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BRIEF COMMUNICATION

Basophils exhibit antibacterial activity through extracellular trap formation

S. Yousefi[1], M. Morshed[1], P. Amini[1], D. Stojkov[1], D. Simon[2], S. von Gunten[1], T. Kaufmann[1] & H.-U. Simon[1]

[1]Institute of Pharmacology, University of Bern; [2]Department of Dermatology, Inselspital, Bern University Hospital, Bern, Switzerland

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Correspondence
Dr. Shida Yousefi, PhD, Institute of Pharmacology, University of Bern, Inselspital, INO-F, CH-3010 Bern, Switzerland.
Tel.: +41 31 6323281
Fax: +41 31 632 4992
E-mail: shida.yousefi@pki.unibe.ch

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Abstract

Basophils are primarily associated with immunomodulatory functions in allergic diseases and parasitic infections. Recently, it has been demonstrated that both activated human and mouse basophils can form extracellular DNA traps (BETs) containing mitochondrial DNA and granule proteins. In this report, we provide evidence that, in spite of an apparent lack of phagocytic activity, basophils can kill bacteria through BET formation.

Extracellular DNA traps formed both by neutrophils and by eosinophils have been demonstrated to be able to bind and to kill bacteria in *vitro* and *in vivo* (1–6). Recently, it has been reported that physiological activation of human and mouse basophils can also lead to the rapid formation of extracellular DNA traps (7). However, whether basophils could exhibit antibacterial activity was unknown. As basophils express toll-like receptors (8, 9) and release antimicrobial factors (10), we hypothesized that activated basophils ought to be able to kill bacteria in the extracellular space through basophil extracellular trap (BET) formation.

Materials and methods

Recombinant human IL-3 and mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) were from R&D Systems Europe Ltd (Abingdon, UK). Human GM-CSF was provided by Novartis Pharma (Nürnberg, Germany), and mouse IL-3 was obtained from PeproTech (London, UK). Human and mouse C5a were purchased from Hycult Biotech (Uden, the Netherlands). Lipopolysaccharide (LPS, 055:B5), 4-hydroxytamoxifen (4-OHT), and LB and BHI media, as well as *Staphylococcus (S.) aureus* (ATCC 25923), were obtained from Sigma-Aldrich (Buchs, Switzerland). Hoechst 33342, MitoSOX Red, mounting medium, Hank's balanced salt solution (HBSS), and RPMI 1640 medium were from Invitrogen (Paisley, UK). X-VIVO™ 15 medium was from Lonza (Verviers, Belgium). GFP-labeled *Escherichia (E.) coli* M91655 was a kind gift of Dr. E. Slack (Zurich, Switzerland). DNase I was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA).

Human basophils were purified as previously described (7). Human blood neutrophils were purified from healthy individuals by Ficoll-Hypaque centrifugation (2, 11). Written informed consent was obtained from all blood donors, and the Ethics Committee of the Canton of Bern approved this study. Mouse basophils were generated from IL-3—dependent, conditional Hoxb8-immortalized myeloid progenitors derived from wild-type C57BL/6 mice (12).

Abbreviations
4-OHT, 4-hydroxytamoxifen; BET, basophil extracellular trap; C5a, complement factor 5a; CFU, colony-forming unit; DAPI, 4,6-diamino-2-phenylindole; DNase I, deoxyribonuclease I; LPS, lipopolysaccharide; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; NET, neutrophil extracellular trap; PI, propidium iodide; PMA, phorbol myristate acetate.

Basophils were seeded on 12-mm glass coverslips in X-VIVO^{TM} 15 medium (Lonza, Walkersville, MD, USA), primed with 10 ng/ml IL-3 for 15 min and subsequently stimulated with 10 nM C5a for 30 min. The total activation time was 45 min after which BET formation was analyzed by confocal laser scanning microscopy (7).

Phagocytosis and bacterial killing assays were performed as previously described (13). To analyze phagocytosis, 0.25×10^6 cells were stimulated and co-cultured with opsonized bacteria in a ratio of 1 : 10. In the bacterial killing assays (CFU assays), 2×10^6 cells were used and the ratio of cells to bacteria was 1 : 5 for human neutrophils/basophils and 1 : 2 for mouse Hoxb8 neutrophils/basophils. For live/dead analysis with flow cytometry, samples were additionally treated with 100 U DNase I for 10 min at 37°C to release the trapped bacteria from the extracellular DNA scaffold. Following centrifugation (100 ***g*** for 5 min), bacteria-containing supernatants were collected and treated with 20 μM propidium iodine (PI) for 5 min. PI uptake was then measured immediately using flow cytometry (BD FACSVerse).

