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Neutrophil Extracellular Traps Downregulate Lipopolysaccharide-Induced Activation of Monocyte-Derived Dendritic Cells

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Polymorphonuclear neutrophils (PMN) play a central role in inflammation and participate in its control, notably by modulating dendritic cell (DC) functions via soluble mediators or cell–cell contacts. Neutrophil extracellular traps (NETs) released by PMN could play a role in this context. To evaluate NET effects on DC maturation, we developed a model based on monocyte-derived DC (moDC) and calibrated NETs isolated from fresh human PMN. We found that isolated NETs alone had no discernable effect on moDC. In contrast, they downregulated LPS-induced moDC maturation, as shown by decreased surface expression of HLA-DR, CD80, CD86, and by downregulated cytokine production (TNF-α, IL-6, IL-12, IL-23), with no increase in the expression of tolerogenic DC genes. Moreover, the presence of NETs during moDC maturation diminished the capacity of these moDC to induce T lymphocyte proliferation in both autologous and allogeneic conditions, and modulated CD4+ T lymphocyte responses to pathogens, fungi, viruses, and protozoa (1, 3–5). Interestingly, the expression and activities of the lymphoid chemokine receptors CCR7 and CXCR4 on moDC were not altered when moDC matured in the presence of NETs. Together, these findings reveal a new role for NETs in adaptive immune responses, modulating some moDC functions and thereby participating in the control of inflammation. The Journal of Immunology, 2014, 193: 000–000.

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Abbreviations used in this article: DC, dendritic cell; MFI, mean fluorescence intensity; MNease, micrococcal nuclease; moDC, monocyte-derived DC; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PI, propidium iodide; PMN, polymorphonuclear neutrophil; rh, recombinant human; SLE, systemic lupus erythematosus; TT, tetanus toxin C fragment.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$5.00 of inflammation (3, 4) and pharmacological agents such as PMA and calcium ionophore (1, 3, 5–7). Netosis was initially described as a suicide-like mechanism, but a concept of “vital netosis” is also emerging (8). NETs are composed of extracellular chromatin decorated by various granule-derived and cytoplasmic proteins. Histones are the most abundant components (~70% of total protein (9)), but several important mediators of immune responses are also present, such as elastase, lactoferrin, calprotectin, myeloperoxidase (MPO), and LL-37 [reviewed in (10)].

The best-known function of NETs is pathogen trapping: this property limits the dissemination of pathogens and exposes them to high local concentrations of various molecules immobilized on the NET chromatin backbone (1, 7, 11) [reviewed in (12, 13)]. However, NET accumulation can also have adverse consequences in vivo and in vivo, such as epithelial and endothelial cell injury (6, 11, 14–16) and thrombus formation (17, 18) [reviewed in (10, 13)]. Moreover, by exposing self-Ags, NETs might be involved in autoimmune disorders, including small-vessel vasculitis, rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus (SLE) (14, 19–21). In particular, during SLE, the association of self DNA with some PMN proteins (LL-37, cathepsin G, elastase, or secretory leukocyte peptidase inhibitor) induces IFN-α production by plasmacytoid dendritic cells (DC) (22–25). This plasmacytoid DC/NETs/IFN-α axis could thus represent an amplification loop of major importance in SLE pathogenesis (16, 23, 24) and illustrates how NETs can modulate DC functions in vivo. Furthermore, on forming complexes with RNA, these PMN proteins are able to activate myeloid DCs from SLE patients, but not from controls (22, 24).

The role of PMN in DC functions is a growing field of investigation [reviewed in (26, 27)] and has been evaluated using blood
PMN or PMN-derived purified mediators. Activated PMN can promote DC migration (28) and also modulate DC maturation and activation through cell–cell contacts (29), eosinocyte release (30), or mediator secretion. Interestingly, several granule-derived mediators such as lactoferrin, LL-37, calprotectins, α-defensins, elastase, bactericidal/permeability-increasing protein, MPO, and proteinase 3 have been shown to either downregulate (31–37) or upregulate (33, 38–41) DC functions, depending on the study.

Few recent studies have evaluated the potential effects of NETs on APCs. Fully activated PMN bearing NETs can stimulate TNF-α and IL-8 release by THP-1 cells (42). NETs recovered after micrococal nuclease treatment of activated PMN can enhance IL-1β and IFN-α production by LPS-activated macrophages (43). In a murine model of autoimmune vasculitis, inflammatory PMN recovered from mouse skin exudates spontaneously produce NETs that have been shown to activate bone marrow–derived DC and to transfer PMN-derived self-Ags to these cells (21). These reports tend to support a proinflammatory effect of NETs on APCs. However, an in vitro study showed no proinflammatory effect of NETs alone on macrophages (44), and aggregated NETs promote the resolution of inflammation in a murine gout model (45). The conflicting effects reported in the literature might be related to technical variations in NET preparation and APC models. In particular, the proinflammatory effects of NETs on APCs were observed using both activated and primed full PMN (21, 42, 43).

