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REVIEW

Extracellular DNA and autoimmune diseases

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Extracellular DNA is secreted from various sources including apoptotic cells, NETotic neutrophils and bacterial biofilms. Extracellular DNA can stimulate innate immune responses to induce type-I IFN production after being endocytosed. This process is central in antiviral responses but it also plays important role in the pathogenesis of a range of autoimmune diseases such as systemic lupus erythematosus. We discuss the recent advances in the understanding of the role of extracellular DNA, released from apoptotic and NETotic cells, in autoimmunity.
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INTRODUCTION

It was long discovered that cytosolic DNA was immune stimulatory and associated with autoimmunity but little was known about the mechanism. Over the last decade, a range of DNA sensors have been identified that recognize DNA, including viral DNA.[1] Following sensor engagement, the adaptor protein stimulator of interferon genes (STING) is stimulated and proinflammatory cytokines released.[2] This response is a key component of host defence against pathogens. However, when perturbed, this response can contribute to susceptibility to autoimmune diseases. Aberrant DNA sensing is implicated in the pathogenesis of systemic lupus erythematosus (SLE) and Aicardi–Goutieres syndrome (AGS).[3] Unlike RNA sensors, which recognize pathogen-specific RNA, the majority of DNA sensors found to date recognize DNA in a sequence-independent manner.[1,4] Under homeostatic conditions, DNA engagement is prevented by accessory proteins such as nucleases that cleave DNA and intracellular regulators for DNA receptors.[5] Together, these prevent the development of an excessive immune response.[6]

DNA sensors and inflammation

DNA sensors can be generally divided into two groups: endosomal membrane receptors and intracellular receptors. Toll-like receptor 9 (TLR9) was the first DNA sensor that was identified. It is still the only endosomal expressed pathogen recognition receptor (PRR) for DNA.[7,8] It is expressed mainly within plasmacytoid dendritic cells (pDC) and to a lesser extent on monocytes, B cells and dendritic cells. It was elegantly demonstrated that TLR9 is activated by CpG DNA (5′ cytosine-phosphate-guanine 3′), which in turn leads to type-I interferon (IFN) production via TLR9 adaptor protein, myeloid differentiation primary response gene 88 (MyD88), and interferon regulatory factor 7 (IRF7).[9–11] Although TLR9 is able to recognize CpG DNA that is enriched in the microbial genome, DNA-protein complexes were also shown to induce proinflammatory cytokine production via TLR9.[12]

In contrast to endosomal PRR, cytosolic DNA sensors are widely expressed in mammalian cells.[4] This enables them to recognize the invading pathogens in the affected cells and mount a prompt immune response. Cytosolic DNA sensors show great redundancy: 13 have been discovered so far. DNA-dependent activator of IFN-regulatory factors (DAI) was the first cytosolic DNA sensor identified. It is essential for viral clearance during cytomegalovirus infection in human fibroblasts. DAI engagement activates *TANK*-binding kinase 1 (TBK1) and the IRF3 complex to provoke type-I IFN production. The function of DAI *in vivo* is still under investigation.[13] Pyrin and HIN domain-containing (PYHIN) family proteins, such as 'absent in melanoma' (AIM2) and interferon-inducible gene 16 (IFI16), function as DNA sensors. They have a DNA binding domain termed the carboxy-terminal HIN domain.[14] AIM2 binds to cytosolic DNA and enables the assembly of the inflammasome with ASC and caspase 1 to induce interleukin-1 beta (IFN-β) production, which is essential for protection against pathogens such as vaccinia virus.[15] The most important DNA sensor that has been found so far is the cyclic-di-GMP-AMP

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(cGAMP) synthetase (cGAS). The cGAS binds directly to dsDNA and the adaptor protein STING to activate TBK1 and IRF3–7. This in turn initiates type-I IFN production. cGAS was shown to be central for IFN-β production during DNA viral infection in mouse and human cells.[16,17] Many other cytosolic DNA receptors including double-strand break repair protein MRE11 (encoded by *MRE11A*), DNA-dependent protein kinase (DNA-PK) and Leucine-rich repeat flightless interacting protein 2 (LRRFIP2) also recognize dsDNA and converge on STING to upregulate type-I IFN expression.[18,19] Another interesting DNA sensor is DNA polymerase III. This converts AT-rich DNA to RNA which then activates RIG-I followed by type-I IFN production.[17] However, the role of RNA polymerase III in DNA sensing is still unclear. The DExD/H-box helicases (DDX) are an emerging group of DNA sensors, which include DDX41, DDX9 and DHX36. DExD/H-box helicase 36 and 9 (DHX36 and DHX9) bind CpG DNA in pDC. This is followed by binding to MyD88 which triggers type-I IFN production.[20] It was also reported that DDX41is an important signalling molecule for dsDNA-dependent responses in addition to its role as an innate DNA sensor.[21] However, it is currently unclear how the DDX family proteins interact with nucleic acids and transduce signals downstream.

