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NADPH Oxidase–Independent Formation of Extracellular DNA Traps by Basophils

Mahbubul Morshed,* Ruslan Hlushchuk, † Dagmar Simon, ‡ Andrew F. Walls, § Kazushige Obata-Ninomiya, ¶ Hajime Karasuyama, † Valentin Djonov, † Alexander Eggel, # Thomas Kaufmann, * Hans-Uwe Simon,* and Shida Yousefi*

Basophils are primarily associated with a proinflammatory and immunoregulatory role in allergic diseases and parasitic infections. Recent studies have shown that basophils can also bind various bacteria both in the presence and the absence of opsonizing Abs. In this report, we show that both human and mouse basophils are able to produce mitochondrial reactive oxygen species and to form extracellular DNA traps upon IL-3 priming and subsequent activation of the complement factor 5 a receptor or FceRI. Such basophil extracellular traps (BETs) contain mitochondrial, but not nuclear DNA, as well as the granule proteins basogranulin and mouse mast cell protease 8. BET formation occurs despite the absence of any functional NADPH oxidase in basophils. BETs can be found in both human and mouse inflamed tissues, suggesting that they also play a role under in vivo inflammatory conditions. Taken together, these findings suggest that basophils exert direct innate immune effector functions in the extracellular space. The Journal of Immunology, 2014, 192: 5314–5323.

B asophils make up <0.5% of all peripheral blood leukocytes but share with mast cells the unique capacity to synthesize various hallmark mediators of allergic inflammation and to express high-affinity IgE receptors (FceRI) (1). Aggregation of FceRI bound to IgE by multivalent Ag leads to basophil activation, granule exocytosis, and mediator release. Besides functioning as effector cells, basophils also may play an important role in the initiation of an inflammatory response (e.g., by recruiting other inflammatory cells) (2, 3). Basophils are also a prime source for the Th2 cytokines IL-4 and IL-13 (1). The phenotype and functions of human basophils are tightly regulated by IL-3, a cytokine produced by various cell types at sites of allergic inflammation. IL-3 is not only an important activation factor for basophils, it is also important for their differentiation and survival (4). In addition, IL-3 primes basophils to produce lipid mediators, such as leukotriene C4 (LTC4) in an allergen-independent manner in response to stimulation with complement factor 5 (C5a) (4). It also has been shown that thymic stromal lymphopoietin (TSLP) can act as a key regulator of basophil hematopoiesis, independent of IL-3 (5).

A role for basophils in innate immunity is suggested by their expression of the functional TLRs 2 and 4 as well as by the possibility of activating these cells by multiple proteases, including Der p1 and hookworm, in a non–IgE-dependent manner (6, 7). Basophils also can recognize microbes in an IgE-dependent manner (8) and release antibacterial factors, such as β-defensin and cathelicidin (9). Previously published work has demonstrated that both neutrophils and eosinophils are capable of killing bacteria extracellularly by forming DNA traps, so-called neutrophil extracellular traps (NETs) and eosinophil extracellular traps (EETs) (10, 11). In this study, we have investigated whether basophils also can form extracellular DNA traps.

Materials and Methods

Reagents

Recombinant human IL-3, rat IgG2a, rabbit IgG, as well as recombinant human eosin and thymic stromal lymphopoietin (TSLP) were from R&D Systems Europe (Abingdon, U.K.). GM-CSF was provided by Novartis Pharma (Nürnberg, Germany). Human and mouse C5a were purchased from Hycult Biotech (Uden, The Netherlands). LPS, dihydrorhodamine-123 (DHR), NBT, and sMLF, as well as Luria–Bertani and BHI media were obtained from Sigma-Aldrich (Buchs, Switzerland). Lipoteichoic acid (LTA) was a gift from Dr. T. Hartung (University of Konstanz, Konstanz, Germany). Platelet-activating factor (PAF) was from Millipore (Zug, Switzerland). Diphenylene iodonium chloride (DPI) and PMA were from Calbiochem (Darmstadt, Germany). Mitochonidine (MitoQ) was from Biotrend Chemicals (Zurich, Switzerland). Propidium iodide (PI) was from Calbiochem (Merck, Darmstadt, Germany). Mitochonidine (MitoQ) was from Biotrend Chemicals (Zurich, Switzerland). Propidium iodide (PI), Hoechst 33342, SYTO13, MitoSOX Red, mounting medium containing DAPI, as well as calcine-AM and ethidium homodimer-1 (EthD-1), HBSS, and RPMI 1640 medium were from Invitrogen (Paisley, U.K.). X-VIVO 15 medium was from Lonza (Verviers, Belgium). Anti-FceRI Ab (CRA1) was from Miltenyi Biotec (Bergisch Gladbach, Germany). APC-conjugated anti-human FceRIα (CRA1) and FITC-conjugated anti-human...
IgE (clone IgE21) Abs were from eBioscience (Vienna, Austria). FITC-conjugated anti-mouse FcεRIα (clone MAR-1), rat anti-mouse mast cell protease 8 (mMCP-8, TUG8), APC-conjugated anti-mouse e-sik (CD117, clone 2B8), APC-conjugated anti-mouse Gr-1 (Ly-6G/Ly-6C, clone RB6-8C5), and APC-conjugated anti-goat rat Abs were from BioLegend (Luzern-Chem, Luzern, Switzerland). Anti-rabbit matrix metallopeptidase 9 (MMP-9) Ab was purchased from Abcam (Cambridge, U.K.). Anti-mouse gp91phox (clone 53; gp91[phox]), APC-conjugated anti-human CCRI (CD193, clone 16A9), and FITC-conjugated secondary Abs (CD123, clone 55F10) were obtained from BD Biosciences (Allschwil, Switzerland). Anti-human histone Ab recognizing the histones H1, H2A, H2B, H3, and H4 was from Millipore (clone H11-4). Polyspecific human IgG was a gift from CSL Behring (Bern, Switzerland).

