Review

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Review

Role of Neutrophil Extracellular Traps in Health and Disease Pathophysiology: Recent Advancements and Future Insights

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Abstract: Neutrophils are the principal trouper of innate immune system. Activated neutrophils undergo a noble cell death termed NETosis and release a mesh-like structure called neutrophil extracellular traps (NETs) as a part of their defensive strategy against microbial pathogen attack. This web-like architecture includes a DNA backbone embedded with antimicrobial proteins like myeloperoxidase (MPO), neutrophil elastase (NE), histones etc. and deploys in the entrapment and clearance of encountered pathogens. Thus NETs play an inevitable beneficial role in the host's protection. However, recent accumulated evidence shows that dysregulated and enhanced NET formation has various pathological aspects including promotion of sepsis, pulmonary, cardiovascular, hepatic, nephrological, thrombotic, autoimmune, pregnancy, cancer diseases etc. and the list is increasing gradually. In this review, we summarize NETs mediated pathophysiology of different diseases, focus on some updated potential therapeutic approaches against NETs and share our future perspectives.

Keywords: neutrophil extracellular traps (NETs); innate immunity; sepsis; lung disease; cardiovascular disease; liver disease; kidney disease; diabetes; COVID-19; coagulopathy and thrombotic microangiopathy; cancer; autoimmunity; preeclampsia; kawasaki disease

1. Introduction to Neutrophil Extracellular Traps (NETs)

1.1. Neutrophil and Innate Immunity

Innate immunity is the first line of defense of the body. The main function of the innate immunity is to protect our body from invading microorganisms and limit the growth and proliferation and finally kill the microorganisms. Polymorphonuclear neutrophils are the most abundant granular leukocytes produced at a rate of 5×10^{10}-10×10^{10} cells per day, contributing approximately 50%–70% of all circulating white blood cells in humans having an average short half-life of 6-8 hours in circulation and key player of the innate immunity [1,2]. They are the first responder of the inflammatory cascades [3,4]. Neutrophils are derived from the bone marrow and enter into the circulation from where they quickly move into the sites of infection or inflammation in response to pathogen attack in the body and roll, adhere to endothelial layer, crawl and thus transmigrated from the vessel and kill the microbes [5–7]. After accomplishment of their role they undergo apoptosis and are cleared by macrophages [8]. As the arsenal of innate immunity neutrophil appear first to the infected site in the body and imply several strategies to eliminate the infection. In fact, neutrophils evolved to fulfill the key role in innate immunity through rapid deployment and effective antimicrobial action against a broad range of pathogens and noninfectious inflammation. Hence they are equipped with multiple weapons that can be deployed as a part of their antimicrobial strategies [9].

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1.2. Antimicrobial actions of Neutrophils:

Neutrophils were recognized as the vanguard of the innate immunity and a vital protector against microbial infection and foreign invasion. In past decades it was thought that when neutrophil encounter invading pathogen in the body, they kill them either by phagocytosis, where they engulf and inactivate the pathogen and rapidly kill them by releasing proteolytic enzymes, antimicrobial proteins or reactive oxygen species (ROS) or by exocytosis, where they de-granulate and release antimicrobial factors in the extracellular space. However, in 2004, Brinkmann et al. described another way of neutrophil derived novel antimicrobial defense mechanism of innate immunity termed NETosis that can participate in pathogen killing extracellularly through the release of NETs [10]. Although neutrophils are classically known to die primarily by apoptosis or necrosis and highly regulated necroptois but NETosis is a cell death program that is distinct from apoptosis and necrosis [11,12].

1.3. Neutrophil Extracellular Traps (NETs)

NETs are released by activated neutrophils and the structure is composed meshwork of decondensed chromatin fibers decorated with antimicrobial granular proteins such as histones, myeloperoxidase (MPO), neutrophil elastase (NE), and cathepsin G etc. This web-like architectural design and composition allow NETs to prevent the dissemination of pathogen in the body. NETs can only be released robustly by matured neutrophils upon stimulation. Immature neutrophils are less potent compared to mature neutrophils at promoting innate immune defenses [13–15] and neutrophils from term and preterm infants fail to form NETs upon activation by inflammatory agonist [16]. Several infectious and sterile stimuli have been reported to trigger NET formation including bacteria [10,12], virus [17], fungi [18,19], parasites [20], pro-inflammatory cytokines like interleukin 8 (IL-8) [21], tumor necrosis factor α (TNF-α) [22], placental micro-debris [23], activated platelets [24], cholesterol crystals, monosodium uric acid [25], immune complexes [26], autoantibodies [27], complements such as C5a [28] and even cancer cells [29–31]. Phorbol esters such as phorbol myristate acetate (PMA), bacterial products such as lipopolysaccharide (LPS) are most widely used non physiological agonist to generate NETs ex vivo [10,32,33].