In addition to protection against pathogens, these DNA sensors also recognize self DNA. Recognition of self-DNA can initiate an inflammatory response (summarized in Figure 1). TLR9 and type-I IFN have been closely associated in SLE from studies in humans and murine models of SLE.[22] Recently, cGAS and STING were identified as the key mediators in Aicardi–Goutieres syndrome.[23] Genetic ablation of cGAS rescued the phenotype in a murine model of Aicardi–Goutieres syndrome.[24] Expression of DAI and AIM2 have also been found to upregulated in patients with autoimmune diseases.[25,26] In summary, cytosolic DNA is sensed by DNA sensors and subsequently activates adaptor proteins, such as STING and MyD88, and the inflammasome, resulting in proinflammatory cytokine production.

Role of type-I IFN

Type-I IFNs are critical anti-viral cytokines that are triggered by DNA sensors.[27] The secreted type-I IFNs bind to IFN receptors to upregulate interferon-stimulated genes (ISGs) via Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway.[28] This changes the cell-intrinsic status and production of interferon stimulated genes (ISGs) to fight against the microbial insult and limit the spread of the pathogens. It also facilitates antigen presentation and natural killer cell cytotoxicity. Furthermore, the adaptive immune system is activated resulting in the generation of activated T cells and high-affinity antibodies.[29–31] The type-I IFN production is tightly regulated by multiple layers of mediators such as some of the ISGs to ensure appropriate immune response.[5,32] In autoimmunity, inappropriate chronic type-I IFN production leads to autoreactive T cells and autoantibody production in some autoimmune animal models.[33] However, it should be noted that type-I IFN also has a protective effect in some autoimmune diseases including inflammatory bowel disease and multiple sclerosis. This might be due to suppression of the production of other proinflammatory cytokine.[34] The effect of type-I IFN in autoimmune disease is probably dependent on the stage of the disease; early type-I IFN production may initiate systemic autoimmunity, whilst later production might limit autoreactive T-cell function.

DNA SENSING AND AUTOIMMUNITY

In mammalian cells, DNA is normally confined to the nucleus. Its presence in the cytosol can induce immune responses.[35,36] DNA is predominantly released into the extracellular space by two mechanisms: cell death and bacterial biofilm. We discuss the role of DNA released from dead cells.

Abnormal response to DNA released from apoptotic cells

Cell death pathways. Death is inevitable but living is not. Multiple types of cell death exist but these can be broadly grouped into programed cell death (PCD) and passive cell demise. PCD plays a key role in development and homeostasis. It is also important in the pathogenesis of autoimmunity. Deficiency in the clearance of DNA/nucleosome derived from either PCD or passive cell death is associated with autoimmunity.

Apoptosis is the most familiar and well-studied PCD pathway. It can be stimulated by both intrinsic and extrinsic signals that activate the caspase protein family.[37] Activated caspases cleave (1) nuclear lamins, resulting in nuclear shrinking and budding; (2) cytoskeleton proteins, resulting in loss of cell shape; and (3) PAK2, which mediates apoptotic body blebbing.[38,39]

During apoptosis, nucleases cleave condensed chromatin into oligonucleosomes.[40] With an increase in the nuclear permeability, oligonucleosomes can migrate into the cytoplasm and fuse with the plasma membrane to form blebs. Apoptotic blebs are about one micron in size and contain DNA fragments (of 500–1000 base pairs), histones and ribonuclear proteins. These apoptotic blebs are then released from the apoptotic cells. Under normal conditions, the blebs are phagocytosed rapidly and under conditions that are anti-inflammatory in nature.[41]