Cells
Written informed consent was obtained from all blood donors, and the Ethics Committee of the Canton of Bern approved this study. Human basophils were purified from healthy donors and atopic patients by negative selection from peripheral blood using the EasySep human basophil enrichment kit (StemCell Technologies, Grenoble, France). Unwanted cells were targeted for removal by tetramer Ab complexes recognizing CD2, CD3, CD14, CD15, CD16, CD19, CD24, CD34, CD36, CD45RA, CD56, glycoprotein A, and dextran-coated magnetic particles. Purity of the isolated basophils was >95% as assessed by Diff-Quik (Medican, Duedingen, Switzerland) staining and light microscopy as well as flow cytometry (Supplemental Fig. 1A).

Human blood neutrophils were purified from healthy individuals by Ficoll–Hypaque centrifugation (12). Briefly, PBMC were separated by centrifugation on Ficoll–Hypaque (Seromed–Fakola, Basel, Switzerland). The lower layer consisting mainly of granulocytes and erythrocytes was treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA [pH 7.3]). The resulting cell populations contained >95% neutrophils as assessed by Diff-Quik staining and light microscopy.

Mouse basophils were generated from IL-3–dependent, conditional Hoxb8-immortalized myeloid progenitors derived from wild-type and NADPH oxidase NOX2-deficient mice (13). NOX2-deficient mice were purchased from The Jackson Laboratory and provided by Dr. K.-H. Krause (Geneva, Switzerland). A total of 2 × 10⁶ cells/ml were passaged in RPMI 1640/GlutaMAX with 10% FCS, 100 U/ml penicillin/100 µg/ml streptomycin, 50 µM 2-ME, mouse IL-3 (added as 10% of WEHI-3B cell-conditioned medium as a source for IL-3), and 100 nM 4-hydroxytamoxifen for 3–4 d. To initiate differentiation into basophils, 2.5 × 10⁶ cells/ml were washed and then cultured in the same medium in the absence of 4-hydroxytamoxifen. At day 6, cells were washed twice with PBS and used for subsequent experiments. Mouse Hoxb8 neutrophils were generated as described previously (13, 14). Hoxb8 basophils and Hoxb8 neutrophils were characterized by flow cytometry (Supplemental Fig. 1B).

Activation of basophils
Cells were seeded on 12-mm glass coverslips in X-VIVO 15 medium (Lonza, Walkersville, MD), primed with 10 ng/ml recombinant human IL-3 for 15 min, and subsequently stimulated with 10 µg/ml anti-FcεRIα Ab or 10 nM CsA for 30 min. Other stimuli were used as follows: 100 nM IL-1α, 20 ng/ml TSLP, 100 nM eosin, 100 nM PAF, and 0.1 mM EDTA [pH 7.3]. The resulting cell populations contained >95% neutrophils as assessed by Diff-Quik staining and light microscopy.

Mouse basophils were generated from IL-3–dependent, conditional Hoxb8-immortalized myeloid progenitors derived from wild-type and NADPH oxidase NOX2-deficient mice (13). NOX2-deficient mice were purchased from The Jackson Laboratory and provided by Dr. K.-H. Krause (Geneva, Switzerland). A total of 2 × 10⁶ cells/ml were passaged in RPMI 1640/GlutaMAX with 10% FCS, 100 U/ml penicillin/100 µg/ml streptomycin, 50 µM 2-ME, mouse IL-3 (added as 10% of WEHI-3B cell-conditioned medium as a source for IL-3), and 100 nM 4-hydroxytamoxifen for 3–4 d. To initiate differentiation into basophils, 2.5 × 10⁶ cells/ml were washed and then cultured in the same medium in the absence of 4-hydroxytamoxifen. At day 6, cells were washed twice with PBS and used for subsequent experiments. Mouse Hoxb8 neutrophils were generated as described previously (13, 14). Hoxb8 basophils and Hoxb8 neutrophils were characterized by flow cytometry (Supplemental Fig. 1B).