1.4. NETosis Mechanism

Two different mechanisms, NADPH Oxidase 2 (Nox 2)-dependent and Nox 2 independent NETosis have been well validated by published literatures. Agonist like PMA, LPS, bacteria such as Pseudomonas aeruginosa etc. induce Nox-dependent NETosis while agonist like calcium ionophores (A231128, ionomycin), uric acid crystals, certain microbes, UV ight etc. triggers Nox-independent NETosis through the formation of different ROS; Nox-ROS and mitochondrial ROS respectively [34–36]. The generated ROS activates Nox-dependent and Nox-independent specific different sets of kinases (MAPK, ERK, p38 and JNK) leading to transcriptional firing and stimulation of MPO. In Nox-independent NETosis histone citrullination is facilitated by a nuclear enzyme protein-arginine deiminase 4 (PAD4) activation [37,38]. MPO trigger the stimulation and translocation of NE from azurophilic granules to the nucleus and decorate the chromatin [39–41]. Ultimately nuclear membrane disintegrates and NETs are released.

1.5. NETosis Pathways

NET formation occurs via two pathways; suicidal NETosis and vital NETosis. Suicidal NETosis represents a cell death pathway that starts with nuclear delobulation, the nuclear membrane disassembly followed by continuous loss of cellular polarization, chromatin decondensation and finally finished with plasma membrane rupture and expulsion of NETs [42]. This lytic slow cell death process usually takes 2-4 hours [33]. On the other hand vital NETosis is cell death independent non-lytic process where expulsion of nuclear chromatin is accompanied by the release of granular proteins and fabrication of these components occur extracellularly leaving active anucleated phagocytic cytoplast having the potency to ingest microorganisms and chemotaxis [42]. Vital

NETosis happens faster within 5-60 minutes depending on the inducer [43]. In vital NETosis the released extracellular DNA by neutrophils could be of either nuclear or mitochondrial [44–46].

1.6. Physiological role of NETs

Decades ago, when the NETs emerged, scientists became very interested observing its antibacterial capacity. Wide range of killing capacities of NETs made the immunologist to work with great haste. NETs conquer over infections by trapping, immobilizing and neutralizing gram-negative and gram-positive bacteria [10], viruses [17] fungi [47], parasites [48,49] and thus it prevents the dissemination of intruding microbes and protects the host. Although NETs trap microbes through charge interaction [50] but some pathogens evade NETs. Many gram positive pathogens including but not limited to pathogens of the Streptococcus and Staphylococcus genera release endonucleases, secreted endonuclease degrades the extracellular DNA scaffold, destroy and evade NETs [51,52]. The escaped bacteria with endonuclease may promote further invasion and spread from the local sites to distant organ and blood stream [53–55]. Again Streptococcus pneumoniae can escape from NETs in a charge dependent manner. This gram-positive bacterial strain expresses anti-phagocytic polysaccharide capsules and lipoteichoic acid which can produce a positive charge on their surface and thus prevent them to be trapped by positively charged NET fibers and histone residues [56]. Interestingly, the beneficial effect of NETosis over phagocytosis is the size of NETs. Large microbes and parasites evade phagocytosis and can prove difficult to clear. But neutrophil has a unique microbe sensing mechanism that allows them to selectively tailor their antimicrobial responses to pathogen on basis of microbial size. The large filamentous form of fungi cannot be removed by phagocytosis. NETs play a significant role to control these large filamentous fungi [57–59].

In addition, NETs also contribute to immunothrombosis. Immunothrombosis is a physiological process having bidirectional role with innate immune system. Although excessive activation of coagulation cascade leads to many clinical conditions including sepsis, disseminated intravascular coagulation (DIC), myocardial infarction and coronavirus disease 2019 (COVID-19) etc. [60] in contrast it also plays an essential protective role in maintaining physiological hemostasis to avoid blood loss and arresting both viral and bacterial infections. However, with discovery of NETs it has been come into light that thrombus is not only for hemostatic purpose stopping bleeding but also takes part in innate immunity. NETs provide large procoagulant surface by activating the contact phase of coagulation [61,62]. Moreover, neutrophils and neutrophils derived micro-particles during NETosis such as DNA, histones and granule proteins provide coagulant activities [61].

