Immune stimulatory effects of neutrophil extracellular traps in granulomatosis with polyangiitis

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ABSTRACT

Objective. *The aim of this study was to analyse the role of netting neutrophils in the pathogenesis of granulomatosis with polyangiitis (GPA), especially their interplay with peripheral blood mononuclear cells (PBMCs).*

Methods. *The amount of cell-free DNA (cfDNA) was determined in sera from GPA patients (pairs active/inactive state of disease, n=18) and from healthy controls (HCs, n=10). Furthermore, we performed in vitro incubation experiments using PBMCs and NETs from patients and HCs for accessing the effect of NETs on PBMC behaviour. We determined proliferation of T- and B-cells (CSFE assay), B-cell maturation (CD38 staining and flow cytometry), production of IgG (ELISpot, ELISA), and secretion of the cytokines IFN-γ, IL-4, IL-10, IL-17A (ELISA).*

Results. *We detected a significant increase in serum cfDNA levels of GPA patients compared to HCs. The concentration of cfDNA was associated with disease activity. NETs of patients and HCs induced proliferation of $CD4^+$ T-cells and $CD19^+$ B-cells and maturation of B-cells. Furthermore, we detected an increase in IL-17A secretion after stimulating PBMCs with NETs. A significant difference between PBMCs from GPA patients and HCs was not detectable.*

Conclusions. *NETs activate PBMCs of HCs and GPA patients. Our findings give supportive evidence that NETosis plays a role in the pathogenesis of GPA.*

Introduction

Granulomatosis with polyangiitis (GPA, Wegener's granulomatosis) is characterised by necrotising small-vessel vasculitis together with neutrophil enriched granulomatous inflammation. Anti-neutrophil cytoplasmic antibodies (ANCA) with specificity for proteinase 3 (PR3) are a defining trait of this disease, which is ranked among the group of ANCA-associated vasculitides (AAV). Although the pathogenesis of AAV is a current subject of intense research (1) the causes of the disease are still unknown.

Neutrophils are the dominant cells in infiltrates within the granulomatous inflammation of GPA. They contain the target antigens for ANCA and are thought to play an active role in GPA pathogenesis (2). Since the discovery of neutrophil extracellular traps (NETs), there has been renewed interest in neutrophils as potential drivers of autoimmunity in GPA (3). During NETosis, a unique type of neutrophils death, NETs are actively released in the form of nuclear chromatin fibres with attached antimicrobial granule proteins (4). NETosis can be triggered by various stimuli (5, 6). It is a physiological defence mechanism in infections, but can also occur in autoimmunity (3).

Neutrophils and their NETs may play an important role in the induction of autoimmune response. Besides the presence of the autoantigens PR3 and myeloperoxidase (MPO) on the NET structure, PR3-ANCA are able to induce NETosis *in vitro* (3). Deposits of NETs in kidney biopsies of AAV patients with acute renal damage as well as circulating MPO-DNA complexes in active vasculitis indicate that NETosis is a trigger for AAV (3). Furthermore, elevated levels of circulating nucleosomes and NET remnants have been detected in AAV (7, 8). Concentration of these remnants is associated with disease activity of AAV patients (8). A subgroup of neutrophils, so-called low-density granulocytes, has been identified, that undergoes spontaneous NETosis *in vitro* and might contribute to the elevated NET remnants in AAV (9). In addition, dendritic cells activated by NETs cause ANCA production and autoimmunity after injection in the murine system (10).

To investigate the role of NETs in the pathogenesis of GPA, we performed *in*

vitro experiments to assess their role as a putative proinflammatory factor influencing the adaptive immune cells in GPA ultimately leading to PR3-ANCA production in plasma cells of GPA patients.

Methods

Patients and ethical approval

GPA patients were classified according to ACR criteria (11) and/or CHCC consensus definition (12, 13) and/or EMA algorithm (14). For assessment of cfDNA levels in sera, 18 patients with systemic disease activity (13 females/5 males; all positive for PR3-ANCA) and 10 healthy controls (HCs, 5 females/5 males) were tested. Patients were tested at two different time points when disease activity was absent/low (BVAS ≤3; mean BVAS 0.2; range 0–2) and high (BVAS >3; mean BVAS 14.2; range 6–28).

