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T Lymphocyte Priming by Neutrophil Extracellular Traps Links Innate and Adaptive Immune Responses

Kati Tillack,* Petra Breiden,* Roland Martin,†* and Mireia Sospedra*†

Polymorphonuclear neutrophils constitute the first line of defense against infections. Among their strategies to eliminate pathogens they release neutrophil extracellular traps (NETs), being chromatin fibers decorated with antimicrobial proteins. NETs trap and kill pathogens very efficiently, thereby minimizing tissue damage. Furthermore, NETs modulate inflammatory responses by activating plasmacytoid dendritic cells. In this study, we show that NETs released by human neutrophils can directly prime T cells by reducing their activation threshold. NETs-mediated priming increases T cell responses to specific Ags and even to suboptimal stimuli, which would not induce a response in resting T cells. T cell priming mediated by NETs requires NETs/cell contact and TCR signaling, but unexpectedly we could not demonstrate a role of TLR9 in this mechanism. NETs-mediated T cell activation adds to the list of neutrophil functions and demonstrates a novel link between innate and adaptive immune responses. The Journal of Immunology, 2012, 188: 3150–3159.

When fighting bacterial infections, cells of the innate immune system recognize pathogen-associated molecular patterns that are not displayed by host tissue. Innate immune cells then secrete cytokines and chemokines that signal danger to other cells and induce inflammation at the site of infection. Polymorphonuclear neutrophils (PMNs) are the first immune cells to be recruited to inflamed tissue (1), to contain and clear infectious organisms, and to direct the extravasation of adaptive immune cells and their activation (2–4). Adaptive immune cells subsequently participate in the elimination of the pathogen and set up memory for the case of reinfection.

The participation of PMNs in adaptive immune responses has not been considered relevant until recently, when it has been recognized that PMNs and T cells may engage in multiple interactions and mutual activation (5). PMNs release chemokines that attract T cells to the site of inflammation (5–7) and also cytokines that influence T differentiation (8). Several cytokines and granule proteins secreted by PMNs such as IFN-γ, TNF-α, cathepsin G, and neutrophil elastase are able to increase T cell proliferation and cytokine production (9, 10), which together enhance adaptive immune responses. Paradoxically, PMNs also secrete mediators such as oxygen species, arginase (11), IL-10, and TGF-β that may suppress T cell activation and proliferation. Despite this evolving evidence, a complete picture about PMN/T cell interactions requires further investigation.

PMNs are endowed with a variety of weapons that enable them to efficiently contain and clear infectious organisms. These include the engulfment and intracellular degradation of microbes (12, 13), production of reactive oxygen species and granule proteins (14), and the recently described release of extracellular chromatin fibers decorated with antimicrobial proteins called neutrophil extracellular traps (NETs) (15). NETs are the most efficient means to contain and eliminate pathogens (16), since they not only trap and kill microbes, but also prevent collateral tissue damage by localizing toxic proteases and reducing their proteolytic activity (17). Furthermore, NETs can modulate immune responses by activating plasmacytoid dendritic cells (pDCs), an APC population specialized in sensing infections. NETs activate pDCs through TLR9, an intracellular receptor that recognizes viral/bacterial DNA (18). Under physiological conditions, pDCs do not respond to self-DNA, most likely because self-DNA lacks CpG motifs found in viral/bacterial DNA and also because self-DNA is rapidly degraded in the extracellular environment and therefore fails to access intracellular TLR9. However, damaged cells can release factors such as the neutrophil antimicrobial peptide LL37 and the high-mobility group box protein 1, which can protect self-DNA from DNase degradation and deliver it to the intracellular component containing TLR9 in pDCs with the consequence of TLR9-mediated activation (19–21). LL37 and high-mobility group box protein 1 are also contained in NETs and hence confer to these structures the potential to activate pDCs via TLR9 (22, 23). NETs activation of pDCs seems to play an important role in the pathogenesis of some autoimmune diseases such as psoriasis (19) and systemic lupus erythematosus (22–25), for which it has been suggested that the large amounts of IFN-γ produced by NETs-activated pDCs can lead to the maturation of myeloid DCs (mDCs) and exert an effect on T cell function. If such an indirect interaction between NETs and T cells were confirmed, it would represent a new NETs-mediated mechanism of communication between PMNs and T cells. Furthermore, TLR9 is expressed in T lymphocytes, and CpG-containing oligodeoxynucleotides (CpG-ODN) can modulate T cell activation (26, 27). NETs may therefore also be able to directly activate T cells through their
TLR9, and this would represent a second and, in this case, direct mechanism of a NETs-mediated communication between PMNs and T cells.

