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From Cell Biology to Tissue Engineering

Review

Neutrophil extracellular traps in tissue pathology

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Summary. Neutrophil extracellular traps (NETs) are innate immune systems against invading pathogens. NETs are characterized as released DNA mixed with cytoplasmic antimicrobial proteins such as myeloperoxidase, proteinase3 and neutrophil elastase. While NETs are thought to have an important role in host defense, recent work has suggested that NETs contribute to tissue injury in non-infectious disease states. Uncontrolled NET formation in autoimmune diseases, metabolic disorders, cancers and thrombotic diseases can exacerbate a disease or even be a major initiator of tissue injury. But spotting NETs in tissues is not easy. Here we review the available histopathological evidence on the presence of NETs in a variety of diseases. We discuss technical difficulties and potential sources of misinterpretation while trying to detect NETs in tissue samples.

Key words: Pathology, Immunostaining, Immunofluorescence, Antibody, NETosis

Introduction

Neutrophils contribute to host defense at sites of tissue injury by patrolling through the circulatory system. In the last two decades, the process of leukocyte recruitment, and in particular neutrophil recruitment, has been elucidated in great detail (de Oliveira et al., 2016). At sites of tissue injury, endothelial cells expose on their surface various molecules, such as selectins, chemokines, and other adhesion molecules, which contribute to the retardation of neutrophil flow and increase neutrophil rolling and adhesion to endothelial cells. More recently, the focus of research has shifted to the effector functions of neutrophils within inflamed tissues. Upon activation, neutrophils undergo degranulation to release the toxic contents of intracellular granules into the extracellular space. Neutrophils also die at sites of injury and their dead cell components are cleared by macrophages. Although pathologists had already characterized as “leuko-cytoclastic” neutrophil involvement in a number of diseases, implying necrotic cell necrosis, the “silent way of death” apoptosis, was anyway considered the predominant route of neutrophil death. In 2004, the formation of neutrophil extracellular traps (NET) was first described as a non-phagocytic mode of bacterial killing (Brinkmann et al., 2004). NET formation involves the extrusion of nuclear chromatin into the extracellular space, which occurs through necrosis, resulting in rupture of the nuclear and plasma membranes and extruded chromatin that is decorated with cytoplasmic granule-derived proteins. Therefore, the term NETosis is used to describe the combination of NET formation and neutrophil death (Desai et al., 2016a).

In the last decade, NET formation has been documented in numerous disease states, and functional studies also suggest that NET formation contributes to the pathogenesis of various experimental disease models. Here, we review the biology of NET formation under normal and pathogenic conditions. Interpretation of these studies is often complicated given that NETs are identified *in vivo* by the presence of circulating neutrophil proteins or by positive immunostaining of neutrophil components in the extracellular space. Therefore, immunostaining techniques to visualize NETs

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and necrotic neutrophils in injured tissues and the technical limitations of these approaches will also be discussed.

What is NET formation?

Phagocytosis was historically regarded as one of the major functions of neutrophils. However, in 2004, Zychlinsky and colleagues discovered that in response to bacterial infection, neutrophils release their genetic material and form web-like structures composed of chromatin fibers (15-17 nm diameter) and decorated with granule enzymes such as neutrophil elastase (NE), myeloperoxidase (MPO), and cathepsin G (50 nm). These structures were called 'neutrophil extracellular traps' (NETs) (Brinkmann et al., 2004). Over the last decade, the formation of NETs has been documented in response to various bacterial, fungal, and viral infections, and in a wide range of organisms from plants to animals, including mice, cattle, horses, fish, cats, rabbits, invertebrates and humans (Vorobjeva and Pinegin, 2014). In addition to neutrophils, extracellular traps are formed by other cell types, including eosinophils (Yousefi et al., 2008), monocytes/ macrophages (Chow et al., 2010), mast cells (von Kockritz-Blickwede et al., 2008), which raises first doubts on a specific mechanism or on misinterpretation of the final product, i.e. extracellular chromatin. Thus, in general, extracellular traps may be referred to simply as 'extracellular traps' (ETs) (Wartha and Henriques-Normark, 2008). Various stimuli, including phorbol myristate acetate (PMA), bacterial pathogens (e.g. *E. coli, S. aureus*, etc.), fungal pathogens (e.g. *C. albicans*, *Aspergillus fumigatus*), lipopolysaccharide (LPS), complement c5a, and various cytokines and chemokines such as tumour necrosis factor α (TNFα) and interleukin-8 (IL-8), can induce NET formation *in vitro*. Schauer, et al. showed that NETs can also be found in non-infectious disease conditions like gout (Schauer et al., 2014). Neutrophils form NETs in response to monosodium urate (MSU) crystals that accumulate in the joints of patients with gout. Again, these data raise doubts about whether all these different stimuli are triggering an identical process or simply produce the same result, io.e. a dead neutrophil with extracellular chromatin, via different molecular mechanisms (Desai et al., 2016a).

Neutrophils, once stimulated, undergo several morphological changes during NETosis. Within an hour of their activation, neutrophils flatten and multiple lobes of the nucleus are lost. These events are followed by chromatin decondensation and granule disintegration. Approximately 2h after stimulation *in vitro*, the plasma membrane ruptures leading to the release of NETs into the extracellular space (Brinkmann et al., 2004). Thus, neutrophils die in the process of forming NETs (Fuchs et al., 2007). However, neutrophils may also form NETs without undergoing cell death (Yipp and Kubes, 2013). In such cases, neutrophils release chromatin through specialized vacuoles, which does not require plasma membrane rupture. This process has been referred to as "vital NETosis".

