Neutrophil Extracellular Traps Promote Angiogenesis
Evidence From Vascular Pathology in Pulmonary Hypertension

Lulwah Aldabbous, Vahitha Abdul-Salam, Tom McKinnon, Lucie Duluc, Joanna Pepke-Zaba, Mark Southwood, Alexander J. Ainscough, Charaka Hadinnapola, Martin R. Wilkins, Mark Toshner, Beata Wojciak-Stothard

Objective—Inflammation and dysregulated angiogenesis are features of endothelial dysfunction in pulmonary hypertension. Neutrophil extracellular traps (NETs), produced by dying neutrophils, contribute to pathogenesis of numerous vascular disorders but their role in pulmonary hypertension has not been studied. We sought evidence of (NETs) formation in pulmonary hypertension and investigated the effect of NETs on endothelial function.

Approach and Results—Plasma and lung tissues of patients with pulmonary hypertension were analyzed for NET markers. The effects of NETs on endothelial function were studied in vitro and in vivo. Patients with chronic thromboembolic pulmonary hypertension and idiopathic pulmonary hypertension showed elevated plasma levels of DNA, neutrophil elastase, and myeloperoxidase. NET-forming neutrophils and extensive areas of NETosis were found in the occlusive plexiform lesions and vascularized intrapulmonary thrombi. NETs induced nuclear factor κB–dependent endothelial angiogenesis in vitro and increased vascularization of matrigel plugs in vivo. Angiogenic responses were associated with increased release of matrix metalloproteinase-9, heparin-binding epidermal growth factor–like growth factor, latency-associated peptide of the transforming growth factor β1, and urokinase-type plasminogen activator, accompanied by increased endothelial permeability and cell motility. NETs-induced responses depended on myeloperoxidase/H₂O₂-dependent activation of Toll-like receptor 4/nuclear factor κB signaling. NETs stimulated the release of endothelin-1 in HPAECs (human pulmonary artery endothelial cells) and stimulated pulmonary smooth muscle cell proliferation in vitro.

Conclusions—We are the first to implicate NETs in angiogenesis and provide a functional link between NETs and inflammatory angiogenesis in vitro and in vivo. We demonstrate the potential pathological relevance of this in 2 diseases of disordered vascular homeostasis, pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension. (Arterioscler Thromb Vasc Biol. 2016;36:2078-2087. DOI: 10.1161/ATVBAHA.116.307634.)

Key Words: cell movement □ endothelial cells □ endothelin-1 □ extracellular traps □ homeostasis □ hypertension, pulmonary □ neutrophils

A new type of neutrophil cell death called NETosis, first identified by Zychlinsky et al,1 has been linked with the pathogenesis of numerous diseases, including atherosclerosis,2 preeclampsia,3 acute lung injury,4 deep vein thrombosis,5 and cancer-associated thrombosis.6 During NETosis, neutrophil extracellular traps (NETs) are formed as a result of reactive oxygen species–triggered decondensation of chromatin dependent on histone hypercitrullination catalyzed by peptidylarginine deiminase 4 (PAD4).7 The cells die releasing a net of chromatin fibers studded with neutrophil secretory granules containing neutrophil elastase and myeloperoxidase.

See accompanying editorial on page 2035

Neutrophils constitute the first line of defense in killing pathogens but are also necessary for tissue repair and the proper development and maintenance of healthy vasculature.7 Neutrophils have been implicated in vascular inflammatory processes in pulmonary hypertension, a condition characterized by progressive thickening of small intrapulmonary arteries because of increased endothelial and smooth muscle proliferation, endothelial dysfunction and in situ thrombosis.8,9 Factors implicated in the pathogenesis of PH such as oxidative stress, interleukin-8 (IL-8), tumor necrosis factor-α or ribonucleoprotein immune complexes can induce NET formation in vitro,1,10-12 but the potential link between the pathophysiology of pulmonary hypertension and NETosis has not been investigated.

The clinical classification of PH categorizes the disease into 5 groups, with pulmonary arterial hypertension (PAH) and chronic thromboembolic pulmonary hypertension (CTEPH) being most common.9,13 PAH may occur either as a primary...
disease of unknown cause (idiopathic PAH [IPAH]), as a result of loss-of-function mutations in the transforming growth factor β/bone morphogenetic protein receptor superfamily (heritable PAH), or as an associated manifestation of other diseases.9 A hallmark of severe PAH is the presence of plexiform lesions, complex vascular formations characterized by disorganized angiogenesis, inflammation, and thrombosis.14 CTEPH is caused by chronic obstruction of major pulmonary arteries by nonresolving thrombi and a concomitant small-vessel arteriopathy.15 Endarterectomized tissues from patients with CTEPH show vessel-like structures in material obtained from distal areas, whereas proximal material is characterized by lower cell density and sometimes the accumulation of fresh thrombotic material.16

In this study, we aimed to establish whether NET formation is a feature of vascular pathology in CTEPH and PAH, study the effects of NETs on pulmonary endothelial and smooth muscle responses in vitro, and investigate the signaling mechanisms involved. We are the first to document NET formation in CTEPH and PAH and show that NETs trigger inflammatory activation of pulmonary endothelial cells and promote endothelial angiogenesis in vitro and in vivo via myeloperoxidase/H$_2$O$_2$/nuclear factor κB (NFκB)/TLR4-dependent signaling.

**Methods**

An expanded Materials and Methods section is available in the online-only Data Supplement.

**Results**

**Markers of NEtosis Are Increased in Plasma and Lung Tissues From PAH and CTEPH Patients**

NET formation (NETosis) is commonly identified by elevated levels of 2 or more markers of NETs, such as circulating DNA, myeloperoxidase, neutrophil elastase, or citrullinated histone 3. CTEPH (n=26) and IPAH (n=52) patients showed increased plasma levels of myeloperoxidase (10-fold increase in CTEPH and 5.6-fold increase in IPAH), neutrophil elastase (7-fold increase in CTEPH and 5.3-fold increase in IPAH), and circulating DNA (1.5-fold increase in CTEPH and 1.5-fold increase in IPAH), compared with plasma from age- and sex-matched healthy volunteers (n=24; Figure 1A through 1C). Elevated levels of NET markers were also found in the independent external cohort of patients with CTEPH from Papworth Hospital (n=21; Figure I in the online-only Data Supplement). As circulating free DNA may come from various cellular sources, the analysis of DNA–myeloperoxidase complexes may provide a more accurate measurement of NET levels in plasma.14 Consistently, both IPAH and CTEPH patient cohorts showed significantly elevated plasma levels of DNA–myeloperoxidase complexes (Figure 1D). Patient characteristics are shown in Table I in the online-only Data Supplement. No correlation between the marker levels and other variables, such as age or drug treatment, was observed (data not shown).