As outlined above, there are at least two possibilities of how PMNs could interact with T cells via NETs production. In this study we have examined whether NETs released by human PMNs are able to exert direct or indirect effects on T cell activation. We found that NETs were able to directly prime T cells by reducing their activation threshold, which increased T cell responses to specific Ags and even to suboptimal stimuli. Priming by NETs required NETs/cell contact and TCR signaling, but unexpectedly we failed to show a role of TLR9 in this mechanism. Our results demonstrate a novel strategy how PMNs are capable of activating adaptive immune responses via NETs.

Materials and Methods

Cell purification and TCC36

Cells were isolated from healthy donors at the Department of Transfusion Medicine, University Medical Center Hamburg–Eppendorf, after informed consent was obtained. PMNs were isolated from blood or Buffy coats using dextran-Ficol, as described previously (28). The purity and viability of PMNs was ≥97% and ≥95%, respectively, as assessed by expression of the neutrophil-specific marker CD16b, annexin V expression, and trypan blue exclusion (Supplemental Fig. 1). PBMCs were isolated by density gradient centrifugation (Supplemental Fig. 1). PMNs were stimulated with CD4+ (2 × 10^6), purified naive CD4+ (2 × 10^6), or purified CD8+ (2 × 10^6) T cells were added to the cultures. As positive control, 1 µg/ml soluble anti-CD3 Ab (OKT3; Ortho Biotech Products, Raritan, NJ) or anti-hCD2/anti-hCD3/anti-hCD28 beads (cell/bead ratio of 2:1) (T cell activation/expansion kit; Miltenyi Biotec) were used (data not shown).

To study the threshold for activation, NETs-primed CD4+ T cells were stimulated with 0.025 µg/ml soluble OKT3 (Ortho Biotech Products) (48 h). NETs-primed TCC36 was seeded in quadruplicate in 96-well plates (25,000 cells/well) together with autologous irradiated PBMCs (1 × 10^5 cells/well) (3000 rad) with or without different concentrations of the specific stimulatory peptide (72 h).

Proliferation assays

Proliferation in the cocultures was measured after 48 h using a Click-it Edu Alexa Fluor 647 flow cytometry assay kit (Molecular Probes/Invitrogen) following the manufacturer’s instructions. Cells were stained with anti-CD3 (PE-Cy7; eBioscience, San Diego, CA), anti-CD4 (allophycocyanin; eBioscience), and anti-CD8 (Pacific Blue; Dako) and analyzed by flow cytometry. Sample acquisition was done with a LSRII (BD Biosciences) flow cytometer and data were analyzed with FACS Diva (BD Biosciences) and FlowJo (Tree Star) softwares.

Proliferation of TCC36 was measured by [3H]thymidine (Hartmann Analytic, Braunschweig, Germany) incorporation using a scintillation beta counter (Wallac 1450; PerkinElmer, Rodgau-Jügersheim, Germany).

Cytokine production

After 24 h coculture, supernatants were collected and cytokine levels were measured by ELISA using the following kits: human IFN-γ (BioLegend); IL-4, IL-10, and IL-2 CytoSet (BioSource International, Camarillo, CA); and IL-17A (eBioscience). Reactions were performed according to the manufacturers’ instructions.

Flow cytometry analysis of surface markers

T cell activation was assessed using the following Abs: anti-CD3 (PE-Cy7; eBioscience), anti-CD4 (allophycocyanin; eBioscience), anti-CD8 (Pacific Blue; Dako), anti-CD25 (PE; eBioscience), and anti-CD69 (FITC; BD Biosciences). DC activation was assessed using the following Abs: anti-CD11c (Pacific Blue; eBioscience), anti-CD14 (Pacific Blue; BD Biosciences), anti-CD19 (Pacific Blue; BD Biosciences), anti-CD56 (Pacific Blue; BD Biosciences), anti-CD11c (PerCP-Cy5.5; BioLegend), anti-CD123 (PE-Cy7; BioLegend), anti-CD40 (PE; Miltenyi Biotec), anti-CD83 (allophycocyanin; BD Biosciences), anti-CD80 (PE; eBioscience), anti-CD86 (FITC; Dako), and anti-HLA class II (FITC; BD Biosciences). FITC (BD Biosciences), PE (BD Biosciences), allophycocyanin (eBioscience), Pacific Blue (Dako), PerCP-Cy5.5 (BD Biosciences), and PE-Cy7 (eBioscience) isotype controls were also used.

