Chapter 6

IgA induces stronger inflammatory responses by neutrophils and monocytes compared to IgG

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Abstract

Neutrophils recognize antibody-opsonized pathogens via Fc receptors and subsequently induce inflammatory responses. Fc gamma receptors (FcγR) recognize immunoglobulin (Ig) G, whereas IgA binds to the Fc receptor FcαRI. Both Fc receptor types are thought to initiate similar signaling pathways, but we previously showed that activation via FcαRI selectively led to leukotriene B4 (LTB4) release with concomitant neutrophil migration. In this study, we investigated neutrophil cellular responses after activation through IgA or IgG in more detail. No differences were observed in uptake of IgG- or IgA-coated beads. Additionally, subsequent release of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) was comparable. However, only IgA stimulation induced release of proinflammatory lipids, cytokines and chemokines. Crosslinking of FcαRI furthermore led to a slower but stronger and more sustained signaling profile, as exemplified by increased intracellular calcium and phosphotyrosine levels. Importantly, enhanced activation through FcαRI seems intrinsic, as stimulation of monocytes with IgA also led to increased TNF-α and IL-6 release when compared with IgG stimulation. These results support that signaling routes of Fcγ receptors differ from those that are initiated by FcαRI, resulting in distinct functional profiles.
Introduction
Neutrophils constitute the most abundant cell type of the polymorphonuclear cell family (PMN) and are one of the first to respond during acute inflammation or infection. When tissues are invaded by bacterial or fungal pathogens, neutrophils are quickly recruited to the site of infection and can clear microorganisms using mechanisms such as phagocytosis, release of enzymes during degranulation, production of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs) \(^1\). Furthermore, neutrophils can secrete anti- and proinflammatory lipids, cytokines and chemokines, and can thereby modulate immune responses. Additionally, they also play a role in antiviral host defense and intracellular pathogen clearance \(^2\).

Microbes are recognized by neutrophils either directly via pattern-recognition receptors or indirectly by Fc receptors that recognize antibody-opsonized pathogens. The family of Fc receptors bind the Fc-tail of immunoglobulins (Ig, also referred to as antibodies). Fc alpha receptor (Fc\(\alpha\)RI) binds IgA-opsonized pathogens while Fc gamma receptors (Fc\(\gamma\)R) bind IgG-opsonized pathogens \(^3\). IgA is the predominant antibody subclass present in mucosal areas and the second most abundant antibody class in the circulation (1-3 mg/ml) after IgG (5-12 mg/ml). Both IgA and IgG antibodies play important roles in immunity and protection against infections by opsonizing pathogens and inducing elimination of microorganisms.

Neutrophils constitutively express two types of activating low-to-medium affinity Fc\(\gamma\)R, namely Fc\(\gamma\)RIIA (CD32A) and Fc\(\gamma\)RIIIB (CD16B) (see Table 1). Fc\(\gamma\)RIIA contains a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) in its intracellular tail to propagate signaling, while Fc\(\gamma\)RIIIB is anchored to the plasma membrane by a GPI moiety. The exact role of Fc\(\gamma\)RIIA and Fc\(\gamma\)RIIIB is highly debated, although Fc\(\gamma\)RIIA is commonly considered as the predominant activating Fc\(\gamma\)R \(^4\). The high affinity receptor Fc\(\gamma\)RI (CD64) is normally not or minimally expressed by neutrophils, but is upregulated during infection \(^5\). Additionally, neutrophils express the low-affinity Fc\(\alpha\)RI (CD89), which is associated with the ITAM-bearing FcR \(\gamma\)-chain to facilitate downstream signaling. Fc\(\alpha\)RI and Fc\(\gamma\)RIIA are thought to induce signaling via similar signaling routes. After crosslinking of Fc receptors, src family kinases are recruited to membrane rafts and aggregate with the receptor complex to phosphorylate tyrosines in the ITAMs \(^6\). Phosphorylated ITAMs subsequently act as docking platforms for signaling molecules such as the family Spleen tyrosine kinases (Syk). Multiple pathways can be induced by activated Syk, leading to cellular activation and calcium release from intracellular stores \(^7,8\). Multimeric stimulation of downstream signaling pathways of Fc receptors on neutrophils leads to inflammatory functions.

