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IgA Enhances NETosis and Release of Neutrophil Extracellular Traps by Polymorphonuclear Cells via Fcα Receptor I

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Polymorphonuclear cells (neutrophils) are the first cells that arrive at sites of infections. According to the current dogma, they are involved in eliminating bacteria, after which they die through apoptosis. We now demonstrate that enhanced IgA-induced phagocytosis of bacteria or beads by neutrophils led to increased cell death. Nuclear changes and positivity for the general cell death marker 7-aminoactinomycin D were observed, but the absence of annexin V membrane staining supported that neutrophils did not die via apoptosis, in contrast to neutrophils that had not phagocytosed bacteria. Moreover, increased release of neutrophil extracellular traps (NETs) was observed, which was most likely due to augmented production of reactive oxygen species after uptake of IgA-opsonized particles. Blocking the IgA Fc receptor FcαRI abrogated phagocytosis and NET formation. Thus, FcαRI triggering on neutrophils resulted in a rapid form of cell death that is referred to as NETosis, as it is accompanied by the release of NETs. As such, IgA may play a prominent role in mucosal inflammatory responses, where it is the most prominent Ab, because it enhanced both phagocytosis of bacteria and formation of NETs, which are effective mechanisms that neutrophils employ to eliminate pathogens. *The Journal of Immunology, 2014, 192: 2374–2383.

Immunoglobulin A is the most produced Ab class in the body (66 mg/kg/day) and the predominant Ab in mucosal areas, where it plays an important role in mucosal defense (1). Mucosal IgA is produced by local plasma cells in the lamina propria as a dimeric molecule (dimeric IgA [dIgA]) in which two IgA molecules are coupled via a protein that is referred to as J chain. It is then transported through epithelial cells and released in the lumen as secretory IgA (SIgA), which plays an important role in mucosal defense as SIgA inhibits binding of micro-organisms to mucosal surfaces, and neutralizes bacterial products (2). SIgA contains an additional molecule, the secretory component (SC), which ensures stability of the complex in the hostile luminal environment. However, SC blocks at least partly the binding site for the IgA Fc receptor FcαRI or CD89 (3). As such, SIgA is a poor opsonin.

FcαRI is a member of the Fc receptor Ig superfamily that also includes Fc receptors for IgG and IgE, and is exclusively expressed on myeloid cells, including neutrophils, eosinophils, monocytes, and Kupffer cells (4–7). Cross-linking of FcαRI induces a multitude of proinflammatory functions that include phagocytosis, respiratory burst, degranulation, Ab-dependent cellular cytotoxicity, Ag presentation, and release of cytokines and inflammatory mediators (4, 8). Few FcαRI-positive cells are observed in mucosal areas in homeostatic conditions (9). In these conditions, dIgA most likely serves as an intermediary molecule, which is released as SIgA after transport through epithelial cells. However, bacteria that are opsonized with dIgA are effectively phagocytosed by polymorphonuclear cells (PMNs) (10). Moreover, we recently demonstrated that cross-linking of FcαRI resulted in PMN recruitment (9, 11), which may play an important role in mucosal immune defense when the lamina propria is invaded by pathogens.

PMNs are the most abundant circulating effector cells of the innate immune system (12). When micro-organisms invade tissues, PMNs rapidly migrate to the site of infection where they play a crucial role in the clearance of pathogens. They are terminally differentiated phagocytes with a short t½ and a primary function in immune defense against bacteria. As such, they are packed with an abundance of granules containing cytotoxic molecules (13). Production of reactive oxygen species (ROS) and release of intracellular antimicrobial proteins are important for killing of pathogens. Furthermore, the release of neutrophil extracellular traps (NETs) has been described as an additional tool in the arsenal of antimicrobial strategies. NETs are web-like structures that are extruded by neutrophils and have been demonstrated to trap and kill bacteria and fungi. NETs contain DNA, histones, and several granular and cytoplasmic antimicrobial proteins, like elastase and myeloperoxidase (MPO) (14, 15).