Microscopy assays

NETs formation was visualized using fluorescence microscopy in PMNs fixed with 4% PFA and blocked overnight with PBS containing 5% donkey serum (The Jackson Laboratory, Bar Harbor, ME) and 0.6% Triton X-100 (Roth), by staining with a primary anti-human myeloperoxidase-specific Ab (Serotec, Oxford, UK) and the DNA dye Hoechst 33258 (Sigma-Aldrich). Staining was performed on cytospin slides prepared from cells cultured in the absence or presence of suboptimal stimuli (100 nM TLR9 antagonist (ODN TTAGGG) (CAYLA-InvivoGen, Toulouse, France), 3 µM herbimycin A (Sigma-Aldrich), 60 µg/ml anti-FLAG

Cell cocultures

PMNs stimulated or unstimulated were seeded on coverslips and placed into tissue culture plates. Autologous PBMCs (3 × 10^6), purified DCs (4 × 10^5), or purified CD8+ (4 × 10^5) T cells were added to the wells. When indicated cells were incubated with PMN-free supernatant containing NETs or were physically separated by a transwell polycarbonate permeable membrane (0.4 µm pore size, Costar; Corning, Acton, MA). Also when indicated 2.5 µM chloroquine (Sigma-Aldrich), 1 µM TLR9 antagonist (ODN TTAGGG) (CAYLA-InvivoGen, Toulouse, France), 3 µM herbimycin A (Sigma-Aldrich), 60 µg/ml anti-FLAG
Results

NETs induce cluster formation, upregulation of the activation markers CD25 and CD69, and phosphorylation of the TCR-associated signaling kinase ZAP70 but not proliferation in CD4+ T cells

To examine the NETs-mediated effect on T cells, purified human CD4+ T cells were cocultured with PMNs releasing NETs (NETting-PMNs) and unstimulated PMNs. We stimulated NET formation by incubating highly pure PMNs with 25 nM PMA for 3 h (Fig. 1A). To assure that we examine the role of NETs and not of other PMA-induced mediators, we also incubated CD4+ T cells with PMNs pretreated with diphenyleneiodonium (DPI) (DPI-PMNs), a NADPH inhibitor of NET formation, before stimulation with PMA. Furthermore, we digested NETs from stimulated PMNs with micrococcal nuclease and incubated purified CD4+ T cells with these cell-free supernatants containing NETs (NETs-supernatant). CD4+ T cells cocultured with NETting-PMNs (Fig. 1B) and NETs-supernatant (data not shown) formed clusters indicative of cell activation or proliferation that were absent when cells were cocultured with unstimulated PMNs (Fig. 1B) or DPI-PMNs (data not shown). As a further sign of activation, CD25 and CD69 expression was upregulated on purified CD4+ T cells cocultured during 24 h with NETting-PMNs and NETs-supernatant, but not on T cells cocultured with unstimulated PMNs or DPI-PMNs (Fig. 1C). To examine whether these effects of NETs on CD4+ T cells involved TLR9 and TCR signaling, CD4+ T cells were cocultured with NETs-supernatant in the presence or absence of two TLR9 inhibitors: chloroquine, which blocks TLR9/DNA interactions in endosomes (33) and the TLR9 antagonist (ODN TTAGGG), or the antibiotic herbimycin A, which inhibits TCR-mediated signaling (34). The upregulation of CD25 and CD69 on CD4+ T cells cocultured with NETs-supernatant remained unchanged in the presence of chloroquine and TLR9 antagonist (ODN TTAGGG), whereas it was reduced by herbimycin A (Fig. 1D), suggesting that the effect of NETs is independent of TLR9, but involves TCR signaling.