Necrosis, unlike programmed cell death, is unplanned and typically associated with inflammation. Necrotic cells result from stress, heat shock or any stimulus that overwhelms the structural integrity of the cell. The process is morphologically different from apoptosis. The cell expands with consequent plasma membrane rupture. This is independent of cell fragmentation or DNA cleavage.[42] Whereas apoptotic cell contents are packed in apoptotic bodies, necrotic cells release DAMPs, which promote inflammation. Necrotic cells are cleared by a process called macropinocytosis, which is slow and incomplete.[43]

Secondary necrosis occurs if the elimination of the apoptotic remnant is not cleared sufficiently. This phenomenon is easily induced *in vitro* but only observed *in vivo* under pathological conditions.[44] Similar to primary necrosis, secondary necrosis is

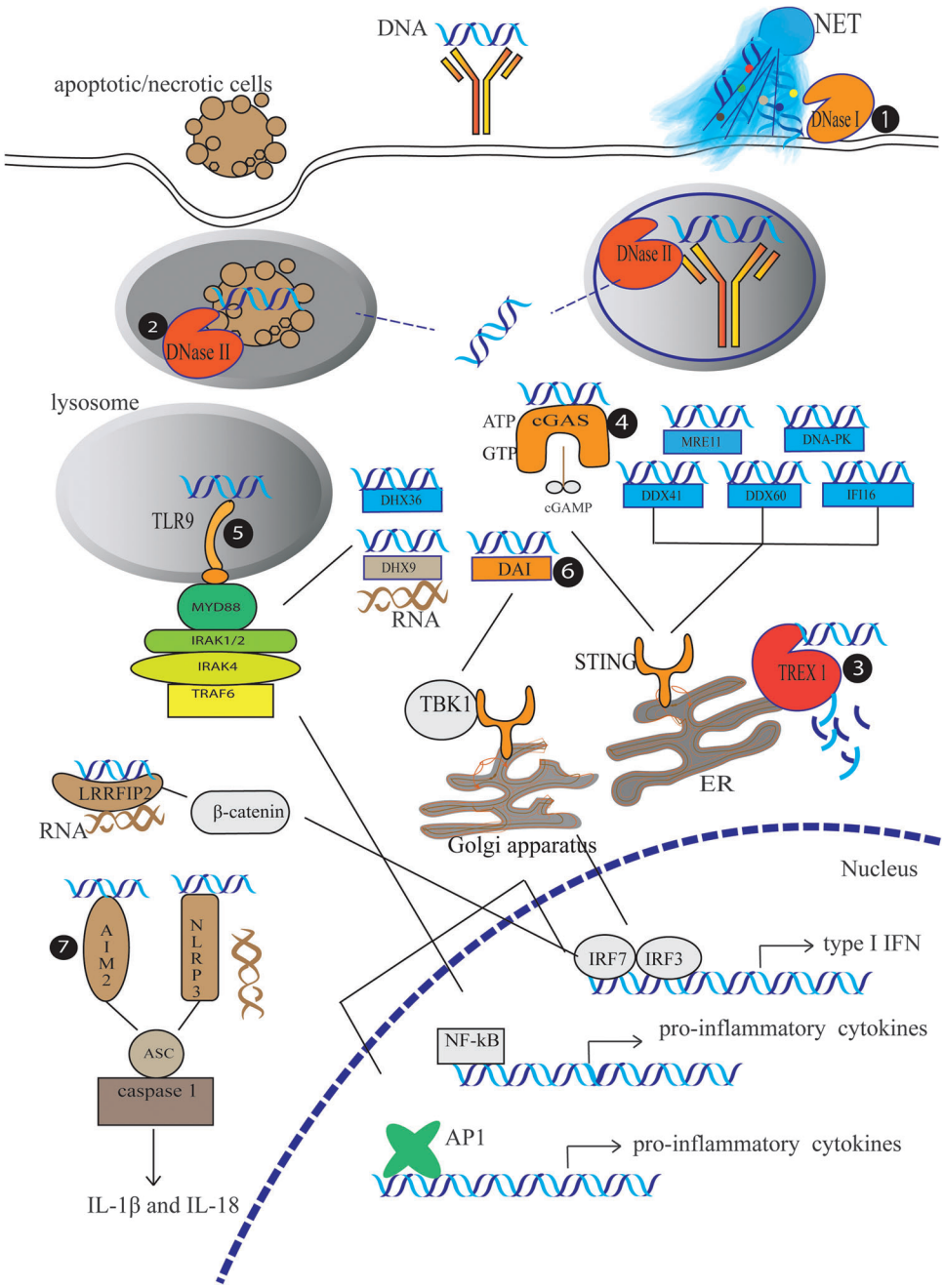

Figure 1 Model of extracellular DNA induced proinflammatory response. DNaseI digests extracellular DNA that is released from either neutrophils undergoing NETosis or apoptotic/necrotic cells. The undigested DNA may form immune complexes with anti-DNA antibodies, which are then phagocytosed following Fc receptor engagement. DNA can also be phagocytosed in the form of microparticles from apoptotic/necrotic cells. The DNA in the lysosome is degraded by DNase II. Undigested DNA in the lysosome may activate TLR9 to stimulate MyD88. This in turn assembles the Myddosome complex which is composed of MyD88, IRAK1, IRAK2 and IRAK4. TRAF6 is then recruited to the complex, leading to the activation of NF-kb and AP-1. RNA helicase DHX36 and DHX9 were found to identify CpG DNA in certain cell types and trigger MyD88 signalling. TREX1 is localized on the endoplasmic reticulum (ER), digests cytosolic DNA to prevent innate immune response. *TREX1* mutations leads to DNA accumulation. This triggers the production of guanosine monophosphate-adenosine monophosphate (cGAMP) by cGAS upon intracellular DNA engagement. cGAMP activates the ER-resident STING, which then is shuttled to ER-Golgi intermediate compartment and the Golgi apparatus. STING initiates TBK1 activation, resulting in the expression of type-I IFNs. Other DNA sensors including double-strand break repair protein MRE11, DNA-dependent protein kinase (DNA-PK), IFNγ-inducible protein 16 (IFI16), protein kinase RNA-activated (PKR), the