The role of extracellular traps in ischemia reperfusion injury

Feilong Zhang1,2†, Yuqing Li1,2†, Jiyue Wu1,2, Jiandong Zhang1,2, Peng Cao1,2, Zejia Sun1,2 and Wei Wang1,2*

1Department of Urology, Beijing Chao-yang Hospital, Capital Medical University, Beijing, China, 2Institute of Urology, Capital Medical University, Beijing, China

In response to strong signals, several types of immune cells release extracellular traps (ETs), which are web-like structures consisting of DNA decorated with various protein substances. This process is most commonly observed in neutrophils. Over the past two decades, ET formation has been recognized as a unique mechanism of host defense and pathogen destruction. However, the role of ETs in sterile inflammation has only been studied extensively in recent years. Ischemia reperfusion injury (IRI) is a type of sterile inflammatory injury. Several studies have reported that ETs have an important role in IRI in various organs. In this review, we describe the release of ETs by various types of immune cells and focus on the mechanism underlying the formation of neutrophil ETs (NETs). In addition, we summarize the role of ETs in IRI in different organs and their effects on tumors. Finally, we discuss the value of ETs as a potential therapeutic target for organ IRI and present possible challenges in conducting studies on IRI-related ETs as well as future research directions and prospects.

KEYWORDS neutrophil, NETs, extracellular traps (ETs), IRI, ischemia reperfusion injury, NETosis

Introduction

Neutrophils are the most abundant immune cells in human blood. They play a central role in the innate immune defense as the first line of defense during infection and inflammation. The main modes of neutrophil function include phagocytosis, degranulation, cytokine release, and the formation of neutrophil extracellular traps (NETs) (1). NET formation is another mechanism of host defense (2). In 2004, neutrophils were first reported to kill pathogens through the formation of extracellular traps (ETs) (3). Since then, a large number of studies have focused on ETs, and it has gradually been discovered that similar to neutrophils, other immune cells (e.g., monocytes/macrophages, mast cells, eosinophils, and basophils) can also release ETs (4–7). Furthermore, researchers from various disciplines are trying to understand the ultrastructure and composition of ETs generated by various immune cells, the cellular
Organ damage caused by ischemia/reperfusion (I/R) involves two major phases, namely, the sterile inflammatory responses due to immune cell infiltration and the oxidative stress and damage to parenchymal cells (e.g., hepatocytes and renal tubular epithelial cells) (15). Parenchymal cells and endothelial cells undergo various types of cell death under conditions of organ ischemia and hypoxia, and necrotic cells release damage-associated molecular patterns (DAMPs), including interleukin (IL)-33, heat shock proteins, histones, and high mobility group box-1 (HMGB1) (16, 17). These DAMPs promote immune cell infiltration and inflammatory factor production, further enhancing the various types of cell death of other parenchymal cells. This induces a pro-inflammatory positive feedback loop, which in turn exacerbates IRI (18, 19). Neutrophils and macrophages are the two most important types of immune cells infiltrating organs during IRI (15, 20). Several recent studies have reported that the ETs released by neutrophils and macrophages are involved in and exacerbate IRI (21, 22).

In this review, we first briefly summarize the various types of immune cells that can release ETs. Afterwards, we focus on the processes and mechanisms involved in the release of ETs from neutrophils, and their role in aggravating IRI in various organs. We further explore the link between NET formation in IRI and tumor progression. Finally, we summarize multiple approaches for the targeted inhibition of ET formation and the clearance of ET components to prevent their deleterious effects, which are of great value for mitigating IRI.

Extracellular traps released from various immune cells

Neutrophils are the most abundant innate immune effector cells in the human immune system. They are also the most classic immune cells known to release ETs, which are web-like structures composed of DNA and granule proteins that are released after cell death (2, 3). NETs capture and kill bacteria and have an important role in the body’s intrinsic immune defense against microbial infections (2, 3). However, the excessive release of ETs by neutrophils results in blood vessel blockage, thrombosis, self-antigen exposure, parenchymal cell damage, and tumor cell metastasis, which in turn disrupt the body’s internal environment and contribute to disease development and progression (8, 12, 13, 23–25). Accumulating evidence has shown that in addition to neutrophils, other innate immune cells — including monocytes/macrophages, mast cells, eosinophils, and basophils — can also release ETs in response to various pathogenic and pro-inflammatory stimuli, which are involved in immune regulation and exert beneficial or harmful effects on the body.

Monocytes/macrophages play an important role in initiating the innate immune defense and regulating inflammation. The ability of monocytes/macrophages to release ETs has attracted increasing attention. Researchers have previously shown that statins induce the release of ETs from monocytes/macrophages by inhibiting the sterol pathway (4). Like neutrophils, macrophages can release ETs to defend the body against attack from various microorganisms, such as Escherichia coli, Bacillus licheniformis, Haemophilus influenzae, Mycobacterium tuberculosis, and Candida albicans (26–28). However, it has also been reported that the macrophage ETs (METs) induced by Mycobacterium massiliense do not have bactericidal activity and instead provide a favorable environment for bacterial aggregation and promote bacterial growth (29). Studies have also confirmed that many inflammatory mediators and chemical stimuli such as interferon-γ (IFN-γ), hypochlorous acid, IL-8, tumor necrosis factor-α, and hydrogen sulfide can also induce METs in vitro (30). The mechanisms of MET formation share some similarities with NETs, including NADPH/Ros-dependent mechanisms, calcium mechanisms and PAD4 mechanisms, which largely depend on the nature of the stimuli (9, 31, 32).

Mast cells are crucial for innate immune responses and are well-known for their role in initiating and maintaining local and systemic allergic responses. However, mast cells also play an equally critical role in host defense against infection, autoimmunity, and inflammatory diseases (33–35). In 2008, researchers first identified the formation of mast cell ETs (MCETs) through reactive oxygen species (ROS)-dependent cell death mechanisms. They found that the intact extracellular meshwork of MCETs can trap and effectively inhibit the growth of pyogenic bacteria (5). These MCETs are known to consist of DNA, histones, trypsin, and the antimicrobial peptide LL-37, of which LL-37 is the major effector molecule controlling Group A Streptococcus infection (36). Some microorganisms such as heat-killed Mycobacterium tuberculosis and Listeria monocytogenes induce the release of microbicidal MCETs by producing large amounts of ROS via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)-dependent mechanism (37, 38). Furthermore, the enhanced activity of the transcription factor HIF-1α induces antimicrobial effects by promoting the formation of MCETs in mice and human cells (39). During the development of psoriasis, IL-17 and IL-1β can induce the formation of MCETs in vivo. IL-17+ mast cells frequently produce IL-17 during the release of ETs, which is closely related to the pathogenesis of psoriasis (40).
Eosinophils are multifunctional cells that play an important role in the defense against parasitic infections, allergic diseases, and the protection of cardiac function after myocardial infarction and autoimmune diseases (41, 42). Eosinophils were first described to release ETs in a ROS-dependent manner in the presence of lipopolysaccharide (LPS) in combination with IL-5 or IFN-γ stimulation (6). The study showed that the release of eosinophil ETs (EETs) was independent of eosinophil death. Further, mitochondrial DNA was rapidly released from cells in a catapult-like manner, which contributed to the maintenance of intestinal barrier function and defense against bacterial infection in inflammatory conditions (6). In contrast, in human allergic diseases, local eosinophils release nuclear DNA traps after cell death (43). Studies have reported that microfilariae can trigger EETs in a Dectin-1-dependent manner, and these extracellular DNA traps can inhibit the motility of microfilariae and contribute to protective immunity against filariae (44). EETs are present in bronchoalveolar lavage fluid (BALF) derived from patients with allergic asthma, where they activate pulmonary neuroendocrine cells through the CCDC25-ILK-PKC pathway and amplify allergic immune responses (45). In tissue samples from patients with eosinophil granulomatosis with polyangiitis, EETs with a bold net of chromatin threads are observed within small-vessel thrombi, providing a scaffold for platelet adhesion (46). In addition, EETs in diseased tissue are believed to induce elevations in cell-free DNA and the formation of immune thrombi, which is closely associated with disease activity (46).