Confocal laser scanning microscopy
Cells were either fixed with 4% formaldehyde, freshly prepared from paraformaldehyde in PBS, or observed live using confocal laser scanning microscopy. The following dyes were used for DNA detection: 0.5 µg/ml PI, 0.5 µM SYTO13, 5 µM MitoSOX Red, and 1 µM Hoechst 33342 (all Invitrogen, Paisley, U.K.). Possible colocalization of granule and nuclear proteins with extracellular DNA was determined using immunofluorescence staining with mouse anti-human basogranulin (BB1; 1:100) (15), rat anti-mouse TUG8 (mMCP-8; 1:100), and anti-human histone (clone H11-4; 1:10,000) Abs. Mitochondrial DNA (mtDNA) was visualized by MitoSOX Red (Invitrogen), added 10 min before fixation (16). Additional immunofluorescence analyses with the same Abs were performed on 5-µm formaldehyde-fixed and paraffin-embedded human and mouse skin sections, respectively, using appropriate secondary Abs labeled with Alexa Fluor 488 (Invitrogen). After washing with PBS, the specimens were mounted in a drop of fluorescence mounting medium (DakoCytomation, Glostrup, Denmark). Slides were analyzed by confocal laser scanning microscopy (LSM 510; Carl Zeiss Micro Imaging, Jena, Germany).

Electron microscopy
For transmission electron microscopy, freshly isolated and activated basophils were fixed in 2.5% (v/v) glutaraldehyde solution buffered with sodium cacodylate (pH 7.4, 540 mM)ostin, prefixed in 1% OsO₄ (buffered with 0.1 M sodium cacodylate [pH 7.4, 340 mM]ostin), dehydrated in ethanol, and embedded in epoxy resin. Thick sections (70–80 nm) were prepared and mounted on copper grids coated with Formvar (polyvinyl formal; Fluka, Buchs, Switzerland). Cells were then stained with lead citrate and uranyl acetate prior to viewing in a Philips EM-400 transmission electron microscope.

For scanning electron microscopy, cells were fixed and processed on the coverslips at all times. The fixative was 2.5% (v/v) glutaraldehyde solution buffered with sodium cacodylate (pH 7.4, 540 mM)ostin. After fixation, cells were postfixed in 1% OsO₄ (buffered with 0.1 M sodium cacodylate [pH 7.4, 340 mM]ostin), dehydrated in ethanol, and underwent critical-point drying. The coverslips were glued onto aluminum stubs, sputtered with gold and examined in a Philips XL-30 FESEM scanning electron microscope.

Determination of cell death
Cell death of activated basophils was assessed by uptake of 450 nM calcein-AM and 450 nM EthD-1 (both from Invitrogen) using live cell imaging techniques under confocal laser scanning microscopy. To analyze basophil viability over longer periods of time, cells were cultured for the indicated times, and cell death was assessed by uptake of ethidium bromide and flow cytometric analysis (11, 16). PMA (50 nM, 100 nM, and 10 µM) was used as a positive control.

Reactive oxygen species measurements
Cells were seeded in clear glass bottom, black wall 96-well microplates (Greiner Bio-One, Frickenhausen, Germany), primed with IL-3 for 15 min, and subsequently activated with CsA or anti-FcεRIα Ab for 30 min or for the indicated times. During cell activation, incubations were with 1 µM DHR (Sigma-Aldrich) or with 5 µM MitoSOX Red. A total of 50 nM PMA was used as control. To block ROS generation, the NADPH oxidase inhibitor DPI (5 µM, except in concentration-dependence experiments) and the flavoprotein inhibitor MitoQ (1 µM) were added to basophils 10 min prior to stimulation. ROS generation was measured at different time points using a SpectraMax M2 plate reader (Bucher Biotec, Zurich, Switzerland) at 488 nm excitation and 530 nm emission for DHR and 510 nM excitation and 580 nM emission for MitoSOX Red (17, 18).

NBT assay
To directly analyze NADPH oxidase activity, granulocytes were seeded on glass coverslips, and the NBT assay was performed as described previously (19). Briefly, cells were stimulated as described above and 0.5 mg/ml NBT was added during the last 20 min of activation. Cells were then washed with PBS and stained with Diff-Quik to label the nuclei. The conversion of NBT to insoluble dark purple formazan deposits was acquired by bright-field microscopy. To determine whether formazan deposits are associated with DNA release by single cells, 5 µM MitoSOX Red was added during the last 10 min of activation. After cell fixation with 4% formaldehyde, 1 µg/ml Hoechst 33342 was added, and samples were mounted on glass coverslips. Fluorescence images are shown next to the corresponding Nomarski images in which black spots represent formazan deposits.

Immunoblotting
Electrophoresis was conducted in Run Blue 4–20% SDS polyacrylamide gels (Invitrogen, Cambridgeshire, U.K.), and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were incubated with blocking solution (PBS containing 0.1% Tween 20 and 5% nonfat dry milk) overnight at 4°C. The membranes were then incubated with anti-mouse gp91phox Ab (1:1000), in blocking solution overnight at 4°C. As a loading control, stripped filters were incubated with anti-GAPDH mAb (1:1000; Millipore). Filters were washed in PBS–Tween (1%) for 30 min and then incubated for 1 h at room temperature. The blots were developed by an ECL technique (ECL kit; GE Healthcare), according to the manufacturer’s instructions.
Flow cytometry
The purity of human basophil preparations was controlled by expression analysis of lineage-associated markers using anti-FcεRⅠx, anti-IgE, anti-IL-3Rα, and anti-CCR3 Abs. For cell surface expression analysis of Hoxb8 basophils and Hoxb8 neutrophils, anti-c-kit, anti-FcεRⅠx, anti-Gr-1 (Ly-6G/Ly-6C), and appropriate isotype-matched control Abs were used. Intracellular staining of these cells was performed according to the BD cell fix and permeabilization kit protocol (BD Biosciences). Anti-TUG8, anti-MMP-9, or isotype-matched control Abs were added. Cells were incubated, followed by staining with appropriate secondary Abs (both 1 h, 4°C). Cells were washed twice and analyzed by flow cytometry (FACSCalibur) and quantitated using FlowJo software (Ashland, OR).