To investigate further the TCR-mediated effect of NETs but also to exclude the possibility that this effect was mediated by PMA that was taken up by neutrophils during stimulation and then released bound to NETs, we analyzed whether NETs induced recruitment of the TCR-proximal tyrosine kinase ZAP70. ZAP70 plays a critical role during early steps of TCR-mediated signaling, but is bypassed by PMA that directly activates protein kinase C without involvement of ZAP70 (35). Purified CD4+ T cells were cocultured for 10 min with unstimulated PMNs, NETting-PMNs, or NETs-supernatant, and ZAP70 (pY319) phosphorylation was measured by flow cytometry. ZAP70 phosphorylation was upregulated in CD4+ T cells cocultured with NETting-PMNs compared with unstimulated PMNs (Fig. 1E), showing an effect of NETs on purified CD4+ T cells that involves TCR signaling and is not mediated by PMA. Furthermore, a putative role of PMA bound to NETs was also excluded by the observation that purified CD4+ T cells stimulated with PMA for 48 h proliferated...
whereas purified CD4+ T cells cocultured with NETting-PMNs or NETs-supernatant did not (Fig. 1F). The lack of proliferation of purified CD4+ T cells cocultured with NETs indicated that the increase in ZAP70 phosphorylation induced by NETs was apparently insufficient to fully activate T cells and support their proliferation.

**T cell priming by NETs lowers the activation threshold**

The changes that NETs exerted on CD4+ T cells suggested that they led to a state of preactivation or priming. We therefore examined whether interaction with NETs reduces the activation threshold of CD4+ T cells. First, we studied the effect of NETs on Ag-specific responses using a previously well-characterized CD4+ T cell clone (TCC36) (29). TCC36 was precultured for 24 h with unstimulated PMNs or NETs-supernatant and then seeded with autologous irradiated PBMCs pulsed or not with its specific target peptide. TCC36 primed by NETs proliferated significantly more vigorously in response to PBMCs loaded with 10 and 1 μg/ml specific peptide than the unprimed TCC36, that is, precultured with unstimulated PMNs (Fig. 2A).

Then, we addressed whether priming by NETs reduces the activation threshold of CD4+ T cells and renders them capable to be activated by suboptimal stimuli. Purified CD4+ T cells were precultured with unstimulated PMNs, NETting-PMNs, DPI-PMNs, or NETs-supernatant for 24 h. Next, T cells were carefully washed and cultured for another 48 h with low concentrations of soluble anti-CD3 Ab (OKT3) (signal 1) in the absence of APC (signal 2), a suboptimal stimulus, which alone is not able to induce proliferation of CD4+ T cells (Fig. 2B). In contrast, 27.2 ± 6.7% of CD4+ T cells precultured with NETting-PMNs or with DPI-PMNs failed to proliferate (Fig. 2B). In both instances, proliferation was obtained in the presence of a T cell-specific Ab (OKT3), indicating that the priming effect of NETs is an Ag-independent process.

Next, we also examined whether DCs, particularly pDCs, precultured with NETs-supernatant (NETs-activated DCs) were able to activate unprimed T cells. Purified CD4+ T cells precultured with unstimulated PMNs or with DPI-PMNs failed to proliferate (Fig. 2B). In contrast, 27.2 ± 6.7% of CD4+ T cells precultured with NETting-PMNs and 13.27 ± 3.5% precultured with NETs-supernatant proliferated (Fig. 2B).

**Resting DCs are able to activate NETs-primed CD4+ T cells in the absence of specific Ag**

Next, we examined whether a more physiologic, but suboptimal stimulus such as the interaction of T cells with resting DCs in the absence of specific Ag, which alone does not induce proliferation of resting T cells, stimulates a response of NETs-primed CD4+ T cells. CD4+ T cells were precultured for 24 h with unstimulated PMNs (unprimed CD4) or with NETs-supernatant (NETs-primed CD4) for 24 h. Simultaneously, purified DCs were precultured with unstimulated PMNs (resting DCs). After this period, both cell types were carefully washed and cocultured for an additional 48 h. NETs-primed purified CD4+ T cells (8.7 ± 1.3%) proliferated and released 2092 ± 459 pg/ml IFN-γ, whereas no activation was observed in unprimed CD4+ T cells (Fig. 3A). To confirm that NETs priming is TLR9 independent, but involves TCR signaling, CD4+ T cells were primed with NETs-supernatant in the presence or absence of the TLR9 inhibitor chloroquine, TLR9 antagonist (ODN TTAGGG), or herbimycin A. Proliferation was not affected in CD4+ T cells primed in the absence of chloroquine or TLR9 antagonist (ODN TTAGGG); however, herbimycin A clearly reduced proliferation (Fig. 3B), confirming that T cell priming by NETs is independent of TLR9 but involves TCR signaling.