Basophils are mainly associated with proinflammatory and immunomodulatory effects in allergic diseases and parasitic infections. Basophils, like neutrophils and eosinophils, can induce the formation of extracellular DNA traps (BETs) under the stimulation of monosodium urate (MSU) crystals (7). Recently, the physiological activation of human and mouse basophils was demonstrated to induce the release of ETs containing mitochondrial DNA and granule proteins. Moreover, BET formation was found to be independent of NOX activity (47). Furthermore, despite lacking phagocytic activity, activated basophils could kill extracellular bacteria by releasing extracellular DNA traps (48). There are relatively few studies on BETs, and substantial research is needed to explore the formation of BETs in different pathological states and their roles in the occurrence and development of diseases.

Mechanisms underlying the formation of neutrophil extracellular traps and NETosis

The mechanism through which immune cells induce the release of ETs is not well understood. It can differ depending on the stimuli and the local microenvironment in which the different immune cells are located. In organ IRI, different infiltrating immune cells are observed at different stages following I/R. The acute phase of I/R is dominated by the inflammatory injury caused by neutrophil infiltration. A deeper understanding of the process of ET release from neutrophils and the mechanism of NET induction is essential for understanding their role and potential impact in IRI.

In 2004, Brinkmann et al. (3) first reported that neutrophils release extracellular trap reticula in response to IL-8, phorbol myristate acetate (PMA), or LPS stimulation. Since then, numerous studies have reported that multiple factors can induce the formation of NETs, including various microorganisms (bacteria, fungi, viruses, and parasites), cytokines, chemicals, metabolites (lipsids, cholesterol, glucose, and MSU crystals), proteases, complement, activated platelets, DAMPs, and hypoxia (19, 49–56) (Figure 1). The specific mechanism through which neutrophils generate NETs also differs according to the different stimuli. The release of NETs accompanied by cell membrane rupture and neutrophil death is called NETosis. Distinct from apoptosis, necroptosis, pyroptosis, and ferroptosis, NETosis is a unique cell death program observed in neutrophils. The main processes involved in NETosis are neutrophil activation, cytoplasmic granule dissolution, neutrophil protease activation, chromatin decondensation and swelling, plasma membrane rupture, and NET release (57, 58).

Studies have reported that the activation of neutrophil surface receptors such as NOX, Toll-like receptors (TLRs), Fc gamma receptor (FcγR), macrophage-1 antigen (Mac-1), programmed death ligand 1 (PD-L1), SIPR2 and dectin-1 may be involved in the initiation of NETosis, which involves the release of ROS (both NOX-derived and mitochondria-derived) or the elevation of intracellular calcium concentrations (59–62) (Figure 1). ROS-related upstream signaling pathways, including protein kinase C (PKC), Raf/MEK/ERK, P38 mitogen-activated protein kinase (MAPK), Src/Syk, and PI3K/Akt, may mediate the NETosis induced by PMA, immobilized immune complexes, microbes, and diphenyl phosphate (DPHP) (51, 63–66). ROS promotes the activation of Akt, which induces PMA-activated neutrophils apoptosis switch to NETosis (65). In addition, the production of ROS activates granzyme myeloperoxidase (MPO), which causes azurophilic granules to release neutrophil elastase (NE) into the cytoplasm. The activated NE is further transported to the nucleus and subsequently synergizes with MPO to promote chromatin decondensation (67–69). During this process, NE binds to and degrades F-actin to block actin dynamics (69). The inhibition of actin disassembly prevents the release of NETs (70). However, recent studies have found that NE transport to the nucleus requires the rearrangement of the actin cytoskeleton and that actin cytoskeleton dynamics are essential for NET formation (71). An increase in intracellular calcium concentrations also induces NET formation (72). Increased intracellular calcium promotes NET formation by directly activating peptidyl arginine deiminase.
4 (PAD4), independent of the ROS pathway (72). Thus, PAD4 is downstream of ROS and calcium signaling during NETosis.

PAD4 induces chromatin decondensation by catalyzing the conversion of arginine residues on histones to citrulline residues and is a key trigger of NETosis (73–75). Of course, NET formation can occur independent of PAD4 (66). Studies have shown that cytosolic LPS and gram-negative bacteria can drive NETosis via a caspase-11-dependent mechanism and the coordination of gasdermin D (GSDMD) function (76). In addition, unilateral ureteral obstruction induces NET formation via a caspase-11/GSDMD-dependent mechanism, which promotes renal inflammation and macrophage-to-myoﬁbroblast transition to facilitate renal ﬁbrosis (77). GSDMD, a pore-forming protein, can be activated upon cleavage by neutrophil proteases during NETosis and localize to the plasma membrane, causing its rupture and the release of decondensed chromatin into the extracellular space (78). The release of NETs seems to be more closely related to pyroptosis, since GSDMD is also a key regulator of pyroptosis. Furthermore, the nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) in ﬂammasome can also contribute to NETosis via a process that is dependent on PAD4, and its inhibition signiﬁcantly attenuates NET formation in a noninfected state (79). Therefore, further studies are needed to better deﬁne the inflammasome pathways acting during NETosis and the possible bridging role between NETosis and pyroptosis. The mechanisms involved in NETosis are very complex, and it is likely that other synergistic or independent cellular and molecular pathways are involved in the induction of NETosis. This also needs to be further explored.

Most stimuli that induce NET formation lead to cell rupture and death, a process that takes several hours. However, as early as 2007, researchers observed that under conditions of sepsis, platelets can induce the rapid release of NETs from neutrophils within minutes through TLR4, enabling the capture of bacteria (80). Subsequently, in response to Staphylococcus aureus infection in human neutrophils, NETs were found to be released into the extracellular space within vesicles through a very rapid (5–60 min), unique mechanism, independent of ROS production by NOX (81). A recent study demonstrated for the ﬁrst time that the rapid (within 5 min) NET release observed after exposure to Staphylococcus aureus is an early event in the antimicrobial response and is dependent on mitochondrial complex III (82). ROS produced by mitochondria and NOX mediate bactericidal activity in neutrophils (82). In addition, tumor-associated aged neutrophils can trigger mitochondria-dependent vital NET formation, which promotes lung metastasis in breast cancer (83). Other stimuli — including gram-positive bacteria (84), parasites (85, 86), and heparin (87) — can also induce rapid NET release. These early/rapid NET release processes have been shown to be independent of cell death, with neutrophils remaining viable for phagocytosis and chemotaxis after NET release. This type of NET release is called “vital NETosis” (23, 88). However, some experts suggest that this nomenclature is inaccurate because “osis” implies death.
and “vital” implies living, making these terms contradictory (89). The term “vital NET formation” is perhaps more accurate. Vital NET formation may be more closely related to infectious diseases (60). However, the rapid release of NETs has not been observed in cases of sterile inflammatory organ injury. In the future, extensive studies will be needed to clarify whether vital NET formation is present and active under conditions of normal tissue repair and organ IRI.

**Extracellular traps in organ ischemia reperfusion injury**

So far, several studies have focused on elucidating the correlation between ET formation and the sterile inflammatory damage induced by organ IRI. ET formation occurs during organ I/R, aggravating organ damage, which induces the formation of more ETs. This leads to a pro-inflammatory vicious cycle and ultimately impairs organ function (14, 19). It is necessary to understand the mechanisms and signaling pathways involved in this pro-inflammatory vicious cycle formed by ETs and IRI. Therefore, we summarized the markers and mechanisms of ETs induction and inhibition in organ IRI (Table 1). In the following sections, we will review, in detail, the link between ET formation and IRI in various organs. Most of the literature focuses on the relationship between NETs and IRI, and this part will also be our focus of attention.