We therefore examined the effects of isolated NETs on monocyte-derived DC (moDC) functions. Large functional NET fragments were prepared from human fresh blood by using the restriction enzyme AluI, as we have previously described (5). These isolated NETs downregulated LPS-induced moDC maturation, as shown by changes in cell surface marker expression and cytokine production, but did not affect the expression or function of selected lymphoid chemokine receptors, or migratory capacity. However, NET-treated mature moDC displayed a decreased capacity to activate CD4+ T lymphocyte proliferation and reduced Th1 and Th17 polarization in favor of Th2 polarization. These results reveal a new and important role for PMN in the regulation of adaptive immune responses, via NETs.

Materials and Methods

Reagents and Abs

Calcium ionophore A23187, LPS from Escherichia coli serotype O55:B5, dexamethasone, tetanus toxin C fragment (TT) from Clostridium tetani, PMA, and AMG3100 were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Imiquimod (R848) was from Invivogen (Toulouse, France). FTTC-, PE-, PE-Cy7−, or allophycocyanin-conjugated mAbs against human CD4, CD40, CD80, CD86, HLA-DR, CD4, CCR5, CD87, and CCRX4, and their respective isotype controls, were from BD Biosciences (Le Pont de Claix, France). CFSE was from Molecular Probes; micrococcal nuclease (MNase), DNase I, and AluI were from New England Biolabs (Evry, France); and annexin V apoptosis detection kit I and recombinant human IL-4, IL-1β, IL-6, IL-12, IL-18, GM-CSF, and TNF-α from R&D Systems.

Cell isolation

PMN, PBMC, monocytes, and naive CD4+ T lymphocytes were isolated from healthy donors’ peripheral blood provided by Etablissement Français du Sang (Rungis, France). Whole-blood centrifugation (20 min at 690 × g) was performed from 65˚C to 95˚C to confirm the presence of a single amplification product. For each target gene, a 3-fold serial dilution of pooled cDNA was amplified for the nonactivated sample. The primer sequences for GAPDH (5) were used for Ag-specific T cell proliferation assays and polarization experiments. Total and naive CD4+ T lymphocytes were confirmed in a purity >95%, based on flow cytometry of CD4 and CD45RA expression, respectively.

NET production and isolation

NETs were produced and isolated, as previously described (5). Briefly, freshly isolated PMN were seeded in 12-well culture plates (1.5 × 106 cells/well) and stimulated with 5 μM A23187 for 3 h at 37˚C with 5% CO2. The cells were carefully washed twice with 1 ml PBS and then treated for 20 min at 37˚C with 20 U/ml restriction enzyme AluI in HBSS to recover large soluble NET fragments. In some experiments, the cells were treated for 20 min at 37˚C with DNase (10 U/ml) or MNase (20 U/ml), two other restriction enzymes. Supernatants were collected and centrifuged at 300 × g for 5 min at 4˚C to remove contaminating cells and debris. NET preparations were then pooled, aliquoted, and stored at −20˚C until use. DNA was quantified in NET samples by using PicoGreen (Molecular Probes), as previously described (5).

Generation of human moDC

Monocytes (2 × 106/ml) were cultured for 5 d in RPMI 1640 Glutamax supplemented with 10% decomplemented FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 550 IU/ml rhGM-CSF, and 550 IU/ml rhIL-4 (R&D Systems, France). On day 5, flow cytometry (FACScalibur flow cytometer and Cell Quest software [BD Biosciences]) confirmed the generation of nonactivated moDC with the following phenotype: CD14neg, CD1a(+) CDS5neg, CD1b(+) CDS3neg, and CD86low as previously described (46, 47).

NET pretreatment and moDC activation

These moDC (106/ml) were washed once with cold RPMI 1640 for 5 min, and incubated with or without AluI-derived NETs (10, 100, or 300 ng/ml DNA), MNase-derived NETs (100 ng/ml DNA), or DNase-derived NETs (100 ng/ml DNA) for 30 min before adding FCS and LPS (25 ng/ml) or not to the medium. Controls were also done using AluI (20 U/ml), DNase (10 U/ml), or MNase (20 U/ml) alone, instead of NETs. In some experiments, moDC were activated with R848, a TR7/TLR8 ligand (10 μg/ml), or PMA (300 nM) instead of LPS. Cells and supernatants were recovered after 24 h at 37˚C, unless otherwise specified. MoDC maturation was evaluated by quantifying both cytokine release (as indicated below) and HLA-DR, CD40, CD80, and CD86 expression. Phenotypic results were expressed as the relative mean fluorescence intensity (MFI), corresponding to the MFI of the specific label/MFI of the isotype control. CCR5, CCR7, and CXCR4 expression was analyzed with a LS-R Fortessa cytometer, and the results were displayed in MFI, as previously described (48). Cell viability was determined by labeling with annexin V and 7-aminoactinomycin D, following the manufacturer’s instructions, followed by analysis on a FACScalibur cytometer.