We previously demonstrated that only IgA, but not IgG immune complexes induced neutrophil migration through the induction of leukotriene B4 (LTB4) release \(^9\). This suggests that IgA and IgG may play distinct roles in inflammatory responses. We therefore now investigated multiple cellular functions after stimulation of Fc\(\alpha\)RI or Fc\(\gamma\)R and we showed that IgA stimulation led to increased phosphorylation of signaling molecules resulting in a distinct proinflammatory profile that was not observed after IgG stimulation.
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Materials and Methods

Isolation and purification human neutrophils

Neutrophils were isolated from healthy donors by standard Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation. Ammonium chloride buffer (155 mmol/L, 10 min, 4°C) was used to lyse the erythrocytes. After washing with PBS, neutrophils were resuspended in RPMI 1640 (Gibco BRL, Paisley, UK), supplemented with Penicillin-Streptomycin-Glutamine (psg) and 10% heat-inactivated fetal calf serum (FCS), hereafter referred to as complete medium. Purity was >90%. Neutrophils were allowed to settle (30 min, 37°C) before experiments. For cytokine and metabolomics experiments, neutrophils were further purified using the EasySep Neutrophil Enrichment kit (Stem Cell Technologies, Ehrenfeld, Cologne, Germany) as described by the manufacturer. Purity was ≥99.5% as assessed by Diff-Quick staining of cytospins using the protocol according to the manufacturer’s guidelines (Medion Diagnostics, Düdingen, Switzerland). All donors gave informed consent, in accordance with the guidelines of the Medical Ethical Committee of VU University Medical Center (The Netherlands).

Isolation of human monocytes from buffy coats

Human monocytes where isolated from buffy coats < 24h after blood collection (Sanquin, Amsterdam, The Netherlands). All donors gave informed consent. Cells from buffy coated were separated with a Lymphoprep density gradient centrifugation and Peripheral Blood Mononuclear Cells (PBMCs) were extracted from the interphase. Monocytes were isolated with CD14-positive beads (Miltenyi Biotec, Leiden, The Netherlands) using MACS cell separation according to standard protocols (Miltenyi Biotec).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>FcγRI</th>
<th>FcεRI</th>
<th>FcγRIIA</th>
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<th>FcγRIIC</th>
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<td>Low</td>
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<td>ITAM (y chain + b chain)</td>
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<td>ITAM</td>
<td>ITAM (y chain)</td>
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<td>On subsets</td>
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<td>Stim.</td>
<td>+</td>
<td>DD</td>
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Table 1. Characteristics of Fc receptors expressed on human neutrophils and monocytes.
Stim., after stimulation; DD, donor dependent.
Quantitative determination of the number of Fc receptors on cell membrane
To determine the number of Fc receptors per cell with an indirect immunofluorescence assay, the Qifikit® was used according the manufacturer’s instructions (Dako, Glostrup, Denmark). In short, $10^5$ cells were incubated with primary mouse antibodies direct against specific Fc receptors or with irrelevant isotype control. Subsequently, FITC-conjugated F(ab’)$_2$ fragments of secondary anti-mouse antibody was added to leukocytes and to the set-up and calibration beads. Flow cytometry was used to determine the fluorescence intensity, and by comparing it to the mean fluorescence intensity of the calibration and set-up beads the amount of bound primary antibody was calculated (FACS Calibur, BD Biosciences). The antibodies CD14-Alexa 647 (1:100, Biolegend, San Diego, CA) and CD15-PE (1:20, Biolegend) were used to gate CD14$^+$CD15$^+$ neutrophils or CD14$^+$CD15$^-$ monocytes from neutrophil or PBMC fraction, respectively.

Coating of latex beads
Carboxylate-modified polystyrene, green fluorescent latex beads (ø1.0 μM, Sigma-Aldrich, St. Louis, MO) or carboxylate-modified polystyrene, non-fluorescent latex beads (ø0.9 μM, Sigma-Aldrich) were washed twice in 2-(N-morpholino)ethanesulfonic buffer (30 mM, pH 6.1). Beads were coated with 2 mg/ml BSA (Roche Diagnostics, Basel, Switzerland), serum IgA (Cappel, MP Biomedicals, Santa Ana, CA) or serum IgG (Sigma-Aldrich) in the presence of N-(3-Dimethylaminopropyl)-N’-acid ethylcarbodimide hydrochloride (200 mM, Sigma-Aldrich). After 2 hours head-over-head incubation at room temperature, beads were washed and resuspended in PBS containing 0.1% BSA.

Phagocytosis assay
Green fluorescent latex beads coated with BSA, IgA or IgG were incubated with cells for 30 min at 37°C in effector:target (ET) ratio’s of 1:60 or 1:150. After washing, fluorescence was measured using flow cytometry (FACS Calibur, BD Biosciences). The phagocytic index was calculated by multiplying the percentage of cells that had taken up beads with mean fluorescence intensity of bead-positive cells.

For imaging flow cytometry experiments, cells were stimulated with coated green fluorescent beads for 30 min and fixed in 4% paraformaldehyde. Results were obtained with ImageStreamX (AMNIS, Merck Millipore, Seattle, WA) and analyzed with supplied software IDEAS (Merck Millipore). Per sample 20.000 cells were measured and number of phagocytosing cells are depicted. A compensation matrix was applied to the acquired images, and properly focused single cells were gated based on the area, aspect/ratio intensity, and bright-field gradient RMS feature using IDEAS v5.0 software (AMNIS). A mask depicting only the intracellular space was designed, and the percentage of FITC$^+$ cells was calculated. Also, a feature was designed to count the number of beads per cell. This feature (spot count) was based on the Peak mask using a spot/background ratio.

