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Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37

Matthieu Ribon[a,b,1,2], Sarra Seninet[a,b,1], Julie Mussard[a,b,3], Mireille Sebbag[c,d,e], Cyril Clavel[c,d], Guy Serre[c,d], Marie-Christophe Boissier[a,b,f], Luca Semerano[a,b,f], Patrice Decker[a,b,*]

[a] University of Paris 13, Sorbonne Paris Cité, Bobigny, France
[b] Inserm UMR 1125, Li2P, Bobigny, France
[c] University of Toulouse, Toulouse, France
[d] Inserm UMR 1056, Toulouse, France
[e] Inserm UMR 1220, Toulouse, France
[f] Avicenne Hospital, Rheumatology Department, AP-HP, Bobigny, France

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ABSTRACT

Objective: Neutrophil extracellular traps (NET), produced by activated polymorphonuclear neutrophils (PMN), are supposed to play a role in the pathogenesis of rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease characterized by anti-citrullinated protein antibodies (ACPA). Indeed, NET contain citrullinated autoantigens and some RA autoantibodies recognize NET. However, the mechanisms by which NET trigger or perpetuate the inflammatory process in RA are hitherto not elucidated. We hypothesized that, in addition to citrullination, NET might also contain stimulatory proteins and directly activate inflammatory target cells, as PMN and macrophages.

Methods: NET antigenic and inflammatory properties were analyzed in 157 healthy donors (HD) and RA patients, the largest analysis reported so far. Primary PMN and monocyte-derived macrophages were isolated and immunoglobulin G (IgG) purified. NET were induced (NETosis), isolated and quantified. NET antigenicity was analyzed by fluorescence microscopy. PMN and macrophages were stimulated with NET with/without ACPA, C1q, LL-37 or lipopolysaccharide (LPS) and cell activation was estimated by flow cytometry and ELISA.

Results: PMN from RA patients produced more NET than HD PMN. We next dissected how NET mechanistically affect inflammatory cells. Particularly, we show for the first time that RA and HD NET activated both resting macrophages and PMN, but importantly RA NET were more stimulatory, leading to secretion of inflammatory cytokines and up-regulation of HLA/CD86/CD11b. IgG from ACPA-positive RA patients specifically recognized RA and even HD NET. Nevertheless, NET-induced cell activation occurs independently of immune complex formation with ACPA. Likewise, endosomal acidification was not required. Notably, we also report that complement C1q increased the NET stimulatory activity on macrophages only, due to higher expression of C1q receptors, which was further supported by the LL-37 antimicrobial peptide. In contrast, NET specifically inhibited interleukin (IL)-6 secretion by LPS-activated macrophages and not PMN, especially with C1q/LL-37. This inhibition was not mediated by NET-derived proteases or LPS neutralization and was associated with the simultaneous induction of IL-10 secretion.

Conclusion: We show that NET possess both pro- and anti-inflammatory properties depending on target cells, their activation levels and C1q/LL-37. Thus, independently of ACPA, NET modulate RA chronic inflammation via this new dual activity we identified. In addition, NET may trigger autoimmunity in RA as ACPA recognize NET antigens but not non-activated PMN. Therefore, we conclude that excess of NETosis together with enhanced NET activity participate to RA pathogenesis at different levels.

* Corresponding author. University of Paris 13, Inserm UMR 1125, Li2P, 1 rue de Chablis, 93017, Bobigny, France.
E-mail address: patrice.decker@univ-paris13.fr (P. Decker).
[1] M.R. and S.S. contributed equally to this work.
[2] Present address: Institut du Cerveau et de la Moelle épinière, Inserm U 1127-CNRS UMR-7225-UPMC-Université Paris 6, Paris, France; matthieu.ribon@icm-institute.org.
[3] Present address: Cancer Research Centre Lyon, UMR INSERM 1052 CNRS 5286, Centre Leon Berard, Lyon, France; mussard.julie@gmail.com.

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1. Introduction

Polymorphonuclear neutrophils (PMN) belong to the first line of defense against pathogens. They are the first cells recruited to most inflammatory sites and sustain inflammation. Moreover, PMN can be activated by endogenous factors. However, excessive PMN activation or impaired resolution of inflammation may be deleterious. Neutrophil extracellular traps (NET) are chromatin fragments composed of DNA and associated proteins expelled by activated PMN. This process, NETosis, was initially described as an innate immunity mechanism against bacteria [1].

Rheumatoid arthritis (RA) is a chronic inflammatory and autoimmune disease leading to joint destruction whose etiology is still unknown. RA affects 0.3–1% of adults and is characterized by the production of the disease-specific anti-citrullinated protein antibodies (ACPA) that form immune complexes (IC) with citrullinated fibrinogen in the synovial tissue of RA patients and activate macrophage [2]. However, the stimuli and the mechanisms triggering ACPA production are still unknown. Particularly, the reason why citrullination, mediated by peptidylarginine deiminases (PAD), leads to ACPA production in RA is unknown. As NETosis is a process associated with citrullination [3] and ACPA recognize citrullinated proteins, NET might play a crucial role in RA pathogenesis. Indeed, NET might be a source of citrullinated autoantigens and neoepitopes [4] and might be either targets for ACPA or at the origin of ACPA induction by favoring the breach of immune tolerance, leading to downstream pathogenic events. Citrullination in NET is, however, less intense than during hypercitrullination [5]. In addition, NETosis is associated with the release of active PAD and the latter is present in RA synovial fluid [6] and might citrullinate local autoantigens. Recently, spontaneous and/or lipopolysaccharide (LPS)-induced NETosis have been shown to be stronger in vitro with PMN from RA patients in comparison to osteoarthritis patients or healthy donors (HD) [7,8] and with PMN from arthritic versus naïve mice [9]. Moreover, RA immunoglobulin (Ig) G stimulate NETosis of RA and HD PMN [7] and recognize citrullinated histone H4 in NET [10]. NETosis-derived products might also correlate with disease activity [11] and different NET pathways might exist in vivo [12]. NET appear thus important in RA, but independently of citrullination, we suggest they might also contain stimulatory proteins.

PMN are activated in RA and depletion of PMN in RA mouse models impairs disease development [13]. PMN, soluble chromatin and chromatin-containing IC are present in joints of RA patients [14,15]. It is tempting to speculate that part of the chromatin might come from NET derived from locally present and activated PMN and that those NET, free or in IC, might be involved in the inflammatory process, as we showed for chromatin on PMN [16,17]. In the latter studies, chromatin but not free DNA activates PMN.

Although a major function of NET is to neutralize bacteria, NETosis may be pathogenic. In systemic lupus erythematosus (SLE), impaired clearance of NET is associated with the production of anti-NET antibodies, nephritis development [18] and interferon-α production by plasmacytoid dendritic cells (DC) [19,20]. However, the clearance of NET from healthy subjects by HD macrophages does not lead to the secretion of pro-inflammatory cytokines [21] and even such NET impair the LPS-mediated activation of HD DC [22]. On the other hand, NET are immunogenic and prime T lymphocytes [23]. Therefore, we aimed at characterizing the role of NET on innate immune cells in RA. Thus, in addition to the pathogenic activity of NET-containing IC, we hypothesized that free NET might directly activate leukocytes in RA, especially key inflammatory cells as macrophages and PMN. We have thus tested and compared the differential antigenic and inflammatory properties of NET from HD and RA patients on target cells from HD and RA patients. We describe new C1q/LL-37-dependent but ACPA-independent and endosomal acidification-independent mechanisms triggering either pro- or anti-inflammatory responses, thus demonstrating a dual activity of NET. Our results suggest that abnormal clearance of NET strongly influences innate immune responses. Particularly, RA PMN produce more NET and more active NET.