Histological analysis of lung tissues from patients with IPAH showed areas of NETosis marked by colocalization of DNA, myeloperoxidase, and citrullinated histone H3, particularly evident in the occlusive plexiform lesions (Figure 1E). IPAH lung tissues also showed increased accumulation of NET-forming, PAD4* neutrophils along the vascular wall and around the remodeled vessels, whereas control tissues showed no staining (Figure 1E). IPAH lung tissues also displayed a marked staining of endothelial L-endoglin, a known regulator of tumor-related angiogenesis and neovascularization19 (Figure IIA in the online-only Data Supplement). Endarterectomy specimens from patients with CTEPH showed a marked colocalization of DNA, myeloperoxidase, and citrullinated histone 3 and the presence of NET-forming PAD4* neutrophils (Figure 1F; Figure IIB in the online-only Data Supplement).

**NETs Promote Proinflammatory and Prothrombotic Responses in Human Pulmonary Artery Endothelial Cells**

Endothelial dysfunction is a key contributor to vascular remodeling in PH, and we investigated the effects of NETs on cultured human pulmonary artery endothelial cells (HPAECs). NETs appeared as large, mesh-like structures showing colocalization of DNA with citrullinated histone H3 and myeloperoxidase (Figure III in the online-only Data Supplement); 2×10$^5$ neutrophils provided ≈2.8 μg of NET DNA. NETs induced =3-fold increase in the activity of the proinflammatory transcription factor, NFκB in HPAECs (Figure 2A). Fragmentation of NETs with DNaseI prevented this response, reinforcing the importance of structural integrity of NETs. To determine whether a direct contact of NETs with the endothelial surface was required, HPAECs were separated from NETs by a porous (0.4-μm pore size) membrane in Transwell dishes (Figure IV in the online-only Data Supplement). The effectiveness of this separation was confirmed by the measurement of DNA content in the bottom chamber of Transwell dishes at different time points (Figure IV in the online-only Data Supplement). The results have shown that a significant activation of NFκB could only be induced by a direct contact of NETs with the endothelial cells (Figure 2B).

Myeloperoxidase/H$_2$O$_2$ signaling drives inflammatory reactions and tissue oxidation and promotes nuclear translocation and activation of NFκB in cells.20-21 In HPAECs, NFκB activation was prevented by myeloperoxidase inhibitor I and catalase, arguing for the major role of myeloperoxidase-induced reactive oxygen species generation in NETs-induced inflammatory signaling. NETs also markedly augmented the

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Nonstandard Abbreviations and Acronyms

- CLIC4: chloride intracellular protein 4
- CTEPH: chronic thromboembolic pulmonary hypertension
- ET-1: endothelin-1
- IPAH: idiopathic PAH
- MMP-9: matrix metalloproteinase-9
- NETs: neutrophil extracellular traps
- NFκB: nuclear factor kappa B
- PAD4: peptidylarginine deiminase 4
- PAH: pulmonary arterial hypertension
- PDGF: platelet-derived growth factor
- TLR4: Toll-like receptor 4

Neutrophil Extracellular Traps in Angiogenesis as if you were reading it naturally.
NFκB-dependent expression of intercellular adhesion molecule-1 (ICAM-1) in HPAECs, an important mediator of leukocyte adhesion and transmigration (Figure 2C).

Consistent with their prothrombotic effects, NETs induced ≈5-fold increase in the surface expression and 1.2-fold increase in total protein expression of von Willebrand Factor (Figure V in the online-only Data Supplement). NETs also markedly increased platelet adhesion to HPAECs under flow, attenuated in the presence of anti–von Willebrand Factor antibodies (Figure VC and VD in the online-only Data Supplement).

**Figure 1.** The levels of neutrophil extracellular trap (NET) markers in plasma of chronic thromboembolic pulmonary hypertension (CTEPH) and idiopathic pulmonary arterial hypertension (IPAH) patients. Concentration of (A) DNA, (B) myeloperoxidase, and (C) neutrophil elastase in plasma of age- and sex-matched healthy controls (n=24), patients with CTEPH (n=26), and patients with IPAH (n=52). D, Quantification of myeloperoxidase (MPO)–DNA complexes. Results are expressed as means±SEM, **P<0.01, ***P<0.001, comparison with healthy controls. E, Histological analysis of sections of PAH lung with plexiform lesions and (F) intrapulmonary thrombus from CTEPH lung. Images show corresponding staining for DNA, citrullinated histone H3 (Cit-H3), MPO and peptidylarginine deiminase 4 (PAD4), merged images and hematoxylin and eosin (H&E) staining, as indicated. Images are representative of n=6. Bar, 50 µm.

NETs Increase Endothelial Angiogenesis In Vitro

The presence of NETs in plexiform lesions and vascularized CTEPH thrombi focused our interest on the potential role of NETs in the regulation of angiogenesis. The effect of NETs on endothelial angiogenesis was studied in a 2-dimensional matrigel tube formation assay, reflecting intussusceptive angiogenesis and in a 3-dimensional spheroid sprouting assay, informing the effect of NETs on sprouting angiogenesis.

NETs (24 hours) significantly increased capillary tube length and network maturity, measured as several completed
NETs induced proangiogenic responses were attenuated in the presence of DNase1, NFκB inhibitor, BAY 117085, and inhibitors of myeloperoxidase/H2O2 (Figure 3A through 3C). Spheroid sprouting assessed by counting the number of sprouts and total spheroid area was significantly increased by NETs (Figure 3D through 3F).

Human angiogenesis microarray analysis of NETs-treated HPAECs revealed a significantly increased expression levels of several proangiogenic factors, including matrix metalloproteinase-9 (MMP-9), platelet-derived growth factor AB/BB (PDGF-AB/PDGF-BB), heparin-binding epidermal growth factor (EGF)-like growth factor, latency-associated peptide of the transforming growth factor β1, and urokinase-type plasminogen activator, compared with untreated controls (Figures VI and VII in the online-only Data Supplement). Microarray analysis of NET components alone revealed only traces of MMP-9 and latency-associated peptide of the transforming growth factor β1 but did not show any other proangiogenic factors found elevated in HPAECs (Figure VI in the online-only Data Supplement).