Two different forms of NETs have been described. First, rapid formation of NETs (within minutes) was reported, which was independent of oxidants (16). During this process PMN nuclei
became condensed, and subsequent separation of the inner and outer nuclear membranes as well as budding of DNA-containing vesicles was observed, after which DNA was released in the extracellular space. This type of NET formation does not require breach of the plasma membrane or cell death. Second, release of NETs after several hours has been shown (14). This process is dependent on the generation of ROS as both the NADPH oxidase enzyme complex and MPO are involved (17–19). Furthermore, generation of these NETs is accompanied by breakdown of the nuclear envelope, decondensation of chromatin, and mixing of nuclear components with antimicrobial proteins. Eventually, the process culminates in cell membrane rupture and expulsion of NETs, resulting in cell death that is referred to as NETosis (17, 18, 20). This is a distinctly different type of cell death compared with apoptosis, which is generally accepted as the main type of cell death involved in PMN clearance after they have executed their antimicrobial functions (12, 21). Apoptosis involves nuclear changes as well. However, these include DNA fragmentation, shrinking of the nucleus without breakdown of membranes (pyknosis), and ultimately nuclear fragmentation. Furthermore, although apoptosis leads to membrane blebbing, the plasma membrane will not rupture, and no leakage of cellular components into the microenvironment will occur.

Because we previously demonstrated that FcαRI potently triggers activation of PMNs, which results in enhanced phagocytosis of IgA-opsonized bacteria as well as PMN migration (4, 7, 9, 11), we now investigated whether FcαRI plays a role in the induction of NETs, as this may represent an important additional defense mechanism in mucosal areas.

Materials and Methods

Isolation of human PMNs

PMNs were isolated from peripheral blood that was obtained from healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) density gradient centrifugation, after which erythrocytes were lysed in ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.11 mM EDTA, 10 min, room temperature). After lysis, PMNs were washed with PBS (B. Braun, Melsungen, Germany). Cells were resuspended in RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with glutamine and antibiotics. Additionally, a low concentration of 1% heat-inactivated (HI) FCS was added (60 min, 70°C), because FCS contains heat-stable nucleases that can interfere or break down NETs (22) (medium hereafter referred to as RPMI 1%). Blood samples were collected according to the guidelines of the Medical Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands), in accordance with the Declaration of Helsinki. All donors gave informed consent.

Culture and labeling of bacteria

Staphylococcus aureus, Escherichia coli, and Salmonella typhimurium were cultured in standard LB medium (NaCl, BD Bacto tryptose, BD Bacto yeast in a ratio of 2:2:1; BD Biosciences, Franklin Lakes, NJ) supplemented with glutamine and antibiotics. Culture and labeling, bacteria were washed twice with 2-(N-morpholino)ethanesulfonic buffer (30 mM, pH 6.1) and resuspended in 2-(N-morpholino)ethanesulfonic buffer with 2 mg/ml BSA (Roche Diagnostics, Basel, Switzerland) or serum IgA (Cappel, MP Biomedicals, Santa Ana, CA) in the presence of N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (Sigma-Aldrich) and incubated for 2 h (overnight). Latex beads were washed twice and resuspended in PBS containing 0.1% BSA.

Detection of IgA opsonization of bacteria

To detect whether bacteria were opsonized with specific IgA, S. aureus, E. coli, and S. typhimurium were incubated with pooled human serum IgA (Cappel; 1 mg/ml; 30 min, 37°C). (Of note, most healthy individuals have Abs against these prevalent bacteria in their serum.) After incubation with or without IgA, bacteria were washed with PBS and further incubated with FITC-conjugated polyclonal rabbit anti-human IgA Ab (Dako, Glostrup, Denmark; 1:50) for 30 min at 4°C. IgA binding to bacteria was analyzed with flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Phagocytosis assays

PMNs were allowed to settle (1 h, 37°C) before experiments. To determine phagocytosis of HI or live bacteria, PMNs (2 × 10⁵) were incubated in glass disposable culture tubes (Duran Group, Wertheim/Main, Germany) for 30 min at 37°C (rotary shaker) in a total volume of 100 μl in the presence of bacteria (FITC labeled, E:T ratio; 1:35) with or without 1 mg/ml serum IgA (Cappel). After 30 min, cells were carefully resuspended in 15 ml PBS and centrifuged for 7 min at 240 × g to remove non-phagocytosed bacteria.

