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Immobile Immune Complexes Induce Neutrophil Extracellular Trap Release by Human Neutrophil Granulocytes via FcγRIIIB and Mac-1

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Canonical neutrophil antimicrobial effector mechanisms, such as degranulation, production of reactive oxygen species, and release of neutrophil extracellular traps (NETs), can result in severe pathology. Activation of neutrophils through immune complexes (ICs) plays a central role in the pathogenesis of many autoimmune inflammatory diseases. In this study, we report that immobilized ICs (iICs), which are hallmarks of several autoimmune diseases, induce the release of NETs from primary human neutrophils. The iIC-induced NET formation was found to require production of reactive oxygen species by NADPH oxidase and myeloperoxidase and to be mediated by FcγRIIIB. Blocking of the β2 integrin macrophage-1 Ag but not lymphocyte function–associated Ag-1 abolished iIC-induced NET formation. This suggests that FcγRIIIB signals in association with macrophage-1 Ag. As intracellular signaling pathways involved in iIC-induced NET formation we identified the tyrosine kinase Src/Syk pathway, which downstream regulates the PI3K/Akt, p38 MAPK, and ERK1/2 pathways. To our knowledge, the present study shows for the first time that iICs induce NET formation. Thus, we conclude that NETs contribute to pathology in autoimmune inflammatory disorders associated with surface-bound ICs. The Journal of Immunology, 2014, 193: 1954–1965.

Polymeronuclear leukocytes are key players in the antimicrobial defense. One important antimicrobial effector mechanism is the release of extracellular fibrous structures, the so-called neutrophil extracellular traps (NETs) (1, 2). NETs are composed of chromatin, histones, and antimicrobial proteins and contribute to pathogen containment (1). To release NETs, activated neutrophils undergo a form of cell death that is distinct from apoptosis and necrosis, the so-called NETosis (1, 3), which is dependent on the formation of reactive oxygen species (ROS) and on NADPH oxidase (NOX) and myeloperoxidase (MPO) (3–7). In addition to ROS-dependent NETosis, few microorganisms and certain stimuli have been reported to induce NETs in an ROS-independent manner (8–11). However, the import of ROS-dependent NETs for host defense is highlighted by patients with chronic granulomatous disease, patients who have mutations that inactive the NOX, or patients completely deficient in MPO whose neutrophils fail to produce ROS and NETs and suffer from severe recurrent infections (3, 12, 13). Besides ROS-producing enzymes, several enzymatic activities (14–19), specific signal transduction events (20), and engagement of β2 integrins (21, 22) are involved in NETosis, indicating a multifactorial process.

Deregulation and defects of NETosis can contribute to inflammation and can lead to severe pathogenesis, because the cytotoxic agents of NETs can cause endothelial and tissue damage (19, 23–26). Additionally, NETs can activate plasmacytoid dendritic cells and thus enhance inflammation (27, 28). A pathophysiological role of NETs has been suggested in allergic, infectious, and autoimmune diseases (29). In autoimmune disorders NETs are not only proposed to potentiate autoimmunity/pathogenesis (effector inflammatory phase) but also to be involved in the allergic immune phase (generation of autoimmune response). Because NETs expose potential autoantigens such as histones, dsDNA, and granule enzymes they have been suspected to support production of autoantibodies and/or immune complexes (ICs) (30–33). The presence of autoantibodies against NET Ags and/or ICs that contain NET components can potentiate a proinflammatory response and further perpetuate an autoimmune inflammation (27, 28, 32, 34). In autoimmune diseases, both soluble and insoluble ICs provoke activation of neutrophils (35–38). Neutrophils isolated from synovial fluid from patients with rheumatoid arthritis (RA) exhibit an enhanced ROS (39, 40) and NET production (34). Pathogenic ICs that are formed on the extracellular matrix and thus are immobilized have been described in several autoimmune diseases (41–43). Whereas soluble ICs (sICs) have been shown to induce NET production by human neutrophils (11, 32, 34, 44), it remains unclear whether immobilized ICs (iICs) are also able to induce NETosis. Thus, the aim of this study was to determine...
whether iICs can stimulate NETosis of primary human neutrophils and to discover the underlying molecular mechanisms. We could clearly show that iICs stimulate the release of NETs in a ROS-dependent manner. Furthermore, we could show that FcγRIIB and its signaling partner macrophage-1 Ag (Mac-1), which initiates Src/Syk signaling, mediate the activation process. As signaling pathways downstream of Src/Syk we identified the P38/ Akt, ERK, and p38 MAPK pathways to be involved in iIC-induced NETosis.

Materials and Methods

Ethics statement

Sample collection was conducted with the understanding and the consent of each participant and was approved by the Ethical Committee of the Medical Faculty of the University of Lübeck (05-124).

Isolation of primary human neutrophils

Peripheral heparinized blood was collected by venipuncture from healthy adult volunteers. Neutrophils were isolated as described previously using Percoll gradient centrifugation (45). Cell purity and viability were >99.9% as determined by morphological examination of Giemsa-stained cytospin slides.

Preparation of immobilized ICs

Plate-bound iICs were formed by using human serum albumin (HSA) Ag (Baxter) and rabbit polyclonal anti–HSA IgG (Sigma-Aldrich, Steinheim, Germany) as described previously (35). Briefly, 20 μg/mL HSA in 50 mM carbonate/bicarbonate buffer (pH 9.6) was coated overnight at 4°C to 96-well Lumitrac 600 (for chemiluminescence-based ROS detection) or Fluotrac 600 (for fluorescence-based NET detection) high-binding plates (Greiner Bio-One, Frickenhausen, Germany). After washing with PBS plus 0.05% Tween 20 (wash buffer), wells were blocked with 10% biotin-free BSA (low LPS; Carl-Roth, Karlsruhe, Germany) in PBS for 1 h at room temperature following 1 h incubation with anti–HSA IgG1 diluted 1:400 in PBS (-10 μg/mL HSA-specific IgG). Finally, IC-coated wells were washed twice with wash buffer and once with assay medium. For microscopy-based experiments ICs were coated as described above by using 100 μg/mL HSA to eight-well ibiTreat μ-slides (Ibidit, Martinisried, Germany) for fluorescence microscopy or to Thermanox coverslips (Nunc/Thermo Scientific, Rockford, IL) for scanning electron microscopy (EM). Uncoated wells, BSA-coated wells, and wells without the HSA Ag (BSA/IgG), without the anti–HSA IgG (HSA), or without has Ag and BSA blocking (IgG only) served as controls. F(ab’2) fragments for preparation of iICs were obtained by using the ImmunoPure F(ab’2) preparation kit (Thermo Scientific) following the manufacturer’s instructions.