NETs Increase Proliferative Activity of HPAECs, Destabilize Intercellular Endothelial Junctions, and Increase Endothelial Cell Motility

NETs stimulated proliferative activity and induced dispersion of intercellular adherens junctions in HPAECs, accompanied by increased endothelial permeability (Figure 4; Figure VIII in the online-only Data Supplement).

To further explain the mechanism of the proangiogenic effects of NETs, we recorded endothelial cell movement and used chemotaxis and migration analysis software to provide images of cell trajectories and compare parameters of cell movement in the untreated and NETs-treated HPAECs. NETs increased the total length of cell trajectories (accumulated distance), cell translocation (distance in a straight line between the start and the end point of each trajectory), and increased the speed of cell movement (Figure 4C through 4G), without affecting directionality (data not shown). In agreement with the results obtained in angiogenesis assays, NETs-induced increase in the endothelial cell migration was attenuated in the presence of DNase1 and myeloperoxidase inhibitor I (Figure IX in the online-only Data Supplement).

Conditioned media from NETs-treated HPAECs contain increased levels of endothelin-1 (ET-1) and increased proliferation of human pulmonary artery smooth muscle cells in vitro. Vasoactive agents released by the activated endothelium, such as PDGF or ET-1, are known stimulators of vascular cell migration and proliferation and may potentially contribute to the vascular remodeling in disease conditions. We measured ET-1 levels in conditioned media from NETs-treated HPAECs and noted a significant increase in ET-1 (≈2.3-fold increase) secreted by these cells (Figure XA in the online-only Data Supplement). Incubation of human pulmonary artery smooth muscle cells with conditioned media from NETs-treated HPAECs significantly enhanced PASMC proliferation in vitro (Figure XB in the online-only Data Supplement). Interestingly, preliminary analysis of CTEPH plasma (Papworth Hospital cohort; n=21) revealed a significant correlation between the levels of ET-1 and the levels of DNA, myeloperoxidase, and neutrophil elastase (Table).

Toll-Like Receptor 4 Mediates the Proinflammatory and Proangiogenic Effects of NETs

Activation of Toll-like receptor 4 (TLR4) by oxidative stress has been implicated in the pathogenesis of PH and was shown to induce inflammatory angiogenesis and increased expression of ICAM-1 and ET-1 in endothelial cells, reminiscent...
of the phenotype induced by NETs. We, therefore, hypothesized that TLR4 may act as a mediator of NETs-induced effects reported in this study.

Incubation of cells with TLR4 inhibitor prevented NETs-induced NFκB activation, tube formation, and spheroid sprouting (Figure 5A through 5C). Western blotting analysis revealed a transient activation of TLR4 signaling, manifested by increased phosphorylation of TLR4 effector, IRAK4, whereas total expression levels of IRAK4 and TLR4 remained unchanged (Figure 5D through 5H).

Our recent studies documented a key role of redox-sensitive CLIC4 (chloride intracellular protein 4) in the regulation of NFκB activation and hypoxia-inducible factor–driven angiogenesis.25 Interestingly, activation of TLR4 by NETs was accompanied by increased expression of CLIC4 in HPAECs, consistent with the proinflammatory and proangiogenic phenotype (Figure 5F).

NETs-induced spheroid sprouting was inhibited by CLIC4shRNA, confirming the importance of this protein in the regulation of endothelial angiogenic responses (Figure XI in the online-only Data Supplement).

NETs Increase Angiogenesis In Vivo

To investigate the effect of NETs on angiogenesis in vivo, matrigel containing equal amounts of either mouse or human NET DNA was injected subcutaneously into mice. Although mouse and human NETs were similar in appearance, human NETs tended to cluster to form larger aggregates (Figure XII in the online-only Data Supplement).

Both mouse and human NETs significantly increased plug vascularization, with human NETs having a more pronounced effect (2-fold increase compared with controls, \( P<0.05;\) Figure 6). Hematoxylin and eosin and DNA staining revealed increased cellularity of plugs containing mouse and human NETs, compared with controls (Figure 6).

Discussion

This report is the first to implicate NETs in angiogenesis and in diseases characterized by disordered vascular homeostasis in the pulmonary vasculature. We demonstrate their role in the regulation of proinflammatory, proangiogenic responses in human endothelial cells via the activation of myeloperoxidase/H₂O₂-mediated TLR signaling.
Proangiogenic effects of NETs were confirmed in 3 different models of angiogenesis, in the matrigel tube formation and spheroid sprouting assays in vitro and in the matrigel plug formation assay in vivo. Angiogenesis is a complex process in which endothelial cells migrate, proliferate, and invade the surrounding tissues to form capillaries. Subsequent migration and proliferation of pericytes, fibroblasts, and smooth muscle cells contribute to vessel maturation. Our study has revealed a complex pattern of responses in NETs-stimulated HPAECs, involving increased endothelial proliferative/metabolic activity, increased motility, and increased endothelial permeability in vitro. These effects are likely to result from the actions of numerous vasoactive agents released by NETs-treated HPAECs, including MMP-9 and urokinase-type plasminogen activator, known to liberate proangiogenic growth factors sequestered to the extracellular matrix26 and stimulators of cell migration and proliferation, such as latency-associated peptide of the transforming growth factor β1, heparin-binding EGF-like growth factor, and PDGF. In addition to the observations reported in this study, NETs were also shown to increase

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Correlation coefficients of all markers from the combined data are shown and those that were significant and highly correlated are marked with an asterisk (P≤0.05, Spearman correlation). CTEPH indicates chronic thromboembolic pulmonary hypertension; ET-1, endothelin-1; MPO, myeloperoxidase; and NE, neutrophil elastase.
cytokine/growth factor release in macrophages. Incubation of HPAECs with NETs significantly increased the expression of ICAM-1 in HPAECs, consistent with other reports. ICAM-1 is an important regulator of angiogenesis and can support VEGF (vascular endothelial growth factor)-induced endothelial cell migration and facilitate recruitment of endothelial progenitor cells. Another factor released by HPAECs, heparin-binding EGF-like growth factor, is mitogenic and chemotactic for vascular smooth muscle cells, keratinocytes, fibroblasts, and cardiomyocytes. Together with PDGF-BB, HB-FGF promotes new vessel formation by recruiting mesenchymal cells during the late phase of angiogenesis.