RNA isolation and real-time PCR analysis of cytokine RNAs in moDC

MoDC (106/ml) were washed once with cold RPMI 1640 for 5 min, and incubated with AluI-derived NETs (100 ng/ml DNA) or AluI alone for 30 min, before adding FCS and LPS (25 ng/ml) or not to the medium. Total RNA was isolated by using TRZol reagent (Invitrogen, Grand Island, NY), and gene (ACTB) expression measured as the fold-change in gene expression using qPCR. Gene expression was confirmed using avian myeloblastosis virus reverse transcriptase (Promega, Lyon, France), following the manufacturer’s protocols. Gene transcripts were amplified using SYBR Green technology on a Bio-Rad CFX96 system. Each reaction was performed with 4 ng cDNA, 0.5 μM each forward and reverse primer, and Sso Advanced SYBR Green Mix (Bio-Rad, Marnes la Coquette, France) in a 10 μl final volume. After 30 s at 95˚C and 44 cycles of amplification (95˚C for 5 s, 60˚C for 5 s), a melting-curve reaction was performed from 65˚C to 95˚C to confirm the presence of a single amplification product. For each target gene, a 3-fold serial dilution of pooled cDNA was analyzed to calculate the amplification efficiency (E). The relative amount of target mRNA was determined using the cycling threshold method ([1 + E]−ΔΔCt), were E is the amplification efficiency. The ratio of each target gene threshold value to the geometric average of the reference genes (ACTB and GAPDH) threshold values was calculated for each sample. Results were expressed as relative gene expression, corresponding to the ratio of the former calculated value for a given sample to the value obtained for the nonactivated sample. The primer sequences for IL-1B, IL-
Quantification of effector and tolerogenic moDC gene expression

MoDC (10^6/ml) were washed once with cold RPMI 1640 for 5 min and incubated with or without Alu-derived NETs (100 ng/ml DNA) for 30 min, before adding FCS and LPS (25 ng/ml) to the medium. After 24 h at 37°C, cells were washed twice with cold PBS, and effector and tolerogenic moDC genes were analyzed, as previously described (47). Total RNA was extracted from moDC with RNeasy minikits and the Qiacube robot (Qiagen, Courtaboeuf, France); cDNAs were obtained by using TaqMan reverse-transcription reagents (Applied Biosystems). mRNA expression was evaluated by quantitative PCR on a 7900HT real-time PCR system (Applied Biosystems) using predesigned TaqMan gene expression assays and reagents, according to the manufacturer’s instructions. The expression of the following genes was assessed, as previously described (47, 50): glucocorticoid-induced leucine zipper (GILZ) (Hs00608272_m1), complement component C1q (C1QA) (Hs00381122_m1), cathepsin C (CATHC) (Hs00175188_m1), myxovirus resistance 1 (MX1) (Hs00858608_s1), normal mucosa of esophagus-specific 1 (NMES1) (Hs00269092_m1), stabilin 1 (STAB1) (Hs01109068_m1), and retinaldehyde dehydrogenase 1 (RALDH1) (Hs00167445_m1). Data were interpreted for target gene expression by comparison with ACTB (Hs99999903_m1) as an endogenous control. The relative amount of each target gene in each sample was calculated by comparison with the calibrator sample (untreated sample), using the cycling threshold method described above.

TNF-α degradation by NETs

A fixed amount of TNF-α (rTNF-α or pooled LPS-activated moDC supernatant, final volume 40 µL) was incubated with increasing concentrations of NETs (0, 10, 30, 100, or 300 ng/ml DNA) for 24 h, and then TNF-α was measured by ELISA, as described above.

MoDC chemotaxis assay

Chemotaxis upon CCL19 or CXCL12 induction was measured in a Transwell assay, as previously described (48). Briefly, 1 x 10^5 MoDC preincubated with NETs (100 ng/ml DNA) were resuspended in 150 µl RPMI 1640 medium supplemented with 20 mM HEPES, 0.2% AB human serum (Sigma-Aldrich) and were added to the upper chamber of a 6.5-mm-diameter, 5-µm pore-size polycarbonate Transwell cell culture insert (Corning Costar, Brumath, France). The same medium (600 µl) with or without CCL19 (1, 10, or 50 nM) or CXCL12 (50 nM), was placed in the lower chamber. HBSS medium that could be quantified by DNA assay, as we have previously described (5).