Data are presented as mean levels ± SEM. Statistical analysis was performed with the two-tailed Student's *t*-test. *P* values <0.05 were considered statistically significant. All statistical analysis was performed using GRAPH PAD software PRISM 5 (La Jolla, CA, USA).

Results and discussion

We performed bacterial killing assays using human basophils co-cultured with *E. coli* or *S. aureus*. Basophils exhibited a significant bacterial killing effect which was reduced in the presence of DNase I (Fig. 1A). The *in vitro* antibacterial activity of human basophils appeared to be as efficient as those mediated by human neutrophils (Fig. 1B). When we additionally stimulated both basophils and neutrophils with cytokines and C5a, we observed a more efficient killing (Fig. 1B) compared to the stimulation with bacteria only (Fig. 1A). To demonstrate DNA nets, we stained with the DNA-binding fluorescent dyes Hoechst 33342 and MitoSOX. Indeed, co-culture of basophils with bacteria resulted in the release of DNA which could be stained with MitoSOX. In contrast, the DNA in the nucleus was stained by Hoechst 333342 only. Such a phenomenon had been previously observed following the activation of neutrophils (2), eosinophils (4), and basophils (7) and was interpreted as an indication that the released DNA was of a non-nuclear origin. Moreover, the released DNA scaffold was able to bind GFP-labeled bacteria (Fig. 1C).

To overcome the limitation in number of primary basophils present in blood or bone marrow, we used conditional mouse Hoxb8-immortalized myeloid progenitor cells to generate large numbers of mouse basophils and neutrophils *in vitro* as described previously (7, 12). To test whether the mouse BETs exhibit antibacterial activity, we co-cultured mature mouse Hoxb8 basophils with *E. coli* or *S. aureus* in the presence or absence of DNase I. As seen with human basophils (Fig. 1A), we observed a significant bacterial killing by mouse Hoxb8 basophils that could be reduced by DNase I treatment of the co-cultures (Fig. 1D). Furthermore, antibacterial activity of mouse basophils was as efficient as that with mouse neutrophils (Fig. 1E). Please note that in these experiments, we additionally stimulated the granulocytes with cytokines and C5a, resulting in an additional killing activity as compared to the cultures in which bacteria were used alone (Fig. 1D).

To demonstrate that bacterial killing occurred extracellularly, we performed live analyses of bacteria and basophil co-cultures using confocal laser scanning microscopy. GFP-labeled *E. coli* took up PI following killing in the extracellular space in co-cultures with either basophils or neutrophils (Fig. 1F, shown by triangles). Moreover, these images also suggested DNA release and bacterial binding to the extracellular DNA scaffolds generated by both basophils and neutrophils (Fig. 1F). It should be noted that in these time-lapse microscopic experiments, neither basophils nor neutrophils demonstrated PI uptake, indicating that cells were viable while they were releasing DNA.

The uptake of PI by dead bacteria was also demonstrated using flow cytometry (Fig. 1G). To release the entrapped dead bacteria within BETs, the co-cultures were briefly treated with DNase I and the amount of dead bacteria could then be subsequently quantified (Fig. 1H). As seen in earlier experiments (Fig. 1B, E), the additional stimulation with IL-3 and C5a increased the killing activity of basophils (Fig. 1H).

Basophils have been reported to lack a functional NADPH oxidase (7, 14). Perhaps, as a consequence, basophils were presumed to be incapable of intracellular bacterial killing (15). To find out whether bacterial killing by basophils was partially due to phagocytosis, we performed phagocytosis experiments using human and Hoxb8 mouse basophils co-cultured with GFP-labeled *E. coli* using flow cytometry. Compared to human neutrophils, phagocytic activity of human basophils was very low or undetectable (Fig. 2A). Similarly, no phagocytosis of GFP-labeled *E. coli* was observed by Hoxb8 mouse basophils as analyzed by live confocal laser scanning microscopy. In contrast, Hoxb8 mouse neutrophils efficiently phagocytozed the bacteria, a process that was enhanced by the stimulation of the cells with physiological agonists, such as combined GM-CSF/C5a or GM-CSF/LPS (Fig. 2B). In these experiments, we observed almost all GFP-labeled *E. coli* to have been taken up in neutrophils within 30 min, while basophils contained no bacteria in their cytoplasm. These data indicate that basophils lack significant phagocytic activity and their killing activity must occur solely in the extracellular space. Moreover, in contrast to neutrophils, additional stimulation of Hoxb8 mouse basophils also failed to induce phagocytosis (Fig. 2C).

Taken together, our data strongly suggest that activated basophils form functional extracellular DNA traps *in vitro* that mediate antibacterial killing activities. In fact, the identification of BETs able to bind and to kill bacteria suggests that basophils function not only as immunomodulatory cells, but also as effector cells in innate immune responses against pathogens. Clearly, such a role could be anticipated at tissue sites of infection, where basophils may be recruited (7). The molecular mechanisms of toxicity, including the nature of the granule proteins that are likely to participate in this process, remain to be identified.