2. Materials and methods

2.1. Experimental design

The overall goal of this study was to compare antigenic and immunoregulatory properties of human NET from HD and RA patients on a large scale. Further experiments were designed to characterize the mechanisms involved, especially by determining the involvement of ACPA, endosomal TLR and two DNA-binding proteins (C1q and LL-37) known to be produced in RA joints. We focused on two pro-inflammatory cell types (PMN and macrophages) involved in RA pathogenesis. All the experiments were performed with primary cells.

2.2. Human samples

157 donors (62 RA patients and 95 HD) have been tested in the present study to delineate the regulatory properties of NETosis. RA patients fulfilled the American College of Rheumatology-European League Against Rheumatism 2010 criteria. Characteristics of the 42 RA patients specifically used to prepare soluble NET are presented in Supplementary Table S1. We endeavored to focus on RA patients who were not treated with biologic therapy.

IgG were purified from a pool of sera from additional ACPA-positive RA patients and from ACPA-negative patients with rheumatic diseases as previously described [2].

2.3. NETosis induction, immunostaining and analysis by fluorescence microscopy

Freshly isolated PMN were seeded on poly-L-lysine-coated (0.001%, Sigma-Aldrich) borosilicate chamber slides (NUNC), settled for 30 min and activated by 50 nM phorbol myristate acetate (PMA) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). After 2 h, supernatants were discarded, NET were fixed with 4% paraformaldehyde and washed. The chamber slides were blocked with 2% bovine serum albumin (Sigma-Aldrich), 2% heat-inactivated goat serum (Eurobio), 0.2% Triton X-100 (Sigma-Aldrich) and NET were stained with 100 μg/ml purified IgG (from either ACPA-positive RA patients or ACPA-negative rheumatic patients or HD) followed by an AlexaFluor568-conjugated anti-human IgG antibody (Life Technologies, catalog number A21090). Alternatively, NET were stained with an anti-histones monoclonal antibody (mAb) (clone H11-4, pan histones, Millipore) followed by an AlexaFluor568-conjugated goat anti-mouse IgG secondary antibody (Life Technologies, catalog number A11004). After washing, NET were also stained with the Sytox green DNA dye (Life Technologies) and analyzed on a Zeiss Axioskop fluorescence microscope. Stainings were evaluated in a blind and independent manner by two persons.

Comparisons of stainings with ACPA-positive and ACPA-negative IgG are based on the percentage of positive stainings and not on the staining intensity, as we consider that the former is more reliable.

2.4. Soluble NET preparation

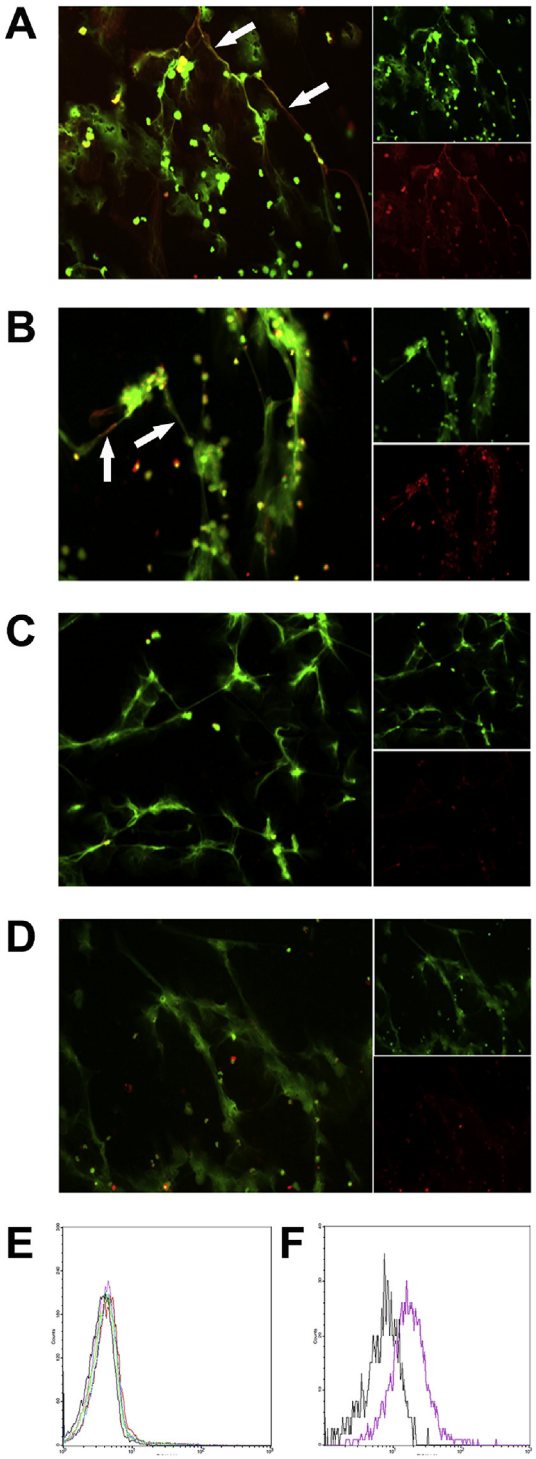
NETosis was induced in vitro by PMA on poly-L-lysine-adherent PMN as described above except that PMN were cultured without FCS. In some cases, NETosis was induced in the presence of 100 μM diphenylene iodonium (DPI, Sigma-Aldrich) to block NETosis. To produce soluble NET, supernatants were discarded after 4 h, chambers were washed twice with phosphate-buffered saline (PBS) and NET were detached from glass by mild deoxyribonuclease 1 (DNase 1) digestion (Sigma-Aldrich, 5 U/ml, 15 min) in PBS. The reaction was stopped by 3 mM EDTA and supernatants containing soluble NET were harvested

and centrifuged at 300 g for 10 min to remove any intact cells. The upper phase was collected and NET were enriched by a second centrifugation step (16,000 g, 10 min, to remove cellular debris) and again the upper phase was harvested and frozen. As a control, the same procedure was followed but without PMN in order to prepare the corresponding NET buffer. NET were quantified by fluorescence in a microplate reader (using PicoGreen, a dye for the quantification of soluble double-stranded DNA, Life Technologies) and by spectrophotometry (NanoDrop technology) and characterized by 16% SDS-PAGE and 1.5% agarose gel. Preparations ranging from 18 to 40 µg/ml (of DNA, as determined by spectrophotometry by measuring optical density at 260 nm) were used in cell cultures.

3. Results

3.1. NET from both RA patients and healthy donors are antigenic and strongly as well as specifically recognized by IgG autoantibodies from ACPA-positive RA patients