Activated neutrophils are a source of MMP9 and VEGF, and therefore, we considered a possibility that some of the proangiogenic factors found in HPAEC lysates may have originated from neutrophil cell/NET fragments internalized by the endothelial cells. However, apart from the traces of MMP9 and latency-associated peptide 1, NET preparations did not contain any of the factors found elevated in NETs-treated HPAECs, consistent with the analyses carried out by others.

Experiments performed in our and other laboratories indicate that NETs can induce a spectrum of activities in cells, depending on the amount of NET material added to the endothelial cell culture. The amount of NETs produced by to NET-forming neutrophils per 10 endothelial cells (0.3-µg/mL NETs DNA) evokes proinflammatory and proangiogenic responses in endothelial cells. However, 10- to 30-fold higher concentrations of NETs enhance endothelial apoptosis, likely to reflect conditions in severe sepsis. Our observations also emphasize the importance of a localized, direct contact of NET fibers with the endothelial cell surface.

The proinflammatory and proangiogenic effects of NETs depended on the myeloperoxidase/H2O2 signaling, resembling the actions of activated neutrophils. H2O2 increases the expression of MMPs 1, 3, and 9 and urokinase-type plasminogen activator in endothelial cells and activates several kinases associated with increased cell proliferation and migration, including Akt, p38 MAPK, and ERK1/2. We focused our attention on TLR4 as a likely mediator of myeloperoxidase/H2O2-induced responses. Myeloperoxidase functions as...
a major enzymatic catalyst for initiation of lipid peroxidation,37 which activates TLR4 in endothelial cells.38 Activated TLR4 can trigger NFκB activity, ICAM-1 expression, and ET-1 release,23 consistent with the phenotype seen in NETs-stimulated HPAECs. In further support of the role of TLR4, TLR4 inhibitors completely prevented NETs-induced NFκB activation and endothelial angiogenesis. NETs induced a rapid (30 minutes–1 hour) activation of TLR4 signaling in NETs-treated HPAECs, corresponding to the timecourse of receptor activation induced by peroxidized lipids.38 Interestingly, in addition to the TLR4 activation, we noted a cotemporary increase in the expression of CLIC4. CLIC4 is activated by reactive oxygen species, and its expression is increased in the endothelium of remodeled vessels and plexiform lesions in human PAH and animal models of PAH.25 CLIC4 gene silencing completely inhibited NET-induced spheroid sprouting, reinforcing the key role of this protein in the regulation of endothelial angiogenesis in vitro.25

Having demonstrated that NETs increase pulmonary endothelial cell motility and angiogenic responses in vitro, we then investigated the angiogenic effects of NETs in vivo. Apart from endothelial cells, the mechanism of NETs-induced angiogenesis in vivo is likely to involve other cell types. For instance, NETs provide a scaffold for thrombus formation by capturing erythrocytes and platelets39 and platelet-derived thrombin is known to enhance the secretion of VEGF and MMP-9 and synergize with VEGF in stimulating endothelial cell proliferation.40

Our results provide arguments for a potential role of NETs-induced signaling in the vascular pathology of CTEPH and PAH. Immunohistochemical staining of lung sections revealed a marked accumulation of NET-forming PAD4+ leukocytes and NET markers at the vascular wall and in the perivascular space of the occlusive plexiform lesions from patients with IPAH. These changes were concurrent with increased expression of L-endoglin, typically found in proliferating endothelial cells during neovessel formation.41 The mechanisms leading to complex vascular formations in PAH are not well understood but numerous studies have documented the presence of continuously proliferating endothelial cells and sprouting vascular channels, accompanied by increased expression of MMP-9 and other mediators of angiogenesis.14 PAD4+ neutrophils and NET markers were also identified in the intrapulmonary thrombi from patients with CTEPH. It is conceivable that NETs may facilitate local pulmonary vascular remodeling by increasing vascular cell proliferation and

![Figure 6. Neutrophil extracellular traps (NETs) induce angiogenesis in vivo. A, Plug vascularization (endomucin fluorescence intensity). Plugs from 5 mice with 6 fields/plug were analyzed (n=5). Results expressed as means±SEM *P<0.05 vs control. B, Representative images of matrigel plugs. C: control mice; M: mice injected with matrigel containing mouse NETs; and H: mice injected with matrigel containing human NETs. C, Representative images of plug vascularization visualized by fluorescent staining of endomucin. DNA (blue), endomucin (green). Hematoxylin and eosin (H&E) staining of plug sections is shown on the right, as indicated. Bar, 10 µm.]
migration. Consistent with this, we observed increased proliferative activity of PASMCs induced by conditioned media from NETs-treated HPAECs and increased levels of ET-1, a potent mitogen for vascular endothelial and smooth muscle cells and a vasoconstrictor implicated in the pathogenesis of PH.\(^3\) NET markers significantly correlated with ET-1 levels in plasma of patients with CTEPH, suggesting that ET-1 may, in addition to the other identified mediators, play a regulatory role in NET-induced responses.

Deficiency of bone morphogenetic protein receptor II signaling in PAH may further augment NET formation/signaling by facilitating neutrophil migration to the diseased lung and provide a positive regulatory feedback loop by TLR4-dependent increase in IL-8, a cytokine known to induce NET formation in vitro.\(^{43,44}\) Future studies should also address the role of NETs in the pathogenesis of PH and investigate whether neutrophils from PAH and CTEPH patients show increased propensity to form NETs. This would not be entirely unexpected because PAH and CTEPH neutrophils respond to stimulation with increased inflammatory mediator generation ex vivo, including increased respiratory burst, elastase secretion, and lipid mediator synthesis.\(^45\) It is important to note that IPAH plexiform lesions form predominantly in the precapillary vessels consisting of endothelial microvascular cells, which may differ in their responses from macrovascular cells used in this study. Another potential avenue for future studies may include a functional link between NET formation and angiogenesis in other conditions characterized by abnormal vascularization, inflammation, and elevated NET markers in plasma and diseased organs/tissues, such as rheumatoid arthritis, cancer, and connective tissue diseases.\(^6,46,47\)

To summarize, we are the first to document that NETs are proangiogenic and demonstrate a potential contributory role of NETs in the vascular pathology of human CTEPH and PAH, characterized by the activation of local proinflammatory and proangiogenic responses in pulmonary vascular endothelium via reactive oxygen species–induced TLR4-mediated signaling.