We first examined whether these isolated NETs could modify moDC maturation, either directly or in response to LPS. MoDC were preincubated with NETs for 30 min in the absence of FCS to avoid NET dismantling by serum DNases (51). Then the culture medium was supplemented with FCS, and LPS was added. After 24 h, the expression of several moDC maturation markers was quantified by flow cytometry, and cytokines were assayed in the supernatants. A first set of experiments showed that the effect of NETs on LPS-induced moDC maturation (in terms of HLA-DR expression and TNF-α release) was only observed when NETs were recovered after Alu digestion, as MNase- and DNase-derived NETs did not modify these moDC markers (Fig. 1A). Moreover, the effect of Alu-derived NETs was concentration dependent and led us to choose a NET concentration of 100 ng/ml DNA for all subsequent experiments (Supplemental Fig. 1).

Fig. 1B shows that the expression of HLA-DR, CD40, CD80, and CD86 was not modified by NETs alone. As expected, LPS alone induced moDC maturation, as shown by increased expression of all the chosen markers. However, this marker upregulation was significantly attenuated when moDC were pretreated with NETs, with the following percentage reductions: HLA-DR (29.3 ± 1.3%), CD80 (18.9 ± 6.2%), CD86 (23.8 ± 2.5%) (Fig. 1B).

In the same experiments, NETs alone had no effect on cytokine release in moDC supernatants (data not shown). In contrast, NETs significantly downregulated LPS-induced cytokine release by moDC, with the following percentage reductions: TNF-α (60.0 ± 7.7%), IL-6 (58.9 ± 7.4%), IL-8 (26.4 ± 10.8%), IL-10 (52.8 ± 11.5%), IL-12p70 (81.1 ± 9.2%), and IL-23 (62.5 ± 8.0%).

Cytokine quantification in moDC and moDC/T lymphocyte culture supernatants

MoDC supernatants were assayed for IL-6, IL-8, IL-10, IL-4, and TNF-α by using a cytokometric bead array (BD Biosciences) with a detection limit of 7.2 pg/ml. IL-23 was quantified by ELISA with the ready-SET-Go kit from eBioscience (San Diego, CA), with a detection limit of 15 pg/ml. In some experiments, TNF-α was quantified by using the optEIA set from BD Biosciences (detection limit 7.8 pg/ml). IL-12p70 was quantified with the Quantikine reagent kit (R&D Systems), with a detection limit of 5 pg/ml. Supernatants of moDC/naïve CD4+ T lymphocyte cocultures were analyzed for IFN-γ, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-17A by using a human cytokine bead kit (Merck Millipore, Darmstadt, Germany) and the Magpix system (Luminex, Austin, TX).

Statistical analysis

Data are expressed as means ± SEM. Differences between groups were evaluated with the Wilcoxon signed rank test or the Mann–Whitney U test (Prism software; GraphPad, La Jolla, CA). The p values <0.05 were considered to denote statistical significance.

Results

NETs prevent full moDC maturation

Recent studies suggest that PMN having undergone netosis (21, 42), and supernatants of activated PMN containing NETs (23, 43), can modulate APC functions. In this study, we evaluated the effects of isolated NETs on moDC. We showed NETs from A23187-activated PMN by using the restriction enzyme AluI, MNase, or DNase, thereby recovering NET fragments in fresh HBSS medium that could be quantified by DNA assay, as we have previously described (5).

The capacity of moDC to activate CD4+ T lymphocytes was compared with two different approaches: activation of allogeneic CD14neg PBMCs and activation of autologous TT-specific CD4+ T lymphocytes. In both approaches, CD4+PBMCs and CD4+ T lymphocytes, respectively, were first labeled with 5 µM CFSE, following the manufacturer’s instructions. In the first approach, moDC pretreated with NETs (100 ng/ml DNA) were incubated with 1.5 x 10^5 CD4+PBMCs from allogeneic or autologous donors at a moDC/PBMC ratio of 1:3 or 1:9, in RPMI 1640 Glutamax supplemented with 10% AB human serum, in round-bottom 96-well plates, in triplicate. On day 6, cells were labeled with propidium iodide (PI) and with an alphaphycocyanin-labeled anti-CD4 Ab. Proliferation of CD4+ T lymphocytes was analyzed by flow cytometry (CD4+ PI- cells). The second approach evaluated the activation of Ag-specific lymphocytes. Autologous CD4+ T lymphocytes (1 x 10^5) were coincubated with moDC pretreated with NETs (100 ng/ml DNA) and preloaded with TT (0.2 µg/ml) during the LPS activation process (moDC:lymphocyte ratios of 1:10 and 1:30). On day 6, cells were stained with PI and analyzed by flow cytometry. Proliferation of CD4+ T lymphocytes was expressed as the percentage of CFSE+ PI-negative cells present in the sample.