**Extracellular traps in liver ischemia reperfusion injury**

Liver IRI is a local sterile inflammatory response driven by innate immunity (15). Liver IRI usually occurs after liver resection and transplantation, and it is one of the main causes of postoperative disease recurrence and poor prognoses (130). Moreover, it is also a key contributor to early organ dysfunction and graft failure after liver transplantation (131). The mechanisms of liver IRI are complex and not fully understood. The overproduction of ROS and subsequent sterile inflammatory cascades are major contributors to tissue damage following liver IRI (132). The generation of ETs is closely related to the excessive production of ROS. ET formation is a newly discovered biological function of immune cells during sterile inflammatory responses, which is involved in the process of liver IRI.

As drivers, neutrophils play an important role in the early stages of liver I/R and are the major amplifiers of liver IRI. Neutrophils are also major contributors to the acute rejection associated with liver transplantation (133, 134). The formation of NETs plays some role in driving liver IRI (135). Excessive NET formation is often observed in the liver tissue and serum from clinical specimens and animal models of liver I/R. These NETs have been shown to be an independent factor of liver IRI (95, 100). During the initial stage of liver I/R, DAMPs (e.g., HMGB1 and histones) released by damaged hepatocytes stimulate the production of NETs through the TLR4 and TLR9-MyD88 signaling pathways (18). The resulting NETs initiate inflammatory responses and exacerbate liver injury, and PAD4 inhibitors and deoxyribonuclease I (DNase I) attenuate DAMP-mediated liver injury by inhibiting NETs (18). In a rat orthotopic liver transplantation model, HMGB1 was found to induce the formation of NETs through the TLR4-/-MAPK signaling pathway (136). This promoted the intracellular translocation of HMGB1 and the M1 polarization of Kupffer cells, which in turn exacerbated acute rejection after liver transplantation (136). IL-33 is also a type of DAMP that drives neutrophil infiltration through its receptor suppression of tumorigenicity 2 (ST2) during the inflammatory response. Huang et al. (50) demonstrated that the IL-33 released by liver sinusoidal endothelial cells during liver I/R induces NET formation via ST2 signaling, which in turn amplifies the inflammatory cascade and sterile inflammatory response in the liver. In addition to DAMPs, other inflammatory mediators and chemicals can also exacerbate liver IRI by inducing NET formation. Using a combination of computerized dynamic network analysis and experimental validation, Tohme et al. (94) identified a central role for IL-17A in the rapid evolution of the inflammatory mediator network in the early phase of liver I/R. IL-17A exacerbates liver injury after I/R by inducing neutrophil infiltration and NET formation (94). Studies have shown that superoxide, a marker of oxidative stress after liver I/R, can induce NET formation in vitro through the TLR4 and NOX signaling cascade (90). In mouse models of liver I/R, pretreatment with allopurinol (superoxide inhibitor) and N-acetylcysteine (ROS inhibitor) results in a reduction of NETs and amelioration of liver injury (90). In addition, acrolein induces the release of NETs through NOX2 and P38 MAPK signaling to aggravate liver IRI in rats (91). In contrast, some physiological inhibitors and chemicals can alleviate liver IRI by inhibiting the formation of NETs. Studies have reported that tissue inhibitor of metalloproteinases-1, a physiological inhibitor of matrix metalloproteinase 9 (MMP9), can reduce the formation of NETs and thus limit the effect of NETs on the liver IRI (92). Diphenyleneiodonium (DPI), a NOX inhibitor, can inhibit the formation of NETs by inhibiting the NADPH/ROS/PAD4 signaling pathway, thereby reducing liver injury and maintaining liver function (137). Tetramethylpyrazine (TMP), the main chemical component of *Ligusticum chuanxiong*, inhibits NET formation during liver I/R by inhibiting NOX.

Further, TMP combined with DPI can effectively attenuate IRI during liver transplantation in the rat (97). Additionally, pretreatment with histidine-rich glycoproteins was found to prevent liver IRI in mice via the inhibition of neutrophil infiltration and NET formation (96). Hydroxychloroquine (HCQ) protects against liver IRI by blocking TLR9 to inhibit
| ETs | Organ | Year | Markers in serum/supernatant | Markers in immunofluorescence | Inducers | Inhibitors | Mechanisms in vitro | Mechanisms in vivo | Refs |
|-----|-------|------|-------------------------------|-------------------------------|----------|------------|---------------------|-----------------|------|
| NETs | Liver | 2015 | MPO-DNA                       | Cit-H3 and H2AX, HMGB1, histones | NA       | NA         | TLR4-/TLR9-MyD88    | TLR4 and TLR9   | (18) |
| NETs | Liver | 2016 | MPO-DNA                       | Cit-H3                       | MPO      | NA         | NA                  | NA              | (90) |
| NETs | Liver | 2017 | MPO-DNA                       | Cit-H3, MPO and NE           | Acrolein | MPO        | NA                  | NA              | (91) |
| NETs | Liver | 2018 | NET-DNA and Cit-H3            | NA                           | TIMP     | NA         | NA                  | NA              | (92) |
| NETs | Liver | 2019 | MPO-DNA                       | Cit-H3, MPO and NE           | Acrolein | MPO        | NA                  | NA              | (93) |
| NETs | Liver | 2020 | MPO-DNA                       | Cit-H3                       | IL-17A   | MPO        | NA                  | NA              | (94) |
| NETs | Liver | 2021 | MPO-DNA                       | Cit-H3, MPO and NE           | HRG      | MPO        | NA                  | NA              | (95) |
| NETs | Liver | 2022 | MPO-DNA                       | Cit-H3                       | IL-17A   | MPO        | NA                  | NA              | (96) |
| NETs | Kidney| 2017 | Extracellular DNA             | Cit-H3 and NE                | Platelets| NA         | NA                  | NA              | (97) |
| NETs | Kidney| 2017 | MPO-DNA                       | Cit-H3 and NE                | Histones | NA         | NA                  | NA              | (98) |
| NETs | Kidney| 2018 | MPO-DNA                       | Cit-H3 and NE                | YW3-56   | NA         | NA                  | NA              | (99) |
| NETs | Kidney| 2020 | MPO-DNA                       | Cit-H3 and NE                | GSK484   | NA         | NA                  | NA              | (100) |
| NETs | Kidney| 2022 | dsDNA and MPO                 | Cit-H3, MPO and NE           | Fcgr2b   | MPO        | NA                  | NA              | (101) |
| NETs | Kidney| 2022 | dsDNA                         | Cit-H3                       | P2RX1    | MPO        | NA                  | NA              | (102) |
| NETs | Kidney| 2022 | dsDNA                         | MPO and NE                   | Candida albicans | NA       | TLR4/dectin1-Syk-NFκB | NA             | (103) |
| NETs | Kidney| 2022 | NA                            | Cit-H3 and MPO               | C3       | MPO        | C3α-C3αR           | C3α-C3αR       | (104) |
| NETs | Intestinal | 2017 | MPO-Histone                   | NA                            | DNase I  | NA         | Extracellular DNA   | NA              | (105) |
| NETs | Intestinal | 2018 | cDNA                          | Cit-H3                       | NA       | MPO        | DNA                | NA              | (106) |
| NETs | Intestinal | 2019 | NA                            | Cit-H3                       | NA       | MPO        | NA                  | NA              | (107) |
| NETs | Intestinal | 2020 | NA                            | Cit-H3                       | NA       | MPO        | Gut microbiota      | TLR4/TRIF      | (108) |
| NETs | Intestinal | 2020 | NA                            | Cit-H3 and MPO               | TXA      | MPO        | ROS/MAPK            | NA              | (109) |
The formation of NETs (95). Recombinant human thrombomodulin prevents NET generation in neutrophils by blocking the TLR4/ERK/JNK and TLR4/NADPH/ROS/PAD4 signaling pathways, thereby preventing rat liver IRI and improving liver function (99). NETs have been shown to interact directly with platelets and exert procoagulant effects in infectious disease models. Studies have shown that NETs generated in mice with liver I/R can directly induce platelet activation via TLR4, leading to a systemic procoagulant state that induces remote organ injury via immunothrombosis (138).