Assessment of neutrophil viability

To exclude an apoptotic- or necrosis-including effect of media, iICs, inhibitors, and blocking Abs, viability of neutrophils was analyzed by flow cytometry using Annexin V<sup>FITC</sup> (Promokine, Heidelberg, Germany) and propidium iodide (PI) (Sigma-Aldrich) staining according to the manufacturer’s instructions. Cells were analyzed by flow cytometry using a FACS Calibur flow cytometer and the CellQuest Pro software (BD Biosciences, San Diego, CA).

Inhibitors, antioxidants, and blocking Abs

Neutrophils were preincubated for 30 min at 37°C in appropriate assay medium with freshly prepared inhibitors or antioxidants prior to exposure to iICs. To inhibit ROS pathways the NOX inhibitor diphenyleneiodonium chloride (DPI, 20 μM, Sigma-Aldrich) and the MPO inhibitor 4-dimethylaminooantipyrine (aminopyrine, 200 μM, Sigma-Aldrich) were used. As antioxidants with ROS scavenging activity l-ascorbic acid (1 or 2 mM, Sigma-Aldrich), or l-buthionine (S,R)-sulfoximine (5-ASA, 0.5 mM, TCI Europe, Eschborn, Germany) were used. Urea (1 mM) served as negative control. For analysis of signaling pathways, neutrophils were preincubated with 30 μM piceatannol (Syk inhibitor, Sigma-Aldrich), 10 μM P2P2 (Src inhibitor, Calbiochem, Darmstadt, Germany), 25 μM LY29004 (PI3K inhibitor, Calbiochem), 10 μM U0126 (ERK inhibitor, Cell Signaling Technology, Beverly, MA), 9 μM VIII (Akt inhibitor, Calbiochem), or 10 μM SB 203580 (p38 MAPK inhibitor, Calbiochem); Solvent (DMSO 1:1000) and untreated neutrophils served as controls.

In studies using Fcy-R blocking Abs, neutrophils were incubated for 50 min at room temperature with 1 μg blocking Abs to FcγRI (anti-human CD64, clone 10.1, BioLegend, San Diego, CA), FcγRII (anti-human CD32, clone AT10, Abcam, Cambridge, U.K.), FcγRIIB (anti-human CD16, clone 3G8, BioLegend), or isotype control Abs (mouse IgG1 isotype, BioLegend) per 10<sup>6</sup> cells/ml. After washing, neutrophils were stimulated with iICs. To block Mac-1 (CD11b/CD18) or lymphocyte function–associated Ag-1 (LFA-1; CD11a/CD18) human neutrophils were preincubated in assay medium for 30 min at 37°C with 20 μg/mL blocking Ab to human CD11b (clone iCRF41, CD11a (clone HI111), or CD18 (clone TS1/18) (all from BioLegend) or a combination of anti-CD11b/α with anti-CD18 prior to stimulation with iICs. Mouse IgG1 isotype (BioLegend) and untreated cells served as controls.

Detection of intra- and extracellular ROS

The sum of intra- and extracellular ROS, mainly H<sub>2</sub>O<sub>2</sub> and MPO-generated metabolites, was measured by using luminol (46, 47). Neutrophils (2 × 10<sup>6</sup>/ml) suspended in medium with freshly prepared inhibitors or antioxidants prior to exposure to iICs. To inhibit ROS pathways the NOX inhibitor diphenyleneiodonium chloride (DPI, 20 μM, Sigma-Aldrich) and the MPO inhibitor 4-dimethylaminooantipyrine (aminopyrine, 200 μM, Sigma-Aldrich) were transferred to iIC-coated 96-well Lumitrac 600 plates (Greiner Bio-One) and ROS-dependent chemiluminescence was analyzed using an Infinite 200 reader and the Tecan i-control 1.7 software (Tecan, Crailsheim, Germany). In some of the experiments, where 20 mM PMA was used as a stimulus, measurement was performed with neutrophils in suspension culture (nonimmobilized) in 96-well Nunc Delta black microwell plates (Nunc, Langensbold, Germany). ROS release was monitored for 1 h every 2 min at 37°C. For statistical analysis the area under the curve (AUC) of each sample was calculated.

Detection of extracellular superoxide

The lucigenin-amplified chemiluminescence assay (46, 48) was performed the same way as the luminol assay, but with 0.2 mM lucigenin (Alexis, Lörantz, Germany) instead of luminol. This assay was used to analyze the extracellular superoxide production.

Detection of NETs

To study the kinetics of NET release of neutrophils, the non–cell-permeable DNA dye Sytox Green (Invitrogen) was used (2, 4, 12). Neutrophils (10<sup>6</sup>/ml) were incubated at 37°C in iIC-coated Fluotrac 600 plates (Greiner Bio-One) in NET medium containing 5 μM Sytox Green. In experiments using PMA as stimulus, neutrophils in suspension culture were incubated in 96-well Nunc Delta black microwell plates (Nunc) and stimulated with 20 nM PMA. Where indicated, cells were pretreated for 15 min with 5 nM TFN-α prior to iIC stimulation. The fluorescence of NET-bound Sytox Green (excitation, 488 nm; emission, 510 nm) was analyzed for 7 h every 5 min at 37°C using an Infinite 200 reader and Tecan i-control 1.7 software (Tecan). For statistical analysis the AUC of each sample was calculated.