Histone citrullination is an important post-translational event during NETosis (Li et al., 2010), since histone deamination results in chromatin decondensation. Histone H3 citrullination is catalyzed by the enzyme peptidyl arginine deiminase 4 (PAD4) (Wang et al., 2009; Li et al., 2010). Despite its importance, whether PAD4 is required to form NETs in all cases is unclear, since NET formation was inconsistently impaired in Pad4-/- mice under different conditions. Thus, PAD4 is likely involved in NET formation in a stimulus-dependent manner. Furthermore, whether PAD4 and histone citrullination are required for vital NET formation has not been determined. Chromatin that is released as a component of NET is decorated with serine-proteases, such as NE and MPO. NE translocates from the cytoplasm to the nucleus where its protease activity is involved in histone degradation (Metzler et al., 2014). Reactive oxygen species (ROS) are an important signaling mediator during NET formation. The activation of NADPH oxidase is essential for NETosis; NET formation is impaired in neutrophils in humans or mice deficient in NADPH oxidase (Fuchs et al., 2007; Rohm et al., 2014; Schauer et al., 2014). ROS may also stimulate MPO-dependent proteolytic activity of NE (Metzler et al., 2014).

Traditional histopathological evidence of NETosis: Karyorrhexis and leukocytoclastic vasculitis

Karyorrhexis, a word derived from the Greek words for nucleus (karyon) and bursting (rhexis), refers to the rupture of the nucleus during cell death leading to the leakage of nuclear contents as it occurs during NETosis. Initial swelling of the nucleus is followed by decondensation of DNA and subsequent rupture of the nuclear and plasma membranes leading to the release of cellular contents into the extracellular space. Karyorrhexis of neutrophils, which can be identified by several methods, is an indicator of catastrophic cell death in several disorders, including vasculitis, acute lung injury, acute kidney injury, etc.

Electron microscopy

Electron microscopy can be applied to identify karyorrhexis in neutrophils by providing detailed information on the nuclear changes that occur during neutrophil cell death (Fuchs et al., 2007), including those during apoptosis, necrosis, or NETosis (Fig. 1).

Live cell microscopy with SYTOX green staining

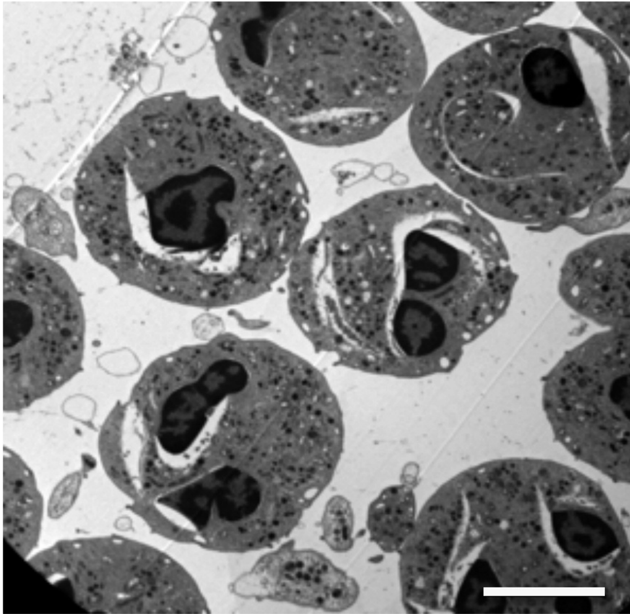
SYTOX green is a cell-impermeable fluorescent DNA dye; therefore, SYTOX green specifically stains extracellular DNA. Peak excitation and emission wavelengths of STYOX green are 504 nm and 523 nm,

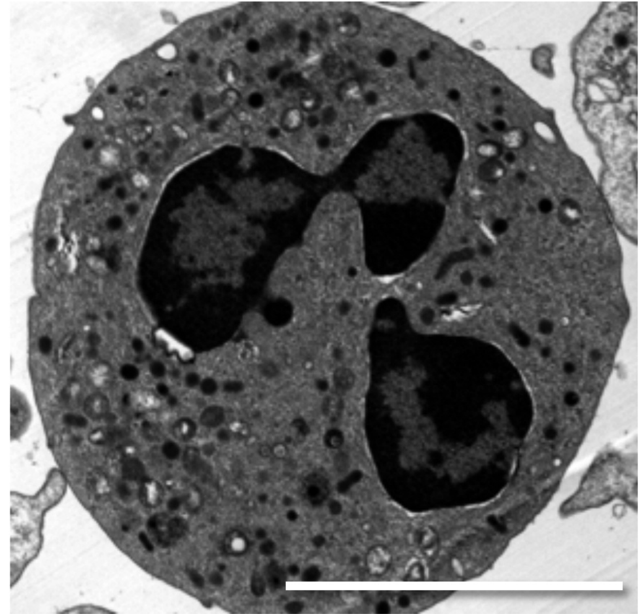
respectively. SYTOX green stains only neutrophils undergoing karyorrhexis/NETosis; apoptotic or live cells are not stained with SYTOX green (Fig. 2). Thus, SYTOX green can be used to quantify the degree of NETosis and necrotic cell death (Desai et al., 2016b).