In a recent study, Lu et al. (100) demonstrated that human umbilical cord-derived mesenchymal stromal cell-derived extracellular vesicles exert nanotherapeutic effects, inhibiting local NET formation by transferring functional mitochondria to intrahepatic neutrophils and repairing their mitochondrial function, thereby attenuating liver IRI in mice. In addition, previous exercise training was found to reduce NET formation during liver I/R and also attenuate liver tissue necrosis (98).

| ETs | Organ | Year | Markers in serum/ supernatant | Markers in immunofluorescence | Inducers | Inhibitors | Mechanisms in vitro | Mechanisms in vivo | Refs |
|-----|-------|------|-------------------------------|--------------------------------|----------|-----------|-------------------|-------------------|------|
| NETs | Intestinal | 2022 | MPO-DNA and dsDNA | Cit-H3 and MPO | HMGBl | NA | NA | TLR4-MyD88 | (112) |
| NETs | Lung | 2020 | CitH3-DNA and NE-DNA | Cit-H3 and NE | mtDNA | NA | TLR9 and PAD4 | TLR9 and PAD4 | (113) |
| NETs | Lung | 2022 | NA | NE and histones | NA | NA | NA | TLR4 and NOX4 | (114) |
| NETs | Cerebral | 2020 | Extracellular DNA | Cit-H3 | NA | NA | NA | PAD4 | (115) |
| NETs | Cerebral | 2022 | NA | Cit3-H4 and MPO | NA | NA | NA | NA | (116) |
| NETs | Cerebral | 2022 | Cit-H3 and MPO-DNA | Cit-H3, MPO and NE | HMGBl | NA | NA | Platelet-neutrophil interactions | |
| NETs | Cerebral | 2022 | NA | NA | PKM2 | NA | STAT3 and NF-kB | NA | (118) |
| NETs | Cerebral | 2022 | NA | Cit-H3, NE and NIMP-R14 | NA | NA | NA | Platelet TLR4 | (119) |
| NETs | Myocardial | 2014 | NA | Cit-H3 | NA | NA | NA | PAD4 | (120) |
| NETs | Myocardial | 2015 | NA | DNA, histone H2B and MPO | NA | NA | NA | NA | (121) |
| NETs | Myocardial | 2018 | NA | Cit-H3 | FN-EDA | NA | NA | TLR4 | (122) |
| NETs | Myocardial | 2018 | NA | Cit-H3 | NA | MKEY | NA | CCL5-CXCL4 | (123) |
| NETs | Myocardial | 2020 | NA | NA | NA | NA | NA | Histones | (124) |
| NETs | Myocardial | 2022 | MPO-DNA | Cit-H3 | Gut microbiota | NA | NA | NA | (125) |
| NETs | Limb | 2013 | NA | H2A/H2B/DNA complex | NA | NA | NA | TLR4 | (126) |
| NETs | Limb | 2016 | NA | H2A/H2B/DNA complex | NA | NA | NA | NA | (127) |
| NETs | Limb | 2020 | NA | Cit-H3 and MPO | NA | NA | NA | PAD4 | (128) |
| NETs | Cutaneous | 2020 | NA | NA | NA | NA | NA | Histones | (124) |
| NETs | Cutaneous | 2022 | NA | Cit-H3 and MPO | NA | IL-36Ra | NA | HMGBl | (129) |

Cit-H3, citrullinated histone H3; cfDNA, cell-free DNA; C3, complement C3; DNase I, deoxyribonuclease I; ETs, extracellular traps; ERK, extracellular regulated protein kinases; ExT, exercise training; FcgR2b, Fc gamma receptor IIb; Fn-EDA, fibronectin splicing variant containing extra domain A; HMGBl, high mobility group box 1; HCQ, hydroxychloroquine; HRG, histidine-rich glycoprotein; IRI, ischemia reperfusion injury; IL, interleukin; IL-36Ra, interleukin-36 receptor antagonist; NETs, macrophage extracellular traps; MPO, myeloperoxidase; MAPK, mitogen-activated protein kinase; MMP9, matrix metallopeptidase 9; MCs, mast cells; MSC-EVs, mesenchymal stromal cell-derived extracellular vesicles; mtDNA, mitochondrial DNA; mCBS, methyl b-cellobioside per-O-sulfate; NETs, neutrophil extracellular traps; NOX, nicotinamide adenine dinucleotide phosphate oxidase; NF-kB, nuclear factor kappa-B; NE, neutrophil elastase; NA, not available; PAD4, peptidyl arginine deiminase 4; P2RX1, purinergic receptor P2X 1; P2Y12, purinergic receptor P2Y 12; PKM2, pyruvate kinase M2; Refs, references; rTM, recombinant thrombomodulin; ROS, reactive oxygen species; ST2, suppression of tumorigenicity 2; Syk, spleen tyrosine kinase; STAT3, signal transducer and activator of transcription 3; SPAs, small polyanions; TLR, Toll-like receptor; TIMP, tissue inhibitor of metalloproteinases; TRIP, TIR-domain-containing adapter-inducing interferon-β; TXA, tranexamic acid.
aggravates liver IRI. Currently, studies on ETs and liver IRI are mainly focused on NETs. However, the liver is an immune organ and contains a large number of innate immune cells. Thus, whether mast cells, eosinophils, and basophils release ETs during liver I/R and participate in the liver injury process deserves further in-depth investigation.

**Extracellular traps in renal ischemia reperfusion injury**

The formation of ETs has a broader role in the pathophysiology of several diseases involving sterile inflammation. In the kidneys, ET formation is a major driver of the self-amplifying cycle of tissue necrosis and inflammation (14). ETs are associated with many renal diseases, such as antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, immune complex glomerulonephritis, acute kidney injury (AKI) and renal fibrosis (14, 77). There exists a close relationship between renal IRI and the formation of NETs, as these events form a positive feedback loop and aggravate the renal necroinflammatory response. Studies have shown that during renal I/R, tubular epithelial cells undergo necrosis and release extracellular DNA, which causes platelet activation. The interaction of activated platelets with neutrophils causes NET formation, leading to a further increase in renal inflammation and tissue damage (101). Pretreatment with clodigodrel, which inhibits platelet aggregation prior to renal ischemia, can significantly reduce the formation of NETs in renal tissue and attenuate IRI in mice (101). Treatment with exogenous DNase I administered intraperitoneally immediately after renal I/R in rats can improve renal function and attenuate renal IRI by degrading extracellular DNA (140).

Consistent with these findings, Nakazawa et al. (19) demonstrated that the tubular epithelial cell necrosis induced during renal I/R occurs prior to the expansion of localized and circulating NETs and increased expression of inflammatory and injury-related genes. In addition, it has been revealed that extracellular histones released from dying tubular epithelial cells are central mediators in NET-related tissue damage and serve as independent accelerator factors during the crescendo of necroinflammation in postischemic kidneys (19). Histones can induce NET formation in neutrophils, and the substances released by NETs further kill tubular epithelial cells and induce NET formation. The death of tubular epithelial cells and the production of NETs show a co-stimulatory interaction, leading to a pro-inflammatory vicious cycle that ultimately leads to renal and distal organ damage (19).