Microscopic assessment of NETs

For visualization of NETs, fluorescence microscopy (FM) and EM were performed. For FM, 10<sup>6</sup> neutrophils/ml in NET medium were incubated for 7 h at 37°C in iIC-coated ibiTreat μ-slides (Ibidit). Following fixation with 4% paraformaldehyde (Sigma-Aldrich), staining of MPO and DNA by using mouse anti-human MPO (1:500; AbD Serotec, Düsseldorf, Germany) and Sytox Green was carried out as described previously (4, 48). Samples were analyzed with the AxioVert A.1 using the the AxioCam HRc and Axioscope (all Carl Zeiss, Jena, Germany) or with the Keyence BZ-9000 software (Keyence, Neu-Isenburg, Germany).

For EM, neutrophils (10<sup>6</sup>/ml) in NET medium were settled on iIC-coated thermowax coverslips (Greiner Bio-One). Following incubation for 7 h at 37°C, the supernatant was removed and samples were fixed with 1 ml Monti-Graziadei solution and processed for EM as described (48). Preparates were examined with an EM505 (Phillips, Eindhoven, The Netherlands).

Western blot analysis

Neutrophils (5 × 10<sup>6</sup>/ml in NET medium) were stimulated for 15 min at 37°C with iICs. In some experiments neutrophils were preincubated for 30 min with 20 μM DPI or 10 μM P2P2. Following iIC stimulation, whole-cell lysates were prepared using TCA as described (49). Western blot analysis was carried out by using Abs against human: phospho-Akt (Thr<sup>308</sup>, p85α/p50γ, ERK1/2, Thr<sup>202</sup>/Tyr<sup>204</sup>, phospho-p38 MAPK (Thr<sup>180</sup>/Tyr<sup>182</sup>, p38 kinase-PI3K p85 (Tyr<sup>185</sup>/P55 (Tyr<sup>196</sup>)), or β-actin (all from Cell Signalling Technology) and probed with HRP-conjugated anti-rabbit or anti-mouse IgG (New England Biolabs, Beverly, MA). The signal
was detected by using Immobilon Western chemiluminescence HRP substrate (Millipore, Billerica, MA) and quantified by using the Fusion Fxt chemiluminescence reader and Bio1D software (Vilber Lourmat, Eberhardzell, Germany). Signals of pAkt, pPI3K, pERK1/2, or pp38 were normalized to β-actin reprobed on the same blots.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism software 6 using the one-way ANOVA followed by a Bonferroni t test for multiple comparisons.

Results

**iICs activate neutrophils and induce the oxidative burst**

Surface-bound iICs were generated by using HSA and rabbit anti-HSA IgG Abs. Immune complexes formed with rabbit IgG have been used previously in several studies with neutrophils (11, 35, 36, 50). Rabbit IgGs bind to human Fcγ receptors in a similar pattern as human IgG (11), and they activate human neutrophils (11, 35, 50). Thus, rabbit IgGs can be used as an equivalent alternative to human IgG. Light microscopy revealed neutrophil spreading in response to iICs (Fig. 1A), indicating activation of these cells. The iIC-induced shape changes were also evident upon flow cytometry in the forward light-scattering properties (Fig. 1B). The oxidative burst was assessed by using two different techniques. iICs induced both the production of MPO-dependent ROS (Fig. 1C) and superoxide (Fig. 1D). Controls (immobilized BSA, HSA, BSA/IgG, and IgG) induced no ROS production. Neither iICs nor the corresponding controls induced apoptosis or necrosis of neutrophils (Supplemental Fig. 1).

**iICs induce ROS-dependent NET release**

Having observed that iICs induce ROS production in neutrophils (Fig. 1), we next assessed the effect of iICs on the formation of NETs. iICs significantly induced NET release by human neutrophils as detected by real-time kinetics (Fig. 2A). Preincubation of neutrophils with TNF-α significantly increased iIC-induced NET production (Fig. 2B). When compared with PMA, a well-characterized inducer of ROS-dependent NETosis, NETs induced by iICs/TNF-α occurred later and to a lesser extent (Fig. 2A).

FM and EM analyses confirmed a NET-inducing effect of iICs (Fig. 2C, 2D). Neutrophils incubated on iICs released complex fibrous or cloud-like structures (Fig. 2C, 2D). Immunohistological staining confirmed that these NET structures (Fig. 2D, arrows) contained decondensed DNA (Fig. 2D, green) and MPO (Fig. 2D, red). Neutrophils under NETosis are characterized by the loss of the multilobated nuclear morphology, chromatin decondensation, and intermixing of DNA and granular proteins (MPO) (Fig. 2D, arrowhead).

Because iICs induced the respiratory burst (Fig. 1) and the release of NETs (Fig. 2), we next aimed to clarify whether NOX- and MPO-dependent ROS were involved in iIC-induced NETosis. Neutrophils were pretreated with the NOX inhibitor DPI or the MPO inhibitor aminopyrine prior to iIC stimulation. A toxic effect of these inhibitors was excluded by annexin V/PI staining (data not shown). The lucigenin-ampfified chemiluminescence assay is a sensitive technique to detect superoxide anions (4, 46). Inhibition of NOX by DPI nearly completely abolished superoxide production (Fig. 3B). Consequently, ROS generated downstream of NOX such as MPO-dependent ROS, which can be measured by the luminol-amplfied chemiluminescence assay (4, 46, 47), were also abolished (Fig. 3A). Aminopyrine exerted a strong inhibitory effect on MPO-dependent ROS as measured by the luminol assay (Fig. 3A) but not on superoxide as measured by the lucigenin assay (Fig. 3B). Lucigenin specifically emits light upon reaction with superoxide, but it is not excited by MPO-derived ROS (46, 51). MPO-derived metabolites are responsible for the excitation of luminol (46). Therefore, these results indicate that aminopyrine specifically inhibits the MPO- but not the superoxide-producing NOX. The Sytox Green assay as well as FM and EM revealed a significant inhibitory effect of both DPI and aminopyrine on the production of NETs in response to iICs (Fig. 3C, 3D).