probable ATP-dependent RNA helicases DDX41 and DDX60, recognize DNA and signal through STING to initiate type-I IFN expression. DNA or RNA is sensed by Leucine-rich repeat flightless interacting protein 2 (LRRFIP2) to activate the β-catenin, resulting in the production of IFN- β. DAI directly recruits TBK1 which leads to type-I IFN production. AIM2 and NLRP3 trigger ASC caspase-1 inflammasome assembly upon dsDNA binding that promote the production of IL-1b and IL-18.

characterized by cytoplasmic swelling, plasma membrane rupture, mitochondria hyperpolarization, oxidative burst and the release of DAMPs as well as oxidized products.[45] Unlike necroptosis, secondary necrosis does not rely on receptor-interacting serine/threonine-protein kinase 1 (RIP1) or mitochondrial dependent reactive oxidative species (ROS) production because neither necrostatin-1 (Nec-1), a RIP1 inhibitor, or knockdown of mitochondrial complex I chaperone affects secondary necrosis.[46] DNA released from necrosis and secondary necrosis is not specifically cleaved in a manner seen during apoptosis. DNA appears as a smear on gel electrophoresis instead of the ladder-like pattern characteristic of apoptosis. Phagocytosis of secondary necrotic cells by phagocytes is enhanced by proteins such as C1q, mannose-binding lectin (MBL) and polyreactive IgM.[47,48]

DNA accumulation and autoimmunity: lessons from mouse models. When the quantity of apoptotic cells overwhelms the regulatory system, autoimmunity can develop. Considerable data support the idea that defective clearance of apoptotic cells leads to autoimmunity.[49,50] For example, a subset of SLE patients show defective apoptotic cell clearance ability with morphologically different macrophages that have impaired phagocyte function.[51,52] In addition, accumulated apoptotic cells were observed in the bone marrow of SLE patients and skin of patients with cutaneous lupus.[53] These findings are further confirmed in mice models. Mice lacking milk fat globule-EGF factor 8 protein (Mfge8), proto-oncogene tyrosine-protein kinase (Mer), or C1q accumulated apoptotic cells and developed an SLE-like disease with anti-nuclear antibodies and glomerulonephritis.[54,55] However, mice lacking CD14 have accumulated apoptotic cells without autoimmunity. And mice that have received apoptotic cell by injection display only transient autoantibody production.[56,57]