Nipponstrangylus brasiliensis infection
Third-stage larvae (L3) of *N. brasiliensis* were washed in PBS (37°C) and injected intramuscularly (i.m.) (500–2,000 larvae) into mice. After 18 d, one half of the mice received a second i.d. inoculation of *N. brasiliensis*, and were sacrificed 2 d later; the other half were sacrificed 18 d after the first inoculation without the second inoculation (20). Skin tissues were removed, fixed with 4% formaldehyde, and embedded in paraffin. Subsequently, sections were prepared for staining analysis. Skin tissues from three individual mice were analyzed for each group.

Quantitative PCR
To remove the extracellular DNA, stimulated cells were briefly vortexed, centrifuged at 1400 rpm for 2 min, and the cellular DNA then isolated with the genomic DNA extraction kit from Macherey–Nagel (Düren, Germany). Quantitative PCR (qPCR) assays were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories). Primers were as follows: for human mitochondrial cytochrome oxidase 1 (*COX1*) (5′-GCC CCC GAT ATG GCG TTG TTC CC-3′ and 5′-GTT GAA CAA CCT GCT GGT CC-3′), for mouse mitochondrial cytochrome oxidase 1 (*COX1*) (5′-GCC CCA GATATA GAA TCC CTG CC-3′ and 5′-GTT CAT CCT GCT GGT CC-3′), for human 18S rDNA (5′-ATC GAA ATG TTC CAG CA-3′ and 5′-GCT TAT GTC TTC ACA ACC CA-3′), and for mouse 18S rDNA (5′-ACC GAG TAC TGG CAG CAG TTA TTT-3′ and 5′-GGG ATT GGT GCA TTC CAT ATG-3′). Primers were synthesized by Microsynth (Balgach, Switzerland). Duplicate real-time PCRs were performed in the iQ5 Multicolor Real-time PCR Detection System (Bio-Rad) with 25 ng extracted genomic DNA and 200 nM of each primer in 25 μl final PCR mix. Cycling variables were 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 55°C.

RT-PCR
For the NOX and phox family of proteins, RT-PCR was performed as described previously (22). Reverse transcription of 2 μg RNA was performed using the Superscript II kit, according to the manufacturer's instructions (Invitrogen). PCR was then performed using Taq DNA Polymerase (Roche, Mannheim, Germany). Primers were as follows: NOX1 (5′-TTA ACA CCA GGC TGA TCC TG-3′ and 5′-CAC TCC ATG AGC ACC AA-3′) (353 bp), NOX2 (5′-AAA TGG TGG CAT GGA TGA TT-3′ and 5′-GGA ATT GGT GCA TTC CAT ATG-3′) (475 bp), NOX3 (5′-CCG CCA GAG CAC TAC ATC TTG GT-3′ and 5′-CCG TGG TGC TTC CAG GGA GAG GAG TA-3′) (432 bp), NOX4 (5′-AAC CCA AGG ACC GTG CAT AA-3′ and 5′-CCC AAA TGT TTC GGC TGT TT-3′) (417 bp), NOX5 (5′-TAC CTC CTC GTG TGG CCT CT-3′ and 5′-GCT CAG GAA AAG GCC TCG TG-3′) (553 bp), p22phox (5′-GTT TGT GGT CCT GCT GGA GT-3′ and 5′-TGG GGC GCT TGT GGA TT-3′) (298 bp), p47phox (5′-GCC AGC CAG CAC TAT GTG TA-3′ and 5′-CCG AGG TCT CCT CAG GTG CG-3′) (453 bp), p67phox (5′-CGA GGC AAC CAG CAG ATG GA-3′ and 5′-CAT GGG AAC ACT GAG CTT CA-3′) (608 bp), and GAPDH (5′-CCC CCT TCT TGA CAG ACC-3′ and 5′-GAG TCC TTC GAT GAC ACC-3′) (300 bp).

Statistical analysis
Data are presented as mean levels ± SEM. Statistical analysis was performed by two-tailed Student t or ANOVA with Bonferroni’s multiple comparison tests. All statistical analysis was done using Graph Pad Prism 5 software (La Jolla, CA). A p value < 0.05 was considered statistically significant.