To substantiate the role of ROS in iIC-induced NET release, we used the antioxidants 5-ASA and L-ascorbic acid. The antioxidant effect of these substances is related to their ability to scavenge ROS (52, 53). Both substances exerted a scavenging effect on intracellular MPO–dependent ROS (Fig. 4A), but only 5-ASA had such an effect on extracellular superoxide (Fig. 4B). In the NET assay, all substances that scavenged MPO-dependent ROS significantly decreased iIC-induced NETosis (Fig. 4C). The inhibitory effect of antioxidants on NET release was also obvious in EM (Fig. 4D) and FM (data not shown) and further confirms that iICs induce NETs in a ROS-dependent manner.

**FIGURE 1.** iICs activate primary human neutrophils and induce the production of ROS. (A and B) Shape change/cell spreading of human neutrophils (2 × 10⁶/ml in NET medium) plated for 30 min at 37°C on immobilized HSA/anti-HSA immune complexes (iICs) as observed by (A) light microscopy (by using the AxioVert A.1 with ×40/0.75 Ph2 EC Plan-Neofluar objective, AxioCam HRc, and AxioVision release 4.8 software; scale bars, 20 μm) and (B) flow cytometry (changes in forward light scatter values) in comparison with neutrophils incubated on uncoated surfaces. Representative real-time kinetics and AUC values of (C) intra-/extracellular MPO-dependent ROS and (D) extracellular superoxide as measured by the luminol- and lucigenin-ampfied chemiluminescence assays, respectively, are shown. Real-time analysis of ROS production was monitored for 1 h at 37°C from neutrophils (2 × 10⁶/ml in CL medium) incubated on iICs and controls (uncoated or plate-bound BSA, HSA, BSA/IgG, IgG); n = 3–10 independent experiments. Values are means ± SEM. ****p < 0.0001 as compared with the uncoated sample.
It is known that activation of neutrophils through ICs is mediated by FcγRs. To check whether iIC-induced NETosis is mediated through FcγRs, neutrophils were stimulated with iICs containing F(ab’)2 fragments of the anti-HSA Ab instead of the full anti-HSA IgG. iICs with F(ab’)2 fragments failed to induce the oxidative burst of human neutrophils (Fig. 5A, 5B) and also failed to stimulate the release of NETs (Fig. 5C, 5D). These results suggest that binding to FcγR is a crucial step for the iIC-induced NET formation.

Resting human neutrophils constitutively express two types of FcγRs on their surface, FcγRIIA (CD32) and FcγRIIB (CD16). FcγRI is expressed by activated neutrophils (54). We investigated whether FcγRI, FcγRIIA, or FcγRIIB is involved in iIC-induced ROS and NET production. For this purpose, neutrophils were incubated with blocking Abs to FcγRI, FcγRIIA, FcγRIIB, or isotype control and production of ROS and NETs in response to iICs was analyzed. Activation of iIC-induced oxidative burst clearly depended on stimulation of both FcγRIIA and FcγRIIB (Fig. 5E, 5F). In the NET assay only blocking of FcγRIIB, but not of other FcγRs, resulted in a decrease of Sytox Green fluorescence, indicating that NET release is mediated solely by FcγRIIB (Fig. 5G). These data indicate that both receptors, FcγRIIA and FcγRIIB, are required for iIC-induced oxidative burst whereas only FcγRIIB is sufficient for iIC-induced NET release.

Syk kinase–dependent signaling pathways are involved in iIC-induced NETosis

Western blot analysis of iIC-stimulated neutrophils revealed an enhanced phosphorylation of PI3K, Akt, ERK1/2, and p38 MAPK (Fig. 6A). To investigate whether these intracellular signaling pathways are involved in NETosis, inhibitors of these signaling molecules and potentially involved upstream pathways (Src, Syk) were used. Neutrophils were pretreated with inhibitors of Src (PP2), Syk (piceatannol), PI3K (LY29004), ERK1/2 (UO126), Akt (VIII), p38 MAPK (SB 203580), or solvent control (DMSO) prior to stimulation with iICs (Fig. 6B–E). A toxic effect of the inhibitors was excluded by annexin V/PI staining and microscopy (data not shown). Inhibition of Syk, Src, and PI3K drastically inhibited iIC-induced ROS production (Fig. 6B, 6C). Blocking of PI3K/Akt, ERK1/2, and p38 MAPK pathways also resulted in diminished ROS production (Fig. 6B, 6C). Treatment of neutrophils with Syk, Src, or PI3K inhibitors also blocked NET production in response to iICs (Fig. 6D, 6E). ERK1/2 inhibition nearly completely abolished NET formation, whereas treatment with inhibitors of Akt and p38 MAPK partially inhibited NET formation in response to iICs (Fig. 6D, 6E).

It has been reported for PMA-induced NETosis that phosphorylation/activation of ERK1/2 and p38 MAPK, which lead to formation of NETs, are mediated by ROS (55). Thus, we next investigated whether ROS are also required for phosphorylation of PI3K, Akt, ERK1/2, or p38 MAPK in iIC-induced NETosis, or whether Src family kinases are the critical upstream component of these signal transduction pathways. To address this question, neutrophils were treated with the NOX inhibitor DPI or the Src inhibitor PP2 prior to stimulation with iICs. Phosphorylation of PI3K, Akt, ERK1/2, and p38 MAPK was investigated by Western blot analysis.
Treatment with the Src-inhibitor PP2 prevented phosphorylation of PI3K, Akt, ERK1/2, and p38 MAPK in iIC-stimulated neutrophils, whereas DPI treatment did not significantly affect their phosphorylation (Fig. 6F–I). These results indicate that activation of PI3K, Akt, ERK1/2, or p38 MAPK results directly from Src family kinases/receptor activation and is not ROS mediated.