We tested whether NET are preferentially recognized by ACPA from RA patients, and whether RA NET are more antigenic. First, typical NET structures (chromatin fibers) were induced in activated PMN, as evidenced by the merged signal upon co-staining of DNA and histones (Fig. 1A, arrows). NET were then stained with IgG purified from either ACPA-positive RA patients or ACPA-negative patients suffering from other rheumatic diseases. In contrast to ACPA-negative IgG (Fig. 1C), ACPA-positive IgG strongly recognize NET structures (Fig. 1B, arrows), i.e. the chromatin filaments, as demonstrated by the merged signal upon DNA staining, whereas we observed no binding of IgG purified from HD on NET (Fig. 1D). NET staining was reproduced with 22 donors (11 RA patients and 11 HD) in 20 independent experiments. The staining with ACPA-positive IgG from RA patients was stronger than with ACPA-negative IgG in 15 out of 22 cases (68%; $p < 0.0001$; 95% confidence interval = 0.45–0.86), suggesting that NET expose antigenic and citrullinated structures at the surface recognized by RA autoantibodies. By comparing NET induced using PMN isolated from RA patients and HD, we observed that ACPA-positive IgG from RA patients recognize both RA NET (in 9 out of 11 patients) and HD NET (in 9 out of 11 HD). This indicates that, once NETosis is triggered, HD NET are as antigenic as RA NET thereby suggesting that the key antigenic event is the induction of NETosis. Among the 11 RA patients tested, 7 were ACPA-positive RA patients and 4 ACPA-negative RA patients. Staining of NET with ACPA-positive IgG was stronger than with ACPA-negative IgG in 6 out of 7 ACPA-positive RA patients and in 3 out of 4 ACPA-negative RA patients (not significant). Therefore, both NET from ACPA-positive RA patients and ACPA-negative RA patients are recognized by ACPA and at similar extent, suggesting there is no strong difference in the level of NET-associated citrullinated proteins between these patients. On the contrary, ACPA-positive IgG do not bind to non-activated PMN (Fig. 1E), indicating that they specifically recognize NET. Gated monocytes (expressing the high affinity IgG receptor CD64) pre-incubated with human immunoglobulins were used as a positive control (Fig. 1F). Those results represent the most detailed comparison of RA autoantibody recognition of HD versus RA NET.



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3.2. RA patients have an increased capacity to produce NET compared with healthy donors

To test whether NET are immunostimulatory, we first established a protocol allowing the isolation of enriched and concentrated NET. Indeed, several protocols and approaches have been described but some of them might not be optimal to analyze the effects of NET on target cells in co-culture. For example, some studies use PMA-activated PMN culture supernatants which actually contain some PMN-derived DNA but also cytokines induced by PMA as well as PMA itself. Others use a protocol similar to ours but did not verify the cytokine content in NET preparations. Some groups directly co-culture target cells with activated PMN to induce NETosis and use NETosis inhibitors to prove the observed effects are NET-dependent; however, we observed that some of those inhibitors not only inhibit NETosis but also cytokine release during PMN activation (Supplementary Fig. S1A). In addition, we observed that co-cultures obtained by adding target cells on surface-adherent NET are also not optimal because part of the PMA or the cytokines secreted by activated PMN bind to plastic/glass (Supplementary Fig. S1B). Recently, it was also reported that NETosis may be confounded with hypercitrullination, e.g. after stimulation by calcium ionophores [5]. In NETosis, citrullination occurs but is relatively low and

Fig. 1. IgG from ACPA-positive RA patients strongly and specifically recognize NET. (**A**) Typical chromatin fibers observed upon NETosis. PMN were freshly isolated from a HD, seeded on poly-L-lysine-coated chamber slides and activated by PMA to induce NETosis. After 2 h, NET (arrows) were stained with a DNA dye (green) and an anti-histones monoclonal antibody followed by an AlexaFluor568-conjugated anti-mouse secondary antibody (red) and analyzed by fluorescence microscopy. The individual fluorescence channels (right) and the merged signals (left) are depicted. (**B-D**) PMN were freshly isolated from a HD and NETosis was induced as in (**A**). After 2 h, NET were stained with a DNA dye (green) and with IgG purified from ACPA-positive RA patients (**B**, arrows) or from ACPA-negative patients with rheumatic diseases (**C**) or from HD (**D**) followed by an AlexaFluor568-conjugated anti-human IgG antibody (red) and analyzed by fluorescence microscopy. NET are the green chromatin filaments. The individual fluorescence channels (right) and the merged signals (left) are depicted. Shown is one representative result from 22 independent donors (11 HD and 11 RA patients) for whom NET have been induced and stained in 20 independent experiments. (**E, F**) Freshly isolated PMN (**E**) and PBMC (**F**) were untreated and stained ex vivo. PMN (**E**) were stained with a PE-conjugated F $(ab')_2$ anti-human IgG alone (purple) or together with ACPA-positive IgG (green), ACPA-negative IgG (blue) or normal IgG (red) and analyzed by flow cytometry. The black histogram represents unstained cells. In (**F**), cells were pre-incubated with human Ig and directly stained with the PE-conjugated F $(ab')_2$ anti-human IgG alone (purple). Monocytes were CD14-gated. The black histogram represents unstained cells.

restricted to some proteins in contrast to leucotoxic hypercitrullination, and the DNA released is associated with histones (as shown in Fig. 1A) in contrast to defective mitophagy. Moreover, classical ("suicidal") NETosis is NADPH oxidase-dependent. We therefore optimized the protocol and included controls to obtain better defined NET, which we call soluble NET, as opposed to NET attached to glass for microscopy analysis. We used the best characterized NETosis inducer (apart bacteria), namely PMA. This protocol (Fig. 2A) was highly reproducible, giving soluble NET composed of DNA from ~100 to ~ 400 base pairs (Fig. 2C) and a characteristic protein content (Fig. 2D). No DNA was detected in supernatants, but only after mild DNase 1 digestion of glass-adherent NET, and NET induction was blocked by the NADPH oxidase inhibitor diphenylene iodonium (DPI, Fig. 2B and Supplementary Fig. S1C), in agreement with a role of reactive oxygen species in NETosis [24]. Both HD and RA soluble NET contain histones as expected (Fig. 2E, top), including low levels of citrullinated histone H3 (Fig. 2E, bottom and Supplementary Fig. S2A). Amounts of citrullinated histone H3 were variable in both HD and RA soluble NET. Altogether, similar NET characteristics were observed with HD and RA PMN. However and importantly, RA PMN produce significantly more soluble NET upon activation than PMN from HD (Fig. 2F, $n = 58$, $p < 0.05$). Interestingly, as shown by the correlation matrix (Supplementary Fig. S3), the concentration of soluble NET prepared in vitro from RA PMN is positively correlated with the concentration of circulating PMN in patients, although NET were always prepared from the same number of PMN. Moreover, the "neutrophil" cluster (represented by the NET concentration and PMN count) appears also positively correlated with the "inflammation" cluster (represented by ESR, CRP and DAS28) in RA patients.

3.3. NET from both RA patients and healthy donors activate healthy donor and RA steady-state PMN as well as macrophages, but RA NET are more stimulatory

We then tested the stimulatory activity of soluble NET on two inflammatory cell types involved in RA pathogenesis. NET activated both macrophages and PMN, as evidenced by the secretion of the pro-inflammatory cytokine interleukin (IL)-8 (Fig. 3A and B (macrophages) and Fig. 3C, D (PMN)) and up-regulation of CD11b (PMN, Fig. 3G) or HLA class I/class II and CD86 (macrophages, Supplementary Fig. S4), indicating that NET are pro-inflammatory. Tumor necrosis factor (TNF) (macrophages and PMN) and IL-6 (macrophages) were also induced by NET, to a lesser but still significant extent, whereas minimal secretion of the immunomodulatory cytokine IL-10 (especially in comparison to IL-8) was observed (Supplementary Fig. S5). Interestingly, soluble NET prepared with PMN from both HD and RA patients induce cell activation indicating that both HD and RA NET have stimulatory properties. In addition, we show that target cells (PMN and macrophages) from both HD (Fig. 3A, C, G) and RA patients (Fig. 3B, D, G) respond to the stimulation by HD or RA NET. These results suggest that a key pathogenic event in RA patients is the triggering of NETosis and the quantity of NET released rather than the ability to respond to NET. As controls, we first verified that the buffers used to enrich soluble NET do not alter the response of target cells (which is the true negative control for estimating NET-induced cell activation) and second that the cytokine detected after PMN and macrophage activation is not simply due to the passive transfer of the cytokine present in NET preparations but is really secreted by target cells (see also Supplementary Fig. S11). For example, IL-8 concentrations measured in the NET purification buffer or in RA NET cultured without target cells are 0 and 0.43 ng/ml respectively in the cultures with HD macrophages (Fig. 3A). By using the NET buffer control, we thus also verified that PMA is not transferred in soluble NET after washes. Next, we have demonstrated that when glass-adherent NET are not detached by DNase 1 treatment after PMA is washed out, no soluble NET (i.e. chromatin fragments) are transferred onto target cells and no activation is induced (Supplementary Fig. S6). Indeed, neither DNA nor proteins (among them histones, bottom) are detected on agarose gel and SDS-PAGE without DNase 1 treatment; and Fig. 1A shows that chromatin fibers (complexes of DNA associated to histones) are observed when NETosis was induced. Likewise, when soluble NET are degraded due to overdigestion by DNase 1 (after PMA is washed out), they lose most of their activating potential (Supplementary Fig. S7). All these controls show that the identified stimulatory activity originates solely from NET. It should also be noted that free mammalian DNA is usually poorly stimulatory. Free DNA can only efficiently trigger activation of innate immune cells when it is forced to enter cells or to reach endosomes or when it is present in immune complexes; or when it is opsonized e.g. by histones or LL-37, like in chromatin and NET as observed in the present study and as previously reported by us.