1.7. Controversial role of NETs

Infect the intervention of NETs is a landmark progression of science. Now the question is whether NETs are too good or too bad for us. NETs are like double edged sword. While NETs have a physiological role in antimicrobial defenses, if dysregulated they also have various pathological aspects that attracted recent attention (Figure 1). In some conditions when it is excessively generated and present, NETs can do harm to the host. The antimicrobial histones and peptides decorating the NET-DNA are directly cytotoxic to tissue and ineffective clearance of NETs results deleterious inflammation of host tissue. The list of disorders implicated by NETs include sepsis, pulmonary, cardiovascular, hepatic, nephrological, thrombotic, autoimmune, pregnancy, cancer diseases etc. and are gradually increasing.

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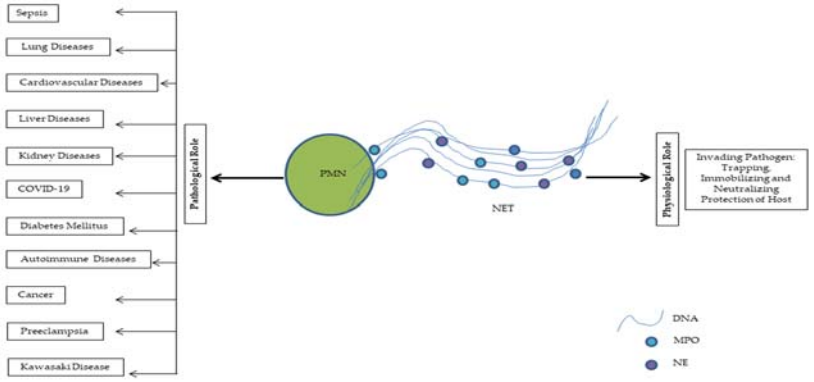

Figure 1. Controversial role of NETs. NET is a powerful weapon of innate immunity and provides protection to the host through clearing the invading pathogens (right part). On the contrary aberrant NET formation causes pathology in numerous ways and attributed to autoimmune, infectious, and non-infectious diseases (left part).

1.8. Detection of NETs

Uncontrolled release of NETs has been reported in several disease pathophysiologies (discussed later). Therefore, precise spotting of NETs in clinical samples could have great potential in the prognosis of disease progression and subsequent consequences. NETs are made up of cell-free DNA (cf-DNA) and anti-microbial proteins like MPO and NE etc. NET detection is very challenging due to its fragile structure, timing of formation and turnover and frequent presence of DNase. Measuring cf-DNA is not a good approach to quantify NETs as the source could also be of apoptosis and necrosis in addition to NETosis [63]. NETs can be visualized and quantified by fluorescence microscopy [64] and flow cytometry [65] tools in human neutrophils in vitro. However, enzyme-linked immunosorbent assay is the handy, highly sensitive and reliable method to quantitatively measure remnants of circulating NETs in plasma [66,67] as well as in cell culture supernatants in vitro [68].

2. NETs in Clinical Settings

2.1. NETs in Sepsis

Sepsis is a dysregulated response to an infection with deleterious effects in host leading to circulatory shock, multiple organ failure syndromes (MODS) and ultimately death. A common cause of death in sepsis is the overwhelming infection in the blood stream and the resulting complications [69]. During sepsis activation of neutrophil with microbial or inflammatory stimuli occur which results in the expulsion of NETs [70]. NETs are essential antimicrobial defense for pathogen clearance in the blood and tissues during infection but at the same time NETs and NET components exert excessive inflammation resulting host tissue damage [42]. Using animal model of sepsis, circulatory NET in the blood stream became evident [24,45,71] and the biomarkers used to check the presence of NETs, were also increased in septic patients [72–74]. NET damages tissue and increase vascular permeability in sepsis. Promotion of neutrophil infiltration occurs in tissues through neutrophil-endothelial cell (EC) interaction resulting increased NET formation [75] and this interaction results excess NET formation which is dependent on activated EC derived IL-8 [76]. This damage is neutralized when incubated with either NAPDH oxidase inhibitors or DNase [76].

Immunothrombosis represents controlled inflammation and coagulation and the major-line of innate immune defense towards intruding infection. In sepsis, dysregulated, sustained and hyper-immunothrombosis leads to DIC complications [77]. Sepsis-induced DIC is detrimental to host and cause organ dysfunctions having a double mortality rate that of septic patients without DIC [78].

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Neutrophils diligently participate in thrombosis associated DIC [79]. Neutrophil released NETs contribute excessive thrombin generation due to its early presence at the onset of DIC [80].