DNaseII. DNA released from dead cells is processed by multiple regulatory mechanisms to prevent inflammation. DNA from the engulfed apoptotic cells is normally transported to the lysosome of phagocytes for degradation. DeoxyribonucleaseII (DNaseII) is the nuclease in the lysosome that degrades DNA. Because huge numbers of cells undergo apoptosis, almost one gram of DNA needs to be cleaved by DNaseII per day. Not surprisingly, DNaseII knockout mice die before or shortly after birth with numerous undigested DNA in the macrophages.[58] *DNaseII* and IFN-I receptor (*IFN-IR)* double knockout mice are born normally although they still have lots of undigested DNA.[59] These double-knockout mice, and mice with induced *DNaseII* knockout after birth, develop an age-dependent rheumatoid arthritis-like disease.[60] Similar to the human rheumatoid arthritis, proinflammatory cytokines including TNF-a, IL-6 and IL-1 are upregulated in the inflamed joints. They stimulate macrophage and fibroblast in the affected joints, followed by pannus formation and tissue destruction.[61]

TREX. Three-prime repair exonuclease 1 (Trex1) is a mammalian 3′-DNA exonuclease. It cleaves cytosolic DNA which might have leaked from the lysosome.[62] Unlike DNase II-deficient mice, TREX-deficient mice develop inflammatory myocarditis, while a TREX mutation in human leads to Aicardi–Goutieres syndrome.[63] A strong proinflammatory cytokine profile is found in the heart, joints and cerebrospinal fluid in Aicardi–Goutieres syndrome possibly due to the cytosolic DNA accumulation.[64] Because both DNase II and TREX-deficient mice display high IFN-α expression, it is plausible that undigested DNA initiates the inflammatory process. Why are different tissues affected in the two deficiencies? This is most likely because DNase II only works in acidic conditions and is expressed in specific organs including bone marrow and spleen. Conversely, TREX1 works in the cytosol and is expressed ubiquitously.[65]

When regulatory mechanisms are impaired, undigested DNA released from dead cells can stimulate proinflammatory cytokine production via an array of mechanisms. Macrophages lacking *Dnase II* or *TREX1* can engulf apoptotic cells. But DNA accumulates within the macrophages. It was observed that TLR9 deficiency did not rescue the *DnaseII* deficient mouse phenotype, indicating the DNA-stimulated immune response in *DnaseII* deficiency is TLR9 independent[66] It is likely that undigested DNA leaks into the cytosol and engages cytosolic DNA sensors. For example, Eyes absent 4 (EYA4) stimulates IFN-β and CXCL10 expression in response to undigested DNA associated with apoptotic cells.[67] And STING is critical for the cytosolic DNA-induced cytokine production. STING deficiency rescues *DnaseII*-dependent embryonic lethality.[68]

DNaseI. DNA may also be released into the extracellular space during cell death in the form of apoptotic bodies, microparticles or naked nucleosomes.[69,70] DNaseI is the predominant nuclease in serum. It was reported that *DNaseI* deficient mice develop an SLE-like disease with anti-DNA antibodies and glomerulonephritis.[71] It should be noted that only 38% of the *DNaseI* deficient mice develop full-blown glomerulonephritis, indicating the presence of compensatory mechanisms such as C1q-mediated apoptotic cell clearance.[71] These observations are consistent with human data. Heterozygous *DNase I* mutations are associated with an SLE-like illness but the DNase I level is not correlated with disease activity.[72]

C1q-deficient mice also develop autoimmunity but this is influenced by genetic background.[55] Deoxyribonuclease I-like 3 (DNaseIL3) is homologous to DNaseI. It digests extracellular DNA. A frameshift mutation of DNaseIL3 was found to be associated with autosomal recessive SLE.[73] *Dnase1l3*-deficient mice display similar phenotype to *Dnase1*-deficient mice: high anti-DNA antibody titres, splenomegaly and strain-dependent glomerulonephritis. Unlike DNaseI, DNaseIL3 can digest membrane-coated DNA such as microparticles that are released from secondary necrotic cells. Taken together, DNaseI and DNaseIL3 act to digest extracellular DNA released from apoptotic cells.[74]

DNA modification. The quantity and nature of DNA can trigger autoimmunity. Extracellular DNA is poorly

immunogenic but certain modifications, such as oxidation and unmethylated CpG, make it a more potent activator.[75,76] Oxidized DNA is commonly found in the ultraviolet (UV)-induced apoptotic cells and is resistant to TREX1 digestion.[77,78] Hence, oxidized DNA could accumulate in the cytosol and engages cGAS to stimulate STING and initiate type-I IFN production. This phenomenon is relevant to SLE and rheumatoid arthritis (RA) patients as high levels of oxidized DNA have been observed in lupus-specific skin lesions after UV exposure and synovial fluid (SF).[79,80] Notably, oxidative DNA damage was abundant in UV-exposed cutaneous lesions in SLE patients and injection of oxidized DNA into the skin of a lupus mouse model (MRL/lpr strain) induced skin lesions.[81]