DAPI and Topro3 staining in paraformaldehyde-fixed cells in vitro

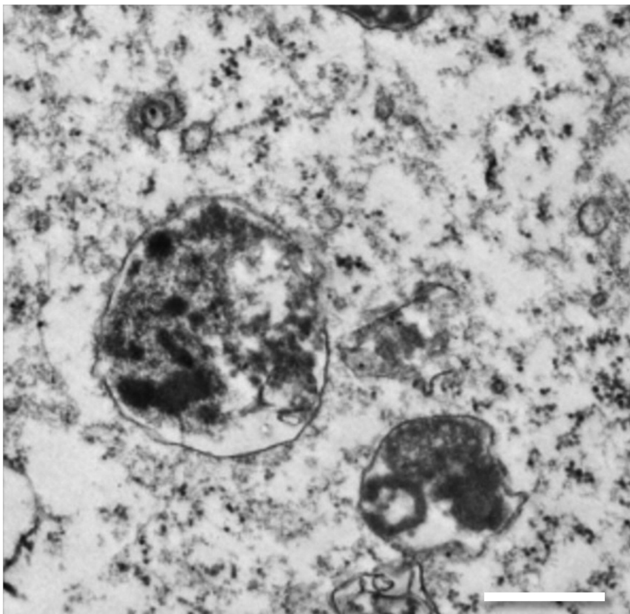
Neutrophil karyorrhexis can be studied using paraformaldehyde (PFA)-fixed cells in addition to live

(a) Unstimulated human neutrophils





(b) PMA stimulated neutrophils



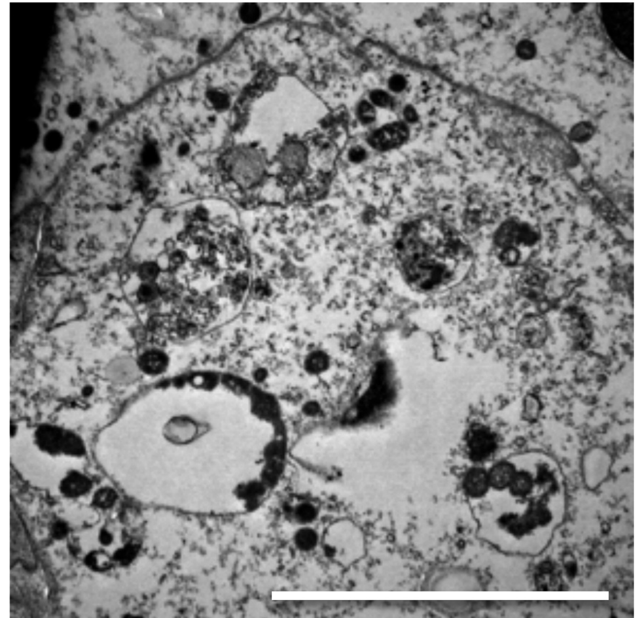


Fig. 1. Transmission electron microscopy. **a.** Unstimulated neutrophils present a well-defined nuclear membrane with lobulation. **b.** PMA stimulated neutrophils show a breakdown of cell and nuclear membrane. The nucleus including chromatin are ruptured to cytoplasmic space and mixed with granular components. Scale bars: 2500 nm.

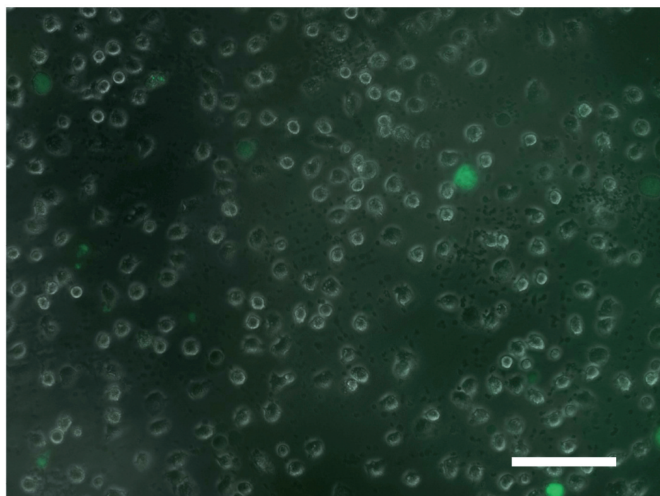
cells. Activated PFA-fixed neutrophils can be stained with cell-permeable DAPI (4',6-diamidino-2-phenylindole) and cell-impermeable TO-PRO-3 to detect cells undergoing NETosis. Peak excitation/emission wavelengths for DAPI and TO-PRO-3 are 350/460 nm and 642/661 nm, respectively. Neutrophils undergoing karyorrhexis are stained with both DAPI and TO-PRO-3. Combined with specific neutrophil markers, such as Ly6B.1, neutrophil elastase, or MPO, DAPI and TO-PRO-3 can also be used to study neutrophil karyorrhexis in paraffin-fixed tissues of solid organs.

Leukocytoclastic vasculitis

The aetiopathology of leukocytoclastic vasculitis, small vessel vasculitis or hypersensitivity vasculitis is not entirely understood but is always associated with neutrophil involvement. Remnants of NETs have been reported in the circulatory system and in renal lesions of patients with small vessel vasculitis (Kessenbrock et al., 2009; O'Sullivan et al., 2015).