Results
Both IgE-dependent and -independent stimulations of human basophils result in extracellular trap formation
Activation of IL-3–primed human basophils through cross-linking of high-affinity IgE receptors resulted in the formation of extracellular DNA nets in a concentration-dependent manner (Fig. 1A, left panel). Basophils primed with IL-3 only, or alternatively, stimulated with anti-FcεRⅠx Ab in the absence of IL-3 priming showed no DNA release (Fig. 1A, right panels). Similarly, we observed DNA release from IL-3–primed basophils following stimulation with C5a, eotaxin, or TSLP but not in the absence of IL-3 priming (Fig. 1B, left panel). Quantitative analysis revealed that 60–80% of the basophils demonstrated evidence for DNA release and no differences in efficacy were observed between anti-FcεRⅠx Ab, C5a, eotaxin, and TSLP when optimal concentrations were used (Fig. 1B, left panel). Interestingly, stimulation with fMLF, PAF, LPS, and LTA also was followed by the formation of extracellular net-like DNA-containing structures that, however, did not require IL-3 priming (Fig. 1B). DNase treatment removed the extracellular structures, indicating that they indeed contain DNA (Supplemental Fig. 2A).

To further visualize the DNA released by human basophils, we applied electron microscopy. Using transmission electron microscopy, we observed multiple mitochondria in basophils (Fig. 1C, upper panels). Upon activation of high-affinity IgE receptors, mitochondria and granules translocated toward the leading edge of the cells and nuclei remained intact during this process (Fig. 1C, lower panels). Moreover, when analyzed by scanning electron microscopy, such IL-3 primed and IgE receptor–activated basophils showed evidence of DNA release, which was not seen in control Ab treated cells (Fig. 1D).

Similar to NETs and EETs (10, 11), we expected colocalization of the extracellular DNA with granule proteins following basophil activation. We stained basophils with anti-basogranulin Ab (23, 24) and PI. Using confocal microscopy, we could demonstrate that basogranulin, a granule protein specifically expressed by basophils, is released following activation of these cells, colocalizing with the extracellular DNA (Fig. 1E, upper panels). In contrast, histones were observed within the nucleus but not in association with the extracellular DNA (Fig. 1E, lower panels). Taken together, these data strongly suggest that activated human basophils are able to form extracellular traps in vitro and that they contain DNA and basogranulin and, perhaps, additional proteins.

Human basophils release mtDNA in a cell death–independent manner
There have been conflicting reports regarding the source of DNA used for extracellular trap formation and whether cell death of neutrophils and eosinophils accompanies trap formation (16, 25). To determine the source of released DNA, we performed quantitative PCR experiments on cellular DNA. We amplified *COX1* as a unique mitochondrial gene and 18S rDNA as a nuclear encoded gene (21). The qPCR data revealed that after IL-3 priming and subsequent activation of IgE or C5a receptors, the mtDNA but not the nuclear DNA (nDNA) content of basophils is reduced (Fig. 2A). In contrast, GM-CSF priming and activation of human neutrophils was not associated with a reduction of the mtDNA content, at least not within a 45-min time period (Supplemental Fig. 2B). Similar data also were observed using mouse neutrophils (Supplemental Fig. 2C).

A possible role for cell death in DNA release was investigated by time-lapse video microscopy following calcine-AM and EthD-1 staining as well as analyzing ethidium bromide uptake by flow cytometry. None of the physiological stimuli causing mtDNA release led to an EthD-1 and ethidium bromide uptake, respectively, at least not within a time period of 48 h (Fig. 2B). Such uptake, however, was observed following exposure to high concentrations...
of PMA (10 μM) (Fig. 2B). In fact, PMA led to ~50% cell death within 45 min. Lower PMA concentrations (100 nM), however, did not induce basophil death within 45 min, but only at later time points, and IL-3 could, at least partially, protect basophils from PMA-induced cell death (Fig. 2B, lower right panel).

**FIGURE 1.** Release of DNA from human basophils following physiological stimulation. (A) Confocal microscopy. DNA release after anti-FceRIα stimulation requires IL-3 priming and is concentration dependent (n = 3). Right, Representative original data. Original magnification ×630. Bars, 10 μm. (B) Confocal microscopy. DNA release following non-IgE-dependent stimulation in the presence and absence of IL-3 priming (n = 4–7). Right, Representative original data. Original magnification ×630. Scale bars, 10 μm. (C) Transmission electron microscopy. Upper panels, Freshly isolated human blood basophils contain multiple mitochondria (arrowheads). Lower panels, Following IgE receptor activation of human basophils, granules and mitochondria are found in close proximity, near the plasma membrane. The nucleus is completely intact. (D) Scanning electron microscopy. DNA fibers (arrowheads) are visualized in IgE receptor–activated basophils. (E) Confocal microscopy. Upper panels, C5a-activated IL-3–primed basophils release DNA together with basogranulin (arrows). Histones, however, are clearly seen in the nucleus. Representative images of three independent experiments are shown. Original magnification ×630. Scale bars, 10 μm.