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Disclosures
None.

References
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Highlights

- Elevated levels of neutrophil extracellular traps markers were found in plasma and lung tissues of patients with idiopathic pulmonary arterial hypertension and chronic thromboembolic pulmonary hypertension.
- Neutrophil extracellular traps induced nuclear factor κB–dependent endothelial angiogenesis in vitro and increased vascularization of matrigel plugs in vivo.
- Neutrophil extracellular traps can induce inflammatory angiogenesis via the activation of myeloperoxidase/H₂O₂–Toll-like receptor 4 signaling.

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MATERIALS AND METHODS

Aldabbous et al. Neutrophil extracellular traps (NETs) promote angiogenesis: evidence from vascular pathology in pulmonary hypertension.

Human plasma collection and measurement of NET components.
Venous blood samples were obtained with local ethics committee approval and informed written consent from healthy volunteers, CTEPH and IPAH patients. Plasma samples were obtained from 24 healthy volunteers, 52 IPAH patients, 26 CTEPH patients (Imperial College Hammersmith Hospital cohort) and an independent, external cohort of 26 CTEPH patients (Cambridge Papworth Hospital). Patients’ characteristics are shown in Supplementary Table I. DNA content was measured using Quant-it PicoGreen kit according to the manufacturer's instructions (Invitrogen, P7589). MPO was measured using Myeloperoxidase Human ELISA Kit (Abcam, #ab119605) according to the manufacturer’s instructions. NE was measured using PMN Elastase Human ELISA Kit (Abcam, #ab119553).

The levels of DNA-MPO complex in plasma were measured with modified ELISA method described in 1. Briefly, ELISA plates (Maxisorp F96, Nunc #442404) were incubated overnight with 5µg/mL of rabbit anti-myeloperoxidase antibody (Abcam #45977, 10 µg/mL). The plates were washed 3 times with 0.1% PBS-Tween (PBST) and 20µL of plasma samples mixed with 80µL of incubation buffer containing peroxidase-labelled anti-DNA mAb (Cell Death ELISAPLUS, Roche, dilution 1:25) per well were added. Following 2 hour incubation and 3 washes in PBST, 100µL of ABTS substrate was added and 405 nm absorbance was read in Glomax™ plate reader.

Staining of human lung sections and endarterectomy specimens.
Paraffin blocks were cut into sections, analysed by immunohistochemistry and mounted in Vectashield containing nuclear stain DAPI (Vector Laboratories, #H-1200). Primary antibodies included rabbit polyclonal anti-neutrophil elastase (Abcam, #ab21595, 1µg/mL), rabbit polyclonal anti-PAD4 (ThermoScientific, #PA5-22317, 10µg/mL). Biotinylated anti-rabbit IgG (Vector Laboratories, #BA-1000, 10µg/mL) was used as a secondary antibody.

For immunofluorescent staining of lung sections and endarterectomy specimens, primary antibodies: rabbit polyclonal anti-citrullinated histone 3 (Abcam, #ab5103, 1µg/mL), goat polyclonal anti-myeloperoxidase (R&D Systems, #AF3667, 10 µg/mL), mouse anti-endoglin (Millipore, # 05-1424) and secondary antibodies: TRITC-labelled donkey anti-rabbit (Jackson Laboratories, #711-025-152, 0.5 µg/mL) and Alexa488 donkey anti-goat antibody (Invitrogen, #A11055, 4 µg/mL), were used. Sections from 6 healthy individuals or patients per group were used in the study (n=6). The slides were mounted in Vectashield containing nuclear stain DAPI (Vector Laboratories, #H-1200). Images were taken under the confocal laser scanning fluorescence microscope (Zeiss LSM-780). Microscopy was performed in the facility for imaging by light microscopy (FILM) at Imperial College London.

Isolation of NETs and quantification of DNA in NET preparations.
Human neutrophils were isolated as described in.2 NET formation was induced by 20 nmol/L phorbol 12-myristate 13-acetate (PMA).3,4 NET formation was induced by overnight stimulation of neutrophils with 20 nmol/L of phorbol 12-myristate 13-acetate (PMA). The NETs layer deposited at the bottom of the culture dish was washed twice in PBS and was collected by vigorous pipetting. The cell debris was removed by centrifugation at 300 x g for 10 minutes and supernatants containing NETs were used for experiments. To measure DNA content, NET samples were digested 10U/mL DNase I (Thermo Scientific B43) in 37°C for 30 minutes and DNA content was measured with picogreen dsDNA kit (Invitrogen, P7589), according to the manufacturer's protocol.
Mouse neutrophils were obtained from bone marrow of 10-week-old male CD1 mice (n=10) according to the method described in 5. Isolation and quantification of mouse NETs was carried out in the same manner as described for human neutrophils.

**Staining of NET markers in NET preparations.** NETs were stained with primary antibodies: rabbit anti-myeloperoxidase antibody (Abcam #45977; 10 µg/mL), and rabbit polyclonal anti-citrullinated histone H3 antibody (Abcam, #5103; 10 µg/mL). TRITC-labelled goat-anti rabbit IgG was used as a secondary antibody (Jackson Laboratories, #111-025-144; 5 µg/mL). The coverslips were mounted in Vectashield containing nuclear stain DAPI (Vector Laboratories, #H-1200). Images of cells were taken under the confocal laser scanning fluorescence microscopy (Leica TCS SP5).

**Cell culture and treatments.**

Human pulmonary artery endothelial cells and human pulmonary artery smooth muscle cells were cultured as previously described 6. NETs were added to cell media at a final concentration of 0.3 µg/mL DNA and the cells were cultured for 4 hours or 24 hours, with or without the inhibitors: 10U/mL DNase I (Thermo Scientific, #B43), 300 nmol/L Myeloperoxidase Inhibitor-I (Calbiochem, #CAS 5351-17-7), the NFκB inhibitor, BAY 117085 (10µmol/L; Sigma) or catalase from bovine liver at 1000 U/mL (Sigma; # C9322)(22). The Toll-like receptor inhibitor (TLRi; InvivoGen,#CLI-095) was added to the cells at the concentration of 10µg/mL.