Stimulation of neutrophils and monocytes to determine induction of cytokines and chemokines
Highly purified neutrophils ($10^6$) were incubated with green-fluorescent latex beads
(E:T 1:60) in 6-well plates for 4 or 24 hours at 37°C. Supernatants were collected. The presence of cytokines/chemokines was determined with Luminex as described by the manufacturer’s instructions (Merck Millipore, Darmstadt, Germany). The following analytes were measured using a Bio-Plex 200 powered by Luminex (Bio-rad): IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-α, IFN-γ, MIP-1α, MIP-1β and MCP-1.

Monocytes (10⁶ in 500 µl) were stimulated in complete medium with fluorescent latex beads coated with BSA, IgA or IgG (E:T ratio 1:60). After incubation at 37 °C for 4, 6 or 24 hours, supernatant of supernatant was collected. Additionally, RNA was extracted using TRIzol and further used as described below. The concentration of IL-6 and TNF-α in the supernatant was determined with ELISA (IL-6 or TNF-α ELISA kit, Sanquin, The Netherlands).

To determine cytokine induction on mRNA level, 10⁷ highly purified neutrophils were incubated with latex beads (ET 1:60) for 4 or 6 hours. Total RNA was extracted using TRIzol reagent as described by the manufacturer (Invitrogen, Life Technologies, San Diego, CA). The concentration and purity of RNA was measured with a Nanodrop (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using the Promega A3500 kit according to the manufacturer’s instructions with some adjustments (Promega, Fitchburg, WI). Each cDNA synthesis enzyme mixture of 20 µl was composed of the following reagents: RNA (1 µg), MgCl₂ solution (25mM, 6 µl), 10X reverse transcription buffer (3 µl), dNTPs (10mM, 2 µl), RNasin ribonuclease inhibitor (40U, 2 µl), AMV Reverse Transcriptase (15U, 0.4 µl), random hexamer primers (0.6 µl) and nuclease-free water. Samples were incubated as followed: 10 min RT, 42ºC for 90 min and 99ºC for 5 min. For Real-Time PCR, a mix of 4 µl SYBR Green mix, 1.8 µl distilled water and 0.2 µl primer mix (containing both the forward and reverse primer, 10 µM each) was added to 2 µl 1:10 diluted cDNA. The primers used are listed in Table 2. The samples were incubated for 10 min at 95ºC, and subsequently 40 cycles of 15 sec 95ºC and 1 min 60ºC. Melt curves were recorded and analysed using a StepOne Real Time PCR System (ThermoFisher Scientific). Genes of interest were normalized against reference genes elongation factor 1a (EF1α) and beta-actin (ACTb). The final value of relative quantification was described as fold change of gene expression in the test sample compared to unstimulated cells.

**Quantitative fluorometric analysis of NET release, ROS production and release of intracellular calcium**

NET release was determined by measuring extracellular DNA after phagocytosis of coated beads, as described previously. Experiments were performed in NET medium, containing RPMI medium and 1% FCS which was heat-inactivated by incubation at 70ºC for 60 min (NET medium). PMNs were incubated with non-fluorescent coated beads (E:T 1:150) for 30 min at 37ºC. After washing, neutrophils were resuspended in NET medium and added to black 96-well plates (FLUOTRAC 200; Greiner Bio-One). PMNs were incubated for 3 h at 37ºC, after which extracellular DNA was detected by adding nucleic acid label SYTOX Green (Invitrogen Life Technologies; 2.5µg/ml). OD was measured using a fluorometer at 480 nm excitation, 520 nm emission (FLUOstar/POLARstar; BMG Labtech, Offenburg, Germany). For calcium flux assays, PMN were loaded with calcium indicator Fluo-4-AM.
IgA vs IgG receptor activation

(3 µM, Invitrogen) in complete medium at 37°C for 30 min. After washing with medium, cells were allowed to settle for 30 min at 37°C. BSA, IgA or IgG were coupled to CNBr-activated sepharose beads according to the manufacturer’s instructions (3 µg/ml, GE healthcare Bio-Sciences, Uppsala, Sweden). Coated beads were added and immediately, fluorescence was measured with a fluorometer. Alternatively, cells were incubated in Ibidi µ-slides (Ibidi, Martinsried, Germany) after labeling. Coated beads were added and cells were monitored in time at 37°C with live cell microscopy (Olympus IX81, Tokyo, Japan). To measure the induction of ROS, PMN were preloaded for 20 min at 37°C with the fluorescent probe 5-(and-6)-chloromethyl-2’7’-dichlorodihydrofluorescein diacetate (Invitrogen Life Technologies; 10 µM), according to the manufacturer’s guidelines. After washing, cells were resuspended in NET medium and allowed to settle for 30 min at 37°C. Non-fluorescent latex beads coated with BSA, IgA or IgG were added (E:T 1:150) to the neutrophils in black 96-well plates (FLUOTRAC 200; Greiner Bio-One). Fluorescence was measured every 5 min for 3 h in a preheated fluorometer at 37°C at 480 nm excitation, 520 nm emission.