NET-specific immunostaining markers in tissues

NETs can be identified by co-localization or complex formation of extracellular chromatin with cytoplasmic proteins, implying nuclear and plasma membrane rupture. Furthermore, a combination of microscopy or flow cytometry (Zhao et al., 2015) and fluorescence staining with a DNA-binding dye (Brinkmann et al., 2012) can be applied to objectively and accurately identify and quantify NETs *in vitro*. However, these same methods cannot be applied to evaluate NETs in tissues because NETs cannot be isolated from tissues under native conditions. Therefore, in fixed tissues, NETs are currently quantified by subjective analysis of co-localized immunofluorescence staining of swollen chromatin DNA with granule proteins (Table 1). However, it is sometimes difficult to distinguish whether a DNA signal reflects *bona fide* NETosis or a false-positive event, such as the accumulation of neutrophils or other routes of cell death. In addition, the swelling of stained samples could represent an artifact of tissue sample processing, which involves cutting, washing, and incubating tissue sections. For these reasons, NET-specific tissue markers have been developed for various diseases (Barnado et al., 2016). As described previously, Li, et al. reported that histone citrullination by PAD4, which catalyzes the conversion of arginine residues to citrulline, induces chromatin decondensation during the process of NET formation (Li et al., 2010). Although it is unclear whether PAD4 is required for NET formation under all circumstances, citrullinated histone (CitH) is always present in neutrophils that will or have formed NETs. Therefore, CitH may be used as a diagnostic marker for NETs in tissues. In particular, in animal models of autoimmune disease, thrombosis, or inflammatory disease, CitH-positive neutrophils were detected in target organs, indicating tissue-specific NETs (Table 1). Tissue-specific NETs are detectable in ischemia/reperfusion models of acute kidney disease and after incisional skin injury (Figs. 3, 4). In addition to CitH-positive neutrophils, NETosis in tissues is characterized by immuno-positive NE staining and swollen DNA in multiplexed immuno-histofluorescence (IHF) analysis.



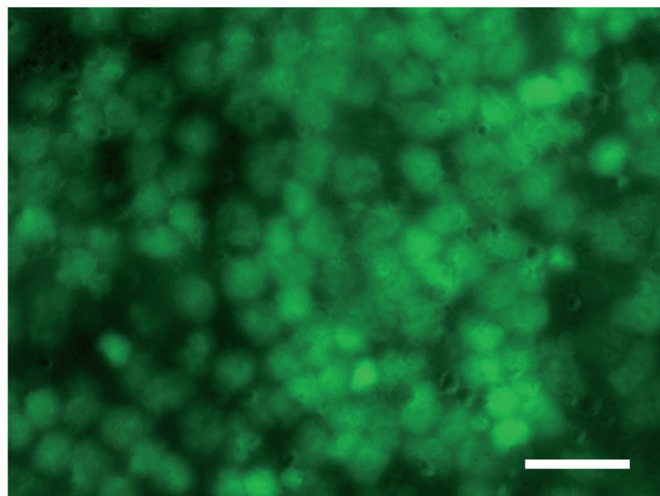


Fig. 2. Sytox green staining to study neutrophil using live microscopy. **a.** Most unstimulated neutrophils show no signal of Sytox green, and the morphologies of cell membrane are maintained. **b.** PMA stimulated neutrophils show the loss of structure with high positivity of Sytox green. Scale bars: 50 μm.

Furthermore, for assessing tissue NET formation, IHF staining of cryosections is likely to be superior to IHC staining of formalin-fixed paraffin-embedded (FFPE) tissues because the procedure to prepare FFPE sections includes a step for deparaffinization-antigen retrieval, which could injure fine DNA structures (Ludyga et al., 2012) and result in the loss of antigenicity of cytoplasmic and nuclear proteins. In contrast, cryosection samples better maintain the structure of DNA and antigenicity of granule proteins. Staining samples for various combinations of antigens in multiplexed IHF analysis will also improve the classification of NETs. For example, citH3/NE-positive cells in which citH3 and NE overlap indicate NET-positive neutrophils, citH3/NE-positive cells in which citH3 and NE do not overlap indicate pre-NETing neutrophils, and citH3-negative/NE-positive cells without swelling indicate infiltrating neutrophils.

Live intravital imaging, combined with extracellular DNA dyes, such as SYTOX green, and granule protein markers, has also been used to identify NETs *in vivo*. Intravital imaging is particularly useful for obtaining time-course and dose-response data, and characterizing the dynamics of specific molecules and the movements of neutrophils *in situ*. Though SYTOX green has been used *in vivo*, its use should be interpreted with caution since it is able to detect necrotic cell DNA and extracellular DNA, thereby making it difficult to distinguish among NET DNA, necrotic extracellular DNA, and intracellular DNA of dead cells. Therefore, it is important to evaluate NETs in tissues with these limitations in mind.

Current evidence of NETosis in mouse models of disease by immunostaining

NETs have been identified in a variety of animal disease models, including models of infection,

Table 1. Current evidence of NETosis in mouse models of disease by immunostaining.