To assess T lymphocyte polarization, moDC pretreated with NETs (100 ng/ml DNA) were cocultured with allogeneic naïve CD4+ T lymphocytes (moDC:T lymphocyte ratio 1:10) for 5 d, as previously described (50). Supernatants were then analyzed for cytokine release, as described below.
NETs did not modify the very low capacity of IL-4 production that could be quantified in LPS-treated moDC from two donors upon seven tested (50 and 57 pg/ml, respectively). We chose to focus on TNF-α production in some subsequent experiments, as TNF-α is one of the earliest cytokines induced by inflammation. As the restriction enzyme AluI cannot be removed from NET preparations, we confirmed that AluI alone did not modify LPS-induced TNF-α production nor HLA-DR expression (Fig. 1A). Moreover, to rule out NET cytotoxicity for moDC, we quantified annexin V and 7-aminoactinomycin D staining of LPS-activated moDC preincubated with increasing NET concentrations (from 10 to 300 ng/ml DNA), and found that NETs were neither proapoptotic nor cytotoxic (Supplemental Fig. 2). Interestingly, the downregulating effect of NETs on moDC maturation was not limited to LPS induction (TLR4 agonist), as TNF-α production was also significantly reduced when moDC were preincubated with NETs before being stimulated with Resiquimod R-848 (TLR7/8 agonist) or PMA (protein kinase C activator) (Fig. 2A). Together, these observations suggest that isolated NETs prevent full moDC maturation in various conditions, in terms of both surface marker expression and cytokine release.

NET-induced downregulation of TNF-α release is not related to a direct effect on LPS or TNF-α.

To detect any indirect effect of NETs on moDC activation, control experiments were performed with TNF-α as the readout. First, moDC were activated with LPS for 5, 30, or 120 min. After one washing step to remove LPS, NETs were added and TNF-α was quantified in the culture supernatants by ELISA (n = 4). *p < 0.05 as compared with LPS-treated moDC (Wilcoxon signed rank test). Immature moDC (NS) were then incubated with AluI-derived NETs (100 ng/ml DNA), LPS (25 ng/ml), or NETs plus LPS (NET/LPS). After 24 h, the expression of cell surface markers was quantified by flow cytometry, and cytokines were assayed in the supernatants by cytometric bead array and ELISA. Data represent the results of six independent experiments. *p < 0.05, **p < 0.01, as determined by Wilcoxon signed rank test.
was assayed in 24-h culture supernatants. As expected, a time-dependent effect of LPS was observed (Fig. 2B). The same time course was maintained when NETs were added after LPS stimulation, but TNF-α levels were always significantly lower (Fig. 2B). These results suggest that NET-induced downregulation of TNF-α release is not due to physical sequestration of LPS by NETs or to TLR4 blockade by NETs, hindering LPS ligation. NETs thus appear to negatively modulate ongoing moDC activation by LPS.

Second, we examined whether the decreased TNF-α levels in NET-treated moDC supernatants might be due to TNF-α internalization or binding to membrane receptors. TNF-α immunostaining of permeabilized cells and flow cytometry showed very low and similar TNF-α expression (both membrane associated and intracellular) by LPS-activated moDC in the presence and absence of NETs (data not shown).

Finally, we examined whether NET-associate proteases might directly degrade TNF-α during its production. For this purpose, 400 pg rhTNF-α (Fig. 2C) or LPS-activated moDC supernatants containing ~300 pg native TNF-α (Fig. 2D) were incubated for 24 h in the presence or absence of increasing amounts of NETs (from 10 to 300 pg/ml DNA), and TNF-α was assayed. TNF-α levels in both conditions were unaffected by NETs, whatever their concentration. Therefore, in our experimental conditions, NETs, and particularly NET-associate proteases, do not seem to directly degrade TNF-α released by activated moDC.

Together, these results suggest that NET-induced downregulation of cytokine release by LPS-activated moDC is not due to LPS sequestration, TLR4 hindrance, intracellular or membrane cytokine sequestration, or extracellular cytokine degradation by NETs.