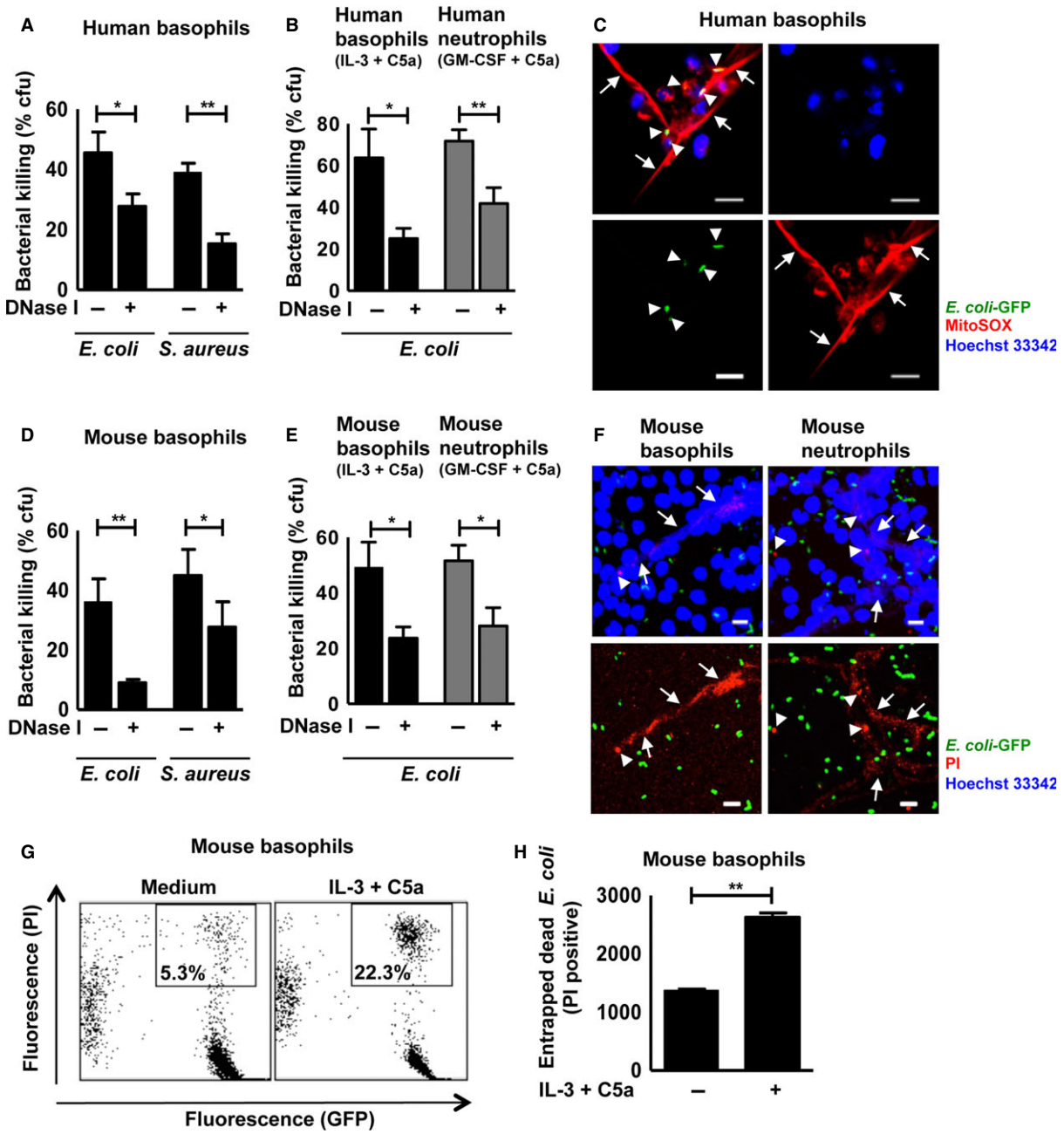

Figure 1 Basophils exhibit antibacterial activity through extracellular trap formation. (A) Bacterial killing (CFU assay). Human basophils exert an antibacterial activity against both *E. coli* and *S. aureus* that can be partially blocked by 100 U/ml DNase I. *, $P < 0.05$, $n = 9$; **, $P < 0.01$, $n = 3$. (B) Bacterial killing (CFU assay). Human basophils and neutrophils increase their antibacterial activity against *E. coli* upon activation with the indicated inflammatory mediators. *$P < 0.05$, $n = 3$; **$P < 0.01$, $n = 7$. (C) Confocal microscopy. GFP-labeled *E. coli*-activated basophils release DNA (red, arrows) able to subsequently bind bacteria (green, triangles). Representative images of three independent experiments are shown. Bars, 10 μm. (D) Bacterial killing (CFU assay). Mouse Hoxb8 basophils exert antibacterial activity against both *E. coli* and *S. aureus* that can be partially blocked by 100 U/ml DNase I. *$P < 0.05$, $n = 4$; **$P < 0.01$, $n = 9$. (E) Bacterial killing (CFU assay). Mouse Hoxb8 basophils and neutrophils increase their antibacterial activity against *E. coli* upon activation with the indicated inflammatory mediators. *$P < 0.05$, $n = 6$ (basophils) and $n = 7$ (neutrophils). (F) Live confocal microscopy. Mouse Hoxb8 basophils and neutrophils were co-cultured with GFP-*E. coli* in the presence of Hoechst 33342 and PI dyes