To better understand the interaction between NETs-primed CD4+ T cells and resting DCs, NETs-primed purified CD4+ T cells and resting DCs were cocultured in the presence or absence of a blocking anti–HLA-DR Ab, the corresponding isotype control, or herbimycin A. The presence of an anti–HLA-DR Ab did not induce a significant reduction of primed CD4+ T cell proliferation (Fig. 3C). However, because class II molecules other than DR are expressed on DCs, that is, HLA-DQ and HLA-DR molecules, we cannot rule out that HLA class II molecules are involved. The presence of herbimycin A completely abrogated the proliferation of primed CD4+ T cells, indicating that activation of primed T cells by DCs requires TCR signaling (Fig. 3C).

**NETs-activated pDCs are not able to activate unprimed T cells**

Next, we also examined whether DCs, particularly pDCs, precultured with NETs-supernatant (NETs-activated DCs) were able to activate CD4+ T cells. Following purification, ~40% of DCs were pDCs and 60% were mDCs. After 24 h coculture with NETs, all mDCs died, whereas pDCs survived and upregulated some maturation and activation markers such as CD40, CD80, CD83 and CD86, but not HLA class II (Fig. 4A). Unprimed CD4+ T cells cocultured with NETs-activated pDCs did not proliferate nor did...
they produce IFN-γ (Fig. 4B). NETs-activated pDCs were able to induce proliferation and IFN-γ release only in NETs-primed CD4+ T cells (Fig. 4B), suggesting that the NETs-mediated activation of pDCs in our in vitro setting does not exert an effect on T cell activation. NETs activation of pDCs seems to play a role in some autoimmune diseases (19, 22–25), and it has been suggested that the large amounts of IFN-α produced by NETs-activated pDCs can activate mDCs and increase Ag presentation to

**FIGURE 3.** Resting DCs are able to activate NETs-primed CD4+ T cells in the absence of specific Ag. (A–C) CD4+ T cell proliferation assessed by EdU incorporation. Histograms represent the proliferation from a representative experiment. Graph represents the percentage of EdU-positive cells and pg/ml IFN-γ (mean values ± SEM) from three or more independent experiments. (A) Purified CD4+ T cells precultured with unstimulated PMNs (unprimed) or with NETs-supernatant (NETs-primed) were cocultured with DCs previously precultured with unstimulated PMNs (resting). *p < 0.014, **p < 0.003. (B) CD4+ T cells precultured with NETs-supernatant in presence or absence of TLR9 inhibitors chloroquine and TLR9 antagonist (ODN TTAGGG) or TCR signaling inhibitor herbimycin A, and then cocultured with resting DCs. *p < 0.05. (C) NETs-primed CD4+ T cells cocultured with resting DCs in presence or absence of a HLA-DR blocking Ab, the corresponding isotype control, or herbimycin A. **p < 0.01.

**FIGURE 4.** NETs-activated pDCs do not exert an effect on unprimed CD4+ T cells. (A) HLA class II, CD40, CD80, CD83, and CD86 expression on gated NETs-activated pDCs. Values show mean fluorescence intensity (MFI) ± SEM. *p = 0.0263, **p = 0.0041, ***p = 0.0004. (B) Purified CD4+ T cells precultured with unstimulated PMNs (unprimed) or with NETs-supernatant (NETs-primed) were cocultured with DCs previously precultured with NETs-supernatant (NETs-activated). CD4+ T cell proliferation was assessed by EdU incorporation. Histograms represent the proliferation from a representative experiment. Graph represents the percentage of EdU-positive cells and pg/ml IFN-γ (mean values ± SEM) from three or more independent experiments. *p < 0.04.
T cells. The loss of mDCs after culture with NETs most likely prevented this indirect effect of NETs-activated pDCs on T cells in our in vitro system.