Alternatively, phagocytosis assays were performed with latex beads. PMNs were incubated in 96 (1 × 10⁵)- or 24 (4 × 10⁵)-well plates with green fluorescent BSA beads or IgA beads (E:T ratios: 1:25, 1:75, 1:150, and 1:300). After 30 min, cells were washed with HBSS (Life Technologies) and analyzed with flow cytometry (FACSCalibur). PMNs were preincubated with anti-FcαRI mAb MIP8α (AbD Serotec, Raleigh, NC; 20 μg/ml) for 20 min on ice to block FcαRI.

Live-cell imaging

PMNs were incubated with bacteria in the absence or presence of IgA for 30 min at 37°C. Cells were washed to remove nonphagocytosed bacteria, after which they were incubated in ibidi μ-slides (ibidi, Martinsried, Germany) (3 × 10⁵/ml well) and assayed with live-cell microscopy (Olympus IX81; Olympus, Tokyo, Japan). When indicated, PMNs were labeled with Oregon Green IL Kit (ENZO Life Sciences, Farmingdale, NY; 1:1000), according to the manufacturer’s instructions (30 min, 37°C). In this case, unlabeled bacteria were used to avoid interference with the microscope (green). In additional experiments, annexin V-FITC (BD Biosciences; 1:200) and 7-aminocinomycin D (7-AAD; Invitrogen Life Technologies, Carlsbad, CA; 1:1000) were added to the medium, and PMNs were monitored in time. Alternatively, nuclei of live PMNs were labeled with blue nuclear label (provided by ENZO Life Sciences; 1:1000), whereas PE-labeled mouse anti-histone H2A/H2B mAbs (AbD Serotec, Raleigh, NC; 20 μg/ml) were added in the medium during recording (a gift of A. Zychlinsky, Max Planck Institute, Berlin, Germany; 1:100).

Diff-Quik and immunofluorescence staining of NETs

PMNs were inserted in Lab-Tek chamberslides (Nalg Nunc, Rochester, NY; 1.5 × 10⁶/well) or on circular glass coverslips (Ø 12 mm) in 24-well plates (4 × 10⁶/well). Chamberslides were coated overnight with 5% BSA in PBS. PMNs were stimulated with bacteria (E:T ratio of 1:35), in the presence or absence of serum IgA (1 mg/ml), for 3 h. Alternatively, PMNs were stimulated with BSA beads or IgA beads. Where indicated, DNase I (Roche, Penzberg, Germany; 20 U/ml) was added during stimulation. After incubation, cells were fixed for 15 min at room temperature with 4% paraformaldehyde, after which the fixative was removed and slides were stained with the Diff-Quik staining protocol according to the manufacturer’s guidelines (Medion Diagnostics, Düdingen, Switzerland). Alternatively, slides were stained with DAPI (Invitrogen Life Technologies; 1:10,000) to visualize DNA or with PE-labeled mouse anti-histone H2A/H2B mAbs (1:50), anti-MPO rabbit polyclonal Ab, or anti-neutrophil elastase rabbit polyclonal Ab (1:50; Calbiochem, Millipore, Billerica, MA). Cells were visualized using a Leica DM6000 microscope (Leica, Solms, Germany).

Scanning electron microscopy

For electron microscopy, coverslips containing PMNs, as described earlier, were fixed with 4% paraformaldehyde, after which all liquid was replaced by McDowell fixative solution (1% glutaraldehyde, 4% paraformaldehyde, 0.01 M sodium cacodilate [pH 7.4]). Afterward, cells were dehydrated in a graded ethanol series and hexamethyldisilazane. Samples were mounted on aluminum SEM specimen mount stubs (Electron Microscopy Sciences) and sputter-coated with gold, using Balzers Union SCD 040. Cells were examined in a scanning electron microscope (Model Phillips 525 with Orion Frame Grabber), operated at 15 kV, with a spot size of 30 nm.
Quantitative fluorometric analysis of NET release