For stimulation and proliferation experiments with peripheral blood mononuclear cells (PBMCs) 4 to 6 patients, all PR3-ANCA positive, and 5 to 7 HCs were tested. The stimulation experiments were performed using age- and gender-matched HCs. Ethical approval (no. 13-262) was obtained from the University of Lübeck, Germany. All patients gave informed content.

Determination of cfDNA in patients' sera

Sera were obtained from donors as stated above. The amount of cfDNA was determined by Leukocare AG, Munich, Germany.

Isolation of neutrophils and PBMCs

PBMCs and neutrophils were isolated from HCs' and GPA patients' EDTA blood by density gradient centrifugation using LSM 1077 (PAA Laboratories GmbH, Cölbe, Germany). Prior to layering over LSM 1077, blood was mixed 1:1 with sterile RPMI 1640 (PAN-Biotech GmbH, Aidenbach, Germany). Cells were separated by centrifugation at **r**oom **t**emperature (RT) for 45 min at 456x g. The layer containing mononuclear cells was removed and washed twice with cold RPMI 1640. For separating PMNs from erythrocytes after density gradient centrifugation, the cell pellet was diluted 1:3 using a 1% polyvinyl alcohol solution as described previously (15). After 20 min at RT the supernatant containing PMNs was removed and washed using ice-cold phosphate buffered saline (PBS, PAN-Biotech GmbH). PBMCs were adjusted to a concentration of $1x10^6$/ml using culture medium (RPMI 1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 5% human serum (HS); all PAN-Biotech GmbH). PMNs were adjusted to a final concentration of $2x10^6$/ml in NET medium (RPMI 7509 (Sigma-Aldrich, St. Louis, MO, USA) containing 10 mM HEPES (Biochrom GmbH, Berlin, Germany) and 0.5% HS) or cell culture medium.

Preparation of NETs

NETs were generated as described by Brinkmann *et al*. (4). Briefly, $2x10^6$ PMNs were transferred into a 12-well plate and stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). After 4 h at 37°C and 5% CO_2, NETs were washed eight times with warm NET medium (RPMI 7509, 10 mM HEPES, 0.5% HS) in order to remove PMA.

Immunocytochemistry

NETosis was induced in PMNs on a round glass cover slip in a 24-well plate as described above. NETs were fixed for 10 min using 4% paraformaldehyde (PFA). After removal of PFA, NETs were blocked for at least one hour at RT using blocking reagent (phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS), all PAN-Biotech GmbH)). NETs were incubated with mouse-anti-human PR3 IgG1 (2 µg/ml, CLB-12.8 (Sanquin, Amsterdam, Netherlands)) or the isotype control (679.1Mc7, Beckman Coulter, Brea, CA, USA) in blocking reagent for 2.5 hours at RT. Antibodies were removed by washing the cover slips with PBS. Afterwards, NETs were incubated with Cy3-conjugated AffiniPure goat anti-mouse IgG antibody (3 µg/ml, Dianova GmbH, Hamburg, Germany) in blocking reagent for 1 h at RT. After washing with PBS, DNA was stained with SYTOX® Green solution (100 µM, Life Technologies, Carlsbad, CA, USA) for 30 min in the dark. NETs were washed with water and embedded into ProLong antifade (Life Technologies). Pictures were taken using the Axioskop 2 and the Axiovision 3.1 software (both Carl Zeiss Microscopy, Jena, Germany).

Stimulation of PBMCs

$1x10^6$/ml PBMCs from HCs or GPA patients were transferred into 12-well plates and stimulated with CpG ODN class B (3.2 µg/ml, Invivogen, San Diego, CA, USA), NETs or $2x10^6$ untreated PMNs, both from HCs or GPA patients. Unstimulated PBMCs served as negative control. Cells were incubated for eight days at 37°C and 5% CO_2. Cells were fed by addition of fresh cell culture medium containing 20 U/ml recombinant interleukin (IL)-2 (R&D Systems, Minneapolis, MN, USA).