The global analysis of all results from experiments testing the activity of NET (all RA and HD NET) demonstrates that NET significantly activate macrophages (Fig. 3E, $p < 0.05$ vs. the NET purification buffer, data pooled from 23 independent experiments) and PMN (Fig. 3F, $p < 0.001$ vs. the NET purification buffer, data pooled from 21 other independent experiments), leading to IL-8 secretion. Then, in the 14 independent experiments (among Fig. 3E and F) in which we compared HD and RA NET on the same target cells from the same donor (HD macrophages or PMN, cell activation estimated by IL-8 secretion, HD and RA NET tested at the same concentration), we have demonstrated that RA NET are more stimulatory than HD NET. Indeed, RA NET induce a stronger IL-8 secretion (Fig. 3H) and thus have a significantly higher activity than HD NET ($p < 0.05$), suggesting that RA NET are potentially more pathogenic. To explain the higher activity of RA NET, we then tested the presence of cytokines in soluble NET (in undiluted soluble NET, Supplementary Fig. S2B). In most soluble NET preparations, cytokine concentrations were low, at least compared to cytokine concentrations measured in cell cultures (where NET are diluted 1:1) and especially in macrophage cultures. IL-8 was detected in both HD and RA NET but at similar concentrations. TNF concentrations were lower than IL-8 concentrations in NET, and TNF was even more concentrated in HD NET than in RA NET ($p < 0.05$). IL-6 was hardly detectable in both HD and RA NET. Likewise, IL-10 was hardly detectable in NET, except in some HD NET ($p < 0.05$). All these results show that cytokine concentrations in NET do not explain the higher activity of RA NET as compared to HD NET.