After isolation, PMNs were allowed to settle (1 h, 37˚C) and cells (1 × 10⁶) were incubated in 96-well plates with nonfluorescent BSA beads or IgA beads (E:T ratios; 1:150 and 1:300) for 30 min at 37˚C. After incubation, cells were washed twice with HBSS and carefully resuspended in RPMI 1% in black 96-well plates (FLUOTRAC 200; Greiner Bio-One). When indicated, DNase I (Roche; 20 U/ml) was added and PMNs were incubated for 3 h at 37˚C, after which extracellular DNA was detected by adding nucleic acid label SYTOX Green (Invitrogen Life Technologies; 2.5 μg/ml). OD was measured using a fluorimeter (FLUOstar/POLARstar; BMG Labtech, Offenburg, Germany) at 480 nm excitation, 520 nm emission.

ROS production and NADPH oxidase inhibition

After isolation, PMNs were allowed to settle (1 h, 37˚C) and cells (1 × 10⁶) 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 incubation, cells were washed twice with HBSS and carefully resuspended in RPMI 1% in black 96-well plates (FLUOTRAC 200; Greiner Bio-One). Cells were stimulated with nonfluorescent BSA beads or IgA beads (E:T ratio; 1:150) or bacteria (E:T ratio; 1:35) in the presence or absence of serum IgA (1 mg/ml). When indicated, the NADPH oxidase inhibitor diphenylene iodonium chloride (DPI; Sigma-Aldrich; 25 μM) was added and PMNs were incubated for 3 h at 37˚C, after which extracellular DNA was detected by adding nucleic acid label SYTOX Green (Invitrogen Life Technologies; 2.5 μg/ml). OD was measured using a fluorimeter (FLUOstar/POLARstar; BMG Labtech, Offenburg, Germany) at 480 nm excitation, 520 nm emission.

Differential cell death of nonstimulated PMNs versus cell death after stimulation with IgA and bacteria was further studied by

Results

IgA induces increased phagocytosis and cell death

To investigate whether targeting of FcγRI on PMNs induces the formation of NETs, PMNs were incubated with bacteria in the presence of serum IgA. dIgA is only produced in mucosal areas and as such difficult to obtain and potentially contaminated with bacterial products after isolation. We therefore used serum IgA, because previous research showed that serum IgA and dIgA are equally capable of activating PMNs (9). Phagocytosis of S. aureus by PMNs was more efficient when IgA was present, compared with phagocytosis of bacteria in the absence of IgA (Fig. 1A, left panels), as previously demonstrated (23). We now demonstrate that when cells were followed in time, great differences were observed in the shape of PMNs that had been incubated with bacteria and IgA compared with either nonstimulated PMNs or PMNs that had been incubated with S. aureus alone. Nonstimulated PMNs had a round shape, which did not alter over time (Fig. 1A, upper panels). PMNs that had been incubated with S. aureus had initially an irregular and elongated shape, but became more round after several hours (Fig. 1A, middle panels). After 16 h, some PMNs were disintegrated, comparable to unstimulated cells. By contrast, expansion in size and disintegration of PMNs that had been incubated with S. aureus and IgA was observed, which increased over time (Fig. 1A [lower panels], 1B, Supplemental Video 1).

IgA induces NETosis and release of NETs

Differential cell death of nonstimulated PMNs versus cell death after stimulation with IgA and bacteria was further studied by

![FIGURE 1. IgA increases phagocytosis and cell death. (A) Histology and time-lapse microscopy images from PMNs that were not stimulated (upper panels), PMNs that were incubated with S. aureus alone (middle panels), or after stimulation with S. aureus in the presence of IgA (lower panels) at different time points. Left column, Illustrates degree of phagocytosis after 30 min of incubation in each condition. This represents t = 0 h, after which the recording was started. The other panels show bright field microscopic images that were taken during live-cell recording at indicated time points. Asterisks (*) indicate disintegrated PMNs. Scale bars, 10 μm. (B) Quantification of the number of disintegrated PMNs at the indicated time points when cells were not stimulated (▲), incubated with S. aureus alone (●), or after stimulation with S. aureus in the presence of IgA (▼). A representative example of eight experiments is shown.]