Determination of cell proliferation using CFSE

PBMCs were labelled using the CellTrace™ CFSE Cell Proliferation Kit (Life Technologies) according to the manufacturer's protocol. PBMCs were adjusted to a concentration of $1x10^6$/ml in preheated PBS containing 0.1 % bovine serum albumin. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was added to a final concentration of 0.5 µM, the suspension was mixed and incubated at 37°C for 10 min. Labelling was stopped by addition of ice-cold RPMI 1640 containing penicillin, streptomycin, L-glutamine and 10% FCS and incubation on ice for 5 min. After centrifugation, cells were washed in RPMI 1640 containing 10% FCS followed by RPMI 1640 containing 5% HS. Afterwards, cells were readjusted to the concentration of $1x10^6$/ml and stimulated as stated above. In addition, PBMCs stimulated with anti-CD3 (100 ng/ml, UCHT1, Biolegend, San Diego, CA, USA) and anti-CD28 (1 µg/ml, CD28.2, Beckman Coulter) served as positive control.

Flow cytometry analysis of surface markers

After eight days, CFSE-labelled cells were harvested, washed with PBS and

stained for CD4, CD19 and CD38 in FACS buffer (PBS, 10% FCS, 0.1% NaN_3) for 30 min at RT using PE-conjugated anti-CD38 (HIT2, Biolegend), PerCP-Cy5.5-conjugated anti-CD19 (SJ25C1) and APC-conjugated anti-CD4 (SK3, both BD Biosciences, Franklin Lakes, NJ, USA) and corresponding isotype controls (APC-, PE- and PerCP-Cy5.5-conjugated mouse IgG_1, κ (MOPC-21; APC- and PerCP-Cy5.5-conjugate from BD Biosciences and PE-conjugate from Biolegend)). After staining, cells were washed with PBS, resuspended in FACS buffer and analysed using a FACSCalibur™ flow cytometer (BD Biosciences) and CellQuest™ Pro software (BD Biosciences). The populations of CFSE-labelled total lymphocytes, CSFE-positive $CD4^+$, or CSFE-positive $CD19^+$ cells were gated and defined as 100% respectively. The border between proliferating and non-proliferating cells within these populations was defined as the minimal turning point between the non-proliferating cells and the cells after the first cleavage within the histographic representation of fluorescence intensity of the CSFE-labelled cells of the positive control.

ELISPot

96-well PVDF plates were used (Merck Millipore, Billerica, MA, USA). Membranes were activated using 35 % ethanol for 1 min and washed with PBS. Membranes were coated with proteinase 3 (2 µg/well, Athens Research and Technology, Athens, GA, USA), a mixture of monoclonal anti-human IgG antibodies (1.5 µg/well, MT91/145, Mabtech AB, Nacka Strand, Sweden) or RPMI 1640 containing 5% FCS, respectively and incubated over night at 4°C. After antigen removal, membranes were washed and blocked for 2 h at 37°C using RPMI 1640 containing 10% FCS. PBMCs were harvested, washed with PBS and cell concentrations were conformed to each other and resuspended in RPMI 1640 containing penicillin, streptomycin, L-glutamine and 10% FCS. 86,700-200,000 cells/well were used. Cells were incubated for 18 to 20 h at 37°C and 5% CO_2. Cells were removed and membranes were washed. A mixture of biotinylated monoclonal anti-human IgG antibodies (100 ng/well, MT78/145, Mabtech AB) was added followed by incubation for 2h at RT. Membranes were washed and 100 µl of a streptavidin-alkaline phosphatase conjugate (1:1000, Mabtech AB) were added to each well followed by incubation for 1 h at RT. The enzyme conjugate was removed and staining was performed using NBT/BCIP solution (Mabtech AB) until blue spots appeared. Substrate was removed by washing with water, plates were dried overnight and the spots were counted using an ELISpot reader (AID Diagnostika GmbH, Straßberg, Germany).

