Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition

Elmar Pieterse, Nils Rother, Marjolein Garsen, Julia M. Hofstra, Simon C. Satchell, Markus Hoffmann, Markus A. Loeven, Hanneke K. Knaapen, Olivier W.H. van der Heijden, Jo H.M. Berden, Luuk B. Hilbrands, Johan van der Vlag

Objective—An excessive release and impaired degradation of neutrophil extracellular traps (NETs) leads to the continuous exposure of NETs to the endothelium in a variety of hematologic and autoimmune disorders, including lupus nephritis. This study aims to unravel the mechanisms through which NETs jeopardize vascular integrity.

Approach and Results—Microvascular and macrovascular endothelial cells were exposed to NETs, and subsequent effects on endothelial integrity and function were determined in vitro and in vivo. We found that endothelial cells have a limited capacity to internalize NETs via the receptor for advanced glycation endproducts. An overview of the phagocytic capacity of endothelial cells for NETs resulted in the persistent extracellular presence of NETs, which rapidly altered endothelial cell–cell contacts and induced vascular leakage and transendothelial albumin passage through elastase-mediated proteolysis of the intercellular junction protein VE-cadherin. Furthermore, NET-associated elastase promoted the nuclear translocation of junctional β-catenin and induced endothelial-to-mesenchymal transition in cultured endothelial cells. In vivo, NETs could be identified in kidney samples of diseased MRL/lpr mice and patients with lupus nephritis, in whom the glomerular presence of NETs correlated with the severity of proteinuria and with glomerular endothelial-to-mesenchymal transition.

Conclusions—These results indicate that an excess of NETs exceeds the phagocytic capacity of endothelial cells for NETs and promotes vascular leakage and endothelial-to-mesenchymal transition through the degradation of VE-cadherin and the subsequent activation of β-catenin signaling. Our data designate NET-associated elastase as a potential therapeutic target in the prevention of endothelial alterations in diseases characterized by aberrant NET release.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:1371-1379. DOI: 10.1161/ATVBAHA.117.309002.)

Key Words: endothelial cells ■ extracellular traps ■ leukocyte elastase ■ lupus nephritis ■ lupus erythematosus, systemic

Neutrophil extracellular traps (NETs) are fibrillar networks of DNA, histones, and antimicrobial peptides that are released from neutrophils during a highly complex cell death signaling pathway termed NETosis.1 NETosis was first described in 2004 as a novel defense mechanism of the innate immune system against intruding pathogens, but during the last years, it has become increasingly clear that NETs can also exert deleterious effects on the host.2 An imbalance between the release and degradation of NETs has been linked to a variety of hematologic disorders, such as transfusion-related acute lung injury,3,4 sickle cell disease,5 thrombosis,6 and myeloproliferative neoplasms.7 Aberrant NETosis has also been associated with autoimmune conditions, such as antineutrophil cytoplasmic antibody–associated vasculitis8 and systemic lupus erythematosus,9 in which NET-associated antigens may become targets of autoantibodies. In systemic lupus erythematosus, the failure to dismantle NETs has been specifically linked to the development of lupus nephritis (LN), but the underlying mechanisms for this association are as yet still unresolved.10

NETs contain an arsenal of cytotoxic proteases, including cathepsin G, proteinase 3, neutrophil serine protease 4, matrix metalloproteinase 9 (MMP9), and neutrophil elastase.11 In addition, NETs are rich in cytotoxic (modified) histones.12 Although NET-associated histones and proteases exert a pivotal role in the elimination of pathogens, they may also injure surrounding tissues and bystander cells. The endothelium may be particularly at risk for NET-mediated damage because endothelial cells are continuously exposed to NETs in NET-driven disorders.13-15 Especially the endothelial cells that...
line the renal glomeruli are thought to be heavily subjected to NETs because circulatory NETs may deposit here in locally high amounts during blood filtration.\(^{16,17}\)

To date, the effects of NETs on endothelial cells still remain poorly characterized. In the present study, we investigated the effects of NETs on macrovascular human umbilical vein endothelial cells (HUVECs) and microvascular glomerular endothelial cells (ciGEnCs) both in vitro and in vivo. We observed that NETs alter endothelial cell–cell contacts through the proteolysis of the intercellular junction protein VE-cadherin by NET-associated elastase, thereby facilitating vascular leakage and the transendothelial passage of albumin. In addition, cultured endothelial cells underwent endothelial-to-mesenchymal transition (EndMT) in response to NETs, which was also dependent on the proteolytic activity of elastase. The relevance of these in vitro findings was further evaluated in vivo, where NET depositions could be demonstrated in renal biopsies of diseased MRL/lpr mice, as well as human LN subjects, in whom the glomerular presence of NETs correlated with the degree of proteinuria and with glomerular EndMT. Collectively, our data reveal that NETs play a crucial role in the induction of vascular leakage and EndMT.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

### Endothelial Cells Internalize NETs in a Receptor for Advanced Glycation Endproduct–Dependent and Clathrin-Dependent Manner