**NETs downregulate cytokine gene transcription in LPS-stimulated moDC**

To better understand these effects of NETs on cytokine production, we evaluated their impact on TNF, IL1B, IL6, IL8, IL10, and IL23A gene transcription. In response to LPS, mRNA accumulation was maximal at 2 h for TNF, IL1B, IL6, and IL23A, and at 4 h for IL8 and IL10 (Fig. 3A). The kinetics were similar in the presence of NETs (Fig. 3A), but the relative amounts of the mRNAs at their respective transcription peaks were significantly reduced, with the following percentage reductions: TNF (42.1 ± 6.6%), IL-1B (45.0 ± 20.1%), IL-6 (41.7 ± 4.6%), IL-8 (22.1 ± 12.4%), IL-10 (28.2 ± 18.3%), and IL-23A (52.3 ± 19.1%) (Fig. 3B). As AluI cannot be removed from NET preparations, we confirmed that AluI alone did not induce TNF, IL-1B, IL-6, or IL-23A gene transcription and did not modify their mRNA accumulation at 2 h in response to LPS (Supplemental Fig. 3).

These results show that NETs negatively impact LPS-induced cytokine production by moDC at both the protein and transcriptional levels.

**The immunoregulatory effect of NETs on moDC is not associated with a tolerogenic gene signature**

We then investigated whether NETs drive LPS-activated moDC toward a tolerogenic phenotype. We thus quantified the expression of NMES1 and MX, two effector-related genes, and also GILZ, STAB1, C1QA, CATC, and RALDH, genes recently shown by Zimmer et al. (47) to be associated with tolerogenic DC. NETs alone induced no significant change in effector-associated gene expression, which, as expected, was upregulated by LPS. The presence of NETs during LPS activation tended to reinforce the effector phenotype, with a trend toward increased NMES1 and MX1 expression (Fig. 4). In addition, tolerogenic markers were downregulated in LPS-activated moDC even when NETs were present during the activation process (Fig. 4), whereas they were significantly upregulated in dexamethasone-treated control moDC, as expected for this positive control (p < 0.05). Together, these results suggest that NETs do not induce a tolerogenic DC gene profile, but might rather enhance the expression of some effector genes in LPS-activated moDC.

**NETs do not affect the migratory capacity of mature moDC in vitro**

We then analyzed the impact of NETs on the rapid and coordinated switch in chemokine receptor expression during DC maturation, and on DC migratory capacity. Using flow cytometry, we first quantified the membrane expression of CCR5, CCR7, and CXCR4, which are involved in DC maturation and homing to lymph nodes. As expected, CCR5 expression was high on immature moDC and was strongly downregulated by LPS activation (Fig. 5A). In contrast, CCR7 and CXCR4 were barely detectable on immature moDC and were upregulated on mature moDC. Interestingly, the...
presence of NETs during moDC maturation did not modulate the expression of CCR5, CCR7, or CXCR4 (Fig. 5A), indicating the relative amount of mRNA at each time point in DC/LPS conditions (dark gray squares) and DC/NET-LPS conditions (light gray squares), as evaluated by quantitative PCR. The curves are representative of four independent experiments. (B) Relative amount of each cytokine mRNA recovered at the transcriptional peak (2 h for TNF, IL-23A, IL-6, and IL-1B, and 4 h for IL-8 and IL-10) is presented (n = 4). *p < 0.05, as determined by Wilcoxon signed rank test.

FIGURE 3. NETs reduce moDC cytokine production at the transcriptional level. After preincubation with NETs for 30 min, moDC were activated with LPS for 2, 4, or 8 h. (A) Representative kinetic curves for cytokine gene expression (TNF, IL-23A, IL-6, IL-1B, IL-8, and IL-10), indicating the relative amount of mRNA at each time point in DC/LPS conditions (dark gray squares) and DC/NET-LPS conditions (light gray squares), as evaluated by quantitative PCR. The curves are representative of four independent experiments. (B) Relative amount of each cytokine mRNA recovered at the transcriptional peak (2 h for TNF, IL-23A, IL-6, and IL-1B, and 4 h for IL-8 and IL-10) is presented (n = 4). *p < 0.05, as determined by Wilcoxon signed rank test.

NETs undermine the capacity of LPS-treated moDC to activate CD4+ T lymphocyte proliferation

To further document the effects of NETs on moDC functions, we evaluated the capacity of these cells to activate CD4+ T lymphocytes. In a first set of experiments, LPS-activated moDC treated with NETs were coincubated with autologous or allogeneic monocyte-depleted PBMC loaded with CFSE. Proliferation was evaluated on day 6 as the percentage of CFSElow CD4+ T cells.

As expected, PBMC activation with PHA resulted in 50% of CFSElow cells, whereas, in the absence of stimulus, 2% of PBMC were CFSElow (Fig. 6A). Preincubation with NETs had no impact on LPS concentration-dependent moDC-induced proliferation of autologous CD4+ T cells. As expected, PBMC activation with PHA resulted in >50% of CFSElow cells, whereas, in the absence of stimulus, <2% of PBMC were CFSElow (Fig. 6A). Preincubation with NETs had no impact on LPS concentration-dependent moDC-induced proliferation of autologous CD4+ T lymphocytes. In allogeneic conditions, LPS-activated moDC induced strong proliferation of CD4+ T lymphocytes, with 67.6 ± 6.9% and 46.6 ± 6.3% of CFSElow cells at moDC:PBMC ratios of 1:3 and 1:9, respectively (Fig. 6A). This proliferation was significantly diminished by NET preincubation (35.2 ± 4.7% and 26.1 ± 3.5% CFSElow cells, respectively).