To investigate whether the iIC-induced signaling events leading to NET release are specific for iIC-induced NETosis or common for ROS-dependent NETosis, we performed signaling-inhibition studies with nonimmobilized neutrophils stimulated with PMA (Supplemental Fig. 2). PMA is the best characterized inducer of ROS-dependent NETosis. PMA-induced NETosis was shown to be mediated through the ERK and p38 MAPK pathways (20, 55). We could observe a role for the ERK but not for the p38 MAPK pathway in PMA-induced ROS and NET production (Supplemental Fig. 2). In addition to ERK we found Syk to be involved in PMA-dependent ROS and NET production (Supplemental Fig. 2). Src and the PI3K/Akt pathways were not directly involved in PMA-induced NET production (Supplemental Fig. 2C). However, blocking of these molecules resulted in a partial decrease in ROS production as measured by the luminol or lucigenin assay (Supplemental Fig. 2A, 2B). These results suggest that receptor/Src activation and the PI3K/Akt pathways are specific for iIC-induced NETosis, whereas Syk and ERK activation are also involved in PMA-induced ROS-dependent NETosis.

**Mac-1 is involved in the FcγRIIIB-mediated NET induction**

FcγRIIIB is a GPI-linked receptor missing transmembrane and cytoplasmic domains (56). The fact that FcγRIIIB-mediated NET release in response to iICs involves Src/Syk pathways (Fig. 5) suggests that FcγRIIIB utilizes signaling molecules associated with other receptors or that other receptors, in addition to FcγRIIIB, are involved in NET induction. FcγRIIA and the β2 integrin Mac-1, which both require Syk-dependent signaling, have been described as signaling partners of FcγRIIIB (57–60). Because we observed that blocking of FcγRIIA did not inhibit the formation of iIC-induced NETs (Fig. 5G), we investigated whether the β2 integrin Mac-1 plays a role. Mac-1 is composed of a β2 subunit (CD18) that is noncovalently associated with an α subunit (CD11b). Whereas blocking of CD18 had no effect on ROS production (Fig. 7A, 7B), we observed an inhibitory effect on extracellular superoxide when CD11b was blocked (Fig. 7B). Blocking of both CD11b or CD18 substantially decreased iIC-induced NET release whereas simultaneous blocking of CD11b and CD18 completely abolished NET formation (Fig. 7C–E). These data suggest that not only FcγRIIIB but also the β2 integrin Mac-1 (CD11b/CD18) is required for iIC-induced NET formation.

In addition to Mac-1, neutrophils constitutively express the β2 integrin LFA-1 (61). LFA-1 is structurally similar to Mac-1, as it is composed of the common β2 subunit (CD18) associated with a respective α subunit (CD11a) (61). Thus, we next addressed the question whether LFA-1 (CD11a/CD18) plays any role in iIC-induced NETosis. Blocking of CD11a, CD18, or LFA-1 (CD11a/CD18) had no effect on iIC-induced ROS production (Fig. 7F, 7G). Blocking of CD11a alone had also no inhibitory effect on iIC-induced NET release whereas simultaneous blocking of CD11b and CD18 completely abolished NET formation (Fig. 7C–E). These data suggest that not only FcγRIIIB but also the β2 integrin Mac-1 (CD11b/CD18) is required for iIC-induced NET formation.
Activation of neutrophils through ICs plays a central role in the pathogenesis of several autoimmune inflammatory diseases. Dysregulation of the release or defects of the clearance of NETs are thought to contribute to autoimmunity and inflammation (29) and have been described for systemic lupus erythematosus (27, 28, 30, 62), small vessel vasculitis (32), MPO/anti-neutrophil cytoplasmic Ab–associated vasculitis (63), lupus nephritis (30), psoriasis (64, 65), RA (34), and gouty arthritis (66). In several autoimmune disease ICs not only occur as circulating sICs, but are formed on extracellular matrix/solid surface and are thus immobilized (41–43). Although both sICs and iICs provoke priming and activation of neutrophils and concomitant tissue damage (11, 35–38, 67, 68), it remained unclear whether iICs are able to induce NETosis. To our knowledge, our data demonstrate for the first time that iICs stimulate the release of NETs from human primary neutrophils.

In response to most stimuli NETs are released during a programmed, lytic cell death, the so-called NETosis, which occurs within hours and is mediated by ROS produced by NOX and MPO (3, 4, 6, 7, 69–71). This cell death has been shown to be involved in autoimmune inflammatory diseases such as RA (34) or systemic lupus erythematosus (27, 72). However, a ROS-independent, rapid (5–60 min) induction of NET release has been recently described upon endocytosis of sICs (11). In contrast, our data show that iICs induce the typical NETosis, which requires hours and depends on ROS. By using pharmacological inhibitors of NOX or MPO as well as by treatment of cells with antioxidants, which scavenge ROS but do not affect the ROS-producing enzymes, we could not only reduce the iIC-induced oxidative burst, but also the release of NETs. Thus, NOX/MPO activity and ROS are important players controlling iIC-induced NETosis.