The accumulated DNA may also form complexes with nuclear proteins to mediate immune responses. The best studied DAMP that is complexed with DNA is high mobility group box 1 (HMGB1), a non-histone nuclear protein that is composed of two DNA binding domains called the A box and B box as well as C terminal tail.[45] HMGB1 is not normally tightly bound to DNA and has considerable mobility inside the cell, translocating from nucleus to cytoplasm depending on the state of the cell. HMGB1 is released from primary necrotic and secondary necrotic cells, either by itself or bound to chromatin.[82] HMGB1 complexed to nucleosome is able to induce IL-β, IL-6 and TNF-α secretion from DC and macrophages. Neither HMGB1 nor nucleosome alone mediates these effects.[83] These processes may be relevant to tissue injury in scleroderma. In this condition, excessive damage of the fibroblast is thought to initiate the fibrosis, the pathological hallmark of the condition.[84] DAMPs released from apoptotic fibroblasts can trigger inflammation.[85] Nucleic acids alone or in complex with autoantibodies against topoisomerase I can stimulate TLRs to produce type-I IFN.[86,87] However, the role of type-I IFN in scleroderma remains unclear.

Anti-DNA antibody. Excessive extracellular nucleosome production can promote the production of anti-DNA antibodies that might further amplify the inflammatory responses. The level of anti-dsDNA antibodies broadly correlates with disease activity in SLE and they can bind to apoptotic cells.[88] Some of these anti-DNA antibodies were demonstrated to inhibit apoptotic cell uptake via Fc receptors.[89] However, polyclonal anti-dsDNA antibodies from SLE patients may form complexes with secondary necrotic cells and promote phagocytosis and IL-8 and IL-1 secretion from granulocytes and monocytes, respectively.[90] The isotype of the anti-dsDNA antibody is important. Anti-DNA IgM antibodies (T15-Nab) form complexes with apoptotic cells and facilitate uptake. This process suppresses the inflammatory cytokine production by macrophages.[91] The seemingly contradictory results are probably caused by two main factors. First, anti-DNA antibodies are highly heterogeneous. They have distinct antigen reactivity as well as antibody isotype. Second, results from studies are likely influenced by the different experimental conditions and their effects on phagocytosis. An improvement would be to focus on the study of individual antibodies derived from SLE patients.

NEUTROPHIL EXTRACELLULAR TRAP AND AUTOIMMUNITY

NET kills pathogens

Neutrophil extracellular trap (NET) secretion is a key mechanism through which neutrophils neutralize pathogens. The pathway is determined by the size of the pathogen. If the pathogen is small enough to be phagocytosed, neutrophil elastase (NE) will be trafficked away from the nucleus, preventing chromatin decondensation, and therefore inhibiting NETosis.[92]

If the pathogen is too big to be phagocytosed, NETosis is initiated. The NET is composed of decondensed neutrophil DNA. DNA in the NET mediates intracellular signal transduction and immune responses that not only contribute to pathogen destruction, but under certain conditions can contribute to inflammation in the setting of autoimmunity.[93] It is still unclear which receptor transduces the signal but reactive oxygen species are critical for NETosis. It drives the protease to migrate from primary granules to the nucleus to degrade histones.[94,95] The chromatin is then further decondensed by NE and myeloperoxidase and released into the cytoplasm.[96,97] Finally, NET are released after plasma membrane rupture. The NET release via cell death is slow while NET release from viable neutrophils is about two to three fold quicker. The decondensed DNA and antimicrobial proteins are wrapped in vesicles that are secreted from viable neutrophils in response to, for example, *Staphylococcus aureus*.[98]

Immunological effects of NET clearance

Like DNA released from apoptotic cells, DNase digests DNA in the NET. Nevertheless, DNase alone may not be sufficient as they cannot degrade the insoluble aggregates formed by DNA complexed with LL37/human neutrophil peptide (HNP). This insoluble aggregate induces type-I IFN production in a TLR9-dependent manner.[99] NETs can be engulfed by professional scavenger cells like monocytes/macrophages, a process facilitated by DNase and complement. Engulfed NETs can be digested in the lysosome without proinflammatory cytokine production.[100] This finding, however, is challenged by other studies that demonstrate that NETs initiate proinflammatory signalling pathways in PBMC, monoctyes and DC.[77] These studies suggest that the DNA content of the NET activates cGAS and TLR9 in PBMC and pDC, respectively, which subsequently initiates type-I IFN production.[101] This makes the clearance of NETs remarkably different from apoptotic cell clearance, which upregulate TGF-β and IL-10 production. One possible explanation for this disparity is that NETs are predominantly produced to combat infection. Hence, in this setting it might be favorable to promote type-I IFN secretion. Many aspects of NET processing/degradation mechanisms are still elusive and remain an active field of research (Table 1).