Recombinant thrombomodulin (rTM) produces anti-inflammatory effects by binding to circulating histones (141). Studies have shown that pretreatment with 10 mg/kg rTM does not ameliorate renal IRI. However, it significantly reduces the accumulation of histones and NETs in the lungs after renal I/R, exerting a protective effect on the lungs (103). PAD4 has been shown to be closely associated with NET formation in many disease models. Studies correlating renal I/R with PAD4 in mice have shown that PAD4-deficient mice do not form NETs during renal I/R, and their renal function is restored 48 h following renal I/R (21). Unlike that of PAD4-deficient mouse-derived neutrophils, the adoptive transfer of wild-type mouse-derived neutrophils to PAD4-deficient mice could restore renal NET formation and impair renal function following renal I/R (21). This cell adoptive transfer experiment confirmed that PAD4 in neutrophils plays a key role in renal IRI and NET formation (21). In addition, PAD4 is also involved in the acute lung injury (ALI) caused by renal I/R. Intraperitoneal injection of GSK484 (a PAD4 inhibitor) before renal I/R attenuates distal lung injury by reducing neutrophil infiltration, NET formation, and inflammatory cytokine secretion (102). Fc gamma receptor Iib (FcgR2b) is associated with systemic lupus erythematosus (SLE), and FcgR2b+/− mice develop age-related lupus features. Thus, they have been used as a representative model for SLE (142). After renal I/R treatment in FcgR2b+/− lupus mice, NETs and apoptosis were found to be significantly induced in FcgR2b+/− kidneys at 24 h post-IRI, and lupus nephritis was aggravated at 120 h post-IRI (104). This process was found to be regulated by spleen tyrosine kinase (Syk) and PAD4 signaling (104). Zhuang et al. (105) systematically compared the transcriptome between IRI kidneys and sham kidneys using RNA sequencing and found that purinergic receptor P2X 1 (P2RX1) was significantly up-regulated in kidneys with IRI. P2RX1 supported the formation of NETs following renal IRI, and these NETs were essential for the impairment of mitochondrial dynamics (105). Meanwhile, in vitro, the activation of P2RX1 promoted platelet ATP release, which subsequently promoted the glycolytic metabolism of neutrophils and NET formation (105). In addition, the oral administration of Candida albicans to mice prior to renal I/R increased systemic inflammation and NETs through the activation of TLR-4 and dectin-1, exacerbating renal IRI (62). In line with these findings, Complement C3 KO mice with renal I/R showed attenuated renal injury when neutrophil infiltration and NET formation were reduced (106).

Whether the ETs causing renal IRI are mainly derived from neutrophils or macrophages has been inconclusive. Nevertheless, most current studies have focused on the NETs promoting renal IRI. Pretreatment with anti-Ly6G IgG can deplete neutrophils in mice with renal I/R, significantly reducing renal NET production and renal injury at 24 h post-reperfusion (106). This depletion experiment demonstrated that neutrophils and their ETs play an important role in promoting renal IRI. However, in a mouse model of rhabdomyolysis, Okubo et al. (143) demonstrated that macrophages and platelets, but not neutrophils, contribute to rhabdomyolysis-induced extracellular DNA release and AKI. During rhabdomyolysis, platelet activation via the hemoglobin (iron) released from necrotic muscle cells enhances NET production by increasing intracellular ROS production and histone citrullination, which further promotes tubular injury.
Whether macrophages and their ETs are involved in IRI independently or act in concert with NETs during renal I/R remains unclear. The numbers and proportions of neutrophils and macrophages in kidneys also vary during the different stages of I/R (20). In the early stage of renal I/R, the renal tissue is predominantly infiltrated by neutrophils, and NETs may play a dominant role during this phase. In the late stage of I/R, with the depletion of neutrophils and repair of renal tissue, the number of macrophages increases gradually. However, whether these increased macrophages can form ETs to continuously promote renal I/R or participate in tissue repair deserves further in-depth investigation. Currently, the research on NETs and METs in renal I/R is still in its infancy. A large number of studies are urgently warranted to explore the role and mechanisms of ETs in renal I/R.

**Extracellular traps in intestinal ischemia reperfusion injury**

Intestinal IRI is a clinical problem that occurs most commonly after acute mesenteric ischemia, traumatic/hemorrhagic or septic shock, burns, and surgery. It can lead to multiple organ dysfunction and mortality in critically ill patients (147–149). Neutrophils may contribute to intestinal IRI by forming ETs (108, 112). After I/R induction in the rat intestine, Wang et al. (108) found that intestinal IRI leads to the excessive release of NETs. These NETs contribute to the early inflammatory response after intestinal I/R and disrupt the intestinal barrier as well as the functional integrity of tight junctions (108). The extracellular DNA released by NETs contributes to organ damage. Treatment with DNase I can disrupt generated NETs and significantly reduce the formation of NETs in the intestine and serum (108). Thus, it inhibits the histopathological changes that occur following intestinal IRI, restores the integrity of the intestinal barrier, and increases the expression of tight junction proteins (108). In addition, therapeutic interventions with DNase I attenuate tissue injury, apoptosis, and oxidative stress after intestinal I/R by inhibiting NET-mediated inflammatory responses (107). In a rat model of traumatic hemorrhagic shock, the early intravenous administration of tranexamic acid attenuated NET formation via the classic ROS/MAPK pathway and prevented the disruption of tight junction proteins (111). Hayase et al. (109) found that the accumulation of extracellular histones and NETs exacerbates remote liver injury after intestinal I/R. In their study, the intraperitoneal injection of 10 mg/kg rTM at the beginning of intestinal I/R in mice neutralized extracellular histones and attenuated the liver tissue injury induced by intestinal I/R (109).

Using intravital imaging technology, Ascher et al. (110) found that the presence of some gut microbes restricted NET formation in I/R-injured mesenteric venules, likely due to diminished neutrophil TLR4 signaling. Furthermore, they also demonstrated that the TLR4/TRIF signaling axis was critically involved in mesenteric IRI-induced NETosis (110). Zhan et al. (112) found that NETosis was enhanced in the lungs after intestinal I/R in C57BL/6J mice and that the deletion of MyD88 attenuated the production of NETs and intestinal I/R-induced lung injury. Treatment with DNase I or a PAD4 inhibitor significantly attenuated intestinal I/R-induced ALI (112). In addition, the HMGB1 released from necroptotic enterocytes during intestinal I/R exacerbated the intestinal I/R-induced ALI by inducing NET formation (112). Therefore, NETs could serve as clinical indicators and therapeutic targets for intestinal IRI. Targeting NETosis and its products could help in attenuating intestinal I/R-induced remote organ injury.

**Extracellular traps in lung ischemia reperfusion injury**

Lung IRI is a common pathological condition, and the resulting inflammatory cascade is thought to play a central role in its pathophysiology (150). Lung IRI usually occurs after lung transplantation and is one of the main factors leading to primary graft dysfunction (PGD) in recipients and early morbidity and mortality after lung transplantation (151, 152). In BALF from human lung transplant recipients, NETs were found to be more abundant among patients with PGD (153). NET formation was increased following either hilar clamp or orthotopic lung transplantation after prolonged cold ischemia (OLT-PCI) (153). Disruption of NETs via the inhibition of platelets or the intrabronchial administration of DNase I reduced lung injury and improved oxygenation (153). In addition, increased mitochondrial DNA (mtDNA, an endogenous DAMP) levels were detected in the BALF of an experimental PGD model induced by OLT-PCI, and it was confirmed that the mtDNA released during lung I/R triggers NET formation via TLR9 signaling, driving lung injury (113). TLR9 deficiency in lung recipients or donors reduces NET formation and lung injury (113). Thus, DNase I treatment may have the dual benefit of both degrading pathogenic NETs and neutralizing NET triggers such as mtDNA. Using intravital imaging, oxidative lipidomics, and transplant models, Li et al. (114) demonstrated that TLR4 signaling and downstream NOX4 expression in vascular endothelial cells during lung I/R mediate neutrophil recruitment to the lungs and increase NET formation. The knockdown of TLR4 expression in vascular endothelial cells results in decreased neutrophil infiltration and NETosis (114).

Treatment with DNase I reduces lung neutrophil extravasation and subpleural NET formation, thus improving graft function (114). However, studies also show that although DNase I treatment can rapidly degrade NETs within the graft, the ensuing release of NET fragments promotes the production of inflammatory factors in human alveolar macrophages by activating the TLR-MyD88 signaling pathway (154). It also initiates the proliferative response of dendritic cells to...
alloantigen-specific CD4+ T cells, preventing lung transplant acceptance (154). In addition, Antunes et al. (155) demonstrated — for the first time — that methoxyxyenol protects lung tissue from inflammation and inhibits LPS-induced neutrophil infiltration and NET formation in ALI mice. At present, the specific mechanism underlying the induction of NETs during lung I/R and the impact of NETs on lung injury or lung transplant rejection remain unclear and need to be explored in depth. In addition, whether alveolar macrophages can also release ETs during I/R and the role they play in lung IRI deserves further investigation.