Normally platelet remains dormant in the circulation [81]. Growing body of evidences reveal that in sepsis the interaction between neutrophils and activated platelets happen simultaneously and that platelet can rapidly mediate neutrophils to make NET in vivo [24,82]. Activated platelets trigger the neutrophils derived NET release in either P-selectin dependent [83,84] or Toll-like receptor (TLR) 4 dependent manner [24,85] which traps bacteria in expense of endothelial tissue damage. Histones along with fragmented DNA are the abundant components of NETs. In acute systemic inflammatory conditions, including sepsis and trauma, circulating histones aggravates micro-circulatory thrombosis, worsen tissue perfusion and contributes significantly to organ injury [85].

2.2. NETs in Lung Diseases

Excessive activation of neutrophils causes MODS and lung, the most sensitive and important organ in systemic inflammation, is the main target [86,87]. Acute lung injury (ALI) is one of the leading causes of death in the ICU. Lung edema, inflammation, hyaline membrane and alveolar damage are the characteristic morphological features of ALI [88]. Acute respiratory distress syndrome (ARDS) is an acute inflammatory lung injury characterized by hypoxemic respiratory failure as a consequence of increased permeability of the endothelial-epithelial barrier, alveolar damage, and pulmonary edema. ALI and its severest form ARDS or chronic obstructive pulmonary disease remains as an important clinical challenge due to complex and ambiguous pathophysiology [89,90]. A massive influx of activated neutrophils is seen to the lung microvasculature, interstitial, and alveolar space and dysregulated inflammatory neutrophils are the key factor to the progression of ALI/ARDS [91–93]. This excessive neutrophil activation and accumulation induces increased formation of NETs along with increased release of proinflammatory mediators [94,95]. NET mediated cytotoxicity on alveolar epithelial cells as well as pulmonary EC is mainly due to protein components of NETs [96]. Histones major components of NETs are too toxic to cells and lung is the most susceptible vital organ to high levels of circulatory histones [97]. NET derived histones were detected from bronchoalveolar lavage fluid (BALF) samples from humans with ARDS [98]. Co-culture of PMA-stimulated neutrophil with EC alter barrier function resulting EC damage which is attributed to NETs and pre-treating EC with DNase, Cl-amidine a PAD 4 inhibitor, and 1-(3-methylbenzoyl)-1H-indazole-3-carbonitrile an NE inhibitor restore the damage [99]. Growing body of evidence suggested that the lung injury in ALI or ARDS is triggered by C5a activated NET release along with histones and enzymes that causes tissue damage [98,100]. NETs also contribute to pathogen induced lung injury in mouse model and human [101].

Cystic fibrosis (CF) is an inherited autosomal recessive disease of the lung due to chronic inflammation of the airways and bacterial colonization leading to death in 90% of patients. CF occurs due to a mutation in CF trans-membrane conductance regulator gene that control the balance of bicarbonate and chloride secretions across the cell surface epithelium of the airways [102] and this mutation increases the susceptibility of these patients to airway bacterial infection mainly by Pseudomonas aeruginosa and Haemophilus influenza and Staphylococcus aureus [103]. Neutrophils are infiltrated to the airway upon bacterial colonization and chronic and show less potency to eliminate microbes instead contributes to lung damage [104]. This colonization induces NET expulsion resulting sputum viscosity and ultimately exacerbating the patient's respiratory capacity [105]. Neutrophil count and extracellular DNA can be used as severity marker for the assessment of inflammation and lung disease severity in CF [106]. High levels of NET components such as MPO and NE enzymes available in CF sputum and BALF are responsible for the damage of airway epithelium and connective tissues that correlate with lung disease severity [96,107,108]. DNase treatments in CF patients diminished this damage and improve pulmonary function [109].

Asthma is a chronic heterogeneous airway inflammatory disorder with symptomatic features of periodic wheezing, coughing and shortness of breath leading to the deterioration of lung function [110]. Asthmatic inflammation was thought to be attributed mainly by eosinophil but recent researches suggested a greater proportion of neutrophilic involvement having worsened disease

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severity with poor treatment outcome with traditional glucocorticoids [111]. Extracellular traps have been marked in the airway of atopic allergic asthmatic patients [112]. In asthmatic patients BALF neutrophil count and IL-8 are reported as the most powerful biomarker to differentiate severe and moderate condition [113]. IL-8 is a neutrophil chemoattractant and established agonist of NETosis. Again in asthmatic patient's plasma activated platelet also rises [114] which is also responsible for induction of NET formation [24].