Animal model		Immunostaining	Section / Fixation	Organ	Ref
Autoimmune disease					
SLE model mice	New Zealand mixed 2328	MPO/DNA(IHF), CitH3/CRAMP (IHC)	CryosectionFFPE	Kidney, Thrombus	Knight et al., 2013
ANCA vasculitis mice	ANCA model induced by NETs	Gr-1 (IHC)	not described	Kidney	Sangaletti et al., 2012
Rheumatoid Arthritis mice	KRN transgenic mice sera induced model	CitH (IHC)	FFPE / PFA after ART	Arthritic leg	Rohrbach et al., 2012
GBM disease mice	anti GBM serum induced model	MPO (IHF)	FFPE / PFA after ART	Kidney	Kumar et al., 2015
Thrombotic disease					
Thrombosis mice	Flow restriction model in IVC	Live imaging by muitiphonton scopy	Live Imaging	Thrombus	von Bruhl et al., 2012
Thrombosis baboon	Iliac vein occlusion by temporary balloon	Histone/DNA/ vWF (IHF)	FFPE / not described	Thrombus	Fuchs et al., 2010
Metabolic Disease					
Diabetes Mellitus	Skin wound in STZ induced DM mice	CitH3/ Ly6G/DNA (IHF)	Cryosection / Zinc	Wound Skin	Wong et al., 2015
Gout	MSU crystral induced tophous	NE/DNA (IHF)	FFPE / PFA after ART	Skin pouch	Schauer et al., 2014
Atheroscrerosis	APO-E knockout mice	MPO/CitH3/DNA (IHF)	Cryosection / PFA	Aorta	Warnatsch et al., 2015
Sepsis					
Sepsis model mice	Histone injection induced model	NE (IHF)		Lung	Clark et al., 2007
Sepsis model mice	LPS injection induced model	Live imaging by NE and Sytox Orange	Live Imaging	Cecum, Liver	Tanaka et al., 2014
Infection disease					
Pneumonia model mice	Pneumococcal Pneumonia infected model	NE/Histone/DNA (IHF)	not described	Lung	Beiter et al., 2006
Cancer					
Metastatic tumor model mice	RIP1-Tag2 model of insulinoma	Gr1/DNA (IHF)	Cryosection / Methanol	Tumor	Cedervall et al., 2015
Other disease					
Ischemic hepatitis model	Ischemic reperfusion injury in liver	CitH3/Ly6G/DNA (IHF)	Not described	Liver	Huang et al., 2015
Lung transplantation	Graft dysfunction model after transplantation	NE/Histone/DNA (IHF)	Cryosection / not described	Lung	Sayah et al., 2015

SLE: systemic lupus erythematosus, MPO: myeloperoxidase, IHF: immunohistofluorescence, CitH3: citrullinated histone3, IHC: immunohistochemistry, PFA: paraformaldehyde, ANCA: anti neutrophil cytoplasmic antibodies, NETs: neutrophil extracellular traps, Gr-1: granulocyte receptor-1 antigen, FFPE: formalin-fixed paraffin-embedded, ART: antigen retrieval treatment, GBM: glomerular basement membrane, IVC: inferior vena cava, vWF: von willebrand factor, STZ: streptozotocin, DM: Diabetes Mellitus, Ly6G: lymphocyte antigen 6 complex locus G, NE: neutrophil elastase, APO-E: apolipoprotein E, LPS: lipopolysaccharide