<table>
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Table 2: qPCR primers.
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**Metabolomic analysis of lipids**

Purified neutrophils (5x10^5 cells in 250 µl) were stimulated with BSA-, IgA- or IgG-coated latex beads in a ratio of 1:60. After 1 or 2 hour stimulation, cells were quenched by addition of 750 µl methanol. Stimulation with ionomycin (2 µM) for 5 min before quenching was used as positive control. Eicosanoids detection and quantification (~120 inflammatory lipids) was performed by a multiple reaction monitoring assay, using ultrahigh performance liquid chromatography (Acquity, Ultra performance LC, Waters), negative mode electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS; TQ-MS, Xevo Waters), essentially as described with the following modifications: sample was extracted by liquid-liquid extraction (LLE) by using ethyl-tetr-butyl ether (MTBE). Chromatographic separation was achieved on a Synergi Hydro RP column (4µm, 2.0mm x 250, phenomenex) column using a flow rate of 0.35 mL/min at 40°C in a 30 min gradient using two solvents: Solvent A: H₂O + 0.1% HAc, Solvent B: 90/10 AcN/Isopropanol. A set of 17 different deuterated internal standards (ISs) were added prior to the extraction step to correct for matrix effects and recovery efficiency. Calibration curves were obtained by plotting the ratio of peak areas of eicosanoids to the ISs against known quantities of eicosanoids leading to a linear regression line (r²>0.99). The limits of detection (LOD) were defined by a signal-to-noise (S/N) ratio of 3:1 and they were in the range of 3pM-1nM depending on the compound. Accuracy and the precision of the method have been tested and resulted to a coefficient variation below 20% for most of the compounds.

**Western blotting**

To prepare crude cell lysates, PMNs were washed and resuspended in RPMI without serum. Subsequently, cells were incubated with anti-CD89 (A59, Becton Dickinson) or anti-CD32 (AT10, Abcam) antibodies for 30 min on ice. Cells were crosslinked using F(ab’)_2 Goat-anti-Mouse fragments (3B, Southern Biotech) at 37°C for a time course between 0 and 20 min. Immediately after the indicated time points, cells were boiled for 10 min at 95°C in pre-heated 2X Laemmli buffer containing 8% 2-mercaptoethanol. Samples were thoroughly vortexed, spun down and kept at -20°C until usage. For western blotting, samples were loaded on a 10% polyacrylamide gel according to standard procedures. Transfer occurred using the semi-dry iBlot system (Novex, Life Technologies, Paisley, U.K.). Membranes were blocked in either 5% milk in PBS-Tween 0.05% or in 5% BSA in PBS-Tween 0.05% for 1 hour. Membranes were incubated O/N with 4G10-HRP (EMD Millipore, Seattle, WA) and GAPD (14C10, Cell Signaling). Membranes were developed using an ECL reagent (ThermoFischer) with a Chemidoc Imaging Touch system (Bio-Rad). Quantification of phosphorylation was determined with ImageJ (U. S. National Institutes of Health, Bethesda, MD). Total phosphorylation at time point 0 was set as 1.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 4 software (GraphPad Software, San Diego, CA). Statistical differences were determined using unpaired Student two-tailed t test (2 groups), or with ANOVA (>2 groups). A p value <0.05 was considered statistically significant.
Results
Crosslinking of FcαRI and FcγRI by IgA- and IgG-coated beads induces neutrophil activation

Previously we showed that stimulation of neutrophils with IgA, but not with IgG led to migration 9. To investigate whether FcαRI and FcγRI expression on neutrophils could account for this difference, we first determined the quantitative number of Fc receptors on the cell surface of neutrophils (Figure 1A). Neutrophils express a high number of medium-affinity FcγRIII (CD16; 529.736±143.019), whereas the expression of FcγRII (CD32) was ten-fold less with 52.637±11.415 molecules per cell. The expression of FcαRI was even lower with 22.563±2.488 molecules per cell, while neutrophils hardly expressed FcγRI (CD64) or FceRI (CD23) (<3.100 molecules/cell).