**Coculture of T cells, NETting-PMNs, and DCs results in T cell activation**

We then asked whether coculture of T cells, DCs, and NETting-PMNs results in T cell activation and whether NETs exert the same effect on CD4+ and CD8+ T cells. We used NETting-PMNs instead of NETs-supernatant as the more physiologic condition. Purified CD4+ or CD8+ T cells were cocultured with unstimulated or NETting-PMNs in the absence or presence of purified DCs. Proliferation and secretion of IFN-γ, IL-17A, IL-4, IL-10, and IL-2 were measured as indicators of T cell activation (Fig. 5). Purified CD4+ and CD8+ T cells cocultured with unstimulated PMNs alone or in the presence of purified DCs did not proliferate or produce cytokines (Fig. 5A, 5B). Similarly, no proliferation or cytokine release was observed upon coculture with NETting-PMNs in the absence of DCs (Fig. 5A, 5B). In contrast, we observed both proliferation and cytokine release when purified DCs were added to the cocultures. We found that 16.2 ± 2.8% of CD4+ T cells and 11.6 ± 1.3% of CD8+ T cells proliferated (Fig. 5A, 5B). Regarding cytokine production, purified CD4+ T cells cocultured with NETting-PMNs and DCs secreted 901 ± 233 pg/ml IFN-γ and 289 ± 99 pg/ml IL-2. Purified CD8+ T cells secreted only IFN-γ (981.6 ± 293 pg/ml) (Fig. 5B). Direct contact of T cells with DCs and NETting-PMNs was required for T cell activation. When CD4+ or CD8+ T cells were physically separated from DCs and NETting-PMNs by a transwell, no activation was detected (Fig. 5A, 5B). The proliferation of CD4+ T cells cocultured with NETting-PMNs and DCs remained unchanged when CD8+ T cells were added (18.5 ± 6.5%); conversely, the proliferation of purified CD8+ T cells cocultured with NETting-PMNs and DCs increased when purified CD4+ T cells were added to the coculture (27 ± 6.7%) (Fig. 5A).

We also addressed whether the ability of NETs to induce T cell proliferation was comparable between naive and memory CD4+ T cells. Purified naive or memory CD4+ T cells were cocultured with unstimulated PMNs or NETting-PMNs in the presence of purified DCs. We found that 14.7 ± 6.8% of naive T cells and 11.1 ± 5% of memory CD4+ T cells cocultured with NETting-PMNs and purified DCs proliferated, whereas neither naive nor memory...
CD4+ T cells proliferated upon coculture with unstimulated PMNs and DCs (Fig. 5C).

Characterization of NETs-mediated T cell activation in PBMCs

Finally, we characterized the activation of CD4+ and CD8+ T cells mediated by NETs in bulk PBMCs, a condition that reflects better the circumstances under which T cells will encounter NETing-PMNs in vivo. PBMCs include, in addition to T cells and DCs, monocytes and B cells that could both influence T cell activation mediated by NETs. PBMCs cocultured with NETing-PMNs formed large clusters that were absent when cells were cocultured with unstimulated PMNs (Fig. 6A) and strongly upregulated CD25 and CD69 expression on both CD4+ and CD8+ T cells (Fig. 6B). Additionally, within the PBMCs, 10.5 ± 1.8% of CD4+ T cells and 32.3 ± 3.1% of CD8+ proliferated after coculture with NETing-PMNs, whereas no proliferation was observed when PBMCs were cocultured with unstimulated PMNs (Fig. 6C). The ability of NETing-PMNs to mediate proliferation in CD4+ and CD8+ T cells showed great interindividual variability (Fig. 6D).

PBMCs cocultured with NETing-PMNs also secreted 1777 ± 272 pg/ml IFN-γ and 93.5 ± 25 pg/ml IL-17A, but IL-4 and IL-10 did not (Fig. 6D). No cytokine secretion was detected when PBMCs were cocultured with unstimulated PMNs.

NETs and not other factors mediate T cell activation in PBMCs

To confirm that NETs and not other factors mediated the substantial T cell activation in bulk PBMCs, we included a broad range of additional controls. First, to discard that residual contaminating PMA activates PBMCs despite careful washing of NETing-PMNs after PMA stimulation, PBMCs were cocultured with PMNs treated with PMA for only 15 min, a period too short to induce substantial NET production. No upregulation of CD25 and CD69 expression (Fig. 7A), T cell proliferation (Fig. 7B), or production of cytokines (Fig. 7C) was observed under this condition. PMNs activated with fMLP, a stimulus less efficient than PMA in inducing NETs, also failed to mediate T cell activation (Fig. 7). NETing-PMNs, that is, PMNs undergoing NETosis, are dying cells. To exclude an effect of dead cells on T cell activation, we cocultured PBMCs with PMNs dying by other mechanisms than NETosis. We induced apoptosis in PMNs by exposure to UV light for 60 min. Cells were subsequently incubated for additional 16 h to increase the number of dying cells and then cocultured with PBMCs. No activation was detected under these coculture conditions (Fig. 7).