IC-induced activation of neutrophils is mediated by FcγRs that are expressed on the cell surface of most immune cells. Engagement of FcγRs not only triggers several antimicrobial functions such as recognition and phagocytosis of IgG-opsonized pathogens, degranulation, and oxidative burst, but also plays an important role in IC-mediated inflammatory processes and the development of autoimmune diseases (55, 73, 74). Mice deficient in the γ-chain and thus lacking all activating FcγRs have significant defects in IC-dependent effector cell responses (73, 75) and are resistant to induction of various IC-induced autoimmune diseases (74, 76–78). Furthermore, FcR polymorphisms and/or disturbed FcγR expression have been described in patients with autoimmune diseases (74, 79–81). These data indicate that a wide range of inflammatory and autoimmune diseases are triggered by FcγR-mediated activation of inflammatory cells. Activating FcγRs expressed on resting human neutrophils are FcγRIIB and FcγRIIA (74, 82), which both mediate activation of neutrophils by ICs (11, 35, 38, 82–85). The high-affinity ITAM-bearing FcγRI is expressed upon activation of neutrophils by inflammatory stimuli and can bind monomeric IgG (86). We could show that iICs induce a robust respiratory burst through engagement of both FcγRIIA and FcγRIIB. Nevertheless, only engagement of FcγRIIB but not FcγRIIA seems to be required for iIC-induced NETosis. This indicates that NETosis is a multifactorial process that not only requires ROS, but also demonstrates that distinct activation and signaling events are necessary. The GPI-linked FcγRIIB lacks transmembrane and cytoplasmic signaling domains (56) but can signal in association with FcγRIIA and/or the β2 integrin Mac-1, which both initiate Syk-dependent signaling (57–60, 87). We identified Mac-1 and the associated signal molecules Src, Syk, and PI3K to play an essential role in iIC-induced NETosis.

**FIGURE 4.** Antioxidants inhibit the formation of iIC-induced NETs. Human neutrophils were pretreated for 30 min at 37°C with 1 or 2 mM L-ascorbic acid (Asc), 0.5 mM 5-ASA, or 1 mM urea or left untreated (medium) before incubation with iICs. (A) Luminol- and (B) lucigenin-amplified chemiluminescence was then monitored for 1 h at 37°C from 10⁶ neutrophils/ml in CL medium, and (C) fluorescence intensities of NET-bound Sytox Green for 7 h at 37°C from 10⁶ neutrophils/ml in NET medium. AUC values (means ± SEM from three independent experiments) of luminol, lucigenin, and Sytox Green intensities are shown. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with medium control. (D) For EM, neutrophils were fixed after 7 h incubation at 37°C on iICs. Representative EM images for neutrophils treated with urea, 5-ASA, and Asc are shown in comparison with untreated (iICs) and unstimulated (HSA) neutrophils. EM images were taken with an EM505. Scale bars, 10 μm for ×170 magnification and 5 μm for ×2500 magnification.
These results suggest that, as a consequence of iIC stimulation, FcγRIIIB and Mac-1 get activated and that interactions of both receptors induce outside–in signaling pathways leading to NET formation. This assumption is in line with previous studies describing that FcγRIIIB utilizes signaling molecules associated with Mac-1 and/or crosstalk/interactions of both receptors are necessary for neutrophil activation/cytotoxic functions (57–60, 82, 87). Surprisingly, Mac-1 is not involved in NET induction upon endocytosis of sICs, which is mainly mediated by FcγRIIA, whereas FcγRIIIB plays a minor role in this process (11), possibly through its ability to capture ICs and subsequently activate FcγRIIA (11, 87). This suggests that FcγRIIIB can mediate signaling independent of its known signaling partners. Therefore, we cannot rule out that in addition to FcγRIIIB and Mac-1 other routes of activation are involved in iIC-induced NETosis. Nevertheless, our data clearly reveal that the β2 integrin Mac-1, in addition to FcγRIIIB, is a key player in iIC-induced NETosis, similar to NET induction through TLRs, fMLP, or TNF, where engagement of Mac-1 is also essential (21, 88). Mac-1 seems to play a minor role in iIC-induced ROS production, because we observed only a slight inhibition of extracellular superoxide upon Mac-1 blocking and no effect on MPO-dependent ROS.

In addition to Mac-1, neutrophils constitutively express the β2 integrin LFA-1, which is structurally similar to Mac-1, as it is composed of the common β2 subunit (CD18) associated with a respective α subunit (CD11a) (61). It has been reported recently in a mouse model and for primary human neutrophils that NET release in response to LPS-stimulated platelets is mediated via LFA-1 (89). On the contrary, LFA-1 is not involved in NET production induced by TNF-related activation protein–stimulated platelets, which was shown to be mediated by Mac-1 (22). In our present study we could not observe a role for LFA-1 either in iIC-induced ROS or in NET production. This indicates that the β2 integrin Mac-1 but not LFA-1 plays a role in iIC-induced outside–in signaling leading to the formation of NETs.

Downstream of FcγRIIIB and Mac-1 we identified tyrosine kinases, particularly of the Src and Syk families, and PI3K to play a key role in iIC-induced NETosis. These kinases are not only known to be key mediators of FcγR signaling, but they are also required for integrin signaling in neutrophils (58, 90, 91) and thus fit to our model that FcγRIIIB signals in association with Mac-1. Syk pathways have also been shown to be involved in sIC-induced activation of neutrophils and NET formation (11, 44, 92). Syk deficiency or blocking of Syk hinders FcγR-mediated NET formation in response to sICs (11, 44, 93) and, additionally, protects mice almost completely from disease development in a collagen Ab–induced arthritis model (44). Thus, Syk pathways could serve as a target for drug application to affect the development of IC-mediated autoimmune diseases. Interestingly, we observed that Syk is not only involved in integrin-mediated iIC-induced NETosis, but also in PMA-stimulated ROS and NET production. The nonphysiological agent PMA directly activates protein kinase C (94) and thus does not require cell surface receptors such as β2 integrins and bypasses receptor-mediated signaling such as Src and Syk. Indeed, in PMA-induced NETosis we could not observe