Subsequently, several cellular responses after triggering of either FcαRI or FcγRI were investigated. To measure phagocytic capacity, neutrophils were incubated with fluorescent BSA-, IgA- or IgG-coated beads for 30 minutes, after which uptake was measured with flow cytometry. Minimal phagocytosis of BSA-coated beads was observed (Figure 1B). Uptake of IgA- or IgG-coated beads varied greatly between donors, but overall no differences were found (Figure 1B). Imaging flow cytometry revealed that some neutrophils had taken up only a few beads, whereas other had phagocytosed multiple beads (Figure 1C). However, uptake of the number of IgA- or IgG-coated beads was similar (Figure 1D).

Neutrophils generate ROS during phagocytosis and as such, the production of ROS was measured after stimulation of neutrophils with BSA-, IgA- or IgG-coated beads. Minimal ROS production was observed over time in neutrophils after incubation with BSA-coated beads (Figure 1E). In contrast, phagocytosis of IgA-coated beads induced a clear increase in ROS production over time, and a similar increase was observed after phagocytosis of IgG-coated beads (Figure 1E). Next, the release of neutrophil extracellular traps (NETs) was measured. Minimal release of extracellular DNA, indicative of NET formation, was observed after incubation with BSA-coated beads by neutrophils (Figure 1F). By contrast, phagocytosis of IgA- or IgG-coated beads resulted in the formation of NETs (Figure 1F). However, NET formation by neutrophils was not significantly different after IgA or IgG stimulation.

IgA stimulation leads to potent release of cytokines and chemokines

Next, we measured cytokine and chemokine release after stimulating highly purified neutrophils with IgA- or IgG-coated beads. To avoid contamination with eosinophils, basophils, monocytes or lymphocytes, which could be present in the PMN fraction after density gradient centrifugation, neutrophils were further enriched, resulting into a purity of >99.5 % (data not shown). Changes in mRNA levels after stimulation of neutrophils with BSA-, IgA- or IgG-coated beads were measured 4 and 6 hours after stimulation. IgA stimulation resulted in increased mRNA levels of IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4) or TNF-α when compared with IgG stimulation (Figure 2A). No difference in mRNA levels of IL-6 or MCP-1 (CCL2) were detected after IgA or IgG stimulation (Figure 2A). These results were validated on protein level after stimulation for 4 and 24 hours. No significant difference in release of cytokines or chemokines was observed 4 hours...
Figure 1: Neutrophils express higher number of FcγR, but phagocytosis, ROS production and release of NETs was similar after stimulation with IgA or IgG. (A) The quantitative number of Fc receptors on the surface of neutrophils was determined with Qifkit. Each dot represents one individual healthy donor (n = 8). (B) Phagocytosis of BSA- (△), IgA- (●) or IgG-coated (■) beads by neutrophils. Phagocytic index was calculated as the percentage of cells that had taken up beads multiplied by the mean fluorescence intensity of bead-positive cells. Each symbol represents the mean of one healthy donor measured in triplo. (C-D) Phagocytosis of BSA-, IgA- or IgG-coated beads was visualized by imaging stream technology (Ch01: bright-field; Ch02: FITC). (C) Pictures as example of phagocytosis of 1, 2, 3 or >4+ green fluorescent beads by neutrophils. (D) Graphs depict phagocytosis calculated as the number of neutrophils that internalized the depicted number of BSA-, IgA- or IgG-coated beads after 30 min. (E) Induction of reactive oxygen species (ROS) was measured by incubating neutrophils with BSA- (△), IgA- (●) or IgG- (■) coated beads for 3 hours. One experiment of one healthy donor is shown as mean ± SD, where each point represents the mean of triplicates. Figure is representative for four different healthy donors. (F) Quantification of extracellular DNA (reflecting release of NETs) after stimulating neutrophils with BSA- (△), IgA- (●) or IgG- (■) coated beads. Each symbol represents the mean of a healthy donor measured in triplo. n.s., not significant.
after stimulation with BSA-, IgA- or IgG-coated beads (Supplementary Figure 1). However, stimulation for 24 hours resulted in significantly increased release of IL-8 (CXCL8), MIP-1β (CCL4) and TNF-α, increased release of MCP-1 (CCL2) and MIP-1α (CCL3) was also observed, but this was not significantly different (Figure 2B). No IL-6 release was found, confirming the absence of contaminating monocytes (Figure 2B). Additionally, no release of IFN-α2, IFN-γ, IL-1β, IL-4 or IL-12p70 was measured (data not shown). Together, these results indicate that only IgA stimulation led a cytokine and chemokine response.