2.3. NETs in Cardiovascular Diseases

Acute myocardial infarction (AMI) is a life-threatening condition that occurs due to blockade of blood flow to the heart muscle because of thrombus formation occluding one or more coronary artery resulting tissue damage. Neutrophil recruitment has been mentioned to be involved in the development of atherothrombosis [115], coronary thrombi [116] and also used as the prediction of acute coronary events [117]. High levels of NETs are associated with severe coronary atherosclerosis patients [118]. NETs are detected at the advanced atherosclerotic lesion both in human and mice model [119,120] and application of DNase in Ischemia reperfusion injury mice model reduces reperfusion injury [121]. Histone H4 structural components of NET scaffold results atheromatous plaque instability and anti-histone H4 antibody implication results plaque stabilization [122]. A report described that NET could be a potential diagnostic marker in atherosclerosis [123]. Tissue factor is a trans-membrane protein that stimulates coagulation process. NET-associated tissue factor induces thrombogenic potential through platelet activation and increased thrombin generation resulting myocardial infarction [124].

2.4. NETs in Liver Diseases

Liver is the main organ for the clearance of circulating DNA and histones from the body. In Ischemia-reperfusion neutrophils are identified to the site of liver injury and expulsion of damage associated molecular patterns (DAMPs) and extracellular histones from damaged hepatocytes were observed to worsen the hepatic injury through TLR-4 and TLR-9 [125]. These DAMPs activate neutrophils to release NETs which intensifies sterile inflammatory liver injury. Again extracellular histones activate the nucleotide-binding domain, leucine-rich repeat containing protein 3 inflammasome, which further contribute in liver injury [126]. LPS activated platelets and the subsequent NET formation showed disturbed microcirculation and liver damage [24]. In a mouse model of ischemia-reperfusion injury NET mediated amplified inflammation and liver damage found to be restored after DNase or PAD 4 inhibitor treatment [125].

2.5. NETs in Kidney Diseases

The pathophysiology of acute kidney injury involves the renal tubular cell death and auto amplification loop of cell necrosis called necroinflammation [127,128]. Infections, trauma, toxins and ischemia induce a huge neutrophil recruitment in the renal tubule resulting necrosis and apoptosis and release of DAMPs and alarmins. Extracellular free or NET bound or immune complex associated histones are potent mediators of renal epithelial cells necrosis [129,130] and induce further histone release that act as DAMPs [129,131]. These DAMPs and other inflammatory mediators further activate neutrophil to release NETs, which accelerate more surrounding tissue injury. Histones and NETs enhance tubular necrosis and capillary injury [132] and this glomerular injury can be rescued by using PAD-4 inhibitor and anti-histone antibodies [130,133].

2.6. NETs in COVID-19

COVID-19 is a highly contagious respiratory disease caused by SARS-CoV-2 virus and declared as a global pandemic by world health organization. Higher presence of NETs in COVID-19 patients has been marked [134,135]. Neutrophilia, immune dysfunction and hyper-inflammation are the clinical features of severe COVID-19 patients [136,137]. This hyper-inflammation in COVID-19 is attributed by NETs [138]. Excessive production of cytokines termed cytokine storm is the hallmark

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in the pathogenesis of COVID-19 with augmented plasma levels of CCL 2, IFNγ, IFNγ-inducible protein 10, G-CSF, CCL3, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-17, and TNF-α leading to subsequent severe consequences like ALI, ARDS, pulmonary thrombosis and MODS [139–143]. IFN-γ, TNF-α, IL-1β and IL-8 are strong agonist of NET induction [21,22,135]. NETs have been reported to contribute the damage of the alveoli and pulmonary endothelium and immunothrombosis in patients with severe progression of COVID-19 [143–148]. Inflammatory microvascular thrombi having NET components have been identified in the lung, kidneys and hearts of COVID-19 patients [149]. NET components such as genomic DNA and citrullinated histones have been marked to initiate coagulation in COVID-19 patients through thrombin generation resulting poor fibrinolysis and dropped anticoagulant factor by binding to factor XII. NET inhibition in COVID-19 patients is found to compensate the NET-induced inflammation and thrombotic tissue damage linked to COVID-19 ARDS and death [143]. Ultimately there is a negative correlation between increased levels of NETs and decreased survivability in COVID-19 patients [150,151].