Human basophils release mtDNA in a ROS-dependent manner

ROS have been shown to be essential for both NET and EET formation (11, 17, 25). Therefore, we investigated whether basophil extracellular trap (BET) formation is also ROS dependent. As with neutrophils and eosinophils, DPI completely blocked the
release of DNA from activated human basophils (Fig. 3A), suggesting that ROS are also required for BET formation. We, therefore, measured ROS generation following activation of basophils in the presence and absence of DPI. Basophils primed with IL-3 and subsequently activated through high-affinity IgE or C5a receptors showed increased ROS production compared with unstimulated or DPI-treated cells (Fig. 3B). The blocking effect of DPI on ROS production by basophils was concentration dependent. Complete inhibition of activation-induced ROS production consistently was observed at 5 μM DPI, a concentration that was used in all subsequent experiments (Supplemental Fig. 3A, 3B). Taken together, these data suggest that basophils are able to generate ROS following their activation and subsequently allow BET formation.

In contrast to neutrophils and eosinophils, basophils have been reported to lack NADPH oxidase activity (26, 27). DPI is widely used as a general inhibitor of ROS production and blocks not only NADPH oxidase activity, but also the enzymatic activity of several flavoproteins that are part of the mitochondrial oxidation pathway (28). We analyzed mRNA expression of the NADPH oxidase (NOX) family of proteins as well as p22phox, p47phox, and p67phox.

FIGURE 2. BETs contain mtDNA and are formed by viable human basophils. (A) qPCR. Human basophils were activated as indicated and cellular DNA extracted for analysis (n = 3). ***p < 0.001. For comparison, we performed the same experiments in human neutrophils (see Supplemental Fig. 2B). (B) Time-lapse microscopy and flow cytometry. No effect on human basophils viability was observed following short-term (45 min) and long-term (48 h) stimulation with the indicated agonists. Only high concentrations of PMA (10 μM) caused uptake of EthD-1 within 45 min. Representative images of three independent experiments are shown. Original magnification ×630. Scale bars, 10 μm. Flow cytometric viability data are presented as means ± SEM (n = 3).
Basophils did not express two essential components of NADPH cytoplasmic ROS production pathway, namely p47phox and p67phox (Fig. 3C).

We also measured the enzymatic activity of the NADPH oxidase in basophils and neutrophils by NBT assay and observed intracellular dark precipitates indicating respiratory burst activity in activated neutrophils, but not basophils. Even stimulation with PMA did not induce any detectable respiratory burst activity (Fig. 3D). To directly demonstrate the release of DNA in the absence of any detectable respiratory burst activity, we combined the NBT assay with cellular DNA staining using the fluorescent dyes MitoSOX Red and Hoechst 33342. Combining confocal laser scanning and Nomarski (DIC) microscopy allowed the visualization of activated basophils releasing DNA in the absence of any detectable respiratory burst activity. Original magnification ×630. Scale bars, 10 μm. (E) Mitochondrial ROS production that can be blocked by MitoQ is increased in IL-3–primed basophils following high-affinity IgE-receptor and C5a receptor activation (n = 3). *p < 0.05, **p < 0.01. (F) Confocal microscopy. Thirty-minute preincubation with MitoQ completely blocked DNA release from basophils. Representative images of three independent experiments are shown. Original magnification ×630. Scale bars, 10 μm.

**FIGURE 3.** Human basophil extracellular DNA trap release is dependent on mitochondrial ROS. (A) Confocal microscopy. Preincubation with DPI completely blocked DNA release from basophils. Representative images of three independent experiments are shown. Original magnification ×630. Bars, 10 μm. (B) Total ROS production is increased in IL-3–primed basophils following high-affinity IgE-receptor or C5a receptor activation. Increases in ROS can be blocked by DPI (n = 4). (C) RT-PCR. Although basophils express NOX2, the p47phox and p67phox subunits are missing. Freshly isolated human basophils and neutrophils served as sources for mRNA preparation. (D) NBT assay. Purified cells were allowed to adhere to glass coverslips and activated as indicated. Slides were counterstained with Diff-Quik stain. Although activated neutrophils demonstrate dark formazan deposits (arrows), activated basophils from the same donor show no evidence for respiratory burst activity. Original magnification ×630. Scale bars, 10 μm. (E) Mitochondrial ROS production that can be blocked by MitoQ is increased in IL-3–primed basophils following high-affinity IgE-receptor and C5a receptor activation (n = 3). *p < 0.05, **p < 0.01. (F) Confocal microscopy. Thirty-minute preincubation with MitoQ completely blocked DNA release from basophils. Representative images of three independent experiments are shown. Original magnification ×630. Scale bars, 10 μm.

BETs are present in human skin undergoing an inflammatory response

We investigated whether BETs can be detected in skin tissues as a possible consequence of allergic, autoimmune, or other inflammatory conditions. We first counted basophil numbers using anti-basogranulin immunofluorescence analysis. Bullous pemphigoid, eosinophilic folliculitis, and Wells’ syndrome skin biopsies showed higher basophil numbers within inflammatory infiltrates compared with atopic dermatitis or urticarial skin lesions (Fig. 4A). In these tissues, we subsequently searched for BETs. Extracellular DNA was detected in association with basogranulin in specimens of bullous pemphigoid and Wells’ syndrome, but also in atopic dermatitis, urticaria, and eosinophilic folliculitis. The fraction of DNA + basogranulin - releasing basophils was usually found to be 15 and 40% (Fig. 4B, left panel). Similar to EETs in these diseases (31), the long, thin DNA structures often seemed to connect the releasing basophils with other cells, including other...
basophils (Fig. 4B, right panels). In normal skin, we identified a few basophils, but no BET formation.