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**FIGURE 5.** iIC-induced NET release from human neutrophil granulocytes depends on FcγRIIIB. (A and B) ROS production and (C and D) NET release was measured after exposure of neutrophils to immobilized HSA/anti–HSA IgG1 or to HSA/anti–HSA F(ab′)2. AUC values (means ± SEM) are shown for (A) the luminol- and (B) lucigenin-amplified chemiluminescence assays (2 × 10^6 cells/ml) as monitored within 1 h at 37°C and (C) the Sytox Green assay (10^6 cells/ml) as monitored within 7 h at 37°C. Mean AUC values (means ± SEM) of luminol (n = 4), lucigenin (n = 4), and Sytox Green (n = 3) intensities are shown. *p < 0.05, **p < 0.01, ***p < 0.001. (D) NETs were visualized by EM (left panels; using EM505) and FM (right panel) after 7 h at 37°C (10^6 cells/ml). For FM, DNA was stained with Sytox Green and overlay of brightfield and green fluorescence is shown. Images were taken with an AxioVert A1 with ×40/0.75 Ph2 EC Plan-Neofluar objective, AxioCam HRc, and AxioVision release 4.8 software. Scale bars, 5 μm for EM and 20 μm for FM images. HSA-F(ab′)2, HSA/anti–HSA F(ab′)2, HSA/anti–HSA IgG1, HSA/anti–HSA F(ab′)2 immune complex with deleted Fc portion; HSA-IgG1, HSA/anti–HSA F(ab′)2.
Src/Syk-dependent signaling pathways are involved in iIC-induced NET release by human neutrophils. (A) Neutrophils (5 × 10⁶ cells/ml) were incubated with medium, immobilized HSA, IgG, or iICs at 37°C and whole-cell lysates were prepared after 15 min. Whole-cell lysates were separated in 10% SDS-PAGE and Western blots were performed. Phosphorylation of PI3K, Akt, ERK1/2, and p38 MAPK was analyzed by using phospho-PI3K p85 (Tyr458/457), phospho-p44/42 MAPK (ERK1/2, Thr202/Tyr204), phospho-Akt (Thr308), and phospho-p38 MAPK (Thr180/Tyr182) mAbs. Equal loading was shown by reprobing with anti-β-actin Abs. Blots shown are representative of three independent experiments. (B–E) Neutrophils were pretreated for 30 min with 30 µM piceatannol (Syk inhibitor), 10 µM PP2 (Src inhibitor), 25 µM LY29004 (PI3K inhibitor), 10 µM UO126 (ERK inhibitor), 9 µM VIII (Akt inhibitor), 10 µM SB203580 (p38 MAPK inhibitor), or solvent (DMSO 1:1000) before incubation with iICs. ROS production was analyzed by (B) luminol- and (C) lucigenin-amplified chemiluminescence assays (2 × 10⁶ cells/ml in CL medium for 1 h at 37°C). NET release was monitored by (D) Sytox Green assay and visualized by (E) FM after 7 h of incubation with iICs at 37°C (10⁶ cells/ml in NET medium). In (B–E) mean AUC values (means ± SEM from three to five independent experiments) of luminol, lucigenin, and Sytox Green intensities are shown. ***p < 0.01, ****p < 0.0001 as compared with solvent control (DMSO). For FM (E) DNA was stained with Sytox Green and overlay of brightfield and green fluorescence is shown. Scale bars, 20 µm. (F–I) Neutrophils (5 × 10⁶ cells/ml) were preincubated for 30 min with 10 µM PP2 (Src inhibitor), 20 µM DPI (NOX inhibitor), or solvent control (DMSO) prior to 15 min incubation with iICs. Phosphorylation of PI3K (p55), Akt, ERK, and p38 was analyzed by Western blotting of whole-cell lysates. Blots shown are representative of three independent experiments. For quantification the signals of pPI3K, pAkt, pERK, and pp38 were normalized to the β-actin signal detected on the same blot or from the same samples. *p < 0.05, **p < 0.01 as compared with solvent control (DMSO).

In addition to Src and Syk, we identified the PI3K/Akt, ERK1/2, and p38 MAPK pathways to be involved in iIC-induced NETosis. We could show that phosphorylation of PI3K, Akt, ERK1/2, and p38 MAPK results directly from Src family kinases/receptor activation and is not mediated by ROS, as it has been described for p38 MAPK and ERK1/2 upon stimulation with PMA (55). All of these downstream pathways are major signaling pathways used in regulation of neutrophil functions such as chemotaxis, migration, degranulation, and oxidative burst (97–100). The ERK and p38 MAPK pathways are also implicated in ROS-dependent NET formation upon PMA stimulation (20, 55). We could also observe a role for ERK but not for p38 MAPK in PMA-induced ROS and NET production. That we could not detect a diminished NET production upon inhibition of p38 MAPK may be due to our experimental settings. The study describing a role for p38 MAPK was done with immobilized and not suspended neutrophils and another p38 inhibitor was used (55). Moreover, it has been reported by others that the inhibitor we used (SB203580) does not inhibit PMA-induced superoxide production (101, 102). Recently, ERK has been shown to be involved in sIC-induced NET formation mediated by FcyRIIA (11). Because MAPKs, such as ERK1/2 and p38 MAPK, are associated with activation of neutrophils upon FcγR and integrin engagement (93, 103, 104), it is not surprising that we observed a role of these molecules in iIC-induced NETosis. We also identified the PI3K/Akt pathway to play a role in iIC-induced NETosis. This pathway is crucial in the initiation of autophagy (105–107), which has been shown to be required for PMA-induced NET formation (70, 108). Interplay between PI3K signaling, autophagy, and NET formation has been described in acute gout inflammatory arthritis (66) and in sepsis (108). PI3K generates phosphatidylinositol (3,4,5)-trisphosphate, which influences the transcription factor NF-κB (109). NF-κB activation leads to transcription of proinflammatory genes and is relevant in the signaling leading to NET release (110). Moreover, a role for Src family kinases, which are directly associated with receptor activation. However, our results indicate a role of Syk in PMA-induced NETosis. A role for PMA in Syk activation was also described by others. It was shown that PMA induces a protein kinase C–dependent phosphorylation of Syk in human neutrophils (95) and that inhibition of Syk with piceatannol results in a decreased production of intra- and extracellular ROS in response to PMA (96), which is in line with our observations. Thus, we conclude that receptor/Src activation is required for iIC-induced but not PMA-induced NETosis, whereas Syk activation is also involved in PMA-induced ROS-dependent NETosis.
The β3 integrin Mac-1 (CD11b/CD18) but not LFA-1 (CD11a/CD18) is involved in iIC-induced NET release. Human neutrophils were pretreated for 30 min at 37°C with 20 μg/ml blocking Abs specific to CD11a, CD11b, CD18, CD11b plus CD18 (Mac-1), CD11a plus CD18 (LFA-1), isotype Abs, or left untreated (medium) before incubation with iICs. (A–E) Mac-1 blocking studies (n = 3) and (F–I) LFA-1 blocking studies (n = 5). ROS production was analyzed by (A and F) luminol- and (B and G) lucigenin-amplified chemiluminescence assays (2 × 10^6 cells/ml in CL medium for 1 h at 37°C). Fluorescence intensities of NET-bound Sytox Green were detected during a period of 7 h at 37°C from 10^6 neutrophils/ml in NET medium for 1 h at 37°C. Images were taken by using an EM505. Scale bar, 5 μm.