NET and autoimmune diseases

Serum from SLE patients protected NETs from DNase degradation: undegraded NETs were observed in kidney tissue and correlated with active lupus nephritis.[102] Glomerular NETs

Table 1 DNA sensors in autoimmune diseases

DNA sensor	Autoimmune diseases	Cell types involved	Potential mechanisms	References
Dnaso 1	SLE and scleroderma	Macrophages and dendritic cells	Mutation ot Dnase I is related to SLE and Scleroderma Dnasc I treatment prevents autmnunuiutv in mice	58,60,61
Dnasc II	Rheumatoid arthritis	Macrophages	Dnase II and IFNα double knockout mice develop age-dependent rheumatoid arthritis	49–51,55
TREX1	Aicaidi–Goutieres syndromes and SLE	Macrophages	Mutations in *TREX1* gene were found in SLE patients. Loss of function mutation of TREX1 causes Aicardi–Goutieres syndromes TREX1 null mice develop inflammatory myocarditis with ssDNA accumulating in the cytoplasm	53,54
cGAS	Aicanli–Goutieres syndromes, SLE Arthritis and SAVI (STING associated vasculopathy with onset in infancy)		Gain-of-function mutations of the gene encoding STING in hunun patients with early-onset vasculopathy and pulmonary inflammation Deletion of cGAS rescues the Dnase II-deficient mice lacking functional TREX1 or RNaseH2, activation of the cGAS-STING pathway causes the Aicardi–Goutieres syndromes	15,22,23
TLR9	SLE	pDC and B cells	TLR9 piomoted the B cells autoreactivity after chromatin engulfment. TLR9 mediated the type-1 IFN production after DNA immune complex uptake by pDC	21,101
DAI	SLE	Macrophages and dendritic cells	DAI expression is upregulated in some SLE patients and one lupus mouse model. Apoptotic DNA immunization activated macrophages via DAI and DAI inhibition ameliorated SLE syndrome.	24
AIM2	Psoriasis, SLE and inflammatory bowel disease	Keratinocytes, macrophages	AIM2 expression is upregulated in keratinocytes in promote IL-1 release. Cytosolic DNA promote IL-1 release from keratinocytes via AIM2. AMI2 expression is elevated in male SLE patients and inflammatory bowel disease	25

were also observed in patients with ANCA vasculitis.[103] Undigested NETs in SLE appear to promote inflammatory responses through TLR9 dependent and independent pathways. TLR9 recognizes both CpG DNA and NET-associated DNA.[104] The DNA complex in NETs was shown to induce type-I IFN production from pDC in a TLR9-dependent manner, driven by the TLR adaptor MydD88 and IRF7. Only the NET DNA–protein complex but not naked DNA is able to activate TLR9 in pDC due to the stability of the complex.[99] NETs are mainly composed of dsDNA therefore it is not surprising that NET stimulates intracellular DNA sensors. In SLE patients, a group of granulocyte population called low-density granulocytes (LDGs) were found to simulate the production of type-I IFNs.[105,106] LDGs secrete NETs containing LL-37 (a cathelicidin-related antimicrobial peptides), matrix metallopeptidase 9 (MMP9), dsDNA and histones. The dsDNA released from LDGs was noted to be oxidized because of its mitochondrial origin. Oxidized DNA is more resistant to cytosolic DNase and augments type-I IFN production from PBMC.[107,108] DNA in endocytosed NETs is sensed by cGAS, which activates the adaptor protein STING to simulate type-I IFNs. IFN-α secreted in response to NETs enhanced the autoreactivity of the autoreactive B cells.[104,109] In addition to DNA, cathelicidin and other NET-associated proteins engage P2X7 receptor on LPS-primed macrophages. This activates the NLRP3 inflammasome, leading to IL-1 and IL-18 release.[15,110,111] This proinflammatory response appears to be augmented by the lack of NET clearance in SLE patients. A proportion of SLE patients have defective DNase and/or DNase inhibitors that impair NET degradation. The presence of anti-NET antibodies in some patients might also block the access of DNase to the NET-associated DNA.[102]

NETs are also detected in synovial fluid, skin and rheumatoid nodules of RA patients.[80,112] NETs have direct effects on fibroblast-like synoviocytes promoting IL-6 and IL-8 production. This process is diminished with DNase I treatment. These data suggest a role for extracellular DNA in the inflammatory response in RA.