**Activated mouse basophils also form extracellular traps in vitro and in vivo**

We used conditional mouse Hoxb8-immortalized progenitor cells to generate large amounts of mouse basophils in vitro (13). For comparison, we established another Hoxb8-immortalized progenitor cell line to produce large numbers of mouse neutrophils as previously described (13, 14). Upon differentiation, we characterized the immunophenotype of both Hoxb8 basophils and Hoxb8 neutrophils by flow cytometry. Mature mouse basophils, but not neutrophils, expressed the FcεRI (FcεR1α) as well as TUG8 (mMCP-8) (Supplemental Fig. 1B). In contrast, neutrophils, but not basophils, expressed Gr-1 (Ly-6G/Ly-6C) (Supplemental Fig. 1B). In contrast, mouse basophils, like human basophils, did not show any respiratory burst activity upon activation (Supplemental Fig. 4B). Whereas the lack of NADPH oxidase completely prevented DNA release from activated neutrophils, genetic lack of NOX2 did not prevent either IgE-dependent or -independent DNA release from basophils (Fig. 5D). We also demonstrated the release of DNA in mouse basophils lacking any respiratory burst activity, a finding in contrast to the result with mouse neutrophils (Supplemental Fig. 4C). Taken together, these data confirm our results with human basophils suggesting that BET formation occurs independent of NADPH oxidase–derived ROS.

To explore whether mouse basophils can also form extracellular traps under in vivo conditions, we used an infection model with *N. brasiliensis*, which is known to induce basophil infiltration (20). All mice received an i.d. inoculation with *N. brasiliensis*. One set of mice received a second inoculation and were sacrificed 2 d later. The other set was left without a second inoculation. We stained the skin tissues with PI and anti-TUG8 Ab, and observed strong basophil infiltration in tissues that received two i.d. inoculations with *N. brasiliensis* compared with the tissues that were infected one time only (Fig. 6A). Moreover, following two helminth infections, >30% of the infiltrating basophils demonstrated evidence for the formation of BETs, which, furthermore, showed colocalization of DNA with mMCP-8 (Fig. 6B).

**Discussion**

Extracellular DNA traps generated by neutrophils and eosinophils have been implicated in innate immune defense mechanisms against pathogens (10, 11, 17, 32, 33). Our study demonstrates for the first time, to our knowledge, that physiological activation of human and mouse basophils can also lead to the rapid formation of extracellular DNA traps, both in vitro and in vivo. We identified cytokine, IgE, Toll-like, chemokine, and lipid mediator receptors that, upon activation, induced BET formation within minutes. Some of the receptor-mediated signaling mechanisms appeared to be effective in IL-3 primed basophils only, others required no priming. Because BETs appear to contain basophil granule proteins, it seems unsurprising that most physiological agonists able to trigger BET formation are known triggers of basophil degranulation (15, 34–36).

Using microscopic (staining of mtDNA, live cell imaging) and molecular biological techniques (PCR amplification of mitochondrial and nuclear genes, in situ hybridization), we have previously shown that both viable neutrophils and eosinophils released mtDNA rather than nDNA (11, 16, 17). In this paper, we show by electron microscopy that basophils contain mitochondria that, upon cell activation, accumulate at the leading edge in close proximity to the plasma membrane in the absence of any visible nuclear DNA involvement. Moreover, we observed the projection
of thin DNA fibers of activated basophils. The exact molecular mechanisms responsible for the generation of these DNA fibers remain to be investigated.

To compare the amounts of cellular mtDNA and nDNA before and after activation, we applied a quantitative PCR method. We detected a significant depletion of mtDNA, but not of nDNA, within

FIGURE 5. Activated mouse basophils form functional extracellular traps in vitro and in vivo. (A) Confocal microscopy. Hoxb8 basophils release DNA following activation of high-affinity IgE or C5a receptors in the absence of IL-3 priming (n = 3–4). **p < 0.01; ***p < 0.001. Right, Representative original data. Original magnification ×630. Scale bars, 10 μm. (B) Confocal microscopy. Hoxb8 basophils were activated as indicated (total 45 min). Activated basophils release DNA together with MCP-8 (TUG8). Representative images of three independent experiments are shown. Original magnification ×630. Scale bars, 10 μm. (C) Quantitative PCR. Hoxb8 basophils were activated as indicated and cellular DNA extracted for analysis (n = 4). **p < 0.01. For comparison, we performed the same experiments in Hoxb8 neutrophils (Supplemental Fig. 2C). (D) Confocal microscopy. DNA release from NOX2-deficient Hoxb8 basophils (knockout [KO] NOX2 basophils) following high-affinity IgE or C5a receptor stimulation. For comparison, we performed similar experiments in NOX2-deficient Hoxb8 neutrophils (KO NOX2 neutrophils), which were unable to release DNA, even after 100 nM PMA stimulation (n = 3). Right, Representative original data. Original magnification ×630. Scale bars, 10 μm.

