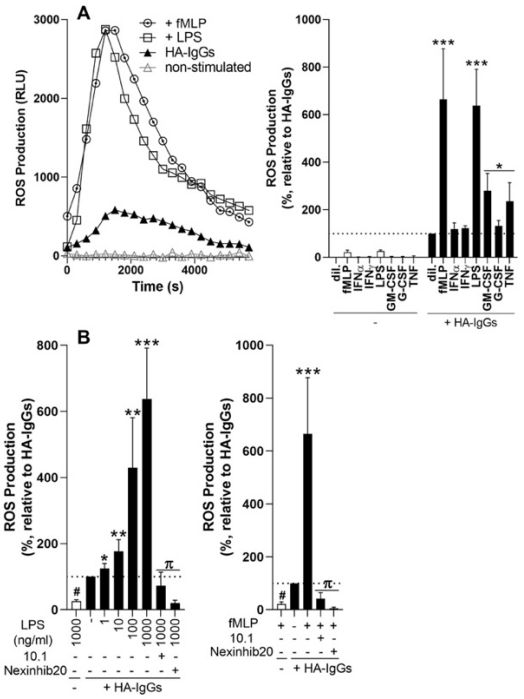

Figure 6. Combinations of fMLP or LPS with HA-IgGs magnify ROS production. Neutrophils were incubated with indicated agonists for 10 min (37°C) before stimulation with HA-IgGs. ROS production was determined. **A)** Typical traces of combinations with fMLP and LPS, representative of n=3 experiments, are shown. **B)** Neutrophils were incubated with indicated concentrations of LPS, or with fMLP (100 nM), then stimulated with HA-IgGs. 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. Significantly lower# or higher* than HA-IgGs alone (*p<0.05, **p<0.01, ***p<0.001). Significantly lowerπ than HA-IgGs+LPS (or +fMLP) p<0.01.

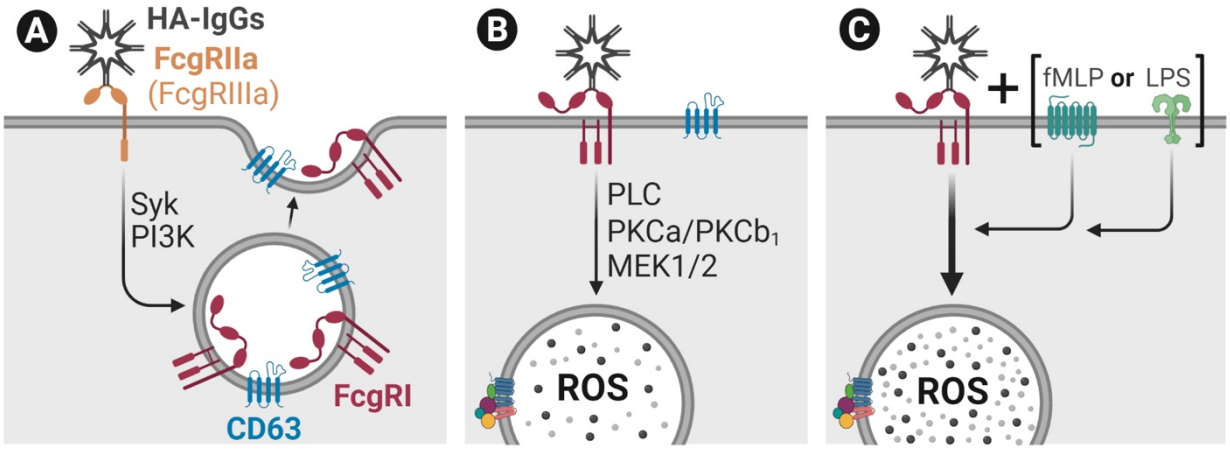

Figure 7. Schematics: HA-IgGs rapidly up-regulate FcgRI expression at the surface of human neutrophils in a FcgRII-dependent fashion and contribute to ROS production. A) Binding of HA-IgGs to FcgRIIa (and, possibly, FcgRIIIa) up-regulate FcgRI surface expression on human neutrophils, from CD63 positive, 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. *Created with BioRender*.

Marker	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 ***
FcgRI (CD64)	**4.61±0.79 ***
FcgRII (CD32)	0.65±0.11 *
FcgRIII (CD16)	0.79±0.14 *

Table 1. Impact of heat-aggregated (HA)-IgGs on neutrophil activation. Human neutrophils were incubated with HA-IgGs (1 mg/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=4 experiments). *$p<0.05$

Compound	Molecular target	Final conc.	Solvent
Cycloheximide	Protein synthesis	10 µg/ml	EtOH
PP-2	Src family of kinase	10 µM	DMSO
Wortmannin	PI3K	200 nM	DMSO
AS605240	PI3K	10 µM	DMSO
U73122	PLC	10 µM	DMSO
Gö6976	PKCa, $PKCb_1$	1 µM	DMSO
U0126	MEK1/2	10 µM	DMSO
PRT-060318	Syk	10 µM	HBSS
SB216763	GSK-3α	10 µM	DMSO
Nexinhib20	Rab27a-JFC1 interaction	10 µM	DMSO
10.1	FcgRI (CD64)	3 µg/ml	Aqueous buffer
IV.3	FcgRIIa (CD32a)	2.5 µg/ml	Aqueous buffer
3G8	FcgRIII (CD16)	4 µg/ml	Aqueous buffer
11.5	FcgRIIIb (CD16b)	2.5 µg/ml	Aqueous buffer
PI-PLC	FcgRIIIb (CD16b)	0.5 U/ml	Aqueous buffer
Fc-Block	Fc receptors	2.5 mg/100 µl	Aqueous buffer

Table 2. List of compounds, their molecular target, concentrations, and diluting buffers.

Agonist	Final conc.
TNF	100 ng/ml
GM-CSF	$1,4x10^{-9}$ M
G-CSF	$1,4x10^{-9}$ M
IFNα	10^{+3} M
IFNγ	100 ng/ml
LPS	1 µg/ml
fMLP	10^{-7} M

Table 3. List of agonists and final concentrations.