Excessive neutrophil extracellular trap formation in ANCA-associated vasculitis is independent of ANCA

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Neutrophil extracellular traps (NETs) are auto-antigenic strands of extracellular DNA covered with myeloperoxidase (MPO) and proteinase3 (PR3) that can be a source for the formation of anti-neutrophil cytoplasmic autoantibodies (ANCAs). The presence of NETs was recently demonstrated in renal tissue of patients with ANCA-associated vasculitis (AAV). NET formation was enhanced in AAV, suggesting that MPO-ANCA could trigger NET formation, supporting a vicious circle placing NETs in the center of AAV pathogenesis. Here we investigated NET formation in 99 patients with AAV by a novel highly sensitive and automated assay. There was a significant excess of *ex vivo* NET formation in both MPO-ANCA– and PR3-ANCA–positive patients with AAV compared to healthy individuals. Excessive NET formation did not correlate with serum ANCA levels. Likewise, immunoglobulin G depletion had no effect on excessive NET formation in patients with AAV, indicating an ANCA-independent process. Next, we explored the relation of excessive NET formation to clinical disease in ten patients with AAV and showed that excessive NET formation was predominantly found during active disease, more so than during remission. Excessive NET formation was found in patients with AAV hospitalized for disease relapse but not during severe infection. Thus, excessive NET formation in AAV is independent of ANCA, and an excess of *ex vivo* NET formation was related to active clinical disease in patients with AAV and a marker of autoimmunity rather than infection.

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Activated neutrophils have been shown to release neutrophil extracellular traps (NETs), a novel antimicrobial mechanism coexisting with the classical and well-known enzyme degranulation and reactive oxygen species (ROS) production. NETs are net-like structures extruded by neutrophils and consist of decondensed DNA with nuclear and granular proteins, such as histones, proteinase 3 (PR3), and myeloperoxidase (MPO)[1]. They can be released upon stimulation with pathogens,[1–4] immune complexes,[5,6] and chemical compounds and are able to trap and kill pathogens.[1,7,8]

Although NETs clearly act as an important first-line immune defense mechanism, they express potential autoantigens and have therefore been implicated in the pathogenesis of ANCA-associated vasculitis (AAV). Anti-neutrophil cytoplasmic antibodies (ANCAs) against MPO and PR3 have been shown to induce NET release,[8,9] and NETs are present in kidney biopsies of AAV patients.[8,10] Furthermore, in MPO-ANCA–positive AAV a reduced degradation of NETs has been observed, suggesting that ANCAs arise from a possible break of tolerance against NET-derived antigens such as MPO and PR3.[9] The latter was further corroborated by showing that myeloid dendritic cells (mDCs) loaded with extracellular DNA derived from NETotic neutrophils led to MPO-ANCA and PR3-ANCA production with concomitant vasculitis-like renal lesions in lpr/lpr mice.[11] In addition, the pathogenicity of NETs was demonstrated by NET-mediated damage to the vascular endothelium and its surrounding tissues.[12–14] Lastly, levimasole has recently been shown to induce NETs resulting in drug-induced systemic vasculitis strongly resembling AAV.[15] Taken together, these studies provide evidence for a central role for NETs in the pathophysiology of AAV.

Supported by the accumulating evidence that NETs are important to the initiation and perpetuation of vasculitis, the present study applied a newly developed, highly sensitive, and automated assay[16] to quantify *ex vivo* NET formation in PR3- and MPO-ANCA–positive AAV patients to study the

interaction between ANCAs, NET formation, and clinical disease in AAV.

RESULTS

Ex vivo visualization of NET formation in AAV

Upon stimulation with serum from AAV patients, the formation of NETs was visualized by the expression of NET-specific citrullinated histon3 (citH3) and neutrophil elastase (NE) on Hoechst-positive extracellular DNA from healthy neutrophils (Figure 1a). Additionally, inhibition of reduced NAD phosphate oxidase with diphenyleneiodonium (DPI) and peptidyl arginine deiminase (PAD) with Cl-amidine confirmed that AAV-induced extracellular DNA were part of NETs: co-incubation with DPI demonstrated a significant reduction of AAV-associated NET induction (mean reduction ± SEM: 61% ± 10%, $P < 0.01$; Figure 1b) as well as co-incubation with Cl-amidine, which led to a significant reduction of AAV-derived NET formation (mean reduction ± SEM: 40% ± 11%, $P < 0.05$; Figure 1c).