**Co-culture of NETs and HPAECs in Transwell dishes.**

HPAECs infected with AdNFκB-luc were grown to confluence at the bottom of 24-well Transwell dishes (Corning Life Sciences, 0.4 µm pore size, # 3381). NETs were either added directly to the bottom chambers (to remain in direct contact with endothelial cells), or were added to the top chambers of Transwell dishes, separated from the cells by a porous membrane. Following 24 hour incubation, the NFκB activity was measured in luciferase reporter assay. To verify whether the 0.4 µm pore size membrane was effective in separating NETs from cells, DNA concentration in the bottom chamber was measured with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™) at selected timepoints throughout the experiment. In experiments involving HPASMC proliferation, HPAECs were cultured in serum-reduced (0.5% FCS) medium in the top chamber of Transwell dishes, with or without NETs. Conditioned media were collected from the bottom chamber after 24 hour incubation and were added to HPASMCs cultured in 96 well plates (7000 cells/well). The cells were subsequently used for CellTiter 96 proliferation assay

**CLIC4 gene silencing.**

Inhibition of CLIC4 expression was achieved by adenoviral overexpression of CLIC4shRNA. AdCLIC4shRNA (pEQU6-Clic4-shRNA-GFP; 5'-GCCGTAATGTTGAACAGAATT-3', Welgen, Inc) was used at the multiplicity of infection (MOI) 1:100. AdGFP (Vector Biolabs) was used as an adenoviral control.

**NFκB Luciferase Reporter Assay**

Adenoviral NFκB luciferase reporter construct (AdNFκB-Luc) and Luciferase Assay System (Promega) were used to measure NFκB activity 8. Briefly, confluent HPAECs grown in 24-well plates were infected with adenoviruses: AdGFP (adenoviral control) or AdNFκB-luc (NFκB luciferase reporter) at the multiplicity of infection (MOI) 1:100 in serum-free medium. Following 2-hour incubation, the medium was replaced with culture medium containing serum and growth factors. 24 hours post-infection, 100 µL of NET suspension in PBS containing 1.4 µg/ml DNA was added to the cells and incubated for a further 24 hours under normoxic (19% O₂, 5% CO₂) or hypoxic (2% O₂, 5% CO₂) conditions at 37°C. The cells were then lysed using 1x lysis buffer (Promega, # E1500) and 20 µl of each sample was transferred to white 96-well plates (Corning™ 3912) and combined with 100 µl of Luciferase Assay Reagent™ (Promega, # E1500). Luminescence, proportional to the level of NFκB-driven expression of luciferase, was measured in the Glomax™ luminometer.
Western blotting. Protein lysates of HPAECs cultured with NETs for 24 hours were resolved by SDS-PAGE and analysed by western blotting. Blots were probed with rabbit anti-human ICAM-1 monoclonal antibodies (Cell Signaling Technology®, #4915S; 1µg/mL), mouse monoclonal anti-TLR4 (Santa Cruz Biotechnology, #sc-13593), rabbit anti-IRAK-4 (Cell Signaling Technology, #4363P), rabbit anti-p-IRAK-4 (Cell Signaling Technology, #T345/S346) and mouse monoclonal anti-β-actin antibodies (Sigma, # A1978; 0.4 µg/ml), as appropriate. Secondary antibodies included goat anti-rabbit HRP-linked antibody (Sigma, 1µg/mL) and goat anti-mouse HRP-linked antibody (Sigma, 1µg/mL). Membranes were imaged using Luminata Crescendo (Milipore) chemiluminescence reagent in BioRad ChemiDoc Imager and optical density of immunoreactive bands were analysed using ImageJ.

Human Angiogenesis Microarray
Confluent HPAECs were grown in serum-reduced (0.5% FBS) and growth-factor-deprived medium, with or without NETs for 24 hours. Pro-angiogenic cytokine release was studied in a Proteome Profiler™ Human Angiogenesis Array (R&D Systems™, # ARY007). Changes in pro-angiogenic cytokine levels were established by comparing optical density of spots on membranes incubated with untreated HPAEC lysates with corresponding spots on membranes incubated with NETs-treated HPAEC lysates.

Endothelin-1 (ET-1) measurement.
ET-1 levels in cell culture media and patient plasma were measured with QuantiGlo ELISA Human Endothelin-1 Immunoassay (R&D, #QET00B), according to the manufacturer’s instructions. Serum ET-1 levels were measured in duplicate wells using a customized assay (Millipore, UK) using a Luminex 200 analyser and x PONENT software (Luminex Cooperation, Texas, USA).

Endothelial Permeability.
Endothelial permeability was measured in Transwell assay, as previously described. Briefly, HPAECs (10,000 cells/well) were grown to confluence in full media in Transwell-Clear dishes with a 0.4 µm pore size membrane (Corning Life Sciences, # 3381). HPAECs were then treated with NETs for 24 hour in normoxic conditions. Fluorescein isothiocyanate (FITC)-conjugated dextran (Sigma, # 46947; 1g/L) was added to the upper compartment of the Transwell-Clear chamber and 1 hour later the fluorescence of FITC-dextran that passed through the endothelial layer into the bottom chamber of Transwell dishes was measured using a Glomax luminometer (Promega) at 490 nm excitation and 520 nm emission wavelength.

Platelet adhesion under flow.
HPAECs were seeded into the channels of Ibidi VI0.4 and cultured for two days to form a confluent monolayer. The cells were then incubated with NETs for 4 or 24 hours. Washed red blood cells and platelets were prepared as previous described and platelets labelled with DiOC6 and perfused over the HPAECs at 250s. The surface area covered by platelets was determined using Image J and expressed as a percentage of the total endothelial surface area.

Intracellular distribution of F-actin and VE-cadherin and surface expression of vWF.
Immunostaining of VE-cadherin and affinity-staining of F-actin in cells grown on coverslips was performed as in. The cells mounted in Vectashield mountant containing DAPI (Vector Laboratories, #H-1200). To visualise surface deposition of vWF, the cells were fixed without permeabilisation and stained with polyclonal rabbit anti-human von Willebrand Factor antibody (DAKO; # A 0082; 10µg/mL) and fluorescent secondary TRITC goat-anti rabbit antibody (Jackson Laboratories #111-025-144; 10µg/mL). Images of cells were taken under the confocal laser scanning fluorescence microscopy (Leica TCS SP5). Image processing was performed with Adobe Photoshop CS (Adobe Systems, San Jose, CA).