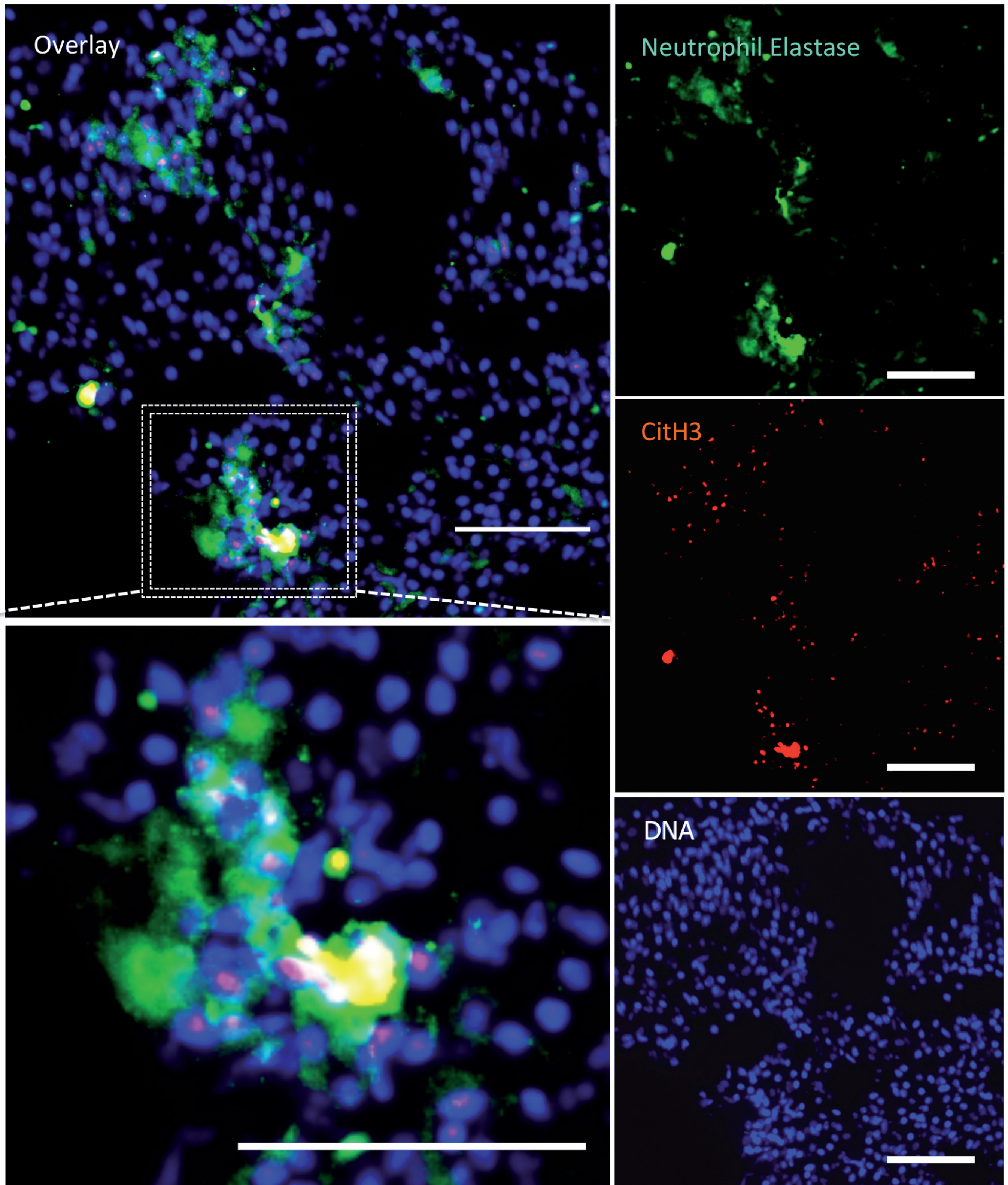


Fig. 3. Tissue NETs in ischemia reperfusion kidney mouse model and incisional skin scar mouse model. In unilateral ischemia (35min)-reperfusion (24hours) kidney model mice, a large amount of neutrophils (NE single positive cells) and NET neutrophils (NE/CitH3 double positive cells) are detected mainly at the boundary zone of the medulla and cortex. Semithin cryosection of each organ stained for NE (Green), CitH3 (Red) and DNA (Blue). NE: neutrophil elastase; CitH3: citrullinated histone 3. Scale bars: 50 μm.