To exclude that PMA-induced mediators other than NETs were responsible for T cell activation, we stimulated PMNs with PMA for 3 h and then fixed them with PFA prior to coculture with PBMCs. After fixation, PMNs are not able to produce any mediators, and only NETs remained on cells. PBMCs cocultured with NETing, PFA-fixed PMNs showed upregulation of CD25 and CD69 expression (Fig. 7A), T cell proliferation (Fig. 7B), and cytokine release (Fig. 7C) similar to those observed with unfixed cells. As an additional control, PBMCs were cocultured with NETing-PMNs, but physically separated by a transwell to avoid cell/cell or NETs/cell contact. No upregulation of CD25 expression, T cell proliferation, or release of cytokines (Fig. 7) was detected under this condition, excluding that soluble mediators produced by PMNs are responsible for T cell activation. Contact between NETs and PBMCs was required for T cell activation, as already observed with purified cells (Fig. 5). Interestingly, some upregulation of CD69 was observed in this condition, suggesting that the expression of this molecule is partially mediated by PMA-inducible soluble factors (Fig. 7A). Finally, as expected, the coculture of PBMCs with DPI-PMNs did not induce T cell activation, whereas the coculture with NETs-superantigens induced proliferation and upregulation of activation markers but not cytokine production (Fig. 7). Proteases released by neutrophils that are eliminated during the careful washing of NETing-PMNs are not eliminated, but concentrated in NETs-superantigens and most likely degraded cytokines (36).

Discussion

The main function of the innate immune system during infection is to rapidly sense microbial pathogens, limit their spread, and eliminate them with minimum collateral tissue damage. The composition of NETs, being fibers of chromatin decorated with antimicrobial proteins, turns them into optimal structures to perform this task (16). The main advantages of NETs are the following: 1) NET fibers trap pathogens and act as physical barriers, preventing microbial spread; 2) NETs render antimicrobial proteins more efficient by concentrating them on the DNA/protein fibers, which keeps them together and allows them to act synergistically; 3) the collateral tissue damage is reduced since proteases do not diffuse into the tissue, and, additionally, binding to NETs reduces the toxic activity of some of these proteases (17); and 5) NETs also allow colocalization of adjuvants and danger signals that can modulate inflammation, for example of self-DNA and LL-37, which are able to activate pDCs. In this study, we describe as a novel function of NETs their ability to directly prime T cells by reducing their activation threshold and in consequence mediate T cell activation. This previously unknown property of NETs demonstrates that their role is not limited to innate immune mechanisms, but is also involved in activating the adaptive immune system.

CD4+ T cell activation mediated by NETs unfolds as a two-step process. In the first step, NETs prime CD4+ T cells by direct contact, which reduces their threshold of activation. CD4+ T cells primed by NETs showed increased Ag-specific responses and can be activated by suboptimal stimuli such as soluble anti-CD3 Ab (signal 1) in the absence of APCs (signal 2) or resting DCs in the absence of specific Ag, which are both not sufficient to induce a response of resting CD4+ T cells. We observed that NETs/T cell contact induced the formation of cell clusters, upregulation of the activation markers CD25 and CD69, as well as some phosphorylation of ZAP70 in CD4+ T cells. These changes were insufficient to fully activate T cells and support T cell proliferation, but they lowered their activation threshold. Unexpectedly, we could not demonstrate a role of TLR9 in this NETs-mediated T cell priming because neither of the two TLR9 inhibitors, the TLR9 inhibitor chloroquine and the TLR9 antagonist (ODN TTAGGG), had an effect. Whether T cell priming mediated by NETs is a DNA activation pathway that is TLR9 independent, such as those induced by CpG in monocytes (37) or nucleosomes in neutrophils (38), requires further investigation. Although TLR9 does not seem to play a role, T cell priming by NETs apparently involves TCR engagement and signaling since they induced phosphorylation of the TCR-associated signaling kinase ZAP70, and herbimycin A, a well-known inhibitor of TCR-mediated signal transduction, strongly reduced T cell priming. Further studies need to determine which of the many components of NETs engage the TCR and induce TCR signaling.