FIGURE 6. Basophil infiltration and extracellular trap formation in mouse inflamed skin. (A) Immunofluorescence. Basophils were identified in N. brasiliensis (Nb)-infected skin using anti-mMCP-8 (TUG8) Ab and the infiltration quantified over time (n = 3). ***p < 0.001. (B) Confocal microscopy. Relative fraction of basophils forming BETs was determined in Nb-infected skin (n = 3–4). ***p < 0.001. Right, Representative original data. Arrows point to BETs. Original magnification ×630. Scale bars, 10 μm.
45 min after basophil activation. Moreover, although the majority of the basophils demonstrated evidence of mtDNA release, 85–90% of the mtDNA was still detectable in the whole cell population even after activation. Similar results were obtained using human and mouse neutrophils. This suggests that the majority of granulocytes still contain functional mitochondria after BET formation, explaining, at least partially, why the formation of extracellular traps does not limit the life span of the cells responsible for their generation (11). Thus, we report in this paper a sensitive method to detect both the amount and source of DNA released in association with extracellular trap formation.

There have been conflicting reports regarding the mechanism(s) of extracellular trap formation in both neutrophils and eosinophils. Although some authors assume that a special form of cell death is required (NETosis in neutrophils (25); EETosis for eosinophils (37)), other groups have either demonstrated that cell death is not required for or have not seen cell death at all in association with extracellular trap formation (11, 16, 17). The reason for these discrepancies may lie in the different systems used for cell activation. From our point of view, non-physiological activation with PMA and/or ionomycin should be avoided in studying the formation of extracellular traps, and hence, conclusions based on such in vitro approaches are unconvincing. Although we agree that within an inflammatory response both extracellular trap formation and cell death can occur concurrently, it has never been convincingly demonstrated that these phenomena depend on each other (32, 33). It should be noted that human basophils treated with high concentrations of monosodium urate crystal (MSU) (200 μg/ml) for 4 h in serum-free medium showed evidence for nDNA release, but no details regarding the molecular mechanism have been provided (38).

In contrast to a requirement for cell death, there seems to be a consensus among researchers in the field that the generation of ROS is required for both NET and EET formation (11, 39). Neutrophils and eosinophils derived from patients suffering from chronic granulomatous disease are unable to form extracellular traps (11, 39), suggesting that a functional NADPH oxidase is required. Using a pharmacological approach, we obtained data suggesting that the formation of a BET also represents a ROS-dependent process. On the other hand, basophils have been reported to lack a functional NADPH oxidase (27). Perhaps, as a consequence, basophils do not seem to be capable of intracellular bacterial killing (26). For other basophil functions, however, a role for ROS has been demonstrated (40, 41). In such a situation, we decided to reanalyze the expression of the NADPH oxidase in human basophils and observed that these cells, in contrast to human neutrophils, do not express p47phox and p67phox, two essential subunits of the enzyme (42, 43). Moreover, NOX2 (p91phox) deficiency prevented the release of DNA from mouse neutrophils, but not from mouse basophils. These data suggest that the formation of BET depends on the generation of ROS, which, however, is not generated by NADPH oxidase.

Indeed, using MitoSOX Red, a mitochondrial ROS fluorescent dye (18), we obtained evidence for mitochondrial superoxide production upon basophil activation. We, therefore, applied MitoQ, which has been characterized as a specific inhibitor of mitochondrial ROS generation (29, 30). Pretreatment of basophils with MitoQ decreased ROS activity in stimulated basophils and completely blocked agonist-induced DNA release. The question remains, why both neutrophils and eosinophils require NADPH oxidase activity for extracellular trap formation and basophils do not. We speculate at this point, that basophils contain higher numbers of mitochondria compared with neutrophils and eosinophils, allowing them to generate the relatively high concentrations of ROS required for BET formation even in the absence of a functional NADPH oxidase. The subsequent signaling events initiated by ROS, which then lead to extracellular trap formation, remain to be determined.

It has previously been reported that the formation of extracellular traps contributes to antibacterial defense in vitro and in vivo (10, 11). Since basophils express TLRs (6, 36) and release anti-microbial factors (9), they had been implicated as an antibacterial defense. Whether BETs are able to mediate an antibacterial activity in the extracellular space remains to be investigated.

We recently developed a technique for generating mouse basophils in nearly unlimited numbers (13). With this method, myeloid progenitor cells are conditionally immortalized using Hoxb8 in the presence of IL-3. Outgrowing cell lines were selected for their potential to differentiate into basophils upon shutdown of Hoxb8 expression. Owing to this technical development, we were enabled to study the mechanisms required for functional BET formation also in the mouse system. The results of our study support the assumption that Hoxb8 basophils closely reflect the biological characteristics of primary mouse basophils.

Previously published work, performed in mice, suggested that basophils mediate adaptive immunity against helminthes (3). The identification of BETs in association with N. brasiliensis infection under in vivo conditions suggests that basophils might be able to exert direct innate immune effector functions against pathogens. Therefore, our data provide additional evidence for a multifaceted and significant role for basophils in both innate and adaptive immune responses to infectious agents.

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