![Figure 2: IgA induces potent cytokine and chemokine release. (A) The fold change in mRNA level of cytokines and chemokines after stimulation of purified neutrophils for 4 (white bars) or 6 hours (black bars) with BSA-, IgA- or IgG-coated beads. The relative gene mRNA level was normalized to reference genes EF1a and beta-actin. Gene expression after BSA stimulation for 4 hours was set as 1. (n = 1) (B) Release of indicated cytokines and chemokines after stimulating purified neutrophils for 24 hours with BSA-, IgA- or IgG-coated beads. Data is presented as mean ± SD and each bar represents the measurements of four different healthy donors. Unstim = Unstimulated. *p < 0.05.](image)
**IgA stimulation induces the secretion of proinflammatory lipids**

Since our previous study showed that IgA stimulation led to the release of LTB4, we performed a pilot experiment with metabolomics analysis to study the release of other lipid mediators. Crosslinking of FcαRI with IgA-coated beads induced upregulation of metabolites that are associated with inflammation, when compared to crosslinking of FcγRI with IgG-coated beads (Figure 3A). IgA potently induced series 2 prostaglandins and leukotrienes when compared to IgG (Figure 3A, respectively green versus red). To verify these results, we measured cyclo-oxygenase 2 (COX-2) induction after stimulation of purified neutrophils with IgA or IgG. COX-2 is responsible for the conversion of arachidonic acid into prostaglandins and thromboxanes. IgA induced upregulation of COX-2 mRNA, especially after 4 hours (Figure 3B). Additionally, we measured the induction of lipooxygenase, which are enzymes catalyzing the biosynthesis of leukotrienes from arachidonic acid. Only stimulation with IgA induced expression of 5-LOX (Figure 3B), which corresponds to the observed LTB4 release after IgA stimulation. Together, these results suggest that only stimulation of neutrophils with IgA, but not IgG induced the release of proinflammatory lipids.

**Crosslinking of FcαRI results in increased signaling**

As IgA complexes induced different cellular responses compared to IgG, we studied signal transduction after neutrophil FcαRI versus FcγRI triggering. As a parameter of signaling, intracellular calcium release was measured. Large beads coated with BSA, IgA or IgG were added to purified neutrophils loaded with the calcium sensitive dye Fluo-4-AM and calcium release was determined with fluorimetric analysis. Both IgA and IgG stimulation led to substantial increase in intracellular calcium (Figure 4A). However, stimulation of neutrophils with IgA led to later but enhanced release of intracellular calcium when compared to IgG stimulation (Figure 4A). Next, real time video fluorescent microscopy recordings were used to determine the kinetics of intracellular calcium release. Addition of IgG-coated beads to Fluo-4-AM loaded neutrophils resulted in one spike of released intracellular calcium, after which neutrophils adhered to the bead. By contrast, addition of IgA-coated beads led to multiple spikes of released calcium (Figure 4B). Furthermore, continued intracellular calcium release coincided with migration of neutrophils to IgA-coated beads, which was not observed with IgG-coated beads (Video 1&2 and Figure 4C). To investigate if phosphorylation of signaling proteins was different after IgA- or IgG-stimulation, FcαRI or FcγRII were crosslinked and western blot analysis was used to detect phosphotyrosine proteins. Crosslinking of FcαRI and FcγRII induced phosphorylation of different proteins (Figure 4D-E). Phosphorylation of tyrosines was rapid and short after FcγRII crosslinking (Figure 4D), while FcαRI crosslinking led to slightly later, but stronger and more sustained phosphorylation pattern (Figure 4E). Quantification of total phosphorylation showed that after FcγRII crosslinking, the level of phosphotyrosine proteins peaked at two minutes and started to decrease at 3 minutes (Figure 4F). In contrast, after FcαRI crosslinking, phosphotyrosine protein levels peaked at 3 minutes and started to decrease after 10 minutes (Figure 4F), indicating that signaling routes of FcαRI and FcγRII are different.
Figure 3: IgA induces inflammatory lipid mediators release. (A) The release of inflammatory lipids after crosslinking of FcαRI or FcγRI after stimulation of purified neutrophils with BSA-, IgA- or IgG-coated beads for 1 or 4 hours. Archidonic acid (blue) is released from cell membrane phospholipids (yellow) by the lipid-cleaving enzymes phospholipases (pink). Eicosanoids (prostaglandins and leukotrienes) are then formed by different routes involving the enzymes COX or LOX (pink). IgA (green) potently induced release of series 2 prostaglandins and leukotrienes when compared to IgG (red). (B) COX-2 (left panel) and 5-LOX (right panel) induction after stimulating purified neutrophils with BSA-, IgA- or IgG-coated beads for 4 (white bars) or 6 (black bars) hours. The relative gene mRNA level was normalized to reference genes EF1a and beta-actin. Gene expression after BSA stimulation for 4 hours was set as 1. (n = 1)
Figure 4: Signaling is increased after crosslinking of FcαRI. (A) Beads coated with BSA- (△), IgA- (●) or IgG (■) were added to calcium dye Fluo-4-AM loaded neutrophils. Intracellular calcium release was quantified using fluorometric analysis. (B-C) Images of neutrophils loaded with the calcium dye Fluo-4-AM. Large IgA- or IgG-coated beads were added and mobilization of intracellular calcium (B) for single cells and migration of neutrophils (C) was detected using live cell fluorescent microscopy. Beads are indicated with dashed line. In (B) numbers reflect time points in seconds. In lower panel fluorescence of pictures is quantified. (D-E) FcαRI or FcγRII on purified neutrophils were crosslinked. Western blot and an anti-phosphotyrosine antibody was used to determine phosphotyrosine proteins. Phosphorylation pattern after crosslinking of FcγRII (D) or FcαRI (E) for indicated time points is shown. (F) Quantification of total phosphorylation as fold change for 2nd antibody control (△), FcαRI crosslinking (●) or FcγRII crosslinking (■). Time point 0 was set as 1.
IgA stimulation induces cytokine release by monocytes