Previously, macrophages were shown to play an important role in clearance of NETs.\(^{18}\) Endothelial cells are also capable to internalize a wide variety of cellular material, that is, senescent neutrophils,\(^{19}\) apoptotic debris,\(^{20}\) and red blood cells.\(^{21}\) Because endothelial cells are continuously exposed to NETs in NET-driven disorders,\(^{13,17}\) we first evaluated whether endothelial cells have the capacity to internalize NETs. Such an endothelial phagocytic clearance may be crucial to avoid uncontrolled NET-mediated endothelial damage. To assess endothelial uptake of NETs, microvascular ciGEnCs or macrovascular HUVECs were incubated with NETs and the culture medium was analyzed for NETs at various time points with the cell-impermeable, DNA-binding dye Sytox Orange. A rapid (\(<15\) minutes) and time-dependent uptake of NETs by both ciGEnCs and HUVECs was observed (Figure 1A), which was more pronounced in ciGEnCs. To further validate internalization of NETs by endothelial cells, ciGEnCs were stained for neutrophil elastase, the most abundant protease within NETs.\(^{13}\) Indeed, NET-treated ciGEnCs showed an intracellular particulate staining for elastase, which was absent in control ciGEnCs (Figure IA in the online-only Data Supplement). Moreover, elastase colocalized with clathrin (Figure IB in the online-only Data Supplement), indicating a receptor-mediated pathway of endocytosis that leads to the formation of clathrin-coated NET-containing endosomes. Besides elastase, NET-derived histones were found in clathrin-coated endosomes after exposure of ciGECs or HUVECs to NETs (Figure 1B, middle panel; Figure IC in the online-only Data Supplement). Because NETs are primarily composed of DNA, we reasoned that the receptor for advanced glycation endproducts (RAGE), a transmembrane receptor of the immunoglobulin super family that was previously shown to promote the uptake of extracellular DNA into endosomes,\(^{22}\) could be the receptor responsible for the internalization of NETs into clathrin-coated endosomes. Indeed, the full digestion of NET-derived DNA with DNase I prevented the uptake of NET-derived histones into clathrin-coated endosomes as observed by immunofluorescence imaging (Figure 1B, bottom panel). Furthermore, in the presence of FPS-ZM1, an antagonist of RAGE, both HUVECs and ciGEnCs were unable to internalize NETs (Figure 1C). The uptake of NETs by ciGEnCs or HUVECs did also not occur at 4°C, indicating that the uptake of NETs is an active process (Figure 1C). In summary, NETs are rapidly internalized by endothelial cells during an active process involving both RAGE and clathrin.

### NET-Associated Elastase Promotes Transendothelial Albumin Passage

Despite a rapid uptake of NETs, the phagocytic capacity of endothelial cells for NETs seemed to be limited. Addition of larger amounts of NETs (\(\geq 250\)-ng NET-derived DNA) to endothelial cells resulted in the saturation of their phagocytic capacity thereby causing a persistent extracellular presence of NETs (Figure 2A). We used HUVECs as a model to study the extracellular effects of NETs on endothelium because the phagocytic capacity of HUVECs for NETs was significantly lower than that of ciGEnCs (Figure 1A). At high NET concentrations (2-µg DNA/mL), a rapid loss of endothelial cell–cell contacts and an impaired endothelial monolayer integrity could be observed (Figure IIA in the online-only Data Supplement), which was not the result of NET-mediated endothelial cell death as measured by Annexin V staining (Figure IIB in the online-only Data Supplement). To further characterize endothelial integrity, the expression of the intercellular (junctional) proteins CD31 and VE-cadherin was analyzed, which revealed that both CD31 and VE-cadherin expression was significantly decreased on NET-treated HUVECs (Figure 2B and 2C). The loss of VE-cadherin expression furthermore coincided with the gradual development of a spindle-shaped, mesenchymal-like phenotype, which was most prominent after 24 hours (Figure 2D). Treatment of ciGEnCs with NETs yielded similar results as obtained for HUVECs (Figure IIC in the online-only Data Supplement). To assess alterations of the endothelial monolayer at a functional level, a transendothelial...
albumin passage assay was performed, which revealed that the transendothelial passage of albumin was significantly increased in NET-treated HUVECs compared with control HUVECs (Figure 2E). Because VE-cadherin and CD31 are known substrates of elastase and MMP9, respectively, we assessed whether we could prevent the loss of these junctional proteins and subsequent transendothelial albumin passage by inhibiting the activity of NET-associated elastase and MMP9. Pre-treatment of NETs with sivelestat, a selective inhibitor of elastase, abolished the NET-induced loss of VE-cadherin expression (Figure 2F and 2G) and reduced transendothelial albumin passage (Figure 2E). The inhibition of MMP9 with anti-MMP9 antibodies could limit CD31 loss (Figure IIID in the online-only Data Supplement), which colocalize with clathrin (middle). Degradation of NET-associated DNA with 5-U/ml DNase I before coincubation prevented the endothelial uptake of NET-associated histones (bottom). C, Blockade of RAGE with 25 µg/ml FPS-ZM1 or coincubation of NETs with ciGEnCs or HUVECs at 4°C abolishes NET uptake measured after 120 min. Scale bars: white=10 µm; yellow=5 µm. *P<0.05, ***P<0.001, compared with control. Statistics: 1-way ANOVA with Bonferroni correction.