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labeling PMNs with Organelle ID, which stains mitochondria (green fluorescence), lysosomes (red fluorescence), and nuclei (blue fluorescence) of live cells (Fig. 2A, Supplemental Video 2). No segregation of fluorescent staining was observed in non-stimulated cells (Fig. 2A, left panels). Over time, membrane blebbing was observed (in this particular cell after 168 min), which is a hallmark characteristic of apoptosis, after which PMNs rapidly regained a round shape. Nuclear changes were observed as well, as the nuclei became pyknotic or fragmented over time. Furthermore, cell membranes became positive for annexin V (green) as a result of the expression of the phospholipid component phosphatidylserine on the outside of the cell membrane, which represents another key characteristic of apoptosis (Fig. 2B, Supplemental Video 3). Ultimately, cells stained positive for the cell death marker 7-AAD, which binds DNA (red) when the integrity of the cell membrane has been disrupted.

Similar changes were observed when PMNs were incubated with *S. aureus* (data not shown). In contrast, when PMNs were incu-

**FIGURE 2.** IgA induces NETosis and not apoptosis. (A) PMNs were labeled with Organelle ID staining and followed over time with live-cell microscopy when cells were not stimulated (left column) or after incubation with *S. aureus* and IgA (right column). Nuclei were stained in blue, and lysosomes or mitochondria were labeled red or green, respectively. Depicted for each time point are bright field images (upper left panels), nuclear staining (upper right panels), mitochondria and lysosomes combined (lower left panels), and overlays of all stainings and bright field with the time indication in min:sec:msec (lower right panels). (B) PMNs were incubated with the apoptosis marker annexin V (green) and a general cell death marker 7-AAD (red) and followed over time with live-cell microscopy. PMNs were either not stimulated (left column) or incubated with *S. aureus* and IgA (right column). Depicted for each time point are overlays of fluorescence and bright field with a time indication in min:sec:msec (left panels), bright field images alone (middle panels), or annexin V/7-AAD staining (right panels). Scale bars, 5 μm. The experiment was repeated three times with similar results.
bated with IgA-opsonized *S. aureus*, shrinkage of the nucleus was accompanied by segregation of lysosomes (red) and mitochondria (green), which were relocated toward the cell membrane (Fig. 2A, right panel, in this particular cell at time point 57.32; see also Supplemental Video 2). Furthermore, nuclear contents then subsequently rapidly diffused throughout the cell. Eventually, the cell membrane disintegrated and the majority of cellular components leaked into the environment, leaving only remnants of the cell. This process was clearly associated with cell death, as PMNs became positive for the general cell death marker 7-AAD (red) (Fig. 2B, Supplemental Video 3). The 7-AAD positivity was not preceded by annexin V membrane staining (green), as occurred in apoptotic cells, but, due to disruption of the cell membrane annexin V, did leak into the cell. Although variation in the onset of cell death was observed in either nonstimulated cells or PMNs that had phagocytosed IgA-opsonized bacteria, on average nonapoptotic cell death occurred at earlier time points (<8 h, compared with apoptosis in nonstimulated cells >8 h).

In addition to PMN cell death at earlier time points, which resembled characteristics of NETosis, such as nuclear disintegration and expansion and subsequent membrane rupture, release of web-like structures was observed when PMNs had phagocytosed IgA-opsonized *S. aureus* (Fig. 3A, 3B). Moreover, bacteria colocalized in this structure, supporting the formation of NETs after IgA-mediated phagocytosis. The formation of web-like structures was not observed when PMNs were not stimulated (Fig. 3A, 3B), and to a lesser extent after activation by *S. aureus* in the absence of IgA. The most prominent component of these structures was DNA (Fig. 3C), and NET formation after phagocytosis of IgA-opsonized bacteria was abrogated in the presence of DNase (Fig. 3A, right panel). NETs furthermore contained histones (Fig. 3D), neutrophil elastase (Fig. 3E), and MPO (Fig. 3F), which are prominent components of NETs (14). Real-time video recording demonstrated that NETs were formed immediately following disintegration of the nucleus (Fig. 3G, Supplemental Video 4). Nonstimulated cells went into apoptosis, as shown by blebbing of the membrane and pyknotic nuclei. However, no NETs were formed, even after prolonged time periods (Fig. 3H, Supplemental Video 4).