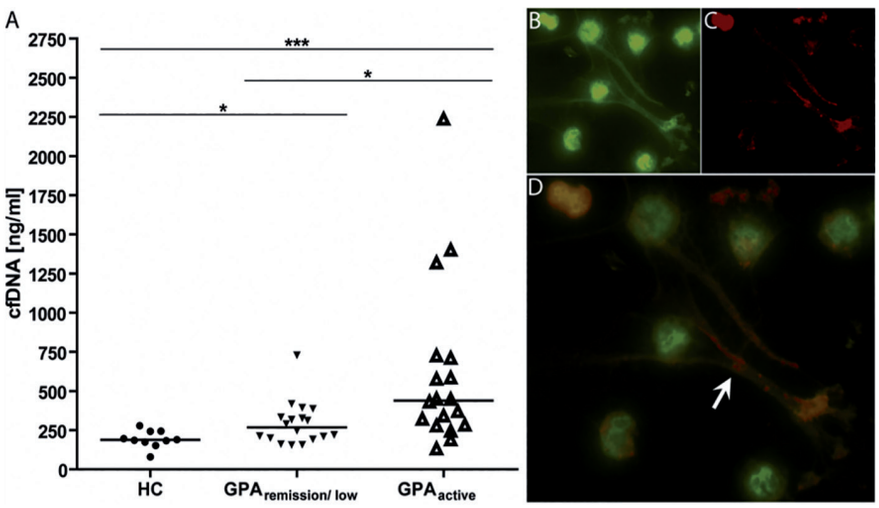

Fig. 1. cfDNA concentrations are elevated in sera of GPA patients compared to healthy controls (**A**) and neutrophil proteinase 3 is present on NETs from GPA patients (**B**-**D**). (A) DNA concentrations (ng/ml) were determined in healthy controls (n=10) and in pairs of GPA patients in remission/ with low disease activity and in active inflamed disease state (n=18); median; *p<0.05, ***p<0.001. (**B**-**D**) NETs were produced *in vitro* on slides. (**B**) DNA staining using SYTOX® Green. (**C**) Immunofluorescence staining of neutrophil proteinase 3 (red). (**D**) Overlay of both pictures showing a co-localisation of NETs and neutrophil proteinase 3 (arrow).

ANCA and cytokine detection in culture supernatant

IL-17A High Sensitivity ELISA (detection limit 0.23 pg/ml) Ebioscience, San Diego, CA, USA), Quantikine® HS ELISA kits for human IL-4 (detection limit 0.25 pg/ml) and IL-10 (detection limit 0.78 pg/ml) and the Quantikine® ELISA kit for human IFN-γ (detection limit 15.6 pg/ml, all R&D Systems) were used for assaying cytokines according to the manufacturers' instructions). Presence of IL-17A was verified by Ganzimmun Diagnostics AG, Mainz, Germany. Concentration of PR3-IgG-ANCA was determined using the Anti-PR3 hs ELISA (Orgentec Diagnostika GmbH, Mainz, Germany) according to the manufacturer's instructions.

Statistical analysis

Calculations of mean values and standard deviations were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Significance was calculated using the two-tailed Mann-Whitney U test.

The relation between cfDNA concentration and BVAS or the individual scores for the organs lung and kidney, respectively, was determined using a generalised mixed model regression in order to take repeated measures for each patient into account. Regression was performed on standardised disease score values using the R package lme4 v. 1.1-12 (16, 17). A random intercept per patient was chosen, supported by lower values of the Akaike's Information Criterion (18) compared to models without random intercept. Models were based on the Poisson distribution for the random component, with a log-link between expected values and the systematic model component. Regression parameters of fixed effect terms were tested for significance using Wald's z-test. All terms were highly significant far below the 0.01% level (data not shown). The influence of measures of