Figure 1. Endothelial cells (ECs) rapidly internalize neutrophil extracellular traps (NETs) through the receptor for advanced glycation end-products (RAGE). A, ECs rapidly internalize NETs, as determined by a decline in Sytox Orange signal after coincubation of NETs with conditionally immortalized glomerular endothelial cells (ciGEnCs; left) and human umbilical vein endothelial cells (HUVECs; right). In these coculture experiments, the cell impermeable dye Sytox Orange was added to culture supernatants (final concentration 100 nmol/L) 5 min before the fluorometry measurement. The obtained value was related to the value obtained from NETs in the absence of endothelial cells (remaining NETs). B, Stimulation of ciGEnCs and HUVECs with NETs for 15-min results in a particulate cytosolic staining pattern for histones (stained with a PAN antibody against all core histones; see Materials and Methods in the online-only Data Supplement), which colocalize with clathrin (middle). Degradation of NET-associated DNA with 5-U/ml DNase I before coincubation prevented the endothelial uptake of NET-associated histones (bottom).

NET-Associated Elastase Promotes EndMT

β-catenin, a dual function protein involved in cell–cell adhesion and gene transcription, is associated with the cytoplasmic tail of VE-cadherin in endothelial cells. It was previously shown that the loss of cadherin-mediated cell–cell adhesions can promote functional β-catenin release and induce β-catenin signaling. Congruent with these reports, NETs promoted the nuclear translocation of functional β-catenin (Figure IIIA and IIIB in the online-only Data Supplement), which was most prominent after 24 hours. The translocated β-catenin most likely is transcriptionally active within the nucleus as demonstrated by immunofluorescence stainings with an antibody specific for transcriptionally active β-catenin, that is, recognizing dephosphorylated Ser37 and Thr41 epitopes (Figure 3A and 3B). Approximately 60% and 70% of NET-treated ciGEnCs and HUVECs, respectively, contained transcriptionally active β-catenin within their nuclei (Figure 3C). To explain the mesenchymal-like phenotype of NET-treated endothelial cells (Figure 2D), the expression of an important target gene of β-catenin, Snail1, which fulfills a key role during EndMT, was analyzed. Indeed, an increased expression of Snail1 was found in both NET-treated HUVECs and ciGEnCs (Figure 3D). Furthermore, the expression of MMP9 and the myofibroblast marker smooth muscle actin, which are in part regulated by Snail1, were also upregulated in both endothelial cell types after exposure to NETs (Figure 3D; Figure IIC in the online-only Data Supplement). Notably, elastase inhibition with sivelestat could completely block NET-induced Snail1, MMP9, and smooth muscle actin expression (Figure 3D; Figure IIC in the online-only Data Supplement).
Glomerular NETs Are Associated With Proteinuria and EndMT in Murine LN

Next, we aimed to extend our in vitro findings to the in vivo situation. Because LN is perceived as a prototype NET-driven disease, we first studied renal biopsies from MRL/lpr mice for the presence of NETs and for markers of EndMT. For this purpose, MRL/lpr mice were classified into 3 groups based on the degree and duration of proteinuria: (1) no proteinuria, (2) short-lived proteinuria, and (3) long-lived proteinuria (see and Methods in the online-only Data Supplement for concise definitions). The glomerular presence of citrullinated histone
H3, an NET-specific marker frequently used in mice, was analyzed (Figure 4A). In contrast to glomeruli of healthy CBA mice, it seemed that glomeruli of MRL/lpr mice in all groups contained NETs, whereby NETs were most prominently present in glomeruli of mice with long-lived proteinuria (Figure 4B). The presence of glomerular NETs coincided with increased cortical Snail1 and MMP9 mRNA expression in MRL/lpr mice, particularly in those with long-lived proteinuria (Figure 4C and 4D). Snail1 protein expression was also specifically upregulated in the glomeruli of proteinuric MRL/lpr mice (Figure 4E), whereas being absent in tubular compartments (Figure IV A in the online-only Data Supplement). Importantly, Snail1 protein expression correlated with cortical Snail1 mRNA expression and with the amount of NET-positive glomeruli in these mice (Figure 4F and 4G). In addition to Snail1, MMP9 protein expression was assessed in glomeruli, whereby double-stainings with CD31 confirmed the actual endothelial origin of MMP9 rather than neutrophil influx being the source of MMP9 (Figure IVB in the online-only Data Supplement). Taken together, the presence of NETs is associated with the glomerular upregulation of the EndMT markers Snail1 and MMP9 in proteinuric MRL/lpr mice.