It was previously reported that secreted bacterial products can induce release of NETs (16). To investigate the involvement of IgA and FcαRI and exclude the influence of these bacterial products, we used HI *S. aureus* in previous experiments. Furthermore, we used different HI bacterial strains, which were incubated with pooled human serum IgA, after which IgA opsonization was determined. A high IgA titer was detected against *E. coli*, whereas IgA opsonization of *S. aureus* and *S. typhimurium* was moderate (Fig. 4A). Nonetheless, uptake of all IgA-opsonized bacteria resulted in NET formation, irrespective of the bacterial strain (Fig. 4B). However, a physiologic scenario most likely also entails phagocytosis of live bacteria. We therefore next studied phagocytosis and NET formation after incubation with either IgA-opsonized HI or live *S. aureus*. Uptake of bacteria was increased in the presence of IgA, but no differences were observed after incubation with either HI or live *S. aureus* (Fig. 4C). Similarly, both IgA-opsonized HI and live bacteria induced release of NETs, which was absent when PMNs were incubated with DNase (Fig. 4D).

**NET release is dependent on FcαRI and production of ROS**

It has been reported that the nonpermeable DNA dye SYTOX Green can be used to quantify NET formation (24). However, this assay proved unsuitable for quantification of NET release after stimulation of PMNs with HI bacteria, as bacterial DNA was stained as well (data not shown). To quantify IgA-induced NET release, we therefore established a phagocytosis assay with IgA-coated latex beads (IgA beads). PMNs and IgA beads were incubated in different E:T ratios. Significantly enhanced phagocytosis was observed when PMNs were incubated with IgA beads compared with phagocytosis of BSA beads, which increased at higher E:T (Fig. 5A). Furthermore, uptake of IgA beads induced release of NETs, as a significant increase in extracellular DNA was detected compared with PMNs that had been stimulated with BSA beads (Fig. 5B, 5C, and data not shown). Addition of DNase furthermore abrogated NET release (Fig. 5B, 5C).

It was previously shown that activation of PMNs via IgA is mediated through FcαRI (7–10), which can be inhibited by blocking mAbs. Addition of the anti-FcαRI mAb MIP8a did not affect nonstimulated cells or PMNs that had been incubated with bacteria alone (data not shown). However, blocking of FcαRI abrogated both phagocytosis of IgA beads and NET formation (Fig. 5A–C). Similarly, phagocytosis of either HI or live IgA-opsonized *S. aureus* and concomitant NET release was inhibited by blocking FcαRI (Fig. 5D).

The activation of NADPH oxidase and the production of ROS are essential for the formation of NETs that are associated with NETosis (17, 18). As such, we investigated ROS production after phagocytosis of IgA-opsonized bacteria and IgA beads. Minimal ROS production was observed over time when PMNs were not stimulated, which was slightly increased when PMNs were incubated with BSA beads or live as well as HI *S. aureus* (Fig. 6A). However, when PMNs were incubated with IgA beads or with live as well as HI *S. aureus* in the presence of IgA, significantly increased ROS production was observed. This most likely explains why phagocytosis of IgA-opsonized particles resulted in enhanced NETosis and release of NETs. Furthermore, when PMNs were incubated with the NADPH oxidase inhibitor DPI, ROS production was abrogated (Fig. 6B). Even though phagocytosis still occurred in the presence of DPI, NET formation was abrogated upon activation with IgA-opsonized *S. aureus* (Fig. 6C), confirming that ROS is required for NET formation.