for 30 min. Arrows indicate the DNA released and triangles *E. coli* showing evidence of uptake of PI following death. Bars, 10 μm. (G) Bacterial killing (flow cytometry). Trapped bacteria were analyzed for their ability to take up PI. (H) Bacterial killing (flow cytometry). The absolute numbers of dead bacteria killed owing to BET. **$P < 0.01$, $n = 3$.

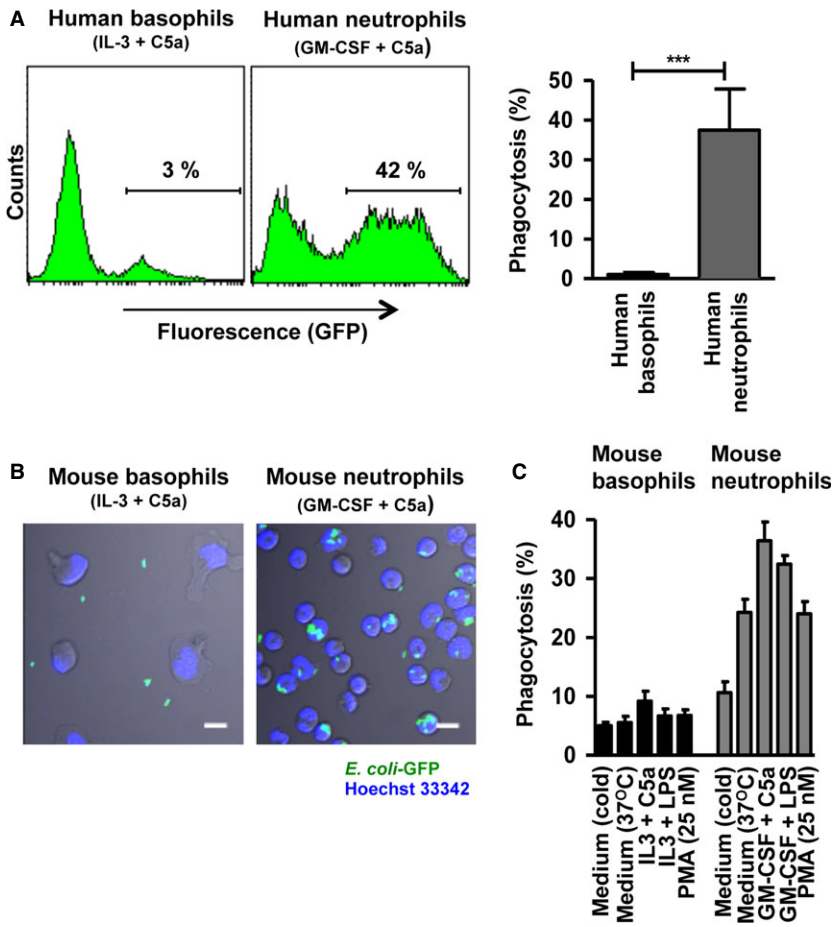

Figure 2 Basophils exert no phagocytic activity**.** (A) Flow cytometry. Phagocytosis of GFP-labeled *E. coli* was assessed. Little or no phagocytic activity was observed by human basophils compared to human neutrophils isolated from same donor. Right: Statistical analysis of five independent experiments. ***$P < 0.001$. (B) Confocal microscopy. Live confocal microscopic analysis of mouse Hoxb8 basophils and neutrophils co-cultured with GFP-labeled *E. coli*, demonstrating the lack of phagocytic activity of basophils. Bars, 10 μm. (C) Flow cytometry. In contrast to Hoxb8 neutrophils, *in vitro* activation does not increase phagocytosis activity of Hoxb8 basophils. Values are means ± SEM of 3 independent experiments.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

S.Y., M.M., P.A., D.St., and D.S. performed experiments; S.Y. and H.U.S. analyzed data, T.K. and S.v.G provided tools, and S.Y. and H.U.S. designed the research and wrote the manuscript.

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