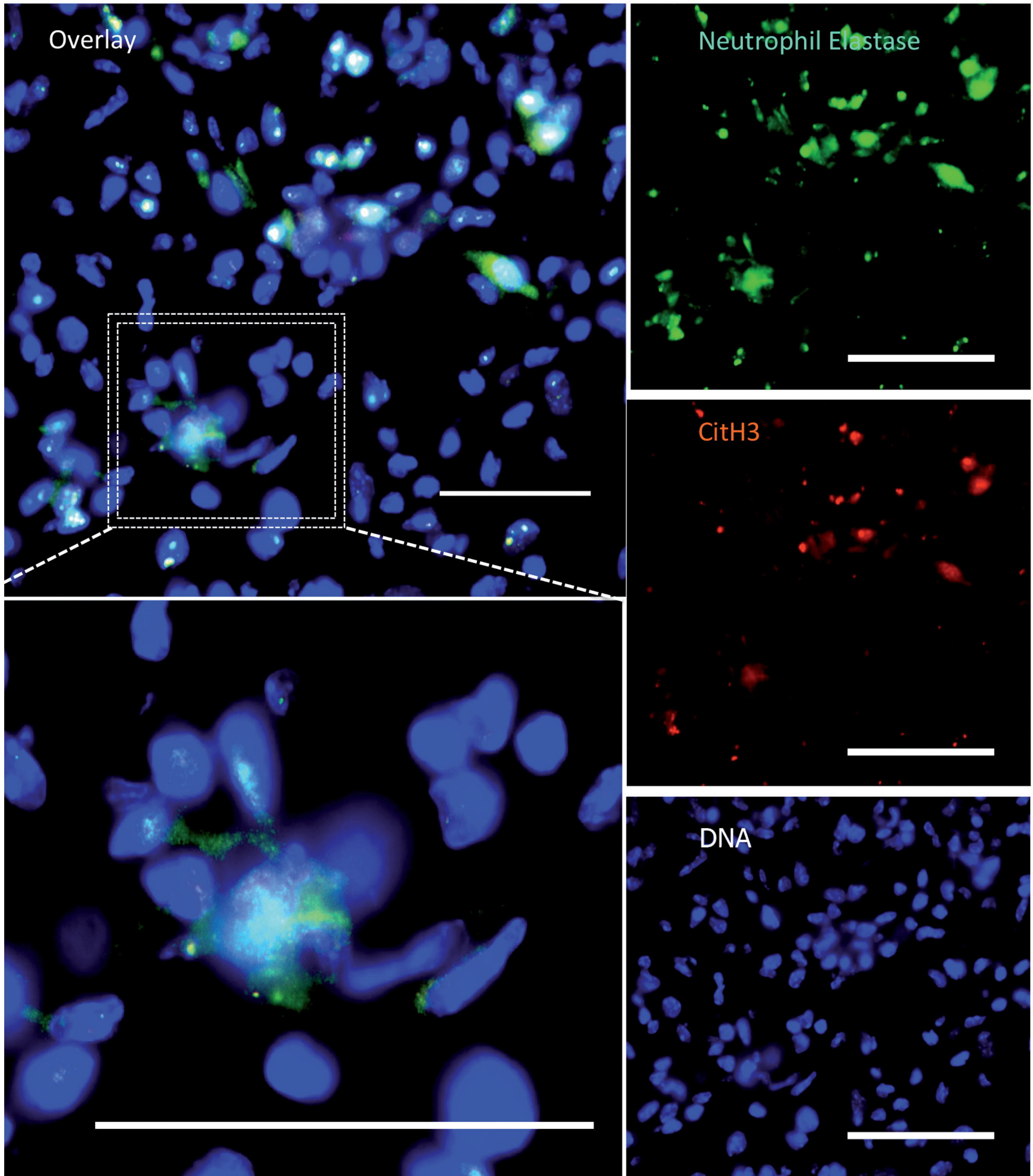


Fig. 4. Tissue NETs in ischemia reperfusion kidney mouse model and incisional skin scar mouse model. In mice skin scar model, 3 days after injury, NETosis are seen. Semithin cryosection of each organ stained for NE (Green), CitH3 (Red) and DNA (Blue). NE: neutrophil elastase; CitH3: citrullinated histone 3. Scale bars: 50 μm.

autoimmune disease, metabolic disease, and malignant tumors (Table 1). NETs in the lung were reported in a case of pneumococcal pneumonitis, which were identified through multiplexed IHF staining for NE, histones, and DNA (Beiter et al., 2006). Alpha-enolase, which is produced by *pneumococci*, binds to the myoblast antigen, 24.1D5, to induce NET formation (Mori et al., 2012). A subtype of *pneumococcus* produces an endonuclease to degrade the DNA scaffold of NETs and facilitate microbial growth at sites of infection, which may lead to sepsis. While, NETs are involved in pathogenesis of sepsis. Tanaka, et al. demonstrated NET formation in specific organs in LPS-induced sepsis animal models by imaging live neutrophils stained with NE and SYTOX green (Tanaka et al., 2014). LPS, which is a component of the outer membrane of Gram-negative bacteria, induces platelet activation and aggregation via TLR4 signalling, which in turn stimulates NET formation (Clark et al., 2007). Xu, et al. reported that intravenous injection of histones into mice induced platelet coagulation and microthrombi formation, which connected with NETs (Xu et al., 2009). Mice rapidly died of the impaired circulation by the robust thrombus with NETs when challenged with high concentrations of histones. Furthermore, Huang, et al. demonstrated NET formation in a hepatic ischemia/treperfusion injury model by imaging live neutrophils stained with CitH/NE markers and SYTOX green (Huang et al., 2015). Hepatic ischemia/reperfusion injury raises circulatory levels of histones, which can directly activate TLR9. NET formation is also involved in the pathogenesis and severity of thrombosis. Flow restriction (von Bruhl et al., 2012) or the insertion of a temporary balloon catheter (Fuchs et al., 2010) in the veins of mice induces thrombus, where the histone components of chromatin bind with coagulation factors to provide the scaffold for NETs. NETs together with platelet-rich red blood cells ensnare invading microbes. These findings indicate that focal association of NETs and microthrombi can be beneficial during infection, but that systemic circulation of NETs unabated is toxic and can be fatal.

NET formation is also involved in the pathogenesis and development of autoimmune diseases (Barnado et al., 2016). In systemic lupus erythematosus (SLE), anti-neutrophil cytoplasmic antibody-associated systemic small-vessel vasculitis (ANCA-SSV), and rheumatoid arthritis, NET components are antigens for each disease-specific autoantibody. Furthermore, autoantibodies can stimulate neutrophils to undergo NETosis, potentially resulting in a *vicious circle* of inflammation. For example, SLE is a heterogenic autoimmune disorder with a broad spectrum of clinical presentations but is characterized by the presence of anti-nuclear autoantibodies. Impairment of deoxyribonuclease I (DNase I) activity by anti-DNase I autoantibodies (Hakkim et al., 2010) may increase the persistence of NETs, leading to sustained inflammation and development of autoantibodies characteristic of SLE. Knight, et al. reported that the administration of a NET inhibitor improved lupus nephritis in a SLE mouse model (Knight et al., 2013), in which tissue-specific MPO/CitH-positive NETs were identified by IHF and IHC, respectively. Furthermore, ANCA-SSV is a systemic disease characterized by the presence of autoantibodies against antigens in the cytoplasm of neutrophils (i.e., NET components); thus, ANCA vasculitis might develop upon prolonged exposure of the immune system to NETs (Nakazawa et al., 2012b), (Sangaletti et al., 2012). Indeed, ANCA binding directly to MPO on the surface of primed neutrophils activate NET formation by intiating histone citrullination via an unknown mechanism (Nakazawa et al., 2014). Tissue-specific NETs were also identified in crescentic glomerulonephritis as swollen Gr-1 (IHF) and DNA-positive neutrophils (22932797). Rheumatoid arthritis is

Table 2. Current evidence of NETosis in human disease by immunostaining.