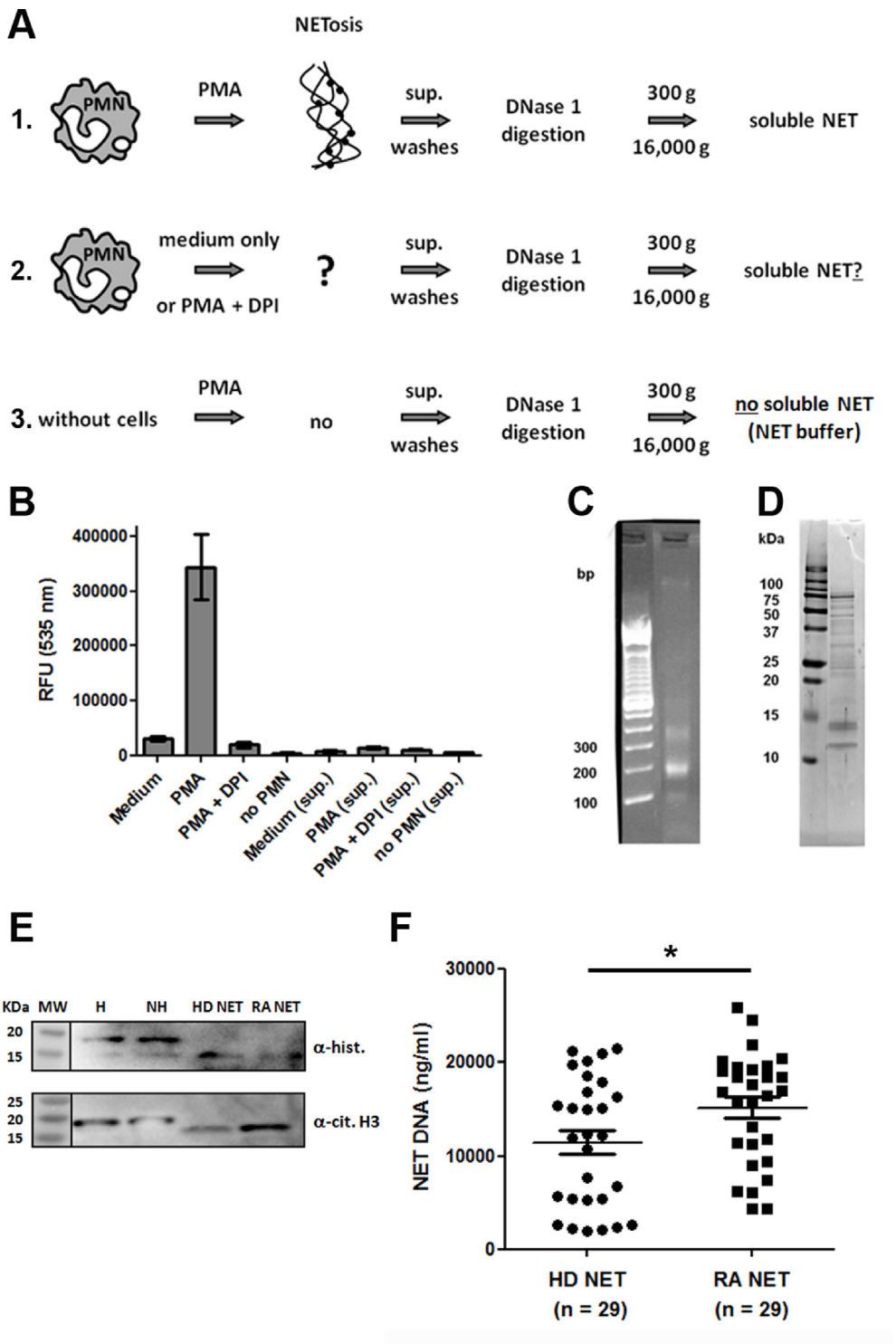

Fig. 2. Characterization of soluble NET and higher yield of production by PMN from RA patients versus healthy donors. (**A**) HD or RA PMN were seeded on poly-L-lysine-coated chamber slides and activated by PMA for 4 h to induce NETosis (**A.1**), or left untreated or activated in the presence of diphenylene iodonium (DPI, 100 μM), a NADPH oxidase inhibitor (**A.2**). As a negative control, soluble NET production was performed without PMN (**A.3**). Culture supernatants (sup.) were collected and chambers were washed. PMN were then treated with deoxyribonuclease 1 (DNase 1), the reaction was stopped by 3 mM EDTA and detached soluble NET were collected and centrifuged at 300 g and 16,000 g. NET were quantified on a fluorometer (**B and F**) using PicoGreen (RFU, relative fluorescence units) and by spectrophotometry and were analyzed on 1.5% agarose gel (**C**) and 16% SDS-PAGE (**D**). In (**C**) and (**D**): left lane, molecular weight markers; right lane, soluble NET; bp, base pair. Shown is one representative experiment of 74 independent experiments (except (**B**), 15 independent experiments testing sup. and DPI) using cells from independent donors (controls or RA patients). Mean and SD are shown. (**E**) Western blot demonstrating the presence of histones (citrullinated or not) in HD and RA soluble NET. Purified histones (H), nucleohistones (NH) as well as soluble NET from a healthy donor (HD NET) or a RA patient (RA NET) were separated by SDS-PAGE, transferred to PVDF membrane and probed for total histones (hist., top) or citrullinated histone H3 (cit. H3, bottom). MW, molecular weight markers; α-, anti. Shown is one representative experiment of 4 independent experiments using 8 pairs of HD and RA NET. As already reported, histones are partially cleaved during NETosis. (**F**) RA PMN produce more NET than HD PMN. Soluble NET were prepared from 58 independent donors (29 HD and 29 RA patients, from the 74 tested as in (**C**) and (**D**)) using freshly isolated PMN (3×10^6 cells in all cases) stimulated with PMA. Concentrations of NET DNA were determined by fluorescence using PicoGreen and according to a standard curve of purified DNA. *, $p < 0.05$ (two-tailed Mann-Whitney test). Mean and SEM are shown. HD, healthy donor; RA, rheumatoid arthritis patient.

3.4. Polyclonal ACPA are not required for strong NET-induced cell activation

Because NET are antigenic (Fig. 1) and PMN as well as macrophages express Fc receptors, we next investigated whether IC formation with ACPA modulates NET activity. As described above, both PMN and macrophages were activated by NET (Supplementary Fig. S8), as shown by IL-8 detection, but in both cases cell activation was not significantly enhanced in the presence of ACPA. Similar results were obtained with both HD and RA NET. Therefore, soluble NET directly activate macrophages or PMN and recognition by ACPA is not required for a strong stimulatory activity of NET.

3.5. The C1q complement protein enhances the NET-induced IL-8 secretion by macrophages

C1q has been shown to bind DNA [25,26] and to deposit onto NET [27]. As C1q is produced in RA synovium [28] and because PMN and macrophages express cell surface C1q receptors, we thus tested whether C1q favors NET-induced cell activation. Interestingly, whereas C1q did not significantly influence PMN activation by NET (Fig. 4A), macrophage activation by NET was significantly much stronger in the

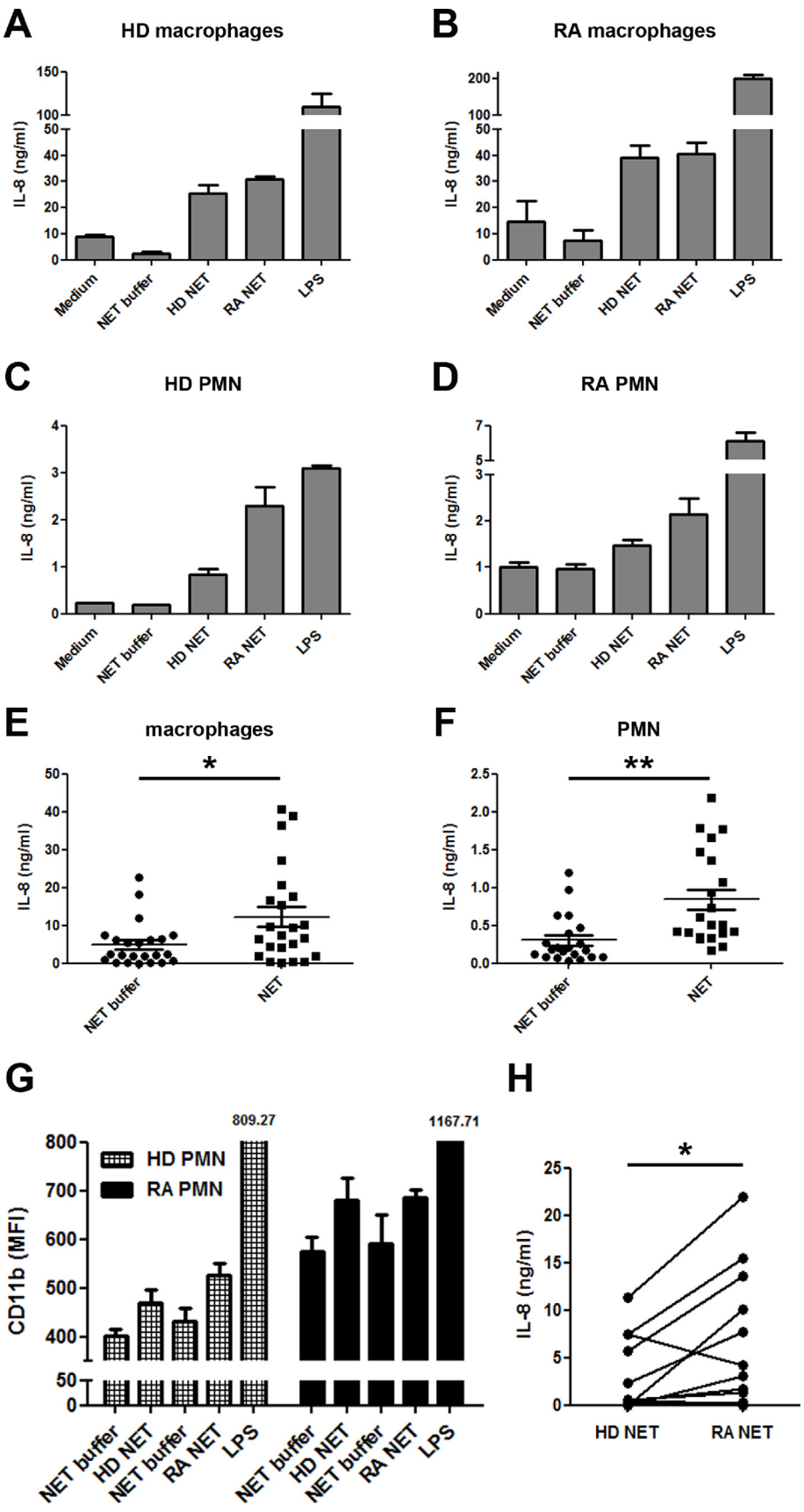

Fig. 3. Soluble NET activate PMN and macrophages and RA NET are more stimulatory than NET from healthy donors. (**A-D, G**) Monocyte-derived macrophages (**A**, **B**) and primary PMN (**C, D, G**) were prepared from HD (**A, C, G**) or RA patients (**B, D, G**) and cultured with soluble NET from HD or RA patients, the NET purification buffer, or LPS. Cell activation was estimated by measuring IL-8 secretion by ELISA (**A-D**) and CD11b up-regulation (on PMN) by flow cytometry (**G**). Shown is one representative experiment of at least 3 independent experiments for each panel. Mean and SD of triplicates are shown. MFI, mean fluorescence intensity. (**E, F**) Pooled data (RA and HD NET) showing the NET-mediated IL-8 induction in macrophages (**E**, $p < 0.05$ (*, two-tailed Mann-Whitney test), $n = 23$ pairs of NET buffer/NET preparations tested on 23 independent HD/RA macrophage donors) and PMN (**F**, $p < 0.001$ (**, two-tailed Mann-Whitney test), $n = 21$ pairs of NET buffer/NET preparations tested on 21 independent HD/RA PMN donors). Each symbol represents one donor tested with one NET preparation or its purification buffer. Mean and SEM are shown. (**H**) HD NET and RA NET were tested by pairs (at the same concentration) on HD macrophages or PMN in 14 independent experiments. IL-8 secretion was determined for each pair of NET and compared by a two-tailed Wilcoxon signed rank test (*, $p < 0.05$). Each line represents a pair of RA NET/HD NET and data are pooled from 10 experiments with macrophages and 4 experiments with PMN. HD, healthy donor; RA, rheumatoid arthritis patient.