2.7. NETs in Coagulopathy and Thrombotic microangiopathy

Thrombosis is a principle cause of morbidity and mortality. Vascular occlusion represents deleterious effects of NETs. DIC is a heterogeneous group of disorder featured by widespread activation of intravascular coagulation [70,152]. The interaction between coagulation and innate immunity results immunothrombosis, NET induced immunothrombosis plays an essential physical role in innate immunity by immobilizing and preventing the dissemination of invading pathogens. Nevertheless exaggerated NETs can initiate abnormal thrombogenicity leading DIC. NETs initiate coagulation cascade and high flow circulating NETs aggregate and stick platelets to its own scaffold and form RBC rich thrombus that serve as a template for thrombus formation and adhere to the vascular endothelium in microvasculature [153]. Electrostatic interaction of the negatively charged cells with positively charged histones in NETs probably contribute to construct the scaffold. This scaffold is responsible for deep vein thrombosis (DVT). In fact, this NET-platelet-thrombin axis promotes intravascular coagulation and microvascular dysfunction [154,155]. Moreover, circulating free DNA suppress fibrinolysis either by accelerating the inactivation of tissue plasminogen activator by PAI-I [156] or by thickening the fibers of fibrin clot in combination with histones [157]. Again, there is a recommendation for NETs to be an important factor of excessive and unbalanced thrombin generation because of its early presence at the onset of DIC [80]. Histones also play a key role in the NET mediated coagulopathy. H3 and H4 of NETs stimulate platelets and induce thrombotic reaction in mice model [158]. Again in an animal model NET and its integral part citrullinated histones H3 was found in thrombi and its intravenous administration induced clot formation [159].

Thrombotic microangiopathy (TMA) is life threatening conditions and causes massive microvascular thrombosis with thrombocytopenia, microangiopathic hemolytic anemia and MODS [160]. High level of serum DNA-histone complexes and MPO in TMA patients implies the involvement of NETs in disease severity [161]. In mice model, treatment with DNase and PAD 4 inhibitor blocks DVT which reflects the involvement of NETs as potential agonist of thrombosis [153,162]. NE is a NET component and in an experimental model of NE-deficient mice, thrombosis was found to be ameliorated [163]. Furthermore, low levels plasma of DNase I activity was reported in thrombotic patients which also passively proof the involvement of NET in thrombosis [164].

2.8. NETs in Diabetes

Diabetes mellitus (DM) is an array of metabolic diseases with clinical features of hyperglycemic condition that arise due to impairment in insulin action, secretion or the two. Hyperglycemia is connected priming neutrophils for oxidative burst and ROS generation. Neutrophils and NETs are involved in the pathogenesis of both insulin deficiency type 1 diabetes (T1DM) and insulin resistance type 2 (T2DM) and subsequent complications [165]. In T1DM patients with disease severity of less than 1 year, circulating protein levels and enzymatic activities of proteinase 3 (PR3), NE and MPO-DNA are found significantly higher compared to healthy controls which indicate the amplified NET formation [166]. In experimental model of T1DM, inhibition of neutrophil function and NETosis

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ablate the progression of diabetes [167]. In T2DM patients elevated level of NETs, neutrophil elastase, mono- and oligonucleosomes and cf-DNA are observed compared to healthy donors [168,169]. NETs are also responsible in pathogenesis of diabetes induced complications. PAD 4, an enzyme taking part in chromatin decondensation during NETosis cascade and citrullinated histones, were markedly elevated in both T1DM and T2DM which implicated poor wound healing in both mice and human and interestingly PAD4 inhibition with Cl amidine and application of DNase restore the wound healing [170,171]. A case control study reported the elevated levels of circulating NET components; DNA-histones and NE are associated with the development diabetes induced retinopathy [172]. NETs are also reported in the sera of diabetic neuropathy [173] and nephropathy [174] patients.