In vitro matrigel tube formation and spheroid sprouting angiogenesis assay
Matrigel tube formation assay was performed in growth factor-reduced Matrigel (Corning™, # 354250), as previously described. Cells were photographed under the Olympus CKX41 microscope.
and tube formation (total tube length and the number of loops) was analysed using Image J 1.48v. Spheroid sprouting assay was carried out as in 11. Briefly, 200 µL HPAEC cell suspension containing 2x10^4 cells/mL were plated into the 96-well Sphera U-bottom microplates (Fisher Scientific, cat no 3151822784) and centrifuged for 5 minutes at 300g. The plates were then incubated for 24 hours at 37°C to allow the formation of spheroids. 100 µL of medium was removed from each well and plates were placed on ice. 100 µL of ice-cold matrigel (Corning™, # 354230) was added to each well and the plates were briefly centrifuged at 4°C for 1 min at 100g to ensure central position of the spheroids. The plates were then placed in 37°C incubator for 1h to allow polymerization of matrigel and 100µL full medium containing additional 10ng/mL basic fibroblast growth factor (FGF; R&D Systems, Abingdon, United Kingdom) was added to each well. Spheroid sprouting was assessed after 48h and 72h post-plating using Image J 1.48v by comparing the surface area covered by spheroids before and after the experiment.

Isolation of NETs from mouse neutrophils.

Mouse NETs were isolated from bone marrow neutrophils of 10 CD1 male 10 week old mice according to the method described in 12. Mice were sacrificed and the bone marrow was flushed out of the tibia and the femur using RPMI supplemented with 5% FCS, glutamine and penicillin/streptomycin and passed through a 70-µm cell strainer to obtain a single cell solution. Cells were pelleted and remaining erythrocytes were lysed using red blood cell lysis buffer (Sigma). The entire bone marrow was resuspended in Hank’s Balanced Salt Solution (HBSS) without CaCl_2/MgCl_2. Mature neutrophils were purified by centrifugation for 30 min at 1,500 x g on a discontinuous Percoll gradient consisting of 52% (v/v), 69% (v/v) and 78% (v/v) Percoll in PBS. Mature neutrophils were recovered from the interphase between 69% and 78% Percoll. Mouse NETs were isolated in the same manner as described for human neutrophils.

Cell proliferation

HPAECs and HPASMCs proliferation was evaluated in CellTiter 96 proliferation assay (Promega).8 To study sparse cell migration, HPAECs were plated at the density of 7000 cells/well in 96-well Nunc® MicroWell™ 96-Well Optical-Bottom Plates (Thermo Scientific). Next day, NETs were added to the cells together with DNA stain, propidium iodide (PI; 50 µg/mL). Live cell movement was recorded for 16 hours on Zeiss Axio Observer Inverted Widefield Microscope with LED illumination and Hamamatsu Flash 4.0 camera using ZEN software with 30 minute time interval. The obtained video recordings were analysed with Image J Cell Tracking and Ibidi Chemotaxis and Migration Tool 2.0 software, to provide images of cell trajectories as well as cell motility parameters including velocity, accumulated distance (total length of cell trajectory), Euclidian distance (distance in a straight line between the start and the end point of each trajectory) and directionality (Euclidian distance: accumulated distance ratio). Further information regarding the method of analysis can be found on the website (http://ibidi.com/software/chemotaxis_and_migration_tool/).

Cell migration was also studied with OrisTM Cell Migration Assay Kit (Platypus Technologies; CMA1.101), according to manufacturer’s instructions. Briefly, HPAECs were plated in 96-well plate with circular inserts provided with the kit. On the following day, circular inserts were removed and NETs were added to the cells at the DNA concentration of 0.3µg/mL. After 24 hour incubation, the cells were fluorescently labelled with CellTracker™ Green BODIPY® Dye (Thermofisher Scientific, # C2102) and images of cells that migrated into the circular “wound” area, were taken under the Olympus IX70 fluorescent microscope equipped with a Peltier CCD camera. The intensity of fluorescence was measured with Image J.

Matrigel plug formation in vivo and quantification of plug vascularization.

Experiments were performed according to the Animals Scientific Procedures Act of 1986 as previously described.13 CD1 male 10 week old mice were anesthetized with an isoflurane gas mixture and injected subcutaneously with the Matrigel Basement Membrane Matrix (BD Biosciences) mixture near the abdominal midline, to induce the formation of a single, solid gel plug. Matrigel preparation included 250 µL Matrigel, 64 U/mL heparin (CP Pharmaceuticals), 80 ng/mL basic fibroblast growth
factor (FGF; R&D Systems, Abingdon, United Kingdom), and PBS to the final volume of 350 µL. In other experimental groups, mouse NETs or human NETs were added to Matrigel preparation to a final concentration of 0.3µg/mL DNA. Following 7 days, mice were killed and plugs were harvested after 7 days, fixed in 4% (wt/vol) paraformaldehyde in PBS for 2 hours at room temperature, transferred to 70% ethanol, embedded in paraffin, and processed for hematoxylin and eosin staining or staining with rat monoclonal anti-mouse endomucin antibodies (Santa Cruz, 65495; 5µg/mL) and secondary, FITC-labelled donkey anti-rat IgG from (Jackson ImmunoResearch #712-095-150; 5µg/mL). Vessels contained in the Matrigel plug were identified by the presence of nucleated cells surrounding a lumen marked by fluorescent staining of endomucin. Images of 6 fields of view /plug in plugs from 5 mice/treatment group were taken using a 10 x objective under the confocal laser scanning fluorescence microscope (Leica TCS SP5). The intensity of fluorescence, corresponding to plug vascularization, was analysed with Image J.

Statistical analysis.
All experiments were repeated at least in triplicate. The data was represented as either a scatter plots or bar graphs, results are expressed as means ± standard error deviation of the mean (SEM) and n is equal to the number of either patient number or the number of experimental repeats. All patient data were tested for normality distribution using D’Agostino-Pearson omnibus normality test. Unpaired t-test were used for normally distributed data. Mann-Whitney test was used for non-normally distributed data and Kruskal-Wallis with Dunn’s comparison was used for multiple groups. In vitro data a one-way ANOVA test was used with Tukey multiple comparison post-test. Unpaired t-test was used in comparisons of angiogenic cytokine expression between control cells and NET-treated cells. All graphs and calculations were performed with GraphPad Prism6 for windows version 6.03 (GraphPad Software Inc).