**Extracellular traps in cerebral ischemia reperfusion injury**

Cerebral IRI, which usually occurs after thrombolysis and recanalization in ischemic stroke, is characterized by massive cell death and neutrophil activation. Novotny et al. (156) detected NETs within the thrombi of 100% (71/71) of patients with acute ischemic stroke (AIS) and confirmed that the abundance of NETs in these thrombi was associated with poor outcomes in these patients. In addition, the level of NETs in the thrombi was also related to the degree of neurological injury (116). With the prolongation of reperfusion, collateral blood flow improved in patients with ischemic stroke, and this was associated with lower levels of NETs in the thrombus (116). These results suggest that targeting NETs in thrombi may enable early neurological protection in AIS patients. In line with this, Denorme et al. (117) found that elevated plasma biomarkers of NETs are associated with worsening stroke outcomes. During AIS, NETs can exert deleterious effects in a platelet TLR4-dependent manner, and the early administration of DNase I can reduce infarct size and improve stroke outcomes after ligature-induced permanent middle cerebral artery occlusion (119). In addition, other studies have shown that platelets can exacerbate cerebral IRI by driving HMGB1 release and NET formation (117). Neonatal NET-inhibitory factor (nNIF) is an endogenous NET inhibitory peptide that blocks the formation of NETs without affecting the other functions of neutrophils (157, 158). Prophylactic therapy with nNIF effectively prevents platelet-induced NET formation and improves short-term and long-term outcomes following ischemic stroke (117). Polymorphonuclear granulocytes (PMNs) migrate into the brain parenchyma and release large amounts of proteases, which are thought to be the main cause of neuronal cell death and reperfusion injury following ischemia (159). However, Enzmann et al. (160) found no PMN infiltration in 25 infarcted brain tissue samples collected from patients with ischemic stroke at early post-infarction time points. Moreover, they found that intravascular PMN aggregation did not correlate spatially with the release of NETs (160). In contrast, studies have shown that neutrophils accumulate around the meninges and blood vessels after cerebral I/R and eventually reach the infarcted brain parenchyma (161). Disruption of the basement membrane and NET formation can be detected 24–48 h after reperfusion (161). In addition, the release of NETs impairs the blood–brain barrier and vascular remodeling during stroke recovery. However, the disruption of NETs using DNase I or the knockdown of PAD4 increases neovascularization and repair and improves functional recovery (115). The components of NETs, i.e., histones and extracellular DNA, are also detrimental during cerebral I/R, and targeting them can attenuate the damage caused by ischemic stroke (162). Studies have also shown that the formation of NETs after cerebral I/R is closely related to the pyruvate kinase M2 (PKM2) gene in myeloid cells, which regulates the post-ischemic inflammatory response of peripheral neutrophils by promoting the phosphorylation of signal transducer and activator of transcription 3 (STAT3) (118). Myeloid cell-specific PKM2−/− mice show reduced formation of NETs and improved cerebral blood flow, and also exhibit reduced thrombotic inflammation following cerebral I/R (118). ML265 is a small molecule that inhibits PKM2 nuclear translocation by inducing its tetramerization (163). ML265 treatment significantly reduces the nuclear translocation of PKM2 and inhibits NETosis after AIS. Additionally, it improves long-term sensorimotor outcomes in mice (118). Currently, DNase I has been identified as the main degrader of NETs following cerebral I/R (164). Studies have shown that differently polarized macrophage subsets can degrade NETs (165). Microglia, a type of macrophage in cerebral, can protect neurons by direct engulfment of invading neutrophil (166). It would be interesting to explore whether reactive microglia can regulate the formation of NETs in cerebral IRI. The link between microglia and NETs in cerebral IRI deserves further in-depth examination.

**Extracellular traps in myocardial ischemia reperfusion injury**

Innate immune cells play an important role in the early response to myocardial IRI. During myocardial I/R in mice, high neutrophil infiltration and NET formation can be observed in the injured myocardial tissue (120). The intraperitoneal injection of recombinant human DNase 1 at 1 h after the induction of a left anterior descending occlusion and 11 h after reperfusion can reduce the infiltration of neutrophils and the formation of NETs in myocardial tissue (120). Additionally, it can reduce the size of the myocardial infarct and improve cardiac function (120). Further, PAD4−/− mice do not produce NETs during I/R and are protected from myocardial IRI (120). Previous studies have demonstrated the potential role of NETs in linking sterile inflammation to thrombosis (167). It was shown that in rats, neutrophil (MPO-positive) density in the left ventricular ischemic zone increases following 45 min of myocardial ischemia and 3 h of reperfusion, and this is accompanied by strong immunostaining for NETs (121). The
intravenous administration of DNase I 5 min before reperfusion reduces I/R-induced neutrophil aggregation, NET formation, and MPO activity (121). In addition, NET-mediated microthrombosis contributes to myocardial "no-reflow." However, DNase I combined with recombinant tissue-type plasminogen activator (rt-PA) reduces myocardial I/R-induced anatomic "no-reflow" and limits infarct size, improving long-term post-infarction left ventricular remodeling (121). Surprisingly, rt-PA treatment alone has no significant effect on the number of NETs (121). Extracellular histones in NETs are highly toxic to tissues (168). Meara et al. (124) described a non-toxic small polyanion (SPA) that interacts electrostatically with histones to displace them from NETs, thereby destabilizing their structure and neutralizing their pathological effects (124). SPAs were found to significantly inhibit rat myocardial IRI in vivo by reducing NET formation and free histone-mediated pathological damage (124). Fibronectin splicing variant containing extra domain A (Fn-EDA), an endogenous ligand of the innate immune receptor TLR4, can promote thrombosis and inflammation (169, 170). In one study, hyperlipidemic apolipoprotein E-deficient mice with Fn-EDA knockout showed less neutrophil infiltration and NET formation compared to WT mice after myocardial I/R. Moreover, they showed reduced cardiomyocyte apoptosis and infarct size. The findings confirmed that Fn-EDA-mediated myocardial IRI requires the involvement of TLR4 (122). The heterodimerization of platelet-derived CCL5 and CXCL4 enhances their ability to activate and recruit inflammatory cells and is involved in the formation of NETs (171). Researchers specifically designed a compound called MKEY (a peptide antagonist) to block the interaction between CCL5 and CXCL4 (172). MKEY was administered intravenously to mice 1 day before myocardial I/R and treatment was continued until 7 days after I/R. The results showed that MKEY treatment significantly reduces the inflammatory response after I/R and the formation of NETs in vivo, while also reducing myocardial infarct size and improving cardiac function (123). The gut microbiota plays a crucial role in cardiovascular disease. During myocardial I/R, the gut microbiota induces the formation of NETs, which can directly lead to the apoptosis of cardiomyocytes and myocardial microvascular endothelial cells, exacerbating myocardial IRI (125). In addition, Tang et al. recently showed that Kruppel-like factor 2 (KLF2)-deficient neutrophils exhibit enhanced NET formation in vitro and are essential for angiotensin II-induced cardiac hypertrophy (173, 174). However, whether KLF2 is involved in myocardial IRI, and whether KLF2 has a negative effect on NET formation during myocardial I/R, warrants further investigation.