2.9. NETs in Autoimmune Diseases

NETs and histones are strongly associated with autoimmune diseases [175]. NET exposure in the presence of B-cells like immune cells develops antibodies directed against self-nucleic acids and cytoplasmic proteins such as MPO and PR 3 [176]. Prolong presence of NETs trigger the production of anti-neutrophil cytoplasmic antibodies and anti-nuclear antibodies and vice-versa which promotes vasculitis and enhances the autoimmune response [177]. NETs have been implicated in numerous autoimmune diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA)[178,179]. RA is a chronic, deleterious systemic autoinflammatory disease of the synovial joints and SLE is a complex and heterogeneous disease with characteristic feature of the systemic, multi-organ inflammation. Elevated propensity of spontaneous NET release along with high ROS, MPO and NE had been identified from isolated neutrophils in patients with RA and SLE compared to healthy controls along with higher nuclear translocation of PAD 4 [179–181]. Moreover, increased levels of NET formation and NET remnants MPO-DNA are also reported in the serum of RA patients [182]. In a rat model of RA, PAD4 inhibition by chloramidine compensated the NET induced inflammation and erosive changes [183]. Another RA model study reports the reduction of joint inflammation and erosion when NETs inhibition was implicated by monoclonal antibodies directed against citrullinated histones [184]. In SLE slow degradation of NETs and complement activation contributes to the disease progression [185]. In a murine model of SLE NET inhibition by DNase I constrain the development of anti-ssDNA and anti-histone antibodies [186] and PAD4 inhibition by chloramidine reduces SLE induced subsequent vascular injury and organ damage [187].

2.10. NETs in Cancer

Inflammation is a trademark of cancer and recent emerging evidences identified presence of neutrophils as infiltrating inflammatory immune cells with tumor [188,189]. Neutrophil plays a controversial dual pro-and anti-tumorigenic role in tumor biology [190,191]. Consequently the effects of NETs with tumor are also found in two reverse regulatory ways: pro-tumor effects that enhance cancer cell proliferation, invasion and metastasis and anti-tumor effects that inhibit proliferation and invasion [192]. In this section we will highlight the pro-tumorigenic role of NETs. Highly elevated levels of NETs deposition is marked in some malignant cancers with Ewing sarcoma [193], Lewis lung carcinoma (LLC) [194], breast cancers [195,196] and lymphoma [197].

Although the detail underlying mechanism is yet to be investigated but several published literature disclosed that NETs promote tumor development by the inhibition of apoptosis and proliferative effects and thus uphold tumor progression and metastasis [198,199]. Circulating tumor cells (CTC) are significantly involved in tumor metastasis. Interestingly due to its sticky mesh like architecture NETs can entrap and adhere CTC and take to an adjacent areas thus expanding tumor cell metastasis. In an animal model of sepsis, significant levels of NET associated tumor cells were clearly pictured upon injection of LLC cells compared to healthy group and cancellation of neutrophils void this effect by lowering CTC adhesion within the liver [200]. A group of researchers further re-confirmed that only NETs but not neutrophils are responsible to augment tumor metastasis [197,201]. Moreover, the interaction of NETs with tumor cells induces a severe cancerous phenotype in cancer cell [192].

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NET components also play significant role in tumor progression [202]. NE has been described to have pro-tumorigenic activity and is found to induce proliferation and migration of tumor cells in vitro [192]. Lung adenocarcinoma model showed increased proliferation of tumor cell line when neutrophils are co-cultured with A549 cells and this proliferation is nullified when co-cultured with NE-/- neutrophils [203]. This pro-tumorigenic role of NE is implicated by the degradation of insulin receptor substrate-1 and subsequent activation of phosphatidaylinositol-4,5-bisphosphate 3-kinase [203,204]. In addition, NE also induce the release of pro-tumor factors like transforming growth factor α, vascular endothelial growth factor and platelet-derived growth factor facilitating interaction with their respective receptors and favoring tumor progression [205].

Matrix metalloproteinase 9 (MMP-9) an integral parts of NETs degrades extracellular matrix and support tumor metastasis [5]. MMPs favors pro-tumorigenic course through tumor cell proliferation, impairing apoptosis, increased angiogenesis, invasion and metastasis [206–208]. In mice model this invasiveness of tumor is reversed in MMP-9-/- mice compared to wild type [207].

Cathepsin G aiding metastasis by increasing angiogenesis and tumor dispersion [209]. In animal model of breast cancer cathepsin G potentiate tumor aggregation in vasculature and form distal tumor emboli [210]. In patients with hepatocellular carcinoma NETs-associated cathepsin G induces tumor cells invasion [211]

2.11. NETs in Preeclampsia

Preeclampsia is a pregnancy disorder with characteristics of inflammation, hypertension, kidney failure and seizures and significant cause of maternal and neonatal mortality worldwide [212]. Placental micro-particle induced neutrophil extracellular DNA lattices are found in preeclampsia [23]. A huge elevation of circulating cf-DNA is measured from the maternal plasma [213]. A published literature indicates inappropriate NET formation as the source of this cf-DNA [214]. Elevated levels of microparticles from preeclampsia patients could trigger NETs in isolated neutrophils though IL-8 dependent manner [215]. Moreover, NET-components have been detected by immunofluorescence staining in the intervillous space of preeclamptic placentae [23,216].Thus exacerbated NETosis lead to placental tissue damage in preeclamptic patients.