NET and anti-NET antibody

NETs were also found to form immune complexes with anti-NET antibodies. Autoantibodies against DNA are specific to SLE and anti-dsDNA antibodies may form immune complexes with NETs. These immune complexes are able to activate pDC to secrete IFN-α and TNF-α in a TLR9-dependent manner.[113] The engagement of Fcgamma receptors by the immune complexes recruits cytosolic endoplasmic reticulum to the phagosome thereby triggering the downstream interferon production.[113] In addition to pDC, other TLR9- and CD32-positive cells including monocytes, B cells and GM-CSF-treated PMNs also respond to DNA–antibody immune complexes.[114] pDC also produce increased amounts of IFN-α in patients with active but not inactive small vessel vasculitis,[103] a phenomenon that correlates with circulating chromatin levels.[103] Hence, it is possible that, in small vessel vasculitis, NETs contribute to IFN-α production by pDC.

NET in animal models

The pathogenic role of NET *in vivo* has been studied in various animal models. Nakazawa and colleagues demonstrated that NETs induced by propylthiouracil and phorbol myristate acetate were relatively resistant to DNase I degradation and had a more compact conformation. When rats were immunised with these abnormal NETs they developed anti-myeloperoxidase antibodies and pulmonary capillaritis.[115] In addition to the direct tissue damage, intact NETs can also act as a source of autoantigens. Myleoid dendritic cell (mDC) can interact and take up NETs. Injection of mDC loaded with NETs to wild-type mice promoted the production of anti-MPO and anti-dsDNA autoantibodies. However, the pathological consequence of the NET uptake remains incompletely understood.[116,117]

Evidence from lupus-prone mice strains remains incomplete. New Zealand mixed 2328 (NZM) mice have enhanced NEtosis which might lead to the NET deposition in the kidney. The inhibition of NET formation by peptidylarginine deiminase (PAD) inhibitor abolished the NET production and reduced anti-dsDNA antibody level in kidney [118] However, it should be noted that the PAD inhibitor did not ameliorate the kidney inflammation. By contrast, disease activity of MRL/lpr lupus-prone mouse disease is exacerbated by the Nox2 deficiency, which inhibits NET formation.[119] The Nox2-deficient mice had defective NET formation but higher titres of anti-nuclear antibodies, proteinuria and more severe glomerulonephritis. More studies using inhibitors within the different NET forming pathways are needed to elucidate the role of NETs in SLE.

CONCLUDING AND PERSPECTIVES

The fundamental function of DNA is to store information in a heritable manner. Foreign DNA is recognized by DNA sensors which can elicit an immune response as part of host defence against pathogens. But inappropriate response to self DNA is associated with autoimmune diseases, such as SLE and AGS. The discovery of DNA-sensing pathways and their contribution to autoimmunity has revealed potential new therapeutic targets. Anti-INF-α and anti-IFNR treatments are under clinical trials. For example, sifalimumab showed promising results in SLE.[120,121] NET inhibition and manipulation of apoptotic cell removal remains in the pre-clinical study area: inhibitors of NADPH oxidase; mitochondrial ROS production; actin cytoskeleton; and PAD enzymes have been studied in lupus models. For example, PAD deficiency prevents NET formation and ameliorate arthritis in an induced arthritis model.[118,122,123]. And, since PPARβ and LXR coordinate apoptotic cell engulfment and induce tolerance in APCs, agonists against PPARβ or LXR, at least in theory, might be beneficial in autoimmunity.[124–126] Clearly, there is a complex regulatory network to prevent an aberrant immune response to host DNA and all the pathways described in our review are important in homeostatic processes and/or response to pathogens and tissue injury. Manipulating these *in vivo* is a major challenge both in terms of expected efficacy (for example, disease-modifying

outcomes in systemic autoimmunity) and in terms of safety (for example, infection risk, impaired homeostatic tissue turnover).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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