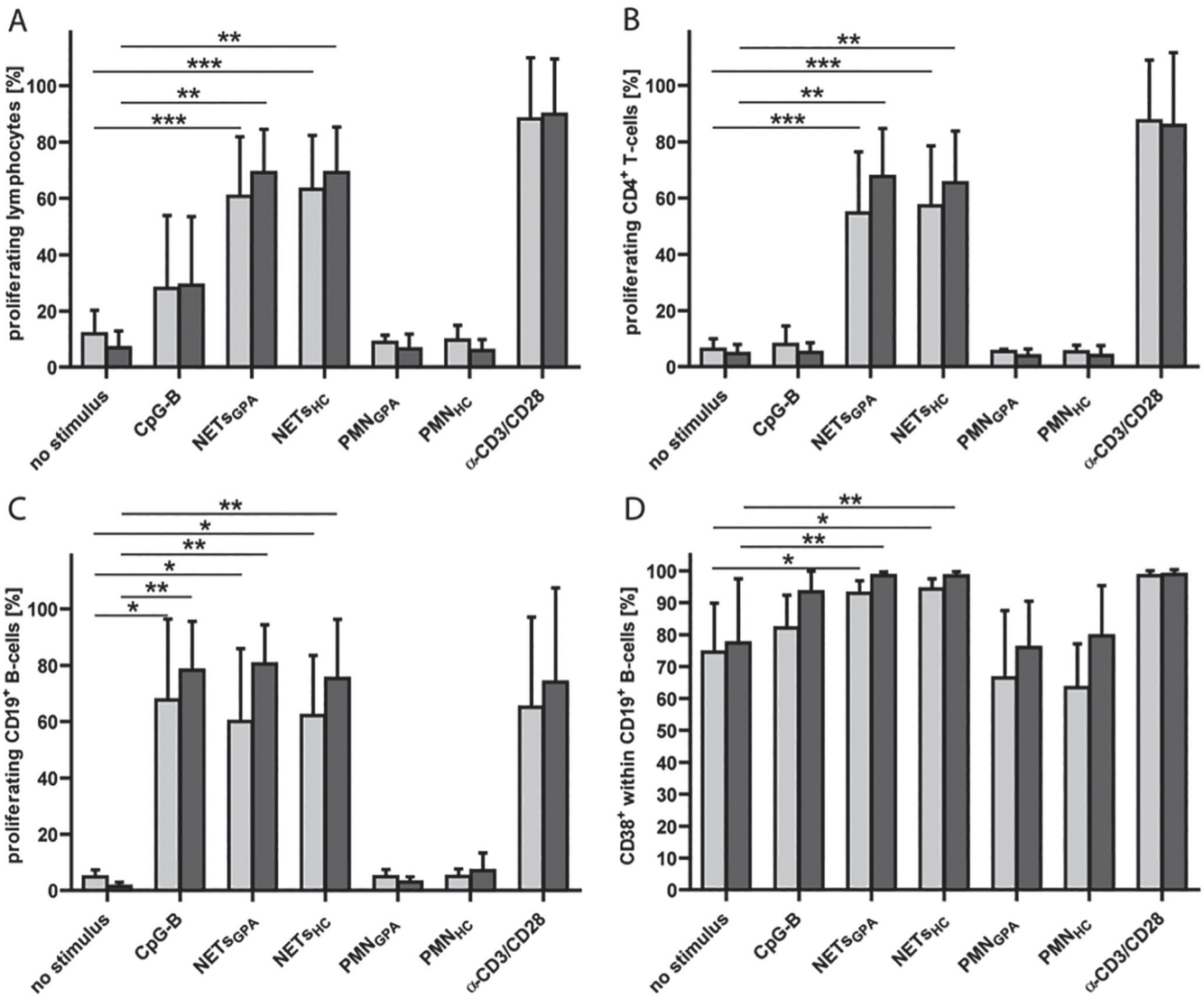

Fig. 2. NETs induce proliferation and maturation of PBMCs. Cells were stimulated with CpG-B, NETs or PMNs from HC or GPA donors, or anti-CD3 and anti-CD28 or left untreated. (**A**) total amount of proliferating lymphocytes (n_{HC}=7; n_{GPA}=6), (**B**) amount of proliferating CD4+ cells (n_{HC}=7; n_{GPA}=6), (**C**) amount of proliferating CD19+ cells (n_{HC}=5; n_{GPA}=6), (**D**) amount of CD38+ cells within population of CD19+ cells (n_{HC}=5; n_{GPA}=6); (percent; light grey, HC; dark grey, GPA; mean ± SD; *p<0.05, **p<0.01, ***p<0.001).

individual patients on parameter estimation was tested with R package influence.ME v0.9-6 (19). Cook's distance was calculated for each patient for all three models. Patient data with associated Cook's distance >1 were removed and the respective model refitted. Estimates remained highly significant for each model.