2.12. NETs in Kawasaki Disease

Kawasaki disease (KD) is an acute multisystem vasculitis syndrome with characteristic feature of febrile illness mainly affects infants and children in the developed countries [217,218]. EC activation and injury is observed in patients with KD [219]. Although the etiology and mechanism is not crystal clear, however, KD patients are very susceptible to acquire coronary artery abnormalities and myocardial ischemia [220,221]. The endothelial glycocalyx is a carbohydrate-rich gel-like layer lining buildup of syndecan, hyaluronic acid, chondroitin sulfate, and heparan sulfate etc. [221]. A recent report demonstrates circulating endothelial glycocalyx proteins syndecan-1 and hyaluronan as predictive biomarker of coronary artery lesions in KD [222]. Interestingly NETs also promote cardiovascular EC damages suggesting that enhanced NET generation may participate to the pathogenesis of KD vasculitis [223]. Again, high levels of proinflammatory mediators including TNF-α, IL-1β and IL-8 have been reported in KD patients which are strong inducer of neutrophils to release NETs [22,218]. The activation of neutrophils respiratory burst assessed by flow cytometry assay using dihydrorhodamine is described in the acute phase of KD [224]. Isolated neutrophils from acute phase KD patient's shows enhanced NET formation compared to healthy controls [225,226]. Moreover, NE plasma level is also higher in acute phase KD [227]. Thus, NET and NET components contribute to KD and subsequent squeals.

3. Evaluation of NETs Inhibition as Therapeutic Targets

NETs are generated from the matured activated neutrophils in response to a wide array of infectious microbial pathogens and sterile stimuli and act as an indispensable protective barrier of innate immunity. Expelled NETs entrap and protect the dissemination of invading microorganisms

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throughout the body either by killing or confining them in its web-like structure. In contrast, aberrant activation of neutrophils and excessive production of NETs exaggerate inflammatory response that is likely to contribute to different diseases including infectious and non-infectious. Thus, NETs act as double-edged sword due to their dual controversial role. Albeit the microbial infection would be eradicated by using broad-spectrum antibiotics in ICU, the issue of stopping the NET induced systemic effects has come forward. Recent evidence suggests that inhibition of NETosis does not hamper the killing capacity of neutrophils other than NETs [228]. The development of blocking NETs is in progress (Table 1) [229]. Inhibition of NETs by DNase and other inhibitors postulates benefits in the context of thrombosis [230], ischemia reperfusion injury [231], SLE [232], CF [107], AMI, stroke, diabetes [233] and cancer [234].

Table 1. Inhibition of NETs as therapeutic approach

References	Year	Results
Martinod *et al* [230]	2014	NET inhibition by DNase I administration showed protective role against thrombosis in vivo murine model of ischemic stroke, myocardial infarction and DVT.
Brill *et al* [163]	2012	Infusion of DNase I protected mice from DVT through inhibition of NETs.
De Meyer *et al* [235]	2012	NET inhibition by DNase I improves ischemic stroke outcome in mice model.
Savchenko *et al* [231]	2014	DNase I therapy against NETs in mice model exerts cardioprotective effects and improve cardiac contractile function.
Papayannopoulos *et al* [107]	2011	DNase I therapy enhances sputum solubilization in CF patients
Wong *et al*[233]	2018	Elevated NETs contribute to diabetes complications and NET disruption by using DNase I therapy improves wound healing.
Patutina *et al* [234]	2011	DNase I treatment inhibits tumor progression and dissemination.

The therapeutic target and the time of initiation of the therapies are an important concern in NET situation. Because NETs are beneficial for the host in the early phase of the infections and become detrimental and backfire in later stage. So, the fine tuning of NET formation throughout the disease course would be the goal for the development of new NET targeted therapies. Early detection of NETs is essential to start the therapy and perhaps a combined therapeutic approach; inhibition of stimulation and damaged tissue repair would be the best option for treatment.

4. Conclusions

From discovery in 2004, NETs have long been recognized as a novel central mechanism of innate immunity for their beneficial physiological role in host defense. But their pathogenic role has attracted recent attention. Several factor including aberrant activation, dysregulation and excessive generation determines their controversial role. The list of NET implicated diseases are gradually expanding ranges from autoimmune disorder to diabetes to cancer [170,232,236]. Although new novel therapeutic strategies are evolving targeting NETs but still, further and more extensive researches are needed to explore their implication in different diseases.

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