We also measured the proliferation of Ag-specific autologous T lymphocytes. Purified CD4+ T lymphocytes were cocultured with LPS-activated moDC loaded with TT and pretreated with NETs. On day 6 of coculture, in the absence of Ag and independently of NET pretreatment, <20% of total cells were

tagonist AMD3100. These findings suggest that moDC migratory capacity, notably in response to lymphoid chemokines, is maintained in the presence of NETs.

FIGURE 4. NETs do not induce the expression of tolerogenic-related genes in activated moDC. MoDC were incubated with dexamethasone (DEX) (1 μg/ml), NETs (100 ng/ml DNA), LPS (25 ng/ml), or NETs plus LPS, for 24 h. Relative expression of genes associated with a tolerogenic profile (GILZ, STAB1, C1QA, CATC, and RALDH1) or an effector profile (MX1 and NMES) was evaluated by quantitative PCR (n = 3).
CFSE\textsuperscript{low}. Conversely, in the presence of TT, and with moDC: T lymphocyte ratios of 1:10 and 1:30, respectively, 37.9 \pm 5.3\% and 23.8 \pm 2.4\% of recovered cells were CFSE\textsuperscript{low}. In keeping with the results obtained in allogenic conditions, moDC maturation in the presence of NETs led to significantly lower proliferation of TT-specific CD4\(^+\) T lymphocytes, with 21.8 \pm 4.4\% and 14.1 \pm 2.8\% of CFSE\textsuperscript{low} cells, respectively, at moDC:T lymphocyte ratios of 1:10 and 1:30 (Fig. 6B).

Collectively, these findings reveal that NETs downregulate the capacity of mature moDC to activate autologous and allogeneic CD4\(^+\) T cell proliferation.

**NETs affect the capacity of LPS-activated moDC to polarize CD4\(^+\) T cells**

To identify the type of effector moDC induced by NETs, we analyzed the polarization of naïve allogeneic CD4\(^+\) T cells cocultured with moDC. MoDC were first incubated for 24 h with medium alone, NETs, LPS, or NETs plus LPS, and then washed and cocultured with allogeneic naïve CD4\(^+\) T lymphocytes. The effector phenotype of activated T lymphocytes was evaluated after 5 d by quantifying cytokines in the culture supernatants (Fig. 7). NETs alone or AluI alone (data not shown) did not induce more cytokine production than medium alone. As expected, LPS treatment of moDC enhanced the secretion of the different cytokines. When moDC were pretreated with NETs, IFN-\(\gamma\), IL-17A, and IL-10 were significantly downregulated. Moreover, a significant increase in IL-5 and IL-13 production was observed. Together, these results suggest that NETs could favor the capacity of LPS-activated moDC to promote effector Th2 responses and hinder polarization toward Th1 or Th17 phenotypes.

**Discussion**

The biological properties of NETs, and especially their immunomodulatory functions, are drawing increasing attention. In this study, we show that NETs influence moDC maturation in vitro: surface markers and cytokine production were both downregulated by NETs, whereas tolerogenic gene expression was not upregulated and chemokine receptor expression and moDC migratory capacity were unaffected. NET-treated moDC displayed a diminished capacity to induce Ag-specific and allogeneic CD4\(^+\) T lymphocyte proliferation, and promoted Th2 CD4\(^+\) T lymphocytes.

Although PMN are well known for their proinflammatory properties, evidence has emerged that PMN also participate in regulating inflammation. Indeed, after target eradication, PMN metabolism shifts toward a proresolutive state, thus damping inflammation and facilitating tissue repair [reviewed in (52)]. In particular, PMN can control DC activation via several mechanisms [reviewed in (26, 27)]. The effects of NETs on APC maturation are unclear (21, 42–44). We thus took advantage of our previously described method for preparing and quantifying isolated functional NETs (5) to study the impact of calibrated NETs on moDC maturation and its functional consequences.