Results

Circulating cell-free DNA in active GPA is up-regulated and correlated with BVAS

We quantified the circulating cell-free DNA (cfDNA) *ex vivo* in the sera of patients with GPA in the active disease phase (BVAS >3) or in remission/with low activity (BVAS ≤3) and in HCs, respectively (Fig. 1A). The concentration of free DNA was significantly increased in the sera of GPA patients in comparison to HCs (median $_{HC}$ = 188 ng/ml (range = 79-279 ng/ml), median $_{GPA\ remission/low}$ = 268 ng/ml (range = 156-728 ng/ml), median $_{GPA\ active}$ = 439.5 ng/ml (range = 132-2237 ng/ml), $P_{HC\ vs.\ GPA\ remission/\ low}$ = 0.0201, $P_{HC\ vs.\ GPA\ active}$ = 0.0004). The amount of cfDNA was elevated in GPA patients with active disease in comparison to corresponding patients in remission ($P_{GPA\ remission/b\ low\ vs.\ GPA\ active}$ = 0.0119). Using a generalised mixed model regression analysis, we detected a highly significant correlation of the cfDNA serum concentration and the BVAS in GPA patients in active disease state and in remission/ with low disease state. cfDNA concentration was furthermore significantly correlated to the separate scores for lung and kidney involvement that are incorporated in the BVAS calculation.

We generated NETs *in vitro* using isolated PMNs from GPA patients and performed an immunofluorescence staining for PR3 (Fig. 1 B-D). We detected PR3 located on the NET structure indicating that PR3 is not only present on NETs derived from healthy individuals, but can also be detected on

in vitro generated NETs derived from GPA patients.

NETs induce proliferation of CD4⁺ T-cells and CD19⁺ B-cells

We performed an *in vitro* CFSE-proliferation assay using PBMCs from GPA patients and HCs incubated for eight days with NETs from HCs and GPA patients and other stimuli. In addition to the negative control without any stimulus, we incubated PBMCs with untreated PMNs from GPA patients and HCs. Neutrophils died within the eight days of incubation and were not able to induce proliferation of PBMCs. After NET-incubation, we observed cell clustering and a significant increase in proliferation of lymphocytes, particularly of CD4⁺ and CD19⁺ lymphocytes compared to negative controls. This proliferation had the same dimension as the proliferation of cells incubated with the positive control. CpG-B is a well-known inducer of T-cell-independent B-cell activation *via* Toll-like receptor (TLR) 9. As expected, only CD19⁺ cells and not CD4⁺ cells proliferated after stimulation with CpG-B. We did not observe any difference between HCs and GPA patients, either in PBMC proliferation or in the capacity of NETs to induce PBMC proliferation (Fig. 2 A-C).

NETs induce maturation of CD19⁺ B-cells

To investigate whether NETs can induce maturation to plasma cells *in vitro*, we additionally performed a flow cytometry analysis using CD38 as a marker for B-cell maturation using the same cells (Fig. 2 A-B-C) incubated with the same stimuli and determined the amount of CD38⁺ cells within the population of CD19⁺ B-cells. When PBMCs of HCs or GPA patients were incubated with NETs, a significant increase in the amount of CD38⁺ B-cells within the population of CD19⁺ B-cells compared to the negative control was observed (Fig. 2D). There was no difference in the induction of maturation between NETs from HCs and NETs from GPA patients nor in B-cell maturation of PBMCs from patients and HCs.