Glomerular NETs Are Associated With Proteinuria and EndMT in Human LN

We next analyzed kidney biopsies from 8 patients with active LN for NET deposits and assessed whether the presence of renal NETs correlated with the degree of proteinuria and associated with glomerular EndMT. To identify NETs in these biopsies, kidney sections were stained for DNA and elastase (Figure 5A). Notably, in contrast to our experience in murine LN kidneys, a reliable staining of human LN biopsies for citrullinated histone H3 could not be obtained (data not shown). Stainings with monoclonal antibodies KM-2 (against triacetylated histone H4 [K8,12,16]34) or BT164 (against trimethylated histone H3 [K27]35) allowed further identification of NETs (Figure V A and VB in the online-only Data Supplement). Notably, these histone modifications were previously shown to be enriched in NETs when compared with unstimulated neutrophils.12 Taken all stainings together, the presence of NETs could be identified in 7 of the 8 renal biopsies, ranging from 18% to 75% NET-positive glomeruli (Figure 5A). There was a positive correlation between the percentage of NET-positive glomeruli and the level of proteinuria (Figure 5B). Because
the impaired DNase I–mediated degradation of NETs was recently linked to the development of LN in patients with systemic lupus erythematosus, we ought to explore whether the impaired degradation of NETs by circulatory DNase I correlated to the glomerular presence of NETs. Plasma from 4 of 8 LN patients degraded NETs poorly in vitro (Figure 5C; Figure VI in the online-only Data Supplement). However, no statistical differences for anti–double-stranded DNA titers, anti-NET antibody titers, the level of proteinuria, or the percentage of NET-positive glomeruli was found when comparing poor degraders with good degraders of NETs (Figure 5D). To replicate the findings from LN mice, Snail1 protein expression was determined in renal biopsies of LN patients with severe proteinuria (>10 g/d) and with minor proteinuria (<1 g/d). It seemed that glomeruli of patients with severe proteinuria showed high glomerular expression of Snail1, whereas those with minor proteinuria showed no evident glomerular Snail1 expression (Figure 5E). Notably, peritubular endothelial cells were negative for Snail1 (Figure IVC in the online-only Data Supplement). In summary, there is a correlation between the presence of glomerular NETs and proteinuria in LN, which in turn associated with glomerular Snail1 expression. However, the NET-degrading capacity of plasma could not be correlated to the presence of NETs in the glomeruli of LN patients.

**Discussion**

In the present study, we demonstrate a novel role for NETs in the transition of endothelial cells toward a mesenchymal phenotype, a process known as EndMT. EndMT is the process of cellular transdifferentiation in which endothelial cells lose their endothelial-specific markers and gain a mesenchymal phenotype. EndMT is important during embryonic vascular development and also plays a role during vascular repair. However, EndMT has also been linked to various pathological conditions, including malignant and fibrotic diseases. It has been suggested that during renal fibrogenesis, mesenchymal cells emerge predominantly via transdifferentiation of local endothelial and epithelial cells. Thus, NETs may facilitate the development of renal fibrosis in the progression.

![Figure 4. Glomerular neutrophil extracellular traps (NETs) associate with proteinuria and glomerular Snail1 expression in murine lupus nephritis (LN).](http://atvb.ahajournals.org/)

- **A** Kidney sections from MRL/lpr and control CBA mice were stained for citrullinated histone H3 (H3Cit) and Ly6G to detect NETs and neutrophils, respectively.
- **B** NETs are present not only in glomeruli of proteinuric MRL/lpr mice (short-lived proteinuria [SP], long-lived proteinuria [LP]) but also in MRL/lpr mice without proteinuria (no proteinuria [NP]).
- **C and D** Snail1 and MMP9 mRNA expression are increased in kidney cortical tissue of proteinuric MRL/lpr mice (SP and LP).
- **E** Snail1 protein expression is increased in glomeruli of proteinuric MRL/lpr mice (SP and LP).
- **F and G** Snail1 protein expression correlates to cortical Snail1 mRNA expression (F) and the amount of NET-positive glomeruli in MRL/lpr mice (G).

Scale bar, 30 µm. *P<0.05, ***P<0.001, compared with CBA; #P<0.05, compared with MRL/lpr NP. Statistics: unpaired Student t test.
of kidney disease by converting glomerular endothelial cells to a mesenchymal phenotype. Similarly, NETs may contribute to the pool of myofibroblasts that arise from lung capillary endothelial cells during the development of pulmonary fibrosis.43 The events that lead to pulmonary fibrosis still remain poorly understood, but the induction of EndMT by NETs may emerge as a potential pathway in diseases that are characterized by a high abundance of NETs within the lung capillaries, such as sickle cell disease5 and transfusion-related acute lung injury.3 Elegant studies recently demonstrated that mice unable to form NETs are indeed protected from the development of fibrosis in the heart and lungs.44

We found that the predominant protease within NETs, neutrophil elastase, is key to the induction of EndMT by NETs. Its mode of action involved the proteolysis of endothelial VE-cadherin, a known substrate of elastase,23 and the subsequent activation of β-catenin signaling. β-catenin is a protein with a dual function that regulates the coordination of cadherin-mediated cell–cell adhesions and the transcription of genes involved in the differentiation of cells toward a mesenchymal phenotype, such as α-smooth muscle actin, vimentin, MMP9, and Snail1.45 It was reported previously that the loss of cadherin-mediated cell–cell contacts can promote junctional β-catenin release and activate β-catenin signaling.27,28 Therefore, the elastase-mediated loss of VE-cadherin seems to be an important initial event in the induction of EndMT through β-catenin signaling. Noteworthy, NET-mediated induction of EndMT is only observed when the concentrations of NETs are high and exceed the limited phagocytic capacity of endothelial cells for NETs. We indeed show that endothelial cells are endowed with a limited capacity to internalize NETs through the endothelial RAGE, which specifically recognizes NET-associated DNA. Interestingly, in addition to its role in the internalization of NETs, RAGE activation is also a trigger for NETosis.46