**Discussion**

Mucosal surfaces cover a vast area in humans of ~400 m², where external influences, like commensal bacteria, food, or inhaled Ags, come into close contact with internal tissues (25). Whereas effective immunological responses against pathogenic microorganisms must be initiated, disproportionate responses against innocuous Ags must be avoided. As such, a delicate equilibrium is required to maintain mucosal homeostasis. IgA plays an important role in this balance. Whereas SlgA prevents invasion of microorganisms without inducing prominent inflammatory responses, dlgA can bind to FcαRI and trigger activation of PMNs (3, 9, 10). This is due to the presence of SC in SlgA, which (partly) blocks the binding site for FcαRI. As dlgA lacks SC, it can act as potent opsonin, and previous studies showed that dlgA is equally active in inducing phagocytosis or PMN migration, compared with serum IgA (9, 10). It has been demonstrated that opsonization of *E. coli*, *Streptococcus pneumonia*, *S. aureus*, *Bordetella pertussis*, *Neisseria meningitides*, and *S. typhimurium* with IgA (dlgA or serum IgA) resulted in enhanced uptake by PMNs (7, 9, 10, 26, 27) (data not shown).

We now show that enhanced uptake of IgA-opsonized bacteria or beads through FcαRI led to NETosis and increased release of NETs, compared with uptake of nonopsonized particles. This was presumably due to enhanced ROS production after uptake of IgA-coated particles, because NET formation was abrogated in the
presence of a ROS inhibitor. Furthermore, NET release was observed after several hours and associated with cell death, which is different from the rapid, oxidant-independent NET release that has also been described (16). Cross-linking of FcαRI by IgA was sufficient to induce NET formation and did not require bacterial components, as both live and HI IgA-opsonized bacteria as well as...
IgA mediated release of NETs. Blocking FcγRI abrogated NET release after incubation of PMNs with IgA-opsonized particles.

The exact function of NETs in vivo is not yet completely elucidated. Bacterial strains that express DNase as virulence factor were shown to break down NETs and disseminate more efficiently (28–30). Furthermore, it was recently shown that NETs, which are formed in the vasculature, efficiently trapped bacteria in the bloodstream, and in vivo removal of NETs by administration of recombinant DNase promoted hypersusceptibility to sepsis (31–33). It was additionally demonstrated that NETs trap and eliminate HIV or pox virus, illustrating that NETs can act as antiviral immune defenses (34, 35). Thus, a role for NETs in early innate immunity is strongly supported. However, NET formation may also play a role in adaptive immune responses, as it was demonstrated that NETs stimulate plasmacytoid dendritic cells (36). Additionally, NETs were shown to prime T cells by reducing their threshold for activation (37).

The ability of NETs to kill micro-organisms is still debated. Several groups have demonstrated that formation of NETs impaired bacterial survival (14, 30, 38–40). However, others showed entrapment of bacteria or Candida albicans by NETs without killing (41). Also, in our experiments, we observed that IgA-induced NETs were able to ensnare live bacteria, but bacterial viability was not diminished (data not shown). As such, it is possible that, in addition to direct cytotoxic effects, NETs also function to contain bacteria for removal by other immune cells like macrophage (42, 43). In fact, dying of PMNs through NETosis may have a second function besides the release of NETs. It is generally accepted that uptake of apoptotic PMNs by macrophages plays an important role in downregulating immune responses, which is essential in preventing excessive inflammation and tissue damage. However, downregulation of immune responses while pathogens are still present may be counterproductive in preventing infectious complications. As such, we hypothesize that induction of NETosis prevents anti-inflammatory signals in macrophages, as it was shown that histones prevent effectorcytosis (uptake of dying host cells) (44). Only after the pathogenic threat has been eliminated do PMNs die through apoptosis and are taken up by macrophages via efferocytosis, which will then result in resolution of inflammation and induction of homeostasis.