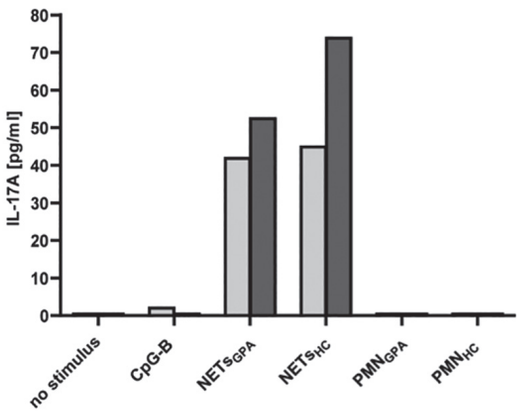

Fig. 3. Incubation of PBMCs with NETs leads to increased amounts of IL-17A in the cell culture supernatants. PBMCs were incubated with the above-mentioned stimuli for eight days. One representative example of all conducted experiments showing a NET-dependent increase of IL-17A in the cell culture supernatant. Experiment was repeated six times with PBMCs from healthy controls and four times with PBMCs from patients with GPA. (pg/ml; light grey, healthy control; dark grey, GPA; n_{HC}=6, n_{GPA}=4).

NETs do not induce the increase of the IgG-secreting plasma cell population

We assessed whether NET-incubation induces plasma cell differentiation including class switch *in vitro* by performing ELISpot assays after incubating PBMCs from GPA patients and HCs with NETs and other stimuli (Fig. S1). Figure S1 shows the relative amount of IgG signals compared to the positive control using CpG-B. The relative amount of IgG-secreting plasma cells within the PBMCs was increased after incubating the cells with CpG-B. However, we neither detected an increase in the amount of IgG secreting plasma cells in HCs, nor in GPA patients after incubation with NETs. Compared to negative controls, we even observed a slight decrease in the amount of IgG secreting cells. Again, no difference was observed in NETs derived from PMNs either from HCs or from GPA patients. ELISpot assays for the specific detection of IgG-PR3-ANCA secreting cells were performed as well. IgG-PR3-ANCA secreting cells were not detectable (data not shown). Additionally, concentration of IgG-PR3-ANCA in the culture supernatant after eight days was determined using ELISA. No increased amount of IgG-PR3-ANCA was detectable.

NETs induce secretion of IL-17 in PBMCs from GPA patients and HCs

To investigate the impact of NETs on cytokine production *in vitro*, we analysed the amount of the cytokines IL-4, IL-10, IL-17A and IFN-γ in the culture supernatants after incubating the PBMCs with the above-mentioned stimuli. We could not detect a NET-dependent induction for IL-4, IL-10, and IFN-γ (data not shown) but observed a NET-dependent increase of IL-17A after incubation of the PBMCs with NETs compared to negative controls (Fig. 3). A difference between PBMCs from HCs and GPA patients or NETs from HCs and GPA patients was not detectable.

Discussion

Recent studies suggest that NET formation triggers vasculitis and promotes autoimmune response against neutrophil components in AAV. The mechanisms involved in this process remain unclear. Here, we have identified a key link between NET formation, T-/B-cell proliferation and maturation, and IL-17 production by PBMCs in GPA and HCs.

We demonstrate that circulating cfDNA is increased in the sera of PR3-ANCA positive GPA patients and observe a correlation between serum cfDNA concentration and disease activity. Clearly, follow-up studies with larger patient cohorts are needed to confirm our initial findings. This study is the first one to investigate serum cfDNA concentration in a patient cohort that includes only GPA patients. Our data is not in line with Wang *et al*. postulating cfDNA is no biomarker for assessing disease activity (20). Different methods for accessing the amount of cfDNA and different patient cohorts (MPO-ANCA positive Asian AAV patients *versus* PR3-ANCA positive European GPA patients) might explain this disagreement. However, our data is consistent with the observations from Söderberg *et al*. showing an increased amount of NET

remnants in the serum of AAV patients associated with disease activity (8). cfDNA or NETs have also been shown to be elevated in the sera of lupus and rheumatoid arthritis patients (21). Our findings suggest that abnormal formation and/or insufficient clearance of NETs may contribute to increase the levels of cfDNA in GPA, thus indicating that NETs/NETosis constitute an important link between cfDNA and active GPA. The detection of cfDNA levels may serve as a marker of disease activity in PR3-ANCA positive GPA and may have its reason in an increased NETosis in these patients.