Excessive *ex vivo* NET formation in AAV does not correlate with serum ANCA levels

Ex vivo NET formation was determined in a large cohort of AAV patients positive for anti-PR3 or anti-MPO. To measure NET formation by serum from AAV patients, we used a novel, highly sensitive, and automated method.[16] Experimental studies supporting the assay's approach to use healthy neutrophils as the basis to quantify excessive NET formation are summarized in Supplementary Figure S1A–C. As such, Figure 2a shows excessive NET formation in PR3-ANCA–positive patients (median fold induction ± interquartile range: 13.5 ± 52) as well as MPO-ANCA–positive patients (38.8 ± 160.1) compared with healthy controls. The difference between MPO-ANCA– and PR3-positive ANCA patients was statistically significant ($P < 0.01$). Unexpectedly, neither PR3-ANCA titers ($R = -0.17$, $P = 0.19$; Figure 2b) nor MPO-ANCA titers ($R = 0.22$, $P = 0.21$; Figure 2c) correlated with the amount of NET formation. NET formation was not found to correlate with serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, or IL-8 (data not shown) or classical markers of inflammation (i.e., C-reactive protein and erythrocyte sedimentation rate ; Supplementary Figure S2).

ANCA IgG is not responsible for excessive NET formation in AAV

To investigate whether ANCA IgG was responsible for the excessive NET formation by AAV serum, we next depleted IgG, and later also IgA, from serum of 10 PR3-ANCA–positive patients, 5 MPO-ANCA–positive patients, and 3 healthy controls. Figure 3a summarizes the serum characteristics demonstrating IgG depletion, including depletion of ANCA IgG. Also, after elution of IgG from the depleting beads, IgG including PR3-ANCA and MPO-ANCA were retrieved. Figure 3b illustrates for 1 representative patient that despite IgG depletion, excessive NET formation remained present and IgG alone was not able to induce NET formation. Quantifying all patients together, for PR3-ANCA–positive patients we observed no significant difference in NET formation after IgG depletion (mean ± SEM fold induction: 8.1 ± 1) compared with whole serum (9.3 ± 2.4; $P = 0.60$). For MPO-ANCA–positive patients we also observed no significant difference in NET formation after IgG depletion (18.3 ± 5.4) compared with whole serum (25.9 ± 6; $P = 0.06$). Moreover, isolated IgG from PR3-ANCA–positive patients was unable to induce NET formation (fold induction ± SEM: 1.5 ± 0.4 for 250 μg/ml and 1.3 ± 0.6 for 25 μg/ml), and neither was isolated IgG from MPO-ANCA–positive patients (1.4 ± 0.7 for 250 μg/ml and 1.1 ± 0.3 for 25 μg/ml) (Figure 3c and d).

To exclude that IgA-derived ANCA was responsible for the observed NET formation in IgG-depleted sera, IgA was subsequently depleted. IgA depletion was confirmed with enzyme-linked immunosorbent assay (ELISA). No significant difference was found after both IgA and IgG depletion (mean fold induction ± SEM: 17 ± 5.5) compared with corresponding serum samples (20.2 ± 10.1; $P = 0.74$; Figure 3e and f).

Next, we investigated whether upregulation of PR3 and MPO expression on neutrophils could influence NET formation by ANCA IgG. To do so, neutrophils were primed with TNF-α, after which PR3 and MPO upregulation was confirmed by flow cytometry (Figure 4a). Incubation of unprimed and TNF-α-primed neutrophils with isolated IgG from AAV patients did not show a significant difference: using 25 ug/ml IgG, the mean fold NET induction ± SEM in unprimed cells was 1.5 ± 0.5 versus 1.5 ± 0.3 ($P = 0.69$) in primed cells. Also, when increasing IgG concentration to 250 ug/ml, the mean fold NET induction ± SEM in primed cells (2.4 ± 0.4) was not significantly different from that in unprimed cells (2.8 ± 1.2, $P = 0.67$). As a positive control, NET formation was observed with whole serum from AAV patients before and after IgG depletion in unprimed as well as primed cells (Figure 4b).

Activated complement pathways are not involved in NET formation in AAV

Because neutrophils of AAV patients are activated through C5a receptor triggering,[17,18] we investigated whether complement activation had a role in excessive NET formation in AAV. Therefore, we pre-incubated healthy neutrophils with a C5aR antagonist, after which NET formation was induced with serum from ANCA-positive AAV patients. Figure 5a shows that excessive NET formation was independent of C5aR inhibition (mean ± SEM of 11 ± 3.8 without and 12.1 ± 3.6 with C5aR inhibition). Next, we pre-incubated serum from ANCA-positive AAV patients with eculizumab, a C5 inhibitor, after which NETs were induced in healthy neutrophils. Neutralization of C5 by 100 ug/ml eculizumab was functionally confirmed (Supplementary Figure S3); however, the abrogation of activated complement with eculizumab did not result in a significant change in NET formation (mean ± SEM of 11 ± 3.8 without and 13.5 ± 4.5 with 10 μg/ml eculizumab and 13.4 ± 4 with 100 μg/ml eculizumab)