Aberrant NET formation has been associated with tissue damage, autoimmunity, and cancer development (45–48). It was recently demonstrated that NETs were involved in transfusion-related acute lung injury (46), neurotoxicity (47), and thrombosis (48). Histones, which are major components of NETs, were furthermore shown to induce epithelial and endothelial cell death (49). Interestingly, it was recently demonstrated that S. aureus can convert NETs into deoxyadenosine, which triggers cell death of immune cells (50). Moreover, as formation of NETs results in exposure of intracellular self molecules, NETs may be involved in the induction of autoimmunity, which is most evident in systemic lupus erythematosus (51, 52). Additionally, NETs are involved in autoimmune vasculitis (53), and impaired NET degradation was associated with lupus nephritis (54). It was recently shown that IgG immune complexes, which are often detected in autoimmune diseases, triggered NET formation (55, 56). We observed release of NETs after phagocytosis of IgG-coated bacteria by PMNs as well (data not shown). As such, IgG-induced NET formation most likely plays a role in systemic immunity, but has also been demonstrated to contribute to development of autoimmunity (55, 56). Furthermore, activation of the classical complement pathway by IgG and additional opsonization of bacteria with complement factors may contribute to activation of neutrophils and NET formation in the circulation or in interstitial fluids where IgG is the main Ab type.
In conclusion, we demonstrated that cross-linking of FcαRI by IgA induced NETosis and release of NETs, which was due to increased ROS production. We propose that this will help to contain infections at mucosal sites. Once bacteria have been able to evade the protective barriers of the mucosal wall, and have infiltrated the intestinal parenchyma, they will be opsonized by dIgA. This will cross-link FcαRI on PMNs that have been recruited into the mucosal tissue, resulting in enhanced NET formation, which can prevent systemic dissemination. Furthermore, induction of NETosis will avoid inappropriate downregulation of immune responses by macrophages at a time at which the infectious threat is still present. Only after pathogens have been cleared will PMNs go into apoptosis, which will signal the end of inflammation, after which anti-inflammatory responses are initiated. As such, our data reinforce the important protective role of IgA to clear infiltrating pathogens and to maintain homeostasis at mucosal sites.

FIGURE 5. Formation of NETs is dependent on FcαRI. (A) Phagocytosis of BSA beads (white bars) or IgA beads in the absence (black bars) or presence of anti-FcαRI blocking Ab MIP8a (gray bars) by PMNs. Phagocytic index was calculated as the percentage of cells that had phagocytosed, multiplied by the geometric mean of FITC-positive cells. Results represent mean ± SEM. Phagocytic index of IgA beads was significantly higher compared with phagocytic index of BSA beads, or IgA beads in the presence of MIP8a. Experiments were repeated four times with similar results. *p < 0.05, **p < 0.01. (B) PMNs were stimulated with IgA beads in the absence (left panel) or presence of DNase (middle panel) or MIP8a (right panel) for 3 h, after which slides were stained with Diff-Quik. Red arrowheads indicate NETs. Scale bar, 10 μm. (C) Quantification of extracellular DNA (which reflects NETs) after 3 h when PMNs were either not stimulated (light gray bars) or stimulated with BSA beads (white bars) or IgA beads (black bars) in the absence (filled bars) or presence of DNase (striped bars). Additionally, PMNs were incubated with IgA beads and MIP8a (dark gray bars). Results represent mean ± SEM. Extracellular DNA was signfiicantly higher after incubation with IgA beads compared with BSA beads or in the presence of DNase or MIP8a. One representative example of four experiments is shown. *p < 0.05. (D) PMNs were stimulated with HI (left panels) or live (right panels) S. aureus and IgA in the absence (upper panels) or presence (lower panels) of MIP8a. After a 3-h incubation period, slides were stained with Diff-Quik. Red arrowheads indicate NETs. Scale bar, 10 μm.

FIGURE 6. Formation of NETs is dependent on ROS production. (A) ROS production was measured over time (3 h) in unstimulated PMNs (○) or after stimulation with HI or live S. aureus in the absence (▽) or presence of IgA (● or ○). Additionally, PMNs were stimulated with BSA beads (▽) or IgA beads (▽). Results represent mean ± SEM. **p < 0.01, ***p < 0.001. (B) PMNs were either not stimulated (○) or stimulated with S. aureus and IgA in the absence (●) or presence (○) of the ROS inhibitor DPI. Results represent mean ± SEM. ***p < 0.001. (C) PMNs were stimulated with S. aureus and IgA in the absence (left panel) or presence (right panel) of DPI for 3 h. Slides were stained with Diff-Quik. Red arrowheads indicate NETs. Scale bar, 25 μm. Experiments were repeated three times with similar results.
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Disclosures

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