We chose to use LPS, a major TLR4 ligand, to induce moDC maturation and assess the effect of NET preincubation on this process. NETs issued from AluI-, MNase-, and DNase-treated PMN were compared, and we chose to use the large NETs from AluI preparations, as smaller MNase- and DNase-derived NETS did not modulate moDC maturation. We can hypothesize that long-size isolated NETs, close to in vivo physiological NETs, better maintain a high local concentration of NET-associated molecules (1) and even enhance their activities. Indeed, it was recently shown that DNA binding increases elastase or cathepsin G function (53). We found that NETs alone or AluI alone had no effect, but that they attenuated LPS-induced upregulation of HLA-DR and some costimulatory molecules (CD40, CD80, and CD86) in a concentration-dependent manner. In addition, TNF-\(\alpha\), IL-6, IL-8, IL-10, and IL-23 production was downregulated in the presence of NETs at both the mRNA and protein levels.
We then examined the mechanisms underlying these regulatory effects on LPS-induced maturation. We first showed that NETs themselves did not have cytotoxic or proapoptotic effects on moDC or degrade TNF-α. We also ruled out a role of indirect effects such as LPS trapping or TLR4 blockade, as previously reported for some PMN proteins present on NETs. Indeed, lactoferrin and bactericidal/permeability-increasing protein can sequester LPS (35, 36), and lactoferrin, cathepsin G, and elastase have been reported to alter LPS binding to TLR4, either by competition or by partial proteolysis of CD14 or TLR4 (39, 54, 55). Interestingly, we found that the NET regulatory effect on moDC was not limited to TLR4 activation, but also occurred when a TLR7/TLR8 ligand or PMA was used.

The active NET component(s) responsible for these regulatory effects on moDC maturation might be one or several NET-associated granular proteins. Indeed, antimicrobial molecules such as LL-37, lactoferrin, and α-defensins can inhibit DC activation (33, 54, 56, 57). In addition, elastase can induce TGF-β (32) and reduce proinflammatory cytokine production by moDC, thus promoting CD4+ lymphocyte T regulatory polarization (31). Recently, Schauer et al. (45) evidenced that aggregated NETs promote the resolution of inflammation in a murine model of gout, via elastase-induced cytokine and chemokine degradation; as we did not evidence such a proteolytic effect of purified NETs on TNF-α in our study, we can assume that other mechanisms are involved. Finally, MPO has been identified as an inhibitor of DC activation and function both in vivo and in vitro, inhibiting adaptive immune responses (37). Besides these granular candidates, NET-associated nuclear proteins such as histones, the most abundant proteins in NETs, might also be involved in the observed regulatory actions (9). The effect of histones on DC activation has not yet been reported, even in their citrullinated form largely present in NETs. NET-associated histones are known to be cytotoxic for endothelia and epithelia (6, 15, 16), but we observed no such cytotoxic effect on moDC in our experimental conditions. Another NET-associated protein candidate is high-mobility group box 1, an important alarmin (23) able to modulate DC functions and induce tolerance (58). The NET components involved in downregulating moDC activation are currently under investigation in our model.

We then documented the functional consequences of NET downregulation of moDC maturation. We first examined the molecular signature of effector and regulatory DCs recently described by Zimmer et al. (50). Although NETs downregulated moDC maturation in response to LPS, the resulting cells exhibited no increase in their expression of pan-regulatory DC markers (C1QA, CATC, and GILZ genes), but rather tended toward DC effector marker expressions (MX1 and NMES1 genes). We then found that NETs did not modify the LPS-induced chemokine receptor expression pattern at the moDC surface (CCR7 and CXCR4) (59) or the migratory capacity of these cells toward the lymphoid chemokines CCL19 and CXCL12 in vitro. This suggests that, even after interaction with NETs at inflammatory sites, DC would maintain their capacity to migrate to lymph nodes.

Finally, we studied the effects of NET-treated moDC on T lymphocyte proliferation and polarization. We observed reduced CD4+ T lymphocyte proliferation when these cells were exposed to moDC preincubated with NETs during their maturation, in both allogeneic and Ag-specific conditions. Conversely, Tillack et al. (60) recently reported that NETs alone can interact directly with
the tCR and thereby enhance Ag-specific T cell proliferation. Together, these results suggest that NETs might differentially modulate T lymphocyte proliferation, through either a direct effect on tCR or an indirect effect via moDC modulation. To investigate the effect of these moDC on CD4+ T cell polarization, we cocultured CD4+ T cells with moDC matured in the presence of NETs and then measured T cell–derived cytokines. A significant decreased IFN-γ, IL-10, and IL-17 production was found, in keeping with the observed decrease in IL-12p70 and IL-23 production by moDC [reviewed in (61)]. These results are also in keeping with those of Doz et al. (62), who recently reported that mycobacteria-infected murine DC inhibited IL-17A production by Th17 CD4+ T cells via neutrophil-derived IL-10. Moreover, Th2 polarization was enhanced in our cocultures, as reflected by significant higher levels of IL-5 and IL-13. MoDC-derived IL-4 was significant higher levels of IL-5 and IL-13. MoDC-derived IL-4 was

**References**


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