Supplemental references.


SUPPLEMENTAL MATERIAL

Aldabbous et al. Neutrophil extracellular traps (NETs) promote angiogenesis: evidence from vascular pathology in pulmonary hypertension.

Supplemental Materials and Methods

**Supplementary Table I.** Patients’ characteristics
Data presented as median (range)

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Figure I. The levels of NET markers in plasma of CTEPH patients (Cambridge cohort, n=21). (A) DNA, (B) myeloperoxidase (MPO) and (C) neutrophil elastase (NE). **p<0.01; ***p<0.001, comparison with healthy controls.
Figure II. Histological analysis of IPAH lung tissues and CTEPH thrombus. (A) L-endoglin (green) and vWF (red) staining in IPAH lung. Enlarged images of the boxed area are shown in the last row. An arrow points to endothelial cells highly expressing L-endoglin, while an arrowhead points to endothelial cells within a well differentiated vessel, showing low level of L-endoglin expression. Staining representative of n=6. (B) Images show corresponding staining for DNA (blue), citrullinated histone C3 (Cit-H3) (red) and a merged image in CTEPH thrombus. Arrows in the enlarged boxed areas point to the areas of NETosis marked by co-localization of DNA and Cit-H3 staining. On the right, the two black-and-white images show details of DNA and Cit-H3 staining. Bar=50µm.
**Figure III.** Images of NETs and NET markers. (A) Images of fluorescent DNA staining in unstimulated human neutrophil cultures (Control) and in PMA-stimulated neutrophil cell cultures (NETs). White arrow points to NETs. Bar=50µm. (B) Images of DNA, MPO and Cit-H3 staining in control, untreated neutrophils and PMA-treated neutrophils, as indicated. The cells were fixed and stained with NET markers without a prior permeabilisation so that only externalised, NETs-associated MPO and Cit-H3 can be seen. Bar=10µm.
**Figure IV.** Separation of NETs and endothelial cells in Transwell dishes. (A) Schematic diagram of the experimental setup. NETs were placed in the top chamber, while HPAECs were grown in the bottom chamber, separated from NETs by a semi-permeable membrane pore size 0.4µm. (B) Changes in DNA concentration in the bottom chamber during 24 hour experiment. Black bar shows DNA concentration in the top chamber (medium with NETs), while grey bars show DNA concentration in the bottom chamber after 2 hours, 4 hours and 24 hours of incubation.
Figure V. NETs promote pro-thrombotic responses in HPAECs. (A) vWF surface expression and (B) total protein expression of vWF in HPAECs treated with NETs for 4 or 24 hours, as indicated. (C) Aggregation of fluorescently labelled platelets on NETs-treated HPAECs (expressed as surface coverage), with or without anti-vWF antibodies, as indicated. (D) Top row: surface deposition of vWF (red) in NETs-treated HPAECs. Cell nuclei are shown in blue. Bottom row: video stills show platelet aggregation on the surface of HPAECs treated with NETs for 4 or 24 hours, as indicated. Flow direction is indicated with an arrow. Bar=10 µm.
Figure VI. The effect of NETs on pro-angiogenic cytokine expression in HPAECs. (A) representative examples of microarray membranes incubated with NETs (0.3µg/mL DNA), cell lysates from untreated (Control) HPAECs and HPAECs treated with NETs, as indicated. A schematic diagram of the microarray layout is shown below. Graph in (B) shows changes in the levels of various pro-angiogenic factors induced by NETs (fold-change compared to untreated controls). n=3.
Figure VII. NETs-induced changes in the pro-angiogenic cytokine expression in HPAECs. (A-E) relative changes in the levels of pro-angiogenic factors: MMP-9, PDGF-BB, HB-EGF, LAP (TGFβ1) and uPA in NETs-treated HPAECs, as indicated; human angiogenesis microarray. *p<0.05; p<0.01; ***p<0.001, comparisons with untreated controls; ##p<0.01, ###p<0.001, comparisons with NETs-treated HPAECs. n=3.
Figure VIII. Changes in distribution of the actin cytoskeleton and vascular endothelial cadherin (VE-cadherin) in HPAECs. HPAECs were left untreated or were treated with NETs for 24 hours, as indicated. Bar=10µm.
Figure IX. Cell migration in HPAECs cultured with NETs with, or without DNase1 or MPO inhibitor for 24 hours, as indicated (Oris assay). In Oris assay, the number of fluorescently-labelled cells that migrated into the circular “wound” area in each well of a 96-well plate, was estimated by measurement of fluorescence intensity and expressed as % of untreated controls. *p<0.05, comparison with untreated controls; **p<0.01, ***p<0.001, comparison with NETs-treated HPAECs, n=3.
Figure X. The effect of HPAEC conditioned media on PASMCs growth and ET-1 release. (A) Conditioned medium from HPAEC/NETs cultures increases proliferative activity of human pulmonary artery smooth muscle cells (SMCs). Conditioned media were collected from the bottom chamber of Transwell dishes, while HPAECs (with or without NETs) were grown in the top chamber. PASMC were then incubated in the conditioned media for 24 hours before the measurement of cell proliferation in MTS assay. **p<0.01, comparison with HPASMCs grown in conditioned medium from untreated HPAECs; n=3. (B) ET-1 levels in conditioned media from HPAECs (24 hour incubation). *p<0.05, n=4.
Figure XI. The effect of CLIC4 gene silencing on NET-induced spheroid sprouting. The graph shows changes in the total spheroid area in cells overexpressing AdGFP (adenoviral control) or AdCLIC4shRNA-GFP 48 hours post-infection. The cells were left untreated or were treated with NETs, as indicated. Representative images of HPAEC spheroids are shown above the graph, while western blots with expression levels of CLIC4 are shown on the right. *p<0.05; **p<0.01; ***p<0.001, comparisons with controls; ### p<0.001, comparison between AdGFP+NETs and AdCLIC4shRNA+NETs. n=3.
Figure XII. Images of human and mouse NETs. DNA staining with Hoechst. Bar=100µm.