In addition to its role in the induction of EndMT, the degradation of VE-cadherin by NET-associated elastase also caused a rapid phase of increased vascular permeability, as demonstrated by transendothelial albumin passage assays. Because the integrity of intercellular junctions, mediated particularly through VE-cadherin, is a prerequisite for maintaining a restrictive endothelial barrier,17,48 NETs may thus play a role in the development of edema and proteinuria by increasing
vascular permeability. Indeed, activated neutrophils have long been associated with vascular leakage and pulmonary edema in transfusion-related acute lung injury and sickle cell disease.\textsuperscript{49,50} Similarly, elastase was previously shown to promote proteinuria in vivo,\textsuperscript{51,52} and inhibition of elastase earlier successfully limited proteinuria in rats.\textsuperscript{53} More recently, Knight et al\textsuperscript{35} found that proteinuria can be reduced in vivo in MRL/lpr mice through the systemic inhibition of NETosis with the peptidylarginine deiminase inhibitor CI-amilidane. In line with this, we found that the degree of proteinuria correlated with the percentage of NET-positive glomeruli in MRL/lpr mice and patients with LN, which was independent from the in vitro capacity of plasma to degrade NETs. The relationship between the in vitro capacity of plasma to degrade NETs and glomerular NET depositions therefore seems complex. This may be explained by the fact that the in vivo formation and deposition of NET-containing immune complexes may cause a drop in the level of antibodies that would normally prevent the degradation of in vitro generated NETs. Alternatively, our data may raise the question whether circulatory endonucleases are actually involved in the degradation of NETs within the kidneys. Hence, >80% of the total endonuclease activity within the kidneys is attributed to locally synthesized DNase I.\textsuperscript{54} Importantly, an acquired, selective downregulation of renal DNase I gene expression has been reported during the progression of both murine and human LN.\textsuperscript{55}

In sum, we propose a model in which excessive NETosis overloads the phagocytic capacity of endothelial cells for NETs, resulting in (1) proteolysis of VE-cadherin, (2) the loss of endothelial cell–cell contacts, (3) the development of vascular leakage, and (4) the induction of EndMT through β-catenin signaling. Future research should be aimed at functional studies in NETosis-deficient mice, or in mice treated with NETosis inhibitors, to functionally link NETosis to EndMT in vivo. Nevertheless, our data implicate that NET-associated elastase is potentially an attractive therapeutic target whose inhibition may limit vascular leakage and procoagulative processes in a plethora of NET-driven inflammatory conditions.

Acknowledgments

We thank the Dutch Working Party on Systemic Lupus Erythematosus for the collection of human material.

Sources of Funding

Research was supported by the Dutch Kidney Foundation (KSBS 12.073), Department of Nephrology Radboudumc PhD program and the Radboudumc Honours Academy.

Disclosures

None.

References

Endothelial cells are endowed with a limited capacity to internalize neutrophil extracellular traps via the receptor for advanced glycation end-products. An excess of neutrophil extracellular traps alters endothelial monolayer integrity and promotes vascular leakage through the degradation of VE-cadherin. Neutrophil extracellular traps convert endothelial cells to a mesenchymal phenotype through the activation of β-catenin signaling.
Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition
Elmar Pieterse, Nils Rother, Marjolein Garsen, Julia M. Hofstra, Simon C. Satchell, Markus Hoffmann, Markus A. Loeven, Hanneke K. Knaapen, Olivier W.H. van der Heijden, Jo H.M. Berden, Luuk B. Hilbrands and Johan van der Vlag

Arterioscler Thromb Vasc Biol. 2017;37:1371-1379; originally published online May 11, 2017; doi: 10.1161/ATVBAHA.117.309002
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/37/7/1371

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2017/05/15/ATVBAHA.117.309002.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Material and methods

Chemicals, antibodies and primers

Chemicals, antibodies and primers used in this study are listed in Tables I, II and III.

Cell culture

Human umbilical vein endothelial cells (HUVECs), isolated from umbilical cords by perfusion and 20 min incubation with trypsin at 37°C, and conditionally immortalized glomerular endothelial cells (ciGENCs) were maintained in fibronectin-coated Corning cell culture flasks (Sigma-Aldrich) in EGM2-medium supplemented with 2% FCS and EGM-2 SingleQuots or EGM-2-MV SingleQuots (Lonza), respectively. HUVECs and ciGENCs were cultured in a humidified incubator with 5% CO2 at 37°C or 33°C, respectively. CiGENCs were allowed to differentiate at 37°C for 1 week as described previously. HUVECs between passages 3 and 8 were used for experiments.

Isolation of neutrophils and induction of NETosis

Neutrophils were isolated from human whole blood by Ficoll density gradient centrifugation using Lymphoprep™ (Stemcell Technologies), as described previously. Isolated neutrophils (3x10^5 cells per cm^2) were seeded in well plates in serum-free RPMI1640 medium (Sigma-Aldrich) supplemented with 100 nM PMA for 4 hours to form NETs. Adherent NETs were washed with pre-warmed PBS and isolated by partially digesting the NETs in fresh DMEM/F12 medium (Life Technologies) supplemented with 5U/mL micrococcal nuclease (MNase). Sytox Orange and N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide were used to quantify DNA content and elastase
activity, respectively, in NET-containing supernatants. The induction of NETosis was confirmed with immunofluorescence microscopy (Suppl. Fig. VII). NETs were stored at -80°C until further use.