Extracellular traps in cutaneous ischemia reperfusion injury

Cutaneous IRI typically occurs due to pressure ulcers, Raynaud’s phenomenon-induced skin ulcers, and skin flap grafts following reconstructive surgery. It is related to the oxidative damage caused by apoptosis, necroptosis, ROS, and excessive generation of pro-inflammatory factors (176–178). NETs have been reported to delay the healing of skin lesions, and pharmacological targeting of NETs can accelerate wound regeneration (179, 180). Methyl β-cellobioside per-O-sulfate (mCBS), a type of SPA, can inhibit NET-associated histone-mediated injury. Further, mCBS injection 5 min before and after cutaneous I/R can consistently and significantly increase the area of skin flap survival (124). IL-36 receptor antagonist (IL-36Ra), encoded by IL36rn, attenuates myocardial IRI though reducing neutrophil recruitment and improving blood flow in mice (181). Tanaka et al. (129) found that in IL36rn<sup>−/−</sup> mice, cutaneous I/R resulted in a significant delay in wound healing and increased inflammatory cell infiltration. Furthermore, they found that compared with WT mice, IL36rn<sup>−/−</sup> mice showed significantly...
greater NET formation in the cutaneous tissue around the IRI after 4 and 72 h of reperfusion (129). The intraperitoneal injection of Cl-amidine (10 mg/kg/day) to inhibit NET formation significantly attenuated the cutaneous IRI in Il36rn−/− mice (129). These results show, to some extent, that NETs are associated with the exacerbation of cutaneous IRI. Skin flap tissue IRI caused by skin flap transplantation is one of the primary reasons for the low success rate of the procedure (176). Hence, there is a need to explore the intrinsic links between NETs and skin flap transplantation-induced IRI along with the related mechanisms. NET targeting could become a new intervention for improving skin flap survival. Furthermore, whether Langerhans cells in the skin can also release ETs and participate in cutaneous IRI is unknown and must be explored in depth.

**Link between ischemia reperfusion injury-induced neutrophil extracellular traps and cancer**

Back in the mid-19th century, Rudolf Virchow discovered leukocyte infiltration in tumor tissue and first proposed the link between inflammation and cancer (182). Today, the close relationship of inflammation with the tumorigenesis and metastasis of most types of cancers has been clarified (183). IRI is a type of sterile inflammatory injury, and I/R-induced sterile inflammation promotes tumor recurrence and metastasis after liver resection or liver transplantation (184, 185). Under inflammatory conditions, the web-like DNA strands released by NETs, which are embedded with various proinflammatory molecules, capture circulating tumor cells (CTCs) and contribute to cancer metastasis (186–188). Yang et al. (189) proposed that NET-DNA is a chemokine that activates the ILK-β-parvin pathway to enhance cell motility and promote cancer metastasis by binding to the transmembrane protein CCDC25 on cancer cells. In this review, we have previously mentioned that a large number of NETs are induced during I/R. Therefore, it would be interesting to understand whether IRI-triggered cancer recurrence and metastasis are closely related to NET formation during I/R. Tohme et al. (190) observed that in patients undergoing hepatectomy for colorectal liver metastases, greater evidence of postoperative NET formation in the serum was associated with a higher risk of recurrence. Subsequently, they induced liver I/R in a mouse model of colorectal liver metastases and found that NETs formed due to IRI promoted the development and progression of liver metastases. Interestingly, this effect could be reversed through local treatment with DNase I or the inhibition of PAD4, which hinder NET formation (190). Ren et al. (191) studied the relationship between IRI-induced NETs and cancer metastasis from the perspective of platelet–neutrophil interactions. Their findings suggested that the surgical stress induced by liver I/R in mice activates platelets and promotes their aggregation within tumor cells via the TLR4-ERK5 axis, which is conducive for the capture of tumor cells by IRI-induced NETs and subsequent distant metastasis (191). Blocking platelet activation or the knockdown of TLR4 protects mice from liver I/R-induced metastasis, and no CTCs are captured by NETs (191). This result suggests that the targeted disruption of the interaction between platelets and NETs may have therapeutic effects, preventing postoperative distant metastases. The relevance of NETs to T cells has been studied to a limited extent. Kaltenmeier et al. (192) induced liver I/R to generate a NET-rich tumor microenvironment (TME) in an established cancer metastasis model. They found that IRI-induced NETs promote tumor growth by enhancing CD4+ and CD8+ T cell exhaustion.

**Targeting extracellular traps for ischemia reperfusion injury treatment**

As mentioned above, ETs are involved in the IRI process in various organs, and the excessive production and/or impaired clearance of ETs can exacerbate IRI. Therefore, strategies for reducing excessive ET formation or enhancing ET degradation...
are expected to improve organ IRI and have obvious therapeutic benefits. Therapeutic approaches for inhibiting the overproduction of NETs by targeting key molecules with various drugs or gene knockout technology have been reported in the literature (Table 2). Among the many approaches used to target intracellular signaling molecules, the drugs rTM and HCQ inhibit NET formation by inhibiting the TLR pathway (95, 99). The inhibition of genes such as IL-33, ST2, IL-36R, P2RX1, PKM2, and complement C3 via gene knockout technology or drugs can reduce NET formation and attenuate IRI (50, 105, 106, 118, 129). In addition, the interaction of platelets and mast cells with neutrophils can promote the formation of NETs. Thus, the inhibition of platelet activation or the elimination of platelets and mast cells can inhibit NET formation and control IRI and IRI-induced tumor metastasis (93, 101, 191). However, these interventions do not directly target NETs.

The inhibition of NET formation via the targeting of NET components could limit the role of NETs more directly. The blockade of ROS production using antibodies or the inhibition of citrullination histones with PAD4 inhibitors can inhibit NET formation and protect against IRI in the liver (90, 137, 138, 190), kidneys (21, 102, 105), intestine (112), lungs (113), cerebrum (115, 117), myocardium (120), limbs (128), and skin (129). The function of NETs largely depends on their reticular DNA structure and the various proteins embedded within them. Enhancing the clearance of NETs by promoting the degradation of extracellular reticular DNA with DNase I may be another effective strategy to reduce IRI (100, 108). One key advantage of DNase I is that it is used clinically and has not shown any toxicity (195). However, in one study, DNase I treatment could not improve limb IRI, although it promoted NET clearance (127). Therefore, in addition to the reticular DNA structure, the multiple proteins embedded within NETs may also contribute to organ damage. Histones are a core factor causing NET-related tissue injuries, and the positive feedback loop between histones and NETs can aggravate distal organ injury after renal IRI (19). Neutralizing histones on NETs significantly attenuates IRI in multiple organs (19, 109, 124, 162). In addition, the antibody-mediated blockade of other NET components — such as MMP9, HMGB1, and PD-L1 — can also limit the function of NETs and improve IRI (92, 112, 192). NE and MPO are also closely related to NET formation (68, 87). Whether targeting agents against NE and MPO could alleviate organ IRI warrants further investigation.

Some treatments with unknown mechanisms of action, such as exercise training and the supplementation of gut microbiota, can also reduce organ IRI by reducing NETs (98, 110). However, gut microbes have been reported to exacerbate myocardial IRI by regulating NET formation (125). The influence of gut microbiota on organ IRI may be dependent on the species of microorganisms, which leads to the conflicting findings. Immunoregulatory methods for reducing NET formation are currently considered one of the primary therapeutic strategies for organ IRI and may further improve patient outcomes. Future studies are needed to identify therapeutic strategies and drugs that target specific pathways of NET induction, generation, and degradation and evaluate them through clinical trials to demonstrate the potential of NETs as therapeutic targets for organ IRI.

Current challenges in the study of extracellular traps and ischemia reperfusion injury

Currently, there are some challenges that hinder research on ETs in organ IRI. First, many drugs and treatments that have been reported to inhibit NET formation also inhibit neutrophil infiltration. However, neutrophil is essential for organ repair, thus, blocking of neutrophil infiltration may be deleterious at the late phase of organ IRI (196). In addition, it is worthwhile to investigate whether the decreased neutrophil infiltration causes the decreased NET formation or whether the drug or treatment itself can alter the ability of neutrophils to produce ETs. Second, in organ IRI, many stimuli can trigger cell necrosis, which involves chromatin release similar to NETs. Many reported detection methods cannot distinguish between the generation of extracellular DNA from the release of ETs and the effects of cell necrosis. The combined immunofluorescence staining of DNA and citrullinated histones helps to distinguish between cell necrosis and other forms of DNA release. NE or MPO, the third most important marker, may enhance the reliability of the results if it is found to be co-localized with DNA and histones on staining (23). In addition, the ET release process can be more directly visualized in real-time using intravital confocal microscopy (50). The NET components NE and MPO are also present in ETs (26, 197). Therefore, whether the ETs affecting organ IRI at various stages are mainly derived from neutrophils needs to be carefully examined. Comparative analysis using the immunofluorescence-based colocalization of immune cell and ET markers or the depletion of corresponding immune cells can help identify the main sources of ETs (106). Finally, accurate quantification of NETs in patient plasma or serum remains a challenge. Recently, Matta et al. (198) developed a new method to reliably detect NETs in patient plasma using multiplex enzyme linked immunosorbent assay (ELISA) (MPO, citrullinated histone H3 and DNA) combined with immunofluorescence smear methods. The techniques for identifying NETosis are complex. Thus, establishing an ELISA for quantifying NET-related components may be practical. However, the quantitative changes in NET formation do not necessarily translate to disease progression or improvement. Therefore, more studies are needed before ETs can be used as a reliable biomarkers for organ IRI and prognosis.
TABLE 2  Targeted anti-extracellular trap interventions to improve outcomes of organ IRI.