FIGURE 7. The β3 integrin Mac-1 (CD11b/CD18) but not LFA-1 (CD11a/CD18) is involved in iIC-induced NET release. Human neutrophils were pretreated for 30 min at 37°C with 20 μg/ml blocking Abs specific to CD11a, CD11b, CD18, CD11b plus CD18 (Mac-1), CD11a plus CD18 (LFA-1), isotype Abs, or left untreated (medium) before incubation with iICs. (A–E) Mac-1 blocking studies (n = 3) and (F–I) LFA-1 blocking studies (n = 5). ROS production was analyzed by (A and F) luminol- and (B and G) lucigenin-amplified chemiluminescence assays (2 × 10^6 cells/ml in CL medium for 1 h at 37°C). Fluorescence intensities of NET-bound Sytox Green were detected during a period of 7 h at 37°C from 10^6 neutrophils/ml in NET medium. (C and H) Representative real-time kinetics and (D and I) normalized mean AUC values (means ± SEM, data normalized to untreated iIC control) of Sytox Green intensities are shown. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with isotype control. (E) Representative EM images of untreated, CD11b-blocked, CD18-blocked, or CD11b plus CD18 (Mac-1)-blocked neutrophils incubated on iICs are shown. Cells (10^6/ml in NET medium) were fixed after 7 h incubation on iICs at 37°C. Images were taken by using an EM505. Scale bar, 5 μm.

because ROX and NETs play a pathophysiological role in several autoimmune diseases (31, 115), inhibition of iIC-induced NET release could result in beneficial therapeutic effects in neutrophil-mediated autoimmune diseases. Because iIC-induced NETosis depends on ROS, any molecule that inhibits generation of ROS or scavenges ROS could be a possible therapeutic agent. We could show that inhibitors of NOX and MPO as well as antioxidants, such as 5-ASA and vitamin C, which act as scavengers against superoxide, hydroxyl radicals, and hypochlorite (48, 52), are able to reduce iIC-induced ROS and NETosis. Thus, these substances may mediate a therapeutic effect in autoimmune diseases. This assumption is supported by studies in which treatment of RA neutrophils with the antioxidant N-acetyl-l-cysteine led to reduced NETosis (34) or in which adjuvant treatment of patients with anti-neutrophil cytoplasmic Ab–associated vasculitis with vitamin C led to reduced superoxide production by neutrophils (116). However, in addition to ROS, targeting of one of the intracellular signaling pathways involved in iIC-induced NETosis (Syk, PI3K/Akt, ERK, p38 MAPK) may be considered as a therapeutic strategy.

In this study, we demonstrate for the first time, to our knowledge, that iICs induce ROS-dependent NETosis of human neutrophils, which is enhanced in the presence of the proinflammatory mediators TNF-α and IL-1β. This indicates that an (auto)inflammatory milieu, characterized by iICs and proinflammatory cytokines, is highly potent regarding the induction of NETs in the absence of microbial stimuli. Our results implicate that FcyRIIIB and Mac-1 engagement, signaling throughout the Src/Syk pathway, and ROS are crucial for the formation of NETs in response to iICs. Additionally, the PI3K/Akt, ERK1/2, and p38 MAPK pathways are involved in the intracellular signaling leading to NET formation. Thus, as compared with sICs, iICs use distinct activation mechanisms to induce NET release from human neutrophils. This demonstrates that the molecular mechanism of NET production is a multifactorial and stimulus-dependent process and thus disease specific.

Because ROI and NETs play a pathophysiological role in several autoimmune diseases (31, 115), inhibition of iIC-induced NET release could result in beneficial therapeutic effects in neutrophil-mediated autoimmune diseases. Because iIC-induced NETosis depends on ROI, any molecule that inhibits generation of ROI or scavenges ROI could be a possible therapeutic agent. We could show that inhibitors of NOX and MPO as well as antioxidants, such as 5-ASA and vitamin C, which act as scavengers against superoxide, hydroxyl radicals, and hypochlorite (48, 52), are able to reduce iIC-induced ROI and NETosis. Thus, these substances may mediate a therapeutic effect in autoimmune diseases. This assumption is supported by studies in which treatment of RA neutrophils with the antioxidant N-acetyl-l-cysteine led to reduced NETosis (34) or in which adjuvant treatment of patients with anti-neutrophil cytoplasmic Ab–associated vasculitis with vitamin C led to reduced superoxide production by neutrophils (116). However, in addition to ROI, targeting of one of the intracellular signaling pathways involved in iIC-induced NETosis (Syk, PI3K/Akt, ERK, p38 MAPK) may be considered as a therapeutic strategy.

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References
IMMUNIZED IMMUNE COMPLEXES INDUCE THE FORMATION OF NETS