The lower activation threshold of NETs-primed T cells has been demonstrated only for CD4+ T cells. The survival of CD8+ T cells precultured with NETs and subsequently incubated with DCs was very low and prevented us from performing priming experiments with CD8+ T cells. It has been reported that the antimicrobial peptide LL-37, which is present in NETs, induces granzyme-mediated apoptosis of cytotoxic T lymphocytes (39), which
could explain the lower survival of \( \text{CD8}^+ \) T cells pre-exposed to NETs, but we did not address this possibility in this study. CD8\(^+\) T cells primed with NETs showed cluster formation and upregulation of CD25 and CD69 expression comparable to that observed in NETs-primed CD4\(^+\) T cells (Supplemental Fig. 2), which suggests a similar behavior of both cell types after NETs priming. Furthermore, only minor differences were found in proliferation and release of IFN-\( \gamma \) between CD8\(^+\) and CD4\(^+\) T cells cocultured

**FIGURE 6.** NETs-mediated T cell activation in PBMCs. (A) Transmission light images of cluster formation of PBMCs cocultured with unstimulated PMNs and NETting-PMNs. Original magnification \( \times 40 \). (B) CD25 and CD69 surface expression after gating on CD4\(^+\) (red) and CD8\(^+\) (blue) T cells. Histograms represent the expression from a representative experiment. Dotted line indicates PBMCs cocultured with unstimulated PMNs; solid line indicates PBMCs cocultured with NETting-PMNs (NETs). Graphs represent mean fluorescence intensity (MFI) ± SEM from five independent experiments. **\( p < 0.01 \), ***\( p < 0.001 \). (C) T cell proliferation in PBMCs cocultured with unstimulated PMNs and NETting-PMNs. T cell proliferation was assessed by EdU incorporation after gating on CD4\(^+\) (red) and CD8\(^+\) (blue) T cells. Graphs represent the percentage of EdU-positive cells (mean values ± SEM) from 21 independent experiments. **\( p < 0.01 \), ***\( p < 0.001 \). Histograms represent the proliferation from a representative experiment. (D) Scatter plot showing T cell proliferation, in which each dot represents one individual donor. (E) IFN-\( \gamma \), IL-17A, IL-4, and IL-10 produced by PBMCs cocultured with unstimulated PMNs and NETting-PMNs (NETs). Values show mean pg/ml ± SEM from five independent experiments. **\( p < 0.01 \), ***\( p < 0.001 \).
with NETting-PMNs and DCs. However, higher proliferation and IFN-γ release were found in CD8+ T cells cocultured with unstimulated PMNs, NETting-PMNs (NETs), PMNs stimulated with PMA for 15 min, PMNs stimulated with FMLP, dying PMNs exposed to UV light, NETting-PMNs fixed with PFA, NETting-PMNs physically separated by a transwell, DPI-PMNs, and NETs-supernatant. Graphs represent mean fluorescence intensity (MFI) ± SEM from five independent experiments. (B) T cell proliferation assessed by EdU incorporation after gating on CD4+ (red) and CD8+ (blue) T cells in PBMCs cocultured as indicated in (A). Graphs represent the percentage of EdU-positive cells (mean values ± SEM) from five independent experiments. (C) IFN-γ, IL-17A, IL-4, and IL-10 produced by PBMCs cocultured as indicated in (A). Values show mean pg/ml ± SEM from five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. Polymorphonuclear neutrophils’ purity and viability.

Neutrophil purity after separation was assessed by expression of the neutrophil-specific marker CD16b. Neutrophil viability after separation was assessed by expression of annexin-V.

Isotype Control

- SSC vs. FSC
- PE vs. FITC
- PE vs. CD16b-FITC
- FITC vs. Pacific Blue
- FITC vs. Annexin-V-Pacific Blue

Apoptotic cells
Supplementary Figure 2. CD8⁺ T cell priming induced by NETs. 

**a)** Transmission light images of cluster formation of purified CD8⁺ T cells co-cultured with unstimulated PMNs and NETting-PMNs (NETs).

**b)** CD25 and CD69 surface expression on purified CD8⁺ T cells co-cultured 24 h with unstimulated PMNs, NETting-PMNs, DPI-PMNs (DPI) and NET-supernatant. Graphs represent MFI 7 SEM from 5 independent experiments. *p<0.05 and **p<0.01.