To determine whether observed differences between FcαRI and FcγR were neutrophil-specific, cellular activation after monocyte stimulation was investigated. We first determined the quantitative number of Fc receptors on the cell surface of monocytes. Only a subset of monocytes (2-13%) express a high number of medium-affinity FcγRIII (indicated with FcγRIII-high; 102.688±3.301), while all monocytes have a moderate expression of FcγRII (38.586±5.378) and FcγRI (16.422±6.497). Low expression of FcαRI (10.988±3.225) was observed, whereas FceRI (<5.000 molecules/cell) was almost absent (Figure 5A).

Subsequently, phagocytic capacity of purified monocytes was measured after adding BSA-, IgA- or IgG-coated beads for 30 minutes. There was no significant difference in phagocytosis of IgA- or IgG-coated beads (Figure 5B). Next, we measured cytokine release after stimulating purified monocytes with BSA-, IgA- or IgG-coated beads. No significant difference in release of IL-6 was observed 4 hours after IgA or IgG stimulation, but a significant increase in IL-6 release was seen 24 hours after IgA stimulation when compared with IgG stimulation (Figure 5C). Additionally, although not significant, increase in TNF-α release was detected 4 and 24 hours after IgA stimulation (Figure 5D).

Figure 5: IgA stimulation of monocytes induces higher cytokine release (A) The quantitative number of Fc receptors on the surface of monocytes was determined with Qifikit. FcγRIII-low/high indicates FcγRIII expression that was low in 98-87% of monocytes and high in 2-13% of monocytes. Each dot represents one individual healthy donor (n = 8). (B) Phagocytosis of BSA- (△), IgA- (●) or IgG-coated (■) beads by monocytes. Phagocytic index was calculated by the percentage of cells that had taken up beads multiplied by the mean fluorescence intensity of bead-positive cells. Each symbol represents the mean of one healthy donor measured in triplo (n = 3). (C–D) Purified monocytes were stimulated with BSA-, IgA- or IgG-coated beads. Release of the cytokine IL-6 (C) or TNF-α (D) was measured in the supernatant after 4 (white bars) or 24 (black bars) hours. Data is presented as mean ± SD and each bar represents the measurements of 2 healthy donors. *p < 0.05. n.s., not significant.
Chapter 6

Discussion

IgA is the dominant antibody subclass present in mucosal areas and the second most abundant antibody class in the circulation after IgG. Both IgA and IgG antibodies play an important role in immunity and protection against infections by detecting and removing pathogens. Whereas IgA is considered to play an important role in mucosal immunity, IgG is mainly considered to have a systemic role.

To investigate cellular activation after Fc receptor triggering, neutrophils were stimulated with IgA- or IgG-coated beads and several cellular functions were tested. No differences in phagocytosis of IgA- versus IgG-coated beads was observed. Similarly, production of ROS after phagocytosis, which is essential for killing of internalized pathogens, was comparable. NETs release is dependent on ROS production. NETs are extracellular web-like structures composed of decondensed chromatin and antimicrobial proteins which trap and kill pathogens. The formation of NETs is a protective mechanism that neutrophils use against infections. However, NETs formation has also been implicated in the pathogenesis of autoimmune diseases. NETs represent an important source of autoantigens and the presence of anti-DNA IgG autoantibodies play an important role in the autoimmune disease systemic lupus erythematosus.

Additionally, recently we demonstrated that IgA immune complexes present in plasma and synovial fluid of rheumatoid arthritis patients induced the release of NETs. We did not observe differences between NETs release after uptake of IgA- or IgG-coated beads, which is consistent with the similarities in ROS production.