**Quantitative reverse-transcription PCR**

Total RNA was extracted with TRIzol® reagent (Ambion). Reverse transcription was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer’s instructions. Quantitative reverse-transcription PCR was performed with FastStart Universal SYBR Green Master (Roche Diagnostics) on a CFX96 real-time PCR detection system (Bio-Rad). Experiments were performed in duplicate and the values were normalized to β2-microglobulin (β2M) or GAPDH using the delta-delta Ct method. Primer sequences are listed in Table III.

**Flow cytometry**

All flow cytometry analyses were performed according to standard procedures 3. Briefly, endothelial cells were detached with 10 mM EDTA and stained with appropriate antibodies. For the assessment of cell death, endothelial cells were taken up in Annexin V-buffer and stained with FITC-conjugated Annexin V according to the manufacturer’s protocol (BioVision). All samples were analyzed on a FC500 flow cytometer with CXP software (Beckman Coulter). Data were analyzed with Kaluza® Flow Analysis Software 1.3 (Beckman Coulter).
**Immunofluorescence imaging**

Endothelial cells cultured on fibronectin-coated slideflasks were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. Slides were blocked with 1% BSA and stained with appropriate antibodies. Slides were embedded in VectaShield mounting medium with DAPI (Vector laboratories). For stainings on murine and human LN material, frozen kidney sections (2 µm) were fixed in ice-cold acetone for 10 minutes and incubated with appropriate antibodies diluted in PBS containing 1% BSA and 0.05% sodium azide. All stainings were analyzed on a Zeiss Axioimager M1 immunofluorescence microscope using standard filter sets.

**Western blotting**

Proteins were extracted with standard RIPA buffer and resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked with 1% BSA and incubated with primary antibodies overnight at 4ºC. Proteins were detected with appropriate HRP-conjugated antibodies. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), according to the provided protocol.

**Degradation assays**

DNA degradation was assessed according to the protocol of Hakkim et al. with modifications. Briefly, NET-associated DNA, calf thymus DNA or nucleosomal DNA from apoptotic cells was adjusted to a concentration of 500 ng/ml and incubated with 10% recalcified plasma. After 180 min of incubation at 37ºC, the remaining DNA was quantified with fluorometry using Sytox Orange. Nucleosomal DNA from apoptotic cells was obtained as described in 5.
**NET ELISA**

Polystyrene microplates were coated overnight with NET-containing supernatants (at a concentration of 2 µg/ml of NET-derived DNA) at 4°C. The next day, coated NETs were incubated with patient or healthy donor plasma (1/100 dilution) for 2 hours at room temperature followed by an 1 hour incubation with HRP-labeled anti-human IgG antibodies. Absorbance was measured at 450 nm after addition of TMB substrate and sulfuric acid.

**Transendothelial albumin passage**

Endothelial cells seeded on polyester membranes (0.4 µm pore size) in tissue culture inserts (Corning Incorporated) were treated with a mixture of NETs and 1 mg/ml FITC-labeled albumin (ratio 1:1) either in the presence or absence of the elastase inhibitor sivelestat. At the indicated time points, aliquots were removed from the well and fluorescence was determined by fluorometry. The amount of albumin passing the endothelial monolayer was determined by a set of standard dilutions, as described previously.

**Animal studies**

Diseased MRL/lpr mice \( (n = 15) \) between 18 to 24 weeks of age were sacrificed and classified into three groups based on their degree of proteinuria: no proteinuria (albustix < 300 µg/ml, \( n = 5 \)), short-lived proteinuria (albustix > 1000 µg/ml, proteinuria < 7 days, \( n = 5 \)) or long-lived proteinuria (albustix > 1000 µg/ml, proteinuria > 14 but < 21 days, \( n = 5 \)). Age-matched CBA mice were included as control \( (n = 5) \). Kidney sections were stained for citrullinated histone H3 and Ly6G to detect NETs and RNA was isolated from kidney cortical tissue using TRIzol® reagent. For
immunofluorescence imaging of glomerular NETs, two independent researchers separately scored the presence of NET-like structures (i.e. extracellular DNA co-localizing with citrullinated histone H3) in a minimum of 10 glomeruli per mouse.

**Human material**

Renal biopsy samples were analyzed from 8 patients (22 – 41 years of age) with active LN, who were included in the Dutch SLE Nephritis Study. All patients fulfilled at least 4 American College of Rheumatology criteria for SLE. Active signs of LN were defined as glomerular hematuria or proteinuria of ≥ 0.5 g/24 hours and confirmed by renal biopsy. Citrated plasma was collected at the time of biopsy retrieval. Two renal biopsies from healthy donor kidneys (not suitable for transplantation for anatomical reasons) were included as control. Clinical parameters, such as proteinuria, anti-dsDNA antibodies and C3 levels, were determined in our diagnostic facility.

**Statistics**

Values are expressed as mean ± SEM. Statistical significance was determined by Student’s t-test or one-way ANOVA followed by Bonferroni correction using GraphPad Prism. P-values less than 0.05 were considered as statistically significant.