Furthermore, we show that NETs induce *in vitro* formation of cell clusters and proliferation of $CD19^+$ B- and $CD4^+$ T-cells of PR3-ANCA positive GPA patients and HCs. We demonstrate that NETs but not unstimulated dying neutrophils are responsible for this process. NETs/T-cell co-culturing induces the formation of cell clusters, upregulation of activation markers, and T-cell proliferation (22). Our results are consistent with these findings. In addition, this is to our knowledge the first study demonstrating not only the activation of T-cells, but also the activation and proliferation of B-cells upon incubation with NETs. We found no differences between the proliferation rates of B- and T-cells from GPA patients and HCs and there was also no difference when using NETs derived from HCs or patients to induce B- and T-cell proliferation. Various reasons may account for this: NETs could be an activator of the adaptive immune system irrespective of the disease. Furthermore, the stimulus to induce NETs may have an influence: The structure of NETs and their immunogenicity seem to be dependent on the stimulus used for NET induction (23). Although there is evidence that ANCA can induce NETs (3) it is not known which factors contribute to the generation of NETs in GPA *in vivo*. Using PMA as a stimulus to induce NETs is rather artificial and may result in the induction of a NET structure, which induces an unspecific activation of adaptive immune cells. There may be differences in the activation and proliferation of immune cells *in vivo* in the milieu of the body that lead to different responses in health and disease.

For the first time, we provide evidence for B-cell maturation upon stimulation with netting neutrophils in patients and controls by the up-regulation of CD38 cell surface expression. We also assessed whether NET-incubation induces plasma cell differentiation including class switch *in vitro* by ELISPOT. There was no increase in the amount of IgG secreting plasma cells in HCs and in GPA patients. No differences were observed in NETs derived either from HCs or from GPA patients. We performed an ELISpot assay for the specific detection of IgG-PR3-ANCA secreting cells which yielded the same results. These results may be a consequence of our artificial stimulus and/or the *in vitro* system. Especially regarding ANCA production there may be some factor or stimulus missing to induce class switch. Additionally, with respect to the results from ELISAs we also need to consider that the system is not sensitive enough to pick up IgG-PR3-ANCA production.

Finally, we investigated the production of TH1/TH2/Th17 cytokines after NET-stimulation and found no increase of the cytokines IL-4, IL-10 and IFN-g whereas there was a NET-dependent increase of IL-17A after incubation of PBMCs with NETs. No difference between PBMCs from HCs and GPA patients and between stimulation with NETs from HCs and GPA patients was detected.

These data support a role for IL-17 in AAV which has already been suggested earlier: IL-17 has been shown to be elevated in the serum of AAV patients (24). Furthermore, a higher level of Th17 cells has been detected in GPA patients (25). IL-17 has been detected in the renal tissue in ANCA-associated glomerulonephritis (26) and seems to promote anti-MPO glomerulonephritis in a murine model (27). This cytokine creates an inflammatory milieu favourable for the induction of vasculitis and autoimmunity. Furthermore, there is evidence of a connection between NET formation and IL-17 production and release: neutrophils seem to release Il-17 through NETs in psoriasis (28). Our data are in line with these findings and support a role for IL-17A in AAV, and in conjunction with NET formation. Barrientos *et al*. (29) who investigated the effect of monocyte-derived dendritic cells (moDC) found that NETs alone had no effect on moDC maturation. Moreover, NETs downregulated LPS-induced moDC maturation, reduced their capacity to induce T-cell proliferation. These NETs stimulated moDC induced a Th2 cytokine profile and suppressed Th1 and Th17 profiles. This data seem to be somewhat conflicting to our data but may be a result of the different stimuli (LPS, not PMA), cells (moDC, not PBMC) and the milieu (cells from HCs and not GPA patients). In summary, we describe the ability of NETing-neutrophils to induce B-cell proliferation and maturation, and IL-17A production by PBMCs. Furthermore, we provide evidence that cfDNA production suggestive of NETosis is increased in PR3-ANCA positive GPA. We demonstrate that NETs are able to induce activation of the adaptive immune system including polarisation towards a Th17 cytokine profile. These data highlight the role of neutrophils in the pathogenesis of this disease.

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