Disease	Immunostaining	Section/Fixation	Organ	Ref
Autoimmune disease				
SLE	MPO/DNA (IHF)	Not described	Kidney	Hakkim et al., 2010
ANCA vasculitis	MPO/CitH3/DNA (IHF)	FFPE / with ART	Kidney	Kessenbrock et al., 2009; O'Sullivan et al., 2015
Rheumatoid Arthritis	MPO/DNA (IHF)	Cryosection / -	Synovial tissue	Khandpur et al., 2013
Psoriasis	NE/DNA (IHF)	Cryosection /Acetone	Skin	Skrzeczynska-Moncznik et al., 2012
Thrombotic disease				
Thrombosis with sepsis	CitH3/DNA (IHF)	FFPE / with ART	Thrombus	Savchenko et al., 2014
Thrombosis with ANCA vasculitis	CitH3 (IHC)	FFPE / with ART	Thrombus	Nakazawa et al., 2012a
Other diseases				
Hyper uric acid syndrome (Gout)	NE/DNA (IHF)	FFPE / with ART	Synovial tissue	Schauer et al., 2014
Preeclampsia	NE/Histone/DNA (IHF)	Cryosection / Acetone	Placental tissue	Gupta et al., 2005

SLE: systemic lupus erythematosus, MPO: myeloperoxidase, IHF: immunohistofluorescence, ANCA: anti neutrophil cytoplasmic antibodies, CitH3: citrullinated histone3, FFPE: formalin-fixed paraffin-embedded, ART: antigen retrieval treatment, IHC: immunohistochemistry, NE: neutrophil elastase, PFA: paraformaldehyde

characterized by autoantibodies against citrullinated proteins, which can stimulate neutrophils to undergo NETosis in the synovial fluid, as determined by Cit-H (IHC) staining (Rohrbach et al., 2012). In anti-glomerular basement membrane (GBM) animal models, glomerular inflammation induces NETs and the release of histones, which are toxic to endothelial cells, podocytes, and parietal epithelial cells, thereby causing severe kidney injury (Kumar et al., 2015). Metabolic diseases, such as diabetes mellitus (DM) and hyperuricemia, are also characterized by high levels of NETs, resulting in chronic inflammation. High glucose levels induce histone citrullination in neutrophils through the upregulation of PAD4 gene expression, regardless of the type of DM (Wong et al., 2015). NET components released in response to high glucose levels can disrupt wound healing through the inhibition of re-epithelialization. Gout attacks (i.e., acute hyperuricemia) are also caused by NET-related inflammation. Monosodium urate (MSU) promotes NET formation independently of ROS by activating NF-κB signalling. Subcutaneous injection of MSU can also induce NETosis (Schauer et al., 2014), which was observed as NE/histone/DNA triple-positive neutrophils (IHF), through receptor-interacting protein kinase (RIPK)1-RIPK3-mixed lineage kinase domain-like (MLKL) signalling (Desai et al., 2016b), which elicits necroptotic cell death. Crosstalk between PAD4 and RIPK1/3 signalling has not been characterized. NETs induced by tumors can have negative collateral effects on distal organs and have been detected by IHF in tissues surrounding an insulinoma as Gr1/DNA-positive neutrophils (Cedervall et al., 2015).

Current evidence of NETosis in human disease by immunostaining

NETs play a significant role in the development and pathogenesis of diverse human diseases as well. In sepsis (Margraf et al., 2008), autoimmune diseases (Zhang et al., 2014) and thrombotic disease (Fuchs et al., 2012), the presence of excessive NETs is correlated with higher mortality. For these NETs detection, plasma DNA (Margraf et al., 2008; Zhang et al., 2014) and circulating MPO (Fuchs et al., 2012) were quantified by PICO green assay, ELISA, respectively. Furthermore, tissue-specific NETs may be used to diagnose specific pathologies if NETs are identified in more diseases. However, NETs are generally identified in formalin-fixed paraffin-embedded (FFPE) tissue sections by immunostaining (Table 2) because of the cost and space advantages of preserving clinical samples by FFPE methods. Kesseonbrock et al. showed the presence of NETs by IHF multiplex staining for MPO, NE, DNA using FFPE samples (Kessenbrock et al., 2009). The proof of the tissue NETs using CitH marker had been demonstrated by IHC mono staining using FFPE samples, because the IHF staining of CitH does not work in some human tissues after formalin fixation (Nakazawa et al., 2012a), nor in some mouse tissues (Kusunoki et al., 2016). Meanwhile, some recent reports have shown that IHF staining of CitH in human FFPE samples was useful for NETs detection (O'Sullivan et al., 2015). Therefore, with the accumulation of further evidence we should address which staining method would be better for human tissue NETs detection and whether the procedure is the same or not in each disease and organ.

The use of fresh frozen samples stained for citH3, neutrophil granule protein markers, and DNA would lead to more reliable NET detection in the future. In addition, therapeutic efficacy has been assessed by tissue-specific NET staining in animal models, which, if translated to humans, would provide another useful therapeutic index.

Summary on NET formation in disease

NETs are important for host defense against local infections, but dysregulated NET formation can result in persistent inflammation, hypercoagulation, and disturbance of wound healing. Inhibition of NETs could be therapeutically beneficial for various diseases, including sepsis, autoimmune diseases, thrombosis, metabolic diseases, and cancer; however, considering the importance of neutrophils to innate immunity, inhibition of NET formation, such as with a PAD4 inhibitor, should not interfere with the ability of the innate immune system to respond to pathogens (Martinod et al., 2015). Therefore, a better understanding of the roles of NETs in the pathogenesis of these diseases will be required to develop specific therapies. This will require improvements in the techniques to identify *bona fide* NETs and the development of reliable NET-specific markers in tissues, such as citrullinated histone.

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