**Study approval**

The local animal ethics committee of the Radboud University Nijmegen approved all animal experiments. All experiments with human material were approved by the institutional medical ethics committees. Written consent was obtained from all patients enrolled in this study.
Table I. Chemicals used in this study.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>From</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPS-ZM1</td>
<td>Merck Millipore</td>
<td>553030</td>
</tr>
<tr>
<td>Sivelestat</td>
<td>Sigma-Aldrich</td>
<td>S7198</td>
</tr>
<tr>
<td>Phorbol myristate acetate (PMA)</td>
<td>Sigma-Aldrich</td>
<td>P1585</td>
</tr>
<tr>
<td>Micrococcal nuclease (MNase)</td>
<td>Worthington Corporation</td>
<td>LS004798</td>
</tr>
<tr>
<td>Sytox Orange</td>
<td>Life Technologies</td>
<td>S11368</td>
</tr>
<tr>
<td>N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide</td>
<td>Sigma Aldrich</td>
<td>M4765</td>
</tr>
<tr>
<td>Phalloidin-TRITC</td>
<td>Sigma-Aldrich</td>
<td>P1951</td>
</tr>
<tr>
<td>Albumin-FITC</td>
<td>Sigma-Aldrich</td>
<td>A9771</td>
</tr>
<tr>
<td>DNase I</td>
<td>Roche</td>
<td>000000010104159001</td>
</tr>
<tr>
<td>4-Nitroquinoline N-oxide (4-NQO)</td>
<td>Sigma-Aldrich</td>
<td>N8141</td>
</tr>
<tr>
<td>Annexin V apoptosis kit</td>
<td>BioVision</td>
<td>640914</td>
</tr>
</tbody>
</table>
Table II. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>From</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD31 (against human)</td>
<td>BD Pharmingen</td>
<td>555446</td>
</tr>
<tr>
<td>Anti-CD31 (against mouse)</td>
<td>BD Pharmingen</td>
<td>553369</td>
</tr>
<tr>
<td>Anti-VE-cadherin</td>
<td>Santa Cruz</td>
<td>sc-6458</td>
</tr>
<tr>
<td>Anti-vimentin</td>
<td>Dako</td>
<td>M0725</td>
</tr>
<tr>
<td>Anti-neutrophil elastase</td>
<td>Abcam</td>
<td>ab21595</td>
</tr>
<tr>
<td>Anti-citrullinated H3</td>
<td>Abcam</td>
<td>ab5103</td>
</tr>
<tr>
<td>Anti- β-catenin</td>
<td>BD Pharmingen</td>
<td>610153</td>
</tr>
<tr>
<td>Anti- β-catenin (active)</td>
<td>EMD Millipore</td>
<td>05-665</td>
</tr>
<tr>
<td>Anti-clathrin</td>
<td>Santa Cruz</td>
<td>sc-6579</td>
</tr>
<tr>
<td>Anti-Ly6G</td>
<td>Biolegend</td>
<td>108406</td>
</tr>
<tr>
<td>Anti- β-actin</td>
<td>Sigma-Aldrich</td>
<td>A5441</td>
</tr>
<tr>
<td>Anti-Snail1</td>
<td>Santa Cruz</td>
<td>sc-28199</td>
</tr>
<tr>
<td>Anti-MMP9 (blocking studies)</td>
<td>Abcam</td>
<td>ab38898</td>
</tr>
<tr>
<td>Anti-MMP9 (mouse stainings)</td>
<td>R&amp;D Systems</td>
<td>AF909</td>
</tr>
</tbody>
</table>
| Anti-agrin                        | Lab collection           | Mouse (MI91): ref 9  
|                                   |                          | Human (JM72): ref 10 |
| Anti-histone                      | Lab collection           | KM-2: ref 5    
|                                   |                          | BT164: ref 11   |
| Anti-histone (pan)                | EMD Millipore            | MABE71         |
Table III. Primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ &gt; 3’)</th>
<th>Reverse (5’ &gt; 3’)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail1</td>
<td>CGAGTGTTTCTTCTGCGCTA</td>
<td>CTGCTGGAAGGTAAACTCTGGA</td>
<td>Human</td>
</tr>
<tr>
<td>Snail1</td>
<td>CGTGTTGGAGTACCTTTCC</td>
<td>GGTACCAGGAGAGATCCC</td>
<td>Mouse</td>
</tr>
<tr>
<td>MMP9</td>
<td>GGACTCGGTCTTTGAGGAGC</td>
<td>CTTTCACGTCGAACTCCAG</td>
<td>Human</td>
</tr>
<tr>
<td>MMP9</td>
<td>GCCGACTTTTGTGGTCCTCC</td>
<td>GTCAAGGTAGCCTCTGCA</td>
<td>Mouse</td>
</tr>
<tr>
<td>SMA</td>
<td>AAAGCAAGTCCTCCAGCGTT</td>
<td>GTTCAAGGTAGCGTCCGTCT</td>
<td>Human</td>
</tr>
<tr>
<td>β2M</td>
<td>ATGAGTGACCTCGGCGTGTG</td>
<td>CCAATGGGCTCTCATTCAAC</td>
<td>Human</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGAAACCTGCAAATGATGAC</td>
<td>TCATTGTCATACAGGAAATGAG</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