| Target molecule/ function | Agents/interventions | Effects | Organ IRI | Refs |
|----------------------------|----------------------|---------|-----------|------|
| **NET components**         |                      |         |           |      |
| DNA                        | DNase I              | Degradation of NETs | Liver (18) (94, 100) (138) (190) | (192) |
|                           |                      |         | Kidney (101) (140) (21) (105) |      |
|                           |                      |         | Intestinal (107, 108, 111, 112) |      |
|                           |                      |         | Lung (113) (114) |      |
|                           |                      |         | Cerebral (117) (119) (115) (162) |      |
|                           |                      |         | Myocardial (120, 121) | (126, 127) |
|                           |                      |         | Limb (19, 103) |      |
|                           |                      |         | Kidney (19, 21) (104) |      |
|                           |                      |         | Cerebral (115, 119) |      |
|                           |                      |         | Limb (128) |      |
|                           |                      |         | Cutaneous (129) |      |
|                           |                      |         | Liver (138, 190) |      |
|                           |                      |         | Kidney (21, 102, 105) |      |
|                           |                      |         | Intestinal (112) |      |
|                           |                      |         | Lung (113) |      |
|                           |                      |         | Cerebral (115, 117) |      |
|                           |                      |         | Myocardial (120) |      |
|                           |                      |         | Limb (128) |      |
| Histones                  | Anti-histones antibody: BWA3 and histones neutralizer: mCB5, rTM | Inhibition of NETs, decreased histones and NETs accumulation | Kidney (109) |      |
|                           |                      |         | Intestinal (109) |      |
|                           |                      |         | Cerebral (162) |      |
|                           |                      |         | Myocardial (124) |      |
| Histone citrullination    | Pan-PAD inhibitors: Cl-amidine, YW3-56, YW4-03 | Inhibition of histone citrullination and NETs formation | Liver (18, 190) | (19, 21) (104) |
|                           |                      |         | Kidney (115, 119) |      |
|                           |                      |         | Limb (128) |      |
|                           |                      |         | Cutaneous (129) |      |
|                           |                      |         | Liver (138, 190) |      |
|                           |                      |         | Kidney (21, 102, 105) |      |
|                           |                      |         | Intestinal (112) |      |
|                           |                      |         | Lung (113) |      |
|                           |                      |         | Cerebral (115, 117) |      |
|                           |                      |         | Myocardial (120) |      |
|                           |                      |         | Limb (128) |      |
| NADPH oxidase             | NADPH oxidase inhibitor: F-apocynin, DPI, TMP ROS inhibitor: N-acetylcyesteine | Inhibition of NETs and inflammatory factors | Liver (91, 97) (99) |      |
| MMP9                      | rAAV8-TIMP-1         | Decreased NETs and leukocyte activation | Liver (92) |      |
| HMGBl                    | HMGBl antagonist: TM Anti-HMGBl antibody | Inhibition of NETs and NET-induced EMT | Liver (193) |      |
| PD-L1                     | anti-PD-L1, PD-L1 KO | Decreased NETs | Liver (192) |      |
| Intracellular signaling molecules | | | | |
| MAPK pathway              | P38 MAPK inhibitor: Naringin ERK/JNK inhibitor: TMP | Inhibition of NETs and inflammatory factors | Liver (91) | (97) |
| NF-κB pathway             | NF-κB inhibitor: BAY11-7082 | Decreased NETs | Kidney (104) |      |
| Syk pathway               | Syk inhibitor: R788 diiodium | | | |
| TLR pathway               | Inhibitor of TLR4 (rTM) and TLR4 KO | Inhibition of NETosis and CTC entrapment by NETs | Liver (99, 191) | (126) |
| IL-33/ST2 pathway         | IL-33 KO and ST2 KO | Decreased NETs and neutrophil infiltration | Liver (50) |      |
| CCL5-CXCL4                | CCL5-CXCL4 blocker: MIKEY | Inhibition of NETs | Myocardial (123) |      |
| IL-36R                    | IL-36R antagonist | Inhibition of NETs | Cutaneous (129) |      |
| P2RX1                     | P2RX1 inhibitor: NF449 | Decreased NETs | Kidney (105) |      |

(Continued)
Conclusion and prospection

NET formation is a double-edged sword. On the one hand, NETs capture and kill bacteria through their reticular DNA traps, playing an important role in the innate immune defense. On the other hand, the excessive production of NETs can affect the development and outcomes of non-infectious diseases, and especially sterile inflammation-related diseases. NET formation appears to be associated with IRI in various organs, especially in the early stages of inflammatory infiltration following I/R. Recently, the role of NETs in liver IRI has also been reported (22). However, the research on ETs at various stages of organ IRI remains in its infancy. In the future, more studies will need to be conducted to explore the roles of ETs generated by various immune cells at different stages of IRI along with the related mechanisms. In addition, it will be necessary to further explore the specific networks regulating ET formation in different microenvironments and the role that the multiple proteins embedded within the reticular DNA traps play in IRI. As numerous proteins present in ETs are investigated, new functions of ETs may emerge. An in-depth understanding of the molecular mechanisms of ET formation could help us inhibit ETs via targeted drugs, and then attenuate IRI. Thus, such research could pave the way for new diagnostic and therapeutic strategies for managing IRI.

Author contributions

FZ and YL wrote the manuscript. FZ, YL and JW contributed to conception, design and interpretation of the manuscript. JZ, PC and ZS revised the manuscript. WW supervised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Pulmonary inflammation caused by neutrophil extracellular traps (NETs) has become a focus of research in recent years. NETs are proinflammatory structures that are formed by the release of DNA from neutrophils after activation. These structures are composed of DNA fibers outside the cell membrane, which entangle other cells and necrosed or apoptotic debris. NETs have been implicated in several pathological conditions, including inflammatory lung diseases, such as acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and asthma. NETs are also involved in the development and progression of various cancer types, such as lung cancer, prostate cancer, and breast cancer, by promoting angiogenesis and tumor cell proliferation. Furthermore, NETs have been shown to play a role in the development of sepsis and multiorgan failure by activating the innate immune system and inducing tissue damage. Despite the growing interest in NETs, their underlying mechanisms remain largely unidentified, and the development of therapeutic strategies to target NETs is still in its infancy. Future research should focus on understanding the pathophysiological roles of NETs in various diseases and developing effective therapies to counteract their pro-inflammatory effects.
NET-works with dire consequences for health.

STAT3 to promote the development of arterial thrombosis.

results of transcription factor HIF-

AD, et al. Inhibition of transcription factor NFAT activity in activated platelets

formation.

secretions and functional attributes.

Mast cells and neutrophils release IL-17 through extracellular trap formation

Increased circulating cell-free DNA in eosinophilic granulomatosis with

IL-33 exacerbates liver sterile inflammation by amplifying neutrophil extracellular trap

Diverse stimuli engage different neutrophil extracellular trap pathways.

Eosinophils improve cardiac function after myocardial infarction.

Gasdermin d plays a vital role in the generation of neutrophil extracellular traps.

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T-cell-mediated release of galectin-10 in eosinophilic granulomatosis with

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Molecular prerequisites for extracellular trap formation. 

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