However, stimulation of neutrophils with IgA resulted in increased production of TNF-α, MIP-1α, MIP-1β, MCP-1 and IL-8, which are potent cytokines and chemokines that attract and activate other immune cells. Additionally, only IgA induced potent release of series 2 prostaglandins and leukotrienes. These lipids are able to exert a wide variety of functions, thereby contributing to a proinflammatory environment. This suggests that although both IgA- and IgG-opsonized pathogens are phagocytosed and destroyed to a similar extent, only IgA stimulation induces an additional orchestrated immune response through secretion of proinflammatory mediators.

This cannot be explained by a higher expression of FcαRI, as we found intermediate to low expression of FcαRI on neutrophils. In contrast, high expression of FcγRIIIB was found, with an intermediate expression of FcγRIIA, and minimal expression of FcγRI and FcεRI, which corresponds with descriptions in existing literature. Neutrophils expresses both FcγRIIA and FcγRIIIB constitutively, but the exact contribution of each IgG receptor to neutrophil activation has been subject to discussion. Triggering of FcγRIIA leads to several proinflammatory processes, including phagocytosis, induction of ROS, calcium mobilization and propagation of signaling. Although FcγRIIIB does not contain a signaling motif, a role for FcγRIIIB in phagocytosis, calcium mobilization, ROS production and NETosis has been described. FcγRIIIB has been proposed to be responsible for initial tethering immune complexes, followed by co-activation of FcγRIIA and FcγRIIIB, resulting in cellular activation. FcγRIIIB co-localizes with FcγRIIA upon stimulation with immune complexes, suggesting that FcγRIIIB transduces signals via FcγRIIA. However, also FcγRIIIB-independent signaling pathways of FcγRIIIB have been described.
As such, IgG stimulation of resting neutrophils is thought to mainly be depend on signaling via FcγRIIA, which contains its own ITAM motif. Crosslinking of FcγRIIA resulted in rapid release of intracellular calcium and tyrosine phosphorylation. Previously, it was thought that activating IgA and IgG Fc receptors signal via a similar downstream signaling routes, ultimately inducing comparable cell activation. However, we observed distinct differences in FcR gamma chain (γ-chain) signaling via FcαRI, like repeated spikes in intracellular calcium releases and slightly later, but stronger and sustained tyrosine phosphorylation. FcαRI signals via FcR γ-chain complexes, which contain an ITAM motif that is similar, but not identical to the ITAM of FcγRIIA (Table 3).

<table>
<thead>
<tr>
<th>Domain</th>
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<th>Sequence</th>
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<tbody>
<tr>
<td>Human γ-chain ITAM</td>
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<td>YMTLNPRAPTDDDKNIYLTL</td>
</tr>
<tr>
<td>Human FcγRIIA ITAM</td>
<td>7</td>
<td>YTGLNTRNQETYETL</td>
</tr>
</tbody>
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Table 3: Characteristics of immunotyrosine associated motifs (ITAMs).

Studies with B-cell transfectants previously showed that FcγRIIA was not capable of inducing cytokine release upon stimulation with IgG, while swapping its ITAM with the ITAM of γ-chain restored its capability to mediate IL-2 production 29,30. Thus, even though some functions, such as phagocytosis and NETs release, can be mediated through both ITAMs, other functions like cytokine release are predominantly mediated via FcγRI γ-chain ITAMs.

However, this cannot completely explain the observed differences, as IgA stimulation of monocytes also induced increased cytokine release compared to stimulation with IgG. In addition to FcγRIIA and FcγRIIIA, monocytes also express the activating high-affinity receptor FcαRI, which signals via the same FcγRI γ-chain that FcγRII uses. As such, it is likely that FcαRI uses additional, yet unidentified, additional signaling routes.

In conclusion, the capacity of IgA to induce a greater cytokine response than IgG is present in both neutrophils and monocytes. IgA induces the secretion of proinflammatory mediators such as cytokines, chemokines and inflammatory lipids, which can trigger the activation and recruitment of other immune cells. This supports that the signaling routes after IgA and IgG triggering are inherently different, and hints towards an intrinsic mechanism of FcαRI to induce more potent cellular responses. As such, is it conceivable that IgA may play a more important proinflammatory role in systemic immunity than previously appreciated.

**Acknowledgements**

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Supplementary Information

Supplementary figure 1: Release of indicated cytokines and chemokines after stimulating purified neutrophils for 4 hours with indicated beads. Data is presented as mean ± SD. Each bar represents the measurements of four different healthy donors.
Supplementary Video 1
Fluo-4-AM loaded neutrophils were stimulated with IgG-coated beads.

https://www.youtube.com/watch?v=seT GnFcjwwE

Supplementary Video 2
Fluo-4-AM loaded neutrophils were stimulated with IgA-coated beads.

https://www.youtube.com/watch?v=PTG41zMHPng
References


24. Zhou, M.J. & Brown, E.J. CR3 (Mac-1, alpha M beta 2, CD11b/CD18) and Fc