Abbreviations:

- MMP9: matrix metalloproteinase 9;
- SMA: smooth muscle actin;
- β2M: beta-2 microglobulin;
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
References


Supplementary Figure 1 – NETs are internalized by endothelial cells into clathrin-coated pits. (A) ciGEnCs incubated with NETs for 15 min show a particulate cytosolic staining pattern for neutrophil elastase (NE), which is absent in ciGEnCs without NETs (control). (B) NE staining in ciGEnCs co-localizes with clathrin after exposure of ciGEnCs to NETs for 15 min. (C) Similar to NE, NET-associated histones are internalized into clathrin-coated pits as well by ciGEnCs after 15 min exposure to NETs (bottom row; magnification at the right). An isotype control staining was included (top row). Scale bars: white = 10 μm; green = 6 μm; yellow = 1 μm.
Supplementary Figure II – Endothelial cell alterations in response to NETs are distinct from apoptosis or cellular activation. (A) After co-incubation with NETs for 4 hours, HUVECs lose their normal ‘cobblestone’ appearance and acquire a spindle-shaped morphology, being fundamentally different from HUVECs treated with 100 nM PMA (‘activation’) or 100 nM of the apoptosis-inducing agent 4-NQO (‘cell death’). (B) Flow cytometry analyses (Annexin V staining) confirmed that the morphological alterations in NET-treated HUVECs were not the result of endothelial cell death. (C) The loss of CD31 and VE-cadherin, and the reorganization of the vimentin cytoskeleton, in ciGENCs (left) and HUVECs (right) occurs in response to NETs but does not occur upon endothelial cell activation with 100 nM PMA for 4 hours. (D) A subset of HUVECs loses CD31 expression in response to NETs, which can be largely prevented by pre-incubation of NETs with 1 μg/ml polyclonal anti-MMP9 antibodies. Scale bars: black = 30 μm; white = 15 μm.
Supplementary Figure III – Junctional β-catenin translocates to the nucleus in response to NETs. (A) HUVECs treated with NETs for 24 hours show an increased and decreased staining pattern of β-catenin in the nucleus or at the membrane, respectively (middle panel). These effects could be abolished by pre-treating NETs with 100 nM sivelestat prior to co-incubation with HUVECs (bottom panel). (B) Quantification of the β-catenin staining intensity at the membrane (left) or in the nucleus (right) after treatment of HUVECs with NETs. (C) Similar to Snail1 and MMP9, smooth muscle actin (SMA) was found upregulated as well in both ciGENCs (left) and HUVECs (right) in response to NETs. Scale bar: 20 μm. ** p < 0.01, *** p < 0.001, compared to control; ^ p < 0.05, compared to NETs. Statistics: unpaired student’s t-test.
Supplementary Figure IV – Snail1 and MMP9 protein expression in peritubular and glomerular endothelial cells. (A) Snail1 protein expression is absent in peritubular endothelial cells in a proteinuric MRL/lpr mouse. (B) Similar to Snail1, MMP9 protein expression in MRL/lpr glomeruli was confirmed by immunofluorescence imaging. As neutrophils also highly express MMP9, we confirmed the endothelial origin of MMP9 in these glomeruli by double stainings with the endothelial marker CD31 and the neutrophil marker Ly6G; as depicted, MMP9 co-localized with the endothelial marker CD31. CBA mice served as control. (C) Snail1 protein expression is absent in peritubular endothelial cells in a LN patient with severe proteinuria. Scale bars: white = 30 μm; yellow = 100 μm; red = 30 μm.
Supplementary Figure V – Glomerular NETs are positive for cell death-associated histone modifications. (A) NETs (white arrows; enlarged at the right panels) contain increased expression of tri-acetylated histone H4 (K8,12,16), as recognized by the lupus-derived monoclonal antibody KM-2, when compared to intact neutrophils (yellow arrow; enlarged at the right panels). (B) Tri-methylated histone H3 (K27), recognized by the lupus-derived monoclonal antibody BT164, is also enriched in NETs (white arrows), when compared to intact neutrophils (yellow arrow). Note the increased size of NETs when compared to intact neutrophils. Scale bars: white = 30 μm; yellow = 15 μm.
Supplementary Figure VI – A subset of LN patients fails to degrade DNA from three different sources. A subset of patients with LN fails to degrade (A) DNA from calf thymus, (B) DNA from apoptotic nucleosomes or (C) DNA from purified NETs. Plasmas from healthy donors (‘NHD’) and SLE patients without LN (‘SLE’) served as control.
Supplementary Figure VII – Induction and isolation of NETs. (A) The induction and isolation of NETs was routinely checked by immunofluorescence microscopy, where NETs were stained for elastase (NE; green) and DNA (red). NETs were induced with 100 nM PMA for 4 hours and isolated with 5U/ml micrococcal nuclease (MNase). (B) Neutrophils loose granularity after stimulation with PMA, as witnessed by conventional light microscopy. (C) DNA content (left) and elastase activity (right) in NET-containing supernatants was quantified with Sytox Orange or N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide, respectively. Scale bar: 20 μm. * p < 0.05, *** p < 0.001, compared to control. Statistics: unpaired student’s t-test.