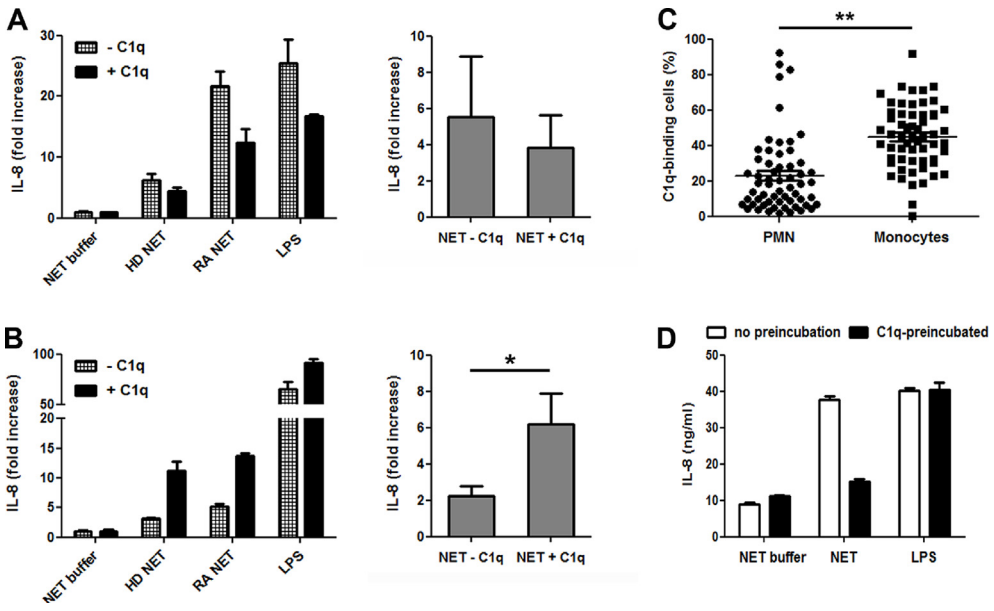

Fig. 4. C1q enhances the NET-induced activation of cells expressing high levels of cell surface C1q receptors. Primary PMN (**A**) and monocyte-derived macrophages (**B**) were prepared from HD and cultured with soluble NET, the NET purification buffer, or LPS in the presence (black bars) or absence (hatched bars) of C1q. Cell activation was estimated by measuring IL-8 secretion by ELISA. Shown is one representative experiment (left) and pooled NET data (right) of at least 6 independent experiments for each panel and using 4 macrophage donors tested with 5 NET preparations and 3 PMN donors tested with 5 NET preparations. Results are presented as fold increases relative to NET buffer. *, $p < 0.05$ (two-tailed Mann-Whitney test). Mean and SD (SEM for pooled data) are shown. (**C**) The C1q-binding capacity of PMN and monocytes was determined in 59 human blood donors (controls and RA patients). For each donor, PMN and monocytes were compared (**, $p < 0.0001$, all monocytes vs. all PMN, two-tailed Mann-Whitney test). Mean and SEM are shown. (**D**) Monocyte-derived macrophages were pre-incubated (black bars) or not (white bars) with an excess of C1q for 2 h and then activated or not with NET or LPS. IL-8 was quantified by ELISA. Shown is one representative experiment out of two (depicting mean and SD). HD, healthy donor; RA, rheumatoid arthritis patient.

presence of C1q (Fig. 4B, $p < 0.05$). Importantly, C1q alone did not activate macrophages. Anew, similar results were obtained with both HD and RA NET.

To explain the different modulatory activity of C1q on NET-mediated activation, we observed C1q binding (which reflects the global expression of all cell surface C1q receptors) with both PMN and monocytes which were used as precursors to prepare macrophages, but the percentage of monocytes expressing C1q receptors was higher than that of PMN (Fig. 4C, 45.1% vs. 23.3%, n = 59 donors, $p < 0.0001$). Results were similar with HD and RA target cells. In addition, we have clearly shown that macrophages express higher amount of C1q receptors than both monocytes and PMN (Supplementary Fig. S9A, $p < 0.005$) and that macrophages acquire high expression of C1q receptors during differentiation from monocytes (Supplementary Fig. S9B, $p < 0.05$).

To support our hypothesis on the involvement of C1q receptors, we have shown that blocking or saturating C1q receptors specifically reduces macrophage activation by NET but not by LPS (Fig. 4D). Thus, our results suggest that C1q facilitates NET-induced activation of cells expressing high levels of C1q receptors, presumably by bridging NET to the target cells.

3.6. LL-37 supports the C1q-enhanced IL-8 secretion by macrophages in response to NET

We next tested whether another co-factor expressed in RA synovial membranes [29] and especially in PMN and macrophages [30], the antimicrobial peptide LL-37, might work in combination with C1q. LL-37 also binds DNA, has both pro- and anti-inflammatory properties and, in particular, is produced by PMN and macrophages, which also express LL-37 receptors. Although LL-37 has little effect on NET-induced IL-8 secretion by macrophages (Fig. 5A), the combination of C1q with LL-37 (Fig. 5B) significantly increases IL-8 production by NET-stimulated macrophages as compared to C1q alone. This LL-37 effect is specific to NET, as LPS activation was not significantly influenced by LL-37, and was not observed with PMN (Supplementary Fig. S10). To verify that LL-37 is active, we showed it directly induces a moderate but significant IL-6 secretion by PMN and, in addition, it inhibits PMN response to LPS (Fig. 5C). Again, to support a role of LL-37 receptors in the recognition of NET by target cells in combination with C1q, we have shown that blocking or saturating LL-37 receptors specifically reduces macrophage activation by NET (Fig. 5D).

Anew, to understand the role of C1q and LL-37, we have tested the presence of LL-37 and C1q in soluble NET (in undiluted soluble NET, Supplementary Fig. S2C). We show that most NET preparations contain LL-37 and C1q, but there is no significant statistical difference between HD and RA NET regarding LL-37 and C1q concentrations, suggesting that these factors are not responsible for the higher activity observed with RA NET. Interestingly, LL-37 concentrations were higher than C1q concentrations when comparing all soluble NET ($p < 0.05$).

3.7. NET-induced cell activation occurs independently of endosomal acidification

The response to NET may require endocytosis and degradation of NET. Moreover, NET DNA may trigger cell activation through endosomal Toll-like receptor (TLR) 9 expressed by PMN and macrophages, which requires endosomal acidification. Cell activation by NET was observed both in the presence and absence of ammonium chloride (NH_4Cl), an inhibitor of endosomal acidification (Supplementary Fig. S11). As a control, activation through endosomal TLR7/8 was completely inhibited by NH_4Cl, whereas triggering of cell surface TLR4 was not influenced. Similar results were obtained with HD and RA PMN or macrophages. Thus, NET-induced cell activation occurs independently of endosomal acidification, suggesting that endosomal TLR7/8/9 are not involved in triggering activation. The incubation of NET without PMN or macrophages confirms that IL-8 is not transferred with NET but is really produced by activated target cells.

3.8. NET are also anti-inflammatory

Finally, we tested whether NET may on the opposite modulate the LPS-induced cell activation and the secretion of other cytokines. Interestingly, NET inhibit IL-6 secretion of LPS-activated macrophages (Fig. 6A) and are therefore also anti-inflammatory. This was observed with both HD and RA NET (both being significant vs. the purification buffer, Fig. 6B) and on both HD and RA macrophages ($p < 0.0001$ for pooled data). Only IL-6 was down-regulated, whereas IL-8 and TNF were normally secreted (Supplementary Fig. S12). Although this effect was not significantly influenced by C1q alone (Supplementary Fig.

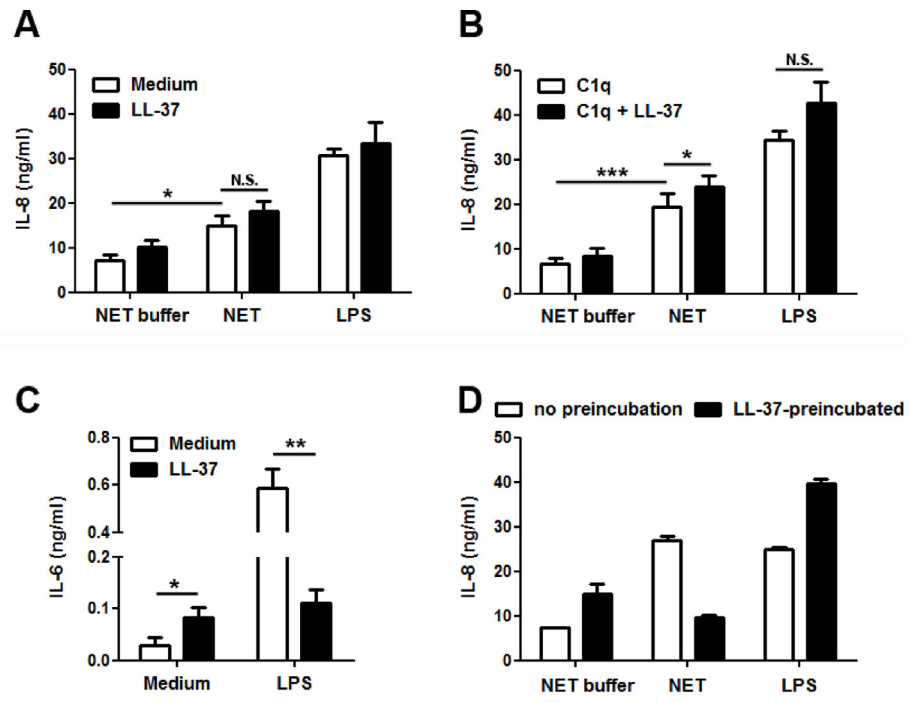

Fig. 5. C1q in combination with LL-37 further enhance macrophage response to NET. Monocyte-derived macrophages (**A, B**) and primary PMN (**C**) were prepared from HD and RA patients. Macrophages were cultured with soluble NET, the NET purification buffer, or LPS in the absence (**A**, white bars) or presence of LL-37 (**A**, black bars), C1q (**B**, white bars) or C1q together with LL-37 (**B**, black bars). PMN were cultured in medium alone or LPS, with LL-37 (**C**, black bars) or not (**C**, white bars). Cell activation was estimated by measuring IL-8 or IL-6 secretion by ELISA. Shown are pooled data from 5 independent experiments (5 macrophage donors tested with 5 NET preparations) and from 3 independent experiments (3 PMN donors). Similar results were obtained with HD and RA cells. Mean and SEM are shown. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$ (two-tailed Mann-Whitney test or two-tailed unpaired *t*-test, with or without Welch's correction); N.S., not significant. (**D**) Monocyte-derived macrophages were pre-incubated (black bars) or not (white bars) with an excess of LL-37 for 2 h and then activated or not with NET or LPS. IL-8 was quantified by ELISA. Shown is one representative experiment out of two (depicting mean and SD).

S13), the NET-mediated inhibition of IL-6 secretion was strongly enhanced in the presence of both C1q and LL-37 (Fig. 6C, $p < 0.01$ for LPS with NET vs LPS with NET, C1q and LL-37). On the contrary, LPS-mediated activation of PMN was not inhibited by NET, as IL-8 and TNF were normally secreted and IL-6 was not inhibited (Supplementary Fig. S14); NET and LPS have even an additive effect. Importantly, the NET-mediated inhibition of IL-6 secretion was accompanied by the simultaneous increased secretion of the immunomodulatory cytokine IL-10 by LPS-activated macrophages (Fig. 6D, $p < 0.05$ for NET vs. the purification buffer), reinforcing the anti-inflammatory potential of NET, although this was not strongly affected by C1q and LL-37.

4. Discussion

In the present study, we demonstrate for the first time that NET are pro-inflammatory and activate steady-state PMN and macrophages. Particularly, we show that, compared to HD, PMN from RA patients produce more NET, and that those NET more potently activate PMN and macrophages. We also show that NET are specifically recognized by ACPA-positive IgG from RA patients and are therefore potentially

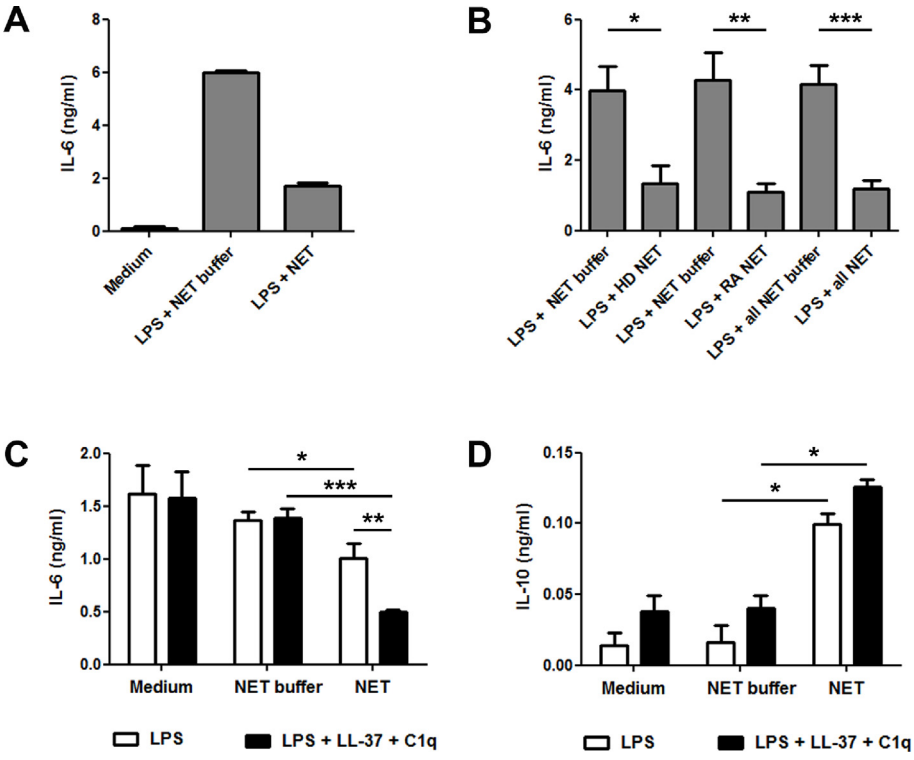

Fig. 6. NET possess an anti-inflammatory activity which is amplified by C1q and LL-37. Monocyte-derived macrophages were prepared from HD or RA patients and cultured in medium only or with soluble NET or the NET purification buffer, in the presence of LPS (**A, B**) or in the presence of LPS with or without C1q and LL-37 (**C, D**). Cell activation was estimated by measuring IL-6 and IL-10 secretion by ELISA. Shown is one representative experiment (**A**) and the 19 data pooled from 11 independent experiments using 13 macrophage donors tested with 12 (HD and RA) NET preparations (**B**). Asterisks indicate $p < 0.05$ for LPS with NET vs. LPS with the purification buffer (*, $p < 0.05$ for HD NET, two-tailed Mann-Whitney test; **, $p < 0.005$ for RA NET; ***, $p < 0.0001$ for all NET; two-tailed unpaired *t*-tests with Welch's correction). Panel (**C**) shows the increase of the NET anti-inflammatory activity by C1q and LL-37, whereas panel (**D**) highlights the simultaneous induction of IL-10. Shown are pooled data from 3 independent experiments (3 macrophage donors tested with 3 NET preparations). *, $p < 0.05$ (two-tailed unpaired *t*-test); **, $p < 0.01$; ***, $p < 0.0001$ (two-tailed unpaired *t*-tests with Welch's correction). Mean and SD (**A**) or SEM (**B, C, D**) are shown. HD, healthy donor; RA, rheumatoid arthritis patient.

antigenic in RA. Importantly, we report a dual activity of NET since we show that NET are also potentially endowed with anti-inflammatory functions, as they inhibit IL-6 secretion and at the same time increase IL-10 secretion by activated macrophages. Moreover, we dissected how NET mechanistically affect inflammatory cells and showed that NET-induced cell activation is independent of IC and of endosomal acidification. Conversely, C1q reinforces the pro- or anti-inflammatory response of macrophages to NET, especially when helped by LL-37. This is, globally, the largest analysis comparing RA and HD NET on RA and HD target cells reported so far.

Despite the NET beneficial role on bacteria, the consequences of uncontrolled NETosis or the potential pathogenic effects of NET in autoimmune diseases are less understood. It has been shown that NET may be involved in fibrosis [31], gout [32], as well as in SLE [33] and in RA [7], mostly promoting inflammation. Nevertheless, at very high concentration as in gout only, NET may participate in the resolution of inflammation [34]. Therefore, immunoregulatory properties of NET need to be further investigated.

In RA, NET might be a source of citrullinated autoantigens and thus trigger ACPA production. If recognized by ACPA, NET might also be involved in downstream pathogenic events, like complement activation by NET-ACPA IC. In our work, the ACPA staining was particularly observed on NET fibers (and not on non-activated PMN) and was significantly much lower with ACPA-negative IgG, whereas IgG from healthy individuals gave no signal. The low signal observed with ACPA-negative IgG is probably due to other antibody specificities present in the serum pool. Interestingly, we clearly show that NET from both HD and RA patients are recognized by ACPA-positive IgG, indicating that once NETosis is triggered, HD NET are as antigenic as RA NET.

We next demonstrated that NET are not only recognized by autoantibodies but are also directly stimulatory and clearly pro-inflammatory. They indeed activate steady-state PMN and macrophages. Particularly, macrophages acquire a phenotype of activated antigen-presenting cells, which may contribute to the breach of tolerance, e.g. by presenting NET-derived antigens. Anew, both HD and RA NET have inflammatory properties, but RA NET are more stimulatory than HD NET. However, concentrations of cytokines, C1q and LL-37 in NET do not explain the higher activity of RA NET. Cells from both HD and RA patients respond similarly to NET, excluding an intrinsic difference in RA cell response to NET. These results suggest that the NETosis process is not fundamentally altered in RA but, rather, that increased levels of NETosis, together with a higher stimulatory activity, probably due to a slightly different composition of NET, may be pathogenic in RA patients.

We also report that NET-ACPA IC formation is not required to trigger strong cell activation, although we have shown that ACPA do bind NET. NET might therefore also be pathogenic in the absence of ACPA, which may happen either in ACPA-negative RA patients or in the early phases of disease, before ACPA are produced. However, ACPA may support cell activation by NET in particular conditions. Particularly, cell activation may be stronger with monoclonal ACPA and vary depending on their antigenic specificities. In addition, we have excluded that endosomal acidification is important for NET-mediated cell activation, suggesting that activation does not occur through recognition by endosomal TLR. On the contrary, we have demonstrated that C1q enhances the NET-induced activation of macrophages with high expression of C1q receptors, which is further potentiated by LL-37. Thus, C1q and LL-37 may behave as NET transporters for macrophages.

Nevertheless, we also show that in some cases NET are anti-inflammatory. This property was only observed with strongly-activated macrophages (after LPS stimulation). IL-6, but not IL-8 nor TNF, was partly inhibited and inhibition was stronger with both C1q and LL-37. On the contrary, IL-10 was induced. According to Schauer et al. [34], we can exclude cytokine degradation by NET-derived proteases as TNF secretion by LPS-activated macrophages was not affected by NET (Supplementary Fig. S12). We can also exclude LPS neutralization by NET as IL-8 secretion by LPS-activated macrophages was not affected by NET, showing that macrophages are truly activated (Supplementary Fig. S12). Likewise, concentrations of cytokines in NET do not explain this anti-inflammatory activity of NET. Thus, a fine control is probably required in vivo to determine whether NET should trigger pro- or anti-inflammatory responses. This regulation might rely on a balance between stimuli, cell types, activation level of target cells and co-factors like C1q and LL-37 that might bind NET, as summarized in Supplementary Fig. S15.

In contrast to some recent studies [21,22], we show that innate immune cells are activated by NET. There are several explanations for this only apparent discrepancy. First of all, and as explained above, we have optimized the preparation of NET, meaning that we used a slightly different protocol (DNase I digestion or no digestion versus other nucleases, PMA versus other stimuli as calcium ionophore), and we have included several controls to prove the immunoregulatory activity of NET (preparations without PMN or with non-activated PMN). Moreover, we have analyzed different cytokines (IL-8 production by macrophages), other pathways (endosomal TLR and ACPA) and especially different target cells (PMN). In addition to these studies, we emphasize a mechanism depending on C1q and LL-37 as well as a dual activity of NET according to their environment. But a particular added value of the present report is the study on a large scale of the antigenicity of NET, the comparison of HD and RA NET as well as HD and RA target cells for the NET immunoregulatory activity. Particularly, all experiments were performed with primary cells and we analyzed and compared two types of pro-inflammatory target cells. But similarly, RA NET were very recently shown to up-regulate HLA class II on DC [9] and fibroblasts [35].

There are a few limitations in our study. Ideally, staining of NET and cell activation assays with NET should be performed with purified ACPA instead of IgG purified from ACPA-positive RA patients in order to determine whether NET form pathogenic IC.

Direct clinical implications arise from these findings because pathogenicity of anti-citrullinated protein immunization has been confirmed in RA [36]. However, not all the involved mechanisms are elucidated, yet. Further studies will be required to determine whether NETosis is pathogenic through its pro-inflammatory activity or by triggering ACPA production or simply as a target of ACPA, or the combination of both. The precise comprehension of NET pathogenic role may help define whether NET may constitute a potential therapeutic target for future treatments. Influence of currently used biologic therapies on NET activity also deserves further studies.

In conclusion, NET are antigenic, immunogenic, pro-inflammatory and therefore potentially pathogenic, even if they may control activation of strongly-stimulated cells. Further experiments will be required to determine whether NET induce ACPA production and what are the downstream consequences of ACPA binding to NET in the pathophysiology of RA, such as complement activation.

Conflicts of interest

None.

Author contributions

All authors were involved in drafting the manuscript. P.D. designed the research, performed part of the experiments, analyzed and interpreted data and wrote the manuscript. M.R. and S.S. performed the experiments, analyzed and interpreted data. J.M. performed part of the experiments and analyzed data. M.S., C.C. and G.S. contributed reagents, analyzed and interpreted data. L.S. and M.C.B. analyzed